



SOCRATIC LECTURES

7TH INTERNATIONAL SYMPOSIUM, LJUBLJANA, 7. MAY 2022

PEER REVIEWED PROCEEDINGS

EDITED BY: VERONIKA KRALJ-IGLIČ

FACULTY OF HEALTH SCIENCES, UNIVERSITY OF LJUBLJANA

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7th International Symposium, Ljubljana, May 7, 2022

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The members of the Organizing Committee of 7th Socratic Lectures: Saba Battelino, Vladimira Erjavec, Darja Feizpour, Tjaša Griessler Bulc, Veronika Kralj-Iglič, Anita Prelovšek, Anna Romolo, Gitta Schlosser, Duško Spasovski, Polonca Trebše.

Program of the Socratic Symposium May 7, 2022, 10:00 – 16:00 (Ljubljana time)

10:05 – 10:35 Plenary lecture: Duško Spasovski: Periacetabular osteotomy of hip

10.40 - 12.40 Scientific sections

Section 1: Medicine organized and moderated by Duško Spasovski and Saba Battelino

- 10:40 - 10:55 **Borut Kovačič, Klemen Stražar, Lenart Zore:** Pelvic osteotomies: experience of University Medical Centre Ljubljana
- 10:55 - 11:10 **Honza Pluhar:** Biomechanical analysis of periacetabular osteotomy. Preliminary results
- 11:10 - 11:25 **Blaž Mavčič:** The role of hip arthroplasty registries and cohort studies in orthopaedic surgery
- 11:25 - 11:40 **Gregor Omejec:** New insights into treatment of patients with carpal tunnel syndrome
- 11:40 - 11:55 **Nejc Steiner:** Surface-based total blood volume calculation for platelet and extracellular vesicle-rich gel preparation
- 11:55 - 12:10 **Domen Vozel:** Use of platelet and small cellular particles rich plasma for closure of skull base
- 12:10 - 12:25 **Sara Bitenc Zore:** Facial nerve reconstructive surgery in otorhinolaryngology and its enhancement by platelet-rich plasma therapy
- 12:25 - 12:40 **Student's examination questions**

Section 2: Veterinary Medicine organized and moderated by Vladimira Erjavec

- 10:40 - 11:00 **Naida Kapo:** Anthelmintic resistance in gastrointestinal nematodes of ruminants
- 11:00 - 11:20 **Tina Gazibarić:** Canine spaying - potential health benefits and risks
- 11:20 - 11:40 **Betka Perc:** Overview of wound healing differences between dogs and cats
- 11:40 - 12:00 **Vladimira Erjavec:** Treating burns in cats and in dogs using medical honey
- 12:00 - 12:20 **Alenka Nemec Svete:** Brachycephalic dogs with brachycephalic obstructive airway syndrome have increased variability in red blood cell size
- 12:20 - 12:40 **Pšenica Kovačič:** The interweaving neoteny of brachycephalic cats in art, evolution and breeding

Section 3: Ecology organized and moderated by Polonca Trebše and Tjaša Griessler Bulc

- 10:40 - 11:10 **Franja Prosenec:** Automated quantification of microplastics - challenges and opportunities
- 11:10 - 11:40 **Ipek Gevinc, Polonca Trebše, Franja Prosenec:** Toxicity assessment of therapeutical peloid mud from Serbian and Slovenian spas
- 11:40 - 12:10 **Lovro Tomazin, Lara Čizmek, Dmitrii Mazur, Polonca Trebše:** Determination of N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine with liquid chromatography and UV-Vis detection
- 12:10 - 12:40 **Katjuša Mežek, Nicola Rhyner, Theo H.M. Smits, Tjaša Griessler Bulc, Ranka Junge, Nadine Antenen:** Characterization of bacterial communities in green wall systems used for greywater treatment



Section 4 : Materials organized and moderated by **Darja Feizpour**

- 10:40 - 11:10 **Zala Jan:** Impact of Corundum Ceramic, TiO₂ and hydroxy apatite nanoparticles on cell line in vitro
- 11:10 - 11:40 **Niharika Rawat:** Fabrication of TiO₂ microflowers and their antibacterial effect against E.coli
- 11:40 - 12:10 **Tamara Črnko:** Additive manufacturing in orthotics and prosthetics
- 12:10 - 12:40 **Darja Feizpour:** Transmission electron microscopy of biomaterials used in total hip arthroplasty

Section 5 : Small Cellular Particles organized and moderated by **Gitta Schlosser**

- 10:40 - 11:00 **Darja Božič:** Effect of temperature and systemic force in centrifuge on small cellular particle count in preparations from blood
- 11:00 - 11:20 **Samo Penič:** Development of sensor for erythrocyte boundary movement during centrifugation
- 11:20 - 11:40 **Barbara Šetina Batič:** Scanning electron microscopy (SEM) and focused ion beam (FIB) for studies of biological specimens
- 11:40 - 12:00 **Marko Jeran:** Measurement of size of erythrocyte extracellular vesicles by using scanning electron microscope images
- 12:00 - 12:20 **Gitta Schlosser, Gabriella Gellen, Gabriella Pocsfalvi:** Proteome of small cellular particles from *Phaeodactylum tricornutum*
- 12:20 - 12:40 **Matic Kisovec, Apolonija Bedina Zavec, Marjetka Podobnik, Matej Hočevar, Darja Božič, David Škufca, Marko Jeran, Ksenija Kogej, Gabriella Pocsfalvi, Aleš Iglič, Veronika Kralj-Iglič:** Electron microscope images of small cellular particles from blood preparations Transmission electron microscopy of small cellular particles from blood preparations

Section 6 : Musicology organized and moderated by **Anita Prelovšek**

- 10:40 - 11:00 **Nelfi Paliska:** Liszt – The funeral tetralogy for Richard Wagner
- 11:00 - 11:20 **Anastasia Kolomiets:** The Titanium 3D Printed Flute: new prospects of additive manufacturing for musical wind instruments design
- 11:20 - 11:40 **Eva Dolinšek:** Claudio Monteverdi in Seconda prattica
- 11:40 - 12:00 **Tatiana Batyr:** J. W. Goethe and music
- 12:00 - 12:20 **Nadia Cannata:** Had we but world enough and time: Representing linguistic memories (or the matter of language).
- 12:20 - 12:40 **Julija Vladimirovna Petrova:** Music in the novel The House of the Dead

Honorary Lectures and Cultural program

13:00-13:20 Andreas Leithner: Medical University of Graz: New technologies in orthopaedics and trauma. What do we need?

13:20-13:35 Marija Ipavec: Government of the Republic Slovenia: Fulfilling the needs of an above knee amputee by prosthesis

13:35 Christoph Willibald Gluck: Melodie. Flute: Anita Prelovšek

13:40 Closing of the symposium

16:00 Live: Gettogether at Caffeteria, Museum of Architecture and Design, Rusjanov trg 7, Ljubljana

Editorial

7th International Symposium Socratic Lectures was held online, 7th May 2022. It was attended by about 150 participants whereas Veterinary medicine and Medicine sections had the biggest number of participants. Symposium was composed of a plenary lecture donated by prof. Duško Spasovski from Belgrade, Serbia, six Scientific sections featuring 33 lectures, a poster section with 5 posters and two honorary lectures donated by prof. Andreas Leithner, Medical University of Graz and dr. Marija Ipavec, Government of Republic Slovenia. As always, it was a pleasure to feel concentrated energy bringing together excellent science and dedicated youth.

Students of the 1st year of Orthotics and prosthetics have entered research by assessing biomechanical parameters of hips that were operated on due to hip dysplasia. For that, the students learned the method HIPSTRESS during the spring semester. They analysed X-ray images of the patients, led by their colleague Honza Pluhar from Technical University in Prague, who was in Ljubljana 4 months within the ERASMUS initiative. Mr Pluhar developed tools and tutored the students to be able to become partners in scientific work. Their effort was topped by meeting with the surgeons at the Socratic lectures symposium.

The proceedings features 24 contributions and 5 posters. In the Proceedings Gallery we are thankful to present illustrations of cats donated by a painter Pšenica Kovačič while the frontpage and the image before the posters were donated by a painter Judit Nyirkos from Budapest, Hungary.

We are happy to see the growth of contents and extent of Socratic Lectures. Our wish has always been to offer a possibility for active participation to those who wish to contribute and are welcoming ideas for the future Symposia.

Veronika Kralj-Iglič and Anna Romolo

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Invited lecture/Review

Pelvic Osteotomies in Children and Adults - Experience of University Medical Centre Ljubljana

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Abstract:

Nowadays, hip dysplasia is recognized and treated mainly conservatively due to good screening program and early detection. There are still some cases found after first year of life, in later childhood and in adults. These cases are treated with pelvic osteotomies to improve femoral head coverage and joint stability and to decrease the hip stress with the final goal to preserve the joint in longer term.

There are three subtypes of pelvic osteotomies. The ones pertaining to first type are redirection osteotomies, which are incomplete osteotomies with the aim to reduce overall volume and redirect the acetabulum by a hinge located between triradiate cartilage and pubic symphysis. This type includes Salter, Pemberton, Dega and San Diego osteotomies. The osteotomies pertaining to the second type are reorientational osteotomies, which are complete osteotomies with the aim to reorient the whole acetabulum. This type includes periacetabular and triple osteotomies. The osteotomies pertaining to the third type are salvage osteotomies which enlarge the acetabulum and medialise the hip center in arthritic and/or incongruent joint and are performed in hips in which the reorientational osteotomy is contraindicated. This type includes shelf and Chiari osteotomies. In University Medical Centre Ljubljana, most of the above-mentioned osteotomies are being performed, however, yearly numbers are small. Redirectional osteotomies have been performed for many years in the Pediatric orthopaedic department and yearly number is under 10 cases. Reorientational osteotomies on young adults are made through minimally invasive approach for periacetabular osteotomy described by Soebale et al. (2015). In selected cases, electromagnetic surgical navigation system is used for more precise positioning of the acetabular fragment.

Keywords: Hip dysplasia, pelvic osteotomy, periacetabular osteotomy, hip preservation, hip pain

1. Definition of Hip Dysplasia

Osteoarthritis is the most common idiopathic disease, with many factors identified which significantly increase the risk of its early onset. Osteoarthritis is defined by thinning and destruction of the articular cartilage and the deformation of anatomical structures around the joint (Harris, 1986). Poorly developed hip joint with deviation in size, shape, mutual proportions or orientation of the acetabulum and femoral head is described as dysplastic hip (Klaue et al., 1991). Hip dysplasia is most commonly found in a pediatric population, however development of the hip joint continues till the young adulthood and dysplastic hips can be found also at later age. It is important to know that untreated or unsuccessfully treated hip dysplasia can eventually lead to cartilage degeneration, presumably due to the pathologically increased stress on smaller articular surface area within the joint. The joint is overloaded on the chondrolabral junction which causes labral injury, chondral delamination and thinning, and rupture of the lig. capitis femoris (lig. teres)(Mavčič et al., 2002). Therefore, symptomatic hip dysplasia represents an important indication for surgical procedures to reduce hip joint stress, before irreversible joint changes may happen. This should at least slow down the pathological processes of the hip cartilage and thereby contribute to functional normalization of the joint biomechanics (Brand et al., 2002, Hadley et al., 1990, Vengust et al., 2001).

Hip dysplasia can be treated conservatively with splica cast in the first year of age. Later hip dysplasia treatment depends on the age of the patient and the level of secondary arthritic changes of the involved hip. In case of well preserved intraarticular cartilage, preservation procedure is indicated. Different pelvic osteotomies are considered from, depending on the type of hip deformation and the patient's age. On the other hand, if the osteoarthritic changes are already present together with intraarticular cartilage damage, pelvic osteotomies may lead to unsatisfactory results and in such cases preservation procedures are indicated. Hip arthroplasty is the procedure of choice and is one of the most common surgeries with excellent outcome. However, solution with artificial hip is still considered inferior to the one with native joint and is indicated in elderly but less optimal for younger active adults.

Pelvic osteotomies are most commonly indicated in children and in young adults with prearthritic hip changes. Dysplasia often results due to some underlying diseases such as developmental dysplasia of hip (DDH), neuromuscular disorders and Legg-Calve-Perthes disease. All mentioned disorders produce pathologic forces on the hip joint and pathological development of the hip joint. Depending of type of deformation and age the choice of the preferred pelvic osteotomy type is made.

2. Types of pelvic osteotomies

There are three subgroups of pelvic osteotomies described in the literature. In the first subgroup, there are redirectional osteotomies with a goal to reduce overall volume and to redirect the acetabulum by a hinge located at the triradiate cartilage and/or pubic symphysis. In the second subgroup, there are reorientational osteotomies which tend to reorient the acetabulum. In the third subgroup, there are salvage osteotomies with the aim to enlarge the acetabulum, medialize the center of rotation and improve the femoral head coverage. The surgical approach for all osteotomies is similar, with the aim to avoid major vessels, nerves and muscles and to decrease soft tissue trauma to minimum. A standard anterior bikini incision is used to approach the pelvis and the hip joint. Proximally, the external oblique muscles are elevated from the iliac crest, and the iliac apophysis is split sharply to expose both the inner and outer tables of the iliac wing to a necessary extent. Distally, the direct head of the rectus femoris tendon is detached from the anterior inferior iliac spine, and tagged for later repair, to expose the hip capsule.

2.1. Salter (innominate) osteotomy

Salter innominate osteotomy was described in 1974 by Salter and Dubos who operated innominate osteotomy in congenital hip dislocations (Salter and Dubos, 1974). This type of surgery is indicated for children with congenital hip dislocation which are older than 18 months. Before that age the preferred treatment is conservative with closed reduction of hip dislocation and casting or bracing

to promote satisfactory remodeling (Gillingham et al., 1999). Also the bones are not mature enough for operation (Salter and Dubos, 1974). The upper age limit is estimated to 6 years since in older children, the joint

incongruity, poor remodeling potential of acetabulum and muscle contractures limit the reduction of the free fragment. However as long as the hip can be concentrically reduced and with good range of motion while reduced, children are the candidates. Salter osteotomy is a type of redirection osteotomy with the aim to reorientate the acetabulum without changing the joint surface or shape. After the surgery the patient is immobilized in a hip spica cast for 6 weeks.

The main goal of the Pemberton osteotomy (Pemberton, 1965) is to reorientate the acetabulum forward and lateral and therefore provide extra coverage of dysplastic anterolateral acetabulum (Pemberton, 1965; Pemberton, 1974) by pericapsular osteotomy around the acetabulum. The surgery is indicated for children 1 to 14 years old, with congenital subluxation of the hip. Before the age of one year, the bone is still too immature and the oldest age is defined by triradiate cartilage plasticity and remodeling potential needed for acetabular displacement (Baki et al., 2016). Postoperatively, hip spica cast is needed for 6 weeks.

2.2. Dega osteotomy

Dega osteotomy is another redirection incomplete osteotomy similar to Pemberton osteotomy. It is most often used in hip dysplasia due to developmental disorders such as spastic cerebral palsy (Mubarak et al., 1992). It was described by Dega et al to treat developmental dislocations of the hip with deficient anterolateral coverage. Dega osteotomy is indicated for children between 4 and 16 years of age. This procedure is not recommended for skeletally mature patients. Postoperatively, patients are maintained in a hip spica cast for 6 weeks.

2.3. San Diego osteotomy

San Diego osteotomy was developed by dr. McNerney (McNerney et al., 2000) for treatment of subluxated hips in children with an open triradiate cartilage in a similar way as Dega osteotomy. It was developed specifically for hip subluxations which resulted from neuromuscular abnormalities and is special in a way it combines osteotomy with muscle-tendon lengthening, open reduction of the hip with capsulorrhaphy and capsular acetabuloplasty. The aim of the osteotomy is to create greater posterior acetabular coverage (McNerney et al., 2000). It is indicated in children between 4 and 15 years of age and can also be performed in cases with femoral head incongruity. It is used mainly for posterolateral acetabular deficiency (Kim et al, 2012). Standard postoperative casting is necessary.

2.4. Bernese periacetabular osteotomy

Bernese periacetabular **osteotomy** is indicated in skeletally mature adult patients with symptomatic acetabular dysplasia. The procedure is also known as periacetabular osteotomy (PAO)(Ganz et al., 1988). It is a reorientation type of osteotomy which combines ischial, pubic and iliac cuts and therefore releases the acetabular fragment free which permits considerable correction of version, lateral coverage and anterior coverage. The ischial osteotomy is incomplete and posterior column is spared which allows postoperative partial weight bearing and enables good healing potential. Free fragment is fixed with 2 or 3 cortical screws. Postoperatively, the patients don't need casting (Millis et al, 2009; Trousdale et al., 1995).

2.5. Triple (Tonis) osteotomy

Triple (Tonis) osteotomy is a complete reorientational osteotomy based on Salter innominate osteotomy to improve restricted movement of the fragment and limited lateralization of the acetabulum. It is a successful procedure for treatment of complex congenital, neuromuscular and teratologic hip conditions (Steel, 1973; Rebello et al., 2009). Unlike Salter and Pemberton osteotomies, three cuts in triple osteotomy allow complete freedom of the acetabular fragment and is used frequently.

2.6. Shelf osteotomy

Shelf osteotomy is a salvage procedure where bone graft is used to increase the femoral head coverage instead of cartilage. It is used in hips with acetabular abnormality or aspherical congruity. Shelf osteotomy was popular in treatment of acetabular dysplasia before reorientation osteotomies

became more popular and proved more effective (Wainwright D, 1976). After the surgery the spica cast is indicated. In contrast to reorientation osteotomies, the shelf osteotomy can be performed in a hip with osteoarthritis changes.

2.7. Chiari osteotomy

Chiari osteotomy is another type of salvage osteotomy described by Chiari (Chiari, 1974) who used medially displaced cancellus bone of the ilium to improve load distribution pattern in the hip joint. It is indicated in young adults when reorientation osteotomy is not possible (Morrissy, 2001). Both shelf and Chiari osteotomies are used in young adults to improve femoral head coverage in slightly osteoarthritis hips when reorientation osteotomies are contraindicated.

3. Experience of University Medical Centre Ljubljana

In Ljubljana Medical Centre at the Department of Orthopaedic Surgery the osteotomies are made for many years, however yearly numbers are small and are decreasing.

Redirectional osteotomies are performed at the Pediatric orthopedic department, yearly under 10 cases and mainly for treatment of hips in children with neuromuscular disorders.

Due to good ultrasound screening program of all newborn hips in the 6th week after birth, the need for later operative treatment of DDH has considerably decreased. Also the x-ray screening protocol for hip migration in children with neuromuscular diseases has decreased the number of hip reconstruction surgeries with pelvic osteotomies.

Some cases which are missed, refractory to conservative treatment or diagnosed to late, still need operative treatment. Since 2015, reorientational periacetabular osteotomies in young adults have been done through a minimally invasive transartorial approach described earlier by Soebale (Soebale, 2013). From 2018 on, electromagnetic surgical navigation system for precise positioning of the free acetabular fragment is applied which shows promising early results (Stražar, 2021).

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Invited lecture/Review

New Insights into Treatment of Patients with Carpal Tunnel Syndrome

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Abstract:

Carpal tunnel syndrome (CTS) is caused by median neuropathy at wrist. Clinically is present with paraesthesia in the first three or four fingers which are more pronounced during the night and at the morning and are improved by shaking or changing the position of the hand. Diagnosis is made by clinical examination and confirmed with nerve conduction studies (NCS). Conservative treatment with wrist splints, physiotherapy and corticosteroid injections are often ineffective or have only short-term effect. Furthermore, corticosteroids injections known to have potential side effects.

Surgical treatment with open surgery or endoscopic release are only known to have long-term effect. However, iatrogenic injuries, scar formation, immobilisation and long rehabilitation is frequently present. Perineural injection therapy with 5% dextrose is highly effective for treatment of patients with CTS. Nevertheless, studies showed only short-term effect. For long-term effect, surgical treatment is advised. US guided minimally invasive carpal tunnel release is promising approach into treatment of patients with CTS with many advantages. It is true US guided procedure, it offers identification of key anatomical structures, only local anaesthesia is required, no tourniquet, immobilisation, wound or sutures or scar formation is present. Therefore, perineural injection therapy with 5% dextrose for short-term effect and US guided minimally invasive carpal tunnel release for long-term effect is recommended.

Keywords: Carpal tunnel syndrome; Treatment; Perineural injection; 5% dextrose; US guided minimally invasive carpal tunnel release

1. Introduction

Carpal tunnel syndrome (CTS) is caused by compression of the median nerve at wrist (Olney, 2001). The reported prevalence and incidence of CTS vary widely according to the diagnostic criteria used in different studies. According to some estimations one in ten people develop clinical signs and symptoms of CTS (Quality Standards Subcommittee of the American Academy of Neurology, 1993). CTS mostly affect women with mean age at diagnosis 50 years. However, these data are based on patients who self-referred to a neurophysiological laboratory or clinic and are therefore intrinsically biased (Stevens, 1997; Jablecki et al., 1993). Results of a postal survey of 3000 individuals randomly selected from the general population register of southern Sweden showed that the prevalence of CTS was similar in men and women with male to female ratio 1:1.4 (Atroshi et al., 1999). However, prevalence was highest in women aged 65–74 years and the prevalence in women was almost four times higher than in men (5.1% vs 1.3%, respectively). Suspected risk factors include diabetes mellitus, menopause, hypothyroidism, obesity, arthritis, and pregnancy (Shiri, 2014; Padua et al., 2010; Pourmemari and Shiri, 2016; Shiri et al., 2015).

The importance of clinical presentation is demonstrated by the fact that the long-accepted gold standard for diagnosis is a comprehensive and accurate clinical history along with the exclusion of other possible causes. The CTS is characterized by intermittent, nocturnal paraesthesias and dysaesthesias that increase in frequency during waking hours (Padua et al., 1999). Subsequently, loss of sensation develops along with weakness and thenar muscle atrophy later in the disease course, which result from extensive axonal degeneration (Padua et al., 1999). This sequence of symptoms is quite typical, rarely occurring in disorders other than CTS. Tinel's sign and Phalen's maneuver are popular diagnostic tests. Results are deemed positive when symptoms are evoked by percussion of the median nerve at the wrist or by forced compressive wrist posture for 1 min, respectively (Brüske et al., 2002). Although these tests are widely used because of ease of performance, their sensitivity and specificity are widely debated. Sensitivity ranges from 42% to 85% for Phalen's maneuver and from 38% to 100% for Tinel's test; specificity ranges from 54% to 98% and from 55% to 100%, respectively (Brüske et al., 2002).

Diagnosis is usually made by clinical examination with presence of typical CTS symptoms. Abnormal findings have lower sensitivity (Mackinnon et al, 2000) but higher specificity (Mackinnon et al, 2000; Gomes et al., 2006) in comparison to symptoms. Clinical diagnosis is confirmed with electrodiagnostic testing (i.e., nerve conduction studies). Extensive work by three US scientific societies (the American Academy of Neurology, the American Association of Electrodiagnostic Medicine and the American Academy of Physical Medicine and Rehabilitation) have provided physicians with recommendations for electrophysiological testing. These recommendations suggest performing median sensory and motor nerve conduction studies across the wrist and in case of normal tests performing comparative, segmental, or comparative and segmental tests, which have been shown to have high sensitivity and specificity (80–90% and >95%, respectively) (Jablecki et al., 2002). When clinical signs are present without electrodiagnostic abnormalities CTS is functionally mild, although there might be severe symptoms.

Various non-surgical treatments are available for management of CTS. The first choice should include patient's education (Huisstede et al., 2014). Changes in habits (eg, limitation of wrist movement and reduction of heavy work activities) should be considered and the use of ergonomically friendly work tools can be useful in reducing median nerve stress. However, there is little adequate evidence about the success of this approach. For example, the effectiveness of ergonomic keyboards in the treatment of CTS is unknown (Buchan and Amirfeyz, 2013; O'Connor et al., 2012). Apart from these interventions, patients should be informed about the standard surgical and non-surgical strategies for treating CTS. Surgical treatment consists of releasing transverse carpal ligament with open surgery or minimally invasive procedures (i.e. endoscopic technique).

2. Non-surgical treatment

Different methods of laser therapy exist. This term encompasses several approaches. Laser therapy is able to improve function, symptoms, and electrodiagnostic measures in short-term. Results of a

randomized controlled study showed that laser treatment is more effective than placebo, especially if it is used in patients with mild to moderate CTS (Lazovic et al., 2014). In another study use of the galliumaluminium-arsenide laser with wrist splint showed higher efficacy than placebo laser therapy with wrist splint, especially in improvement of hand grip strength up to 3 months after treatment (Fusakul et al., 2014). However, results of another randomized trial (Tascioglu et al., 2012) showed that laser therapy was not more effective than placebo. Several studies have compared laser therapy with other non-surgical treatments. In a randomised controlled trial comparing laser therapy with fascial manipulation, laser therapy provided transient, short-term pain relief, whereas fascial manipulation showed pain improvement and function (BCTQ) over a longer time period (>3 months) (Pratelli et al., 2015). In another study, high-intensity laser therapy was compared with TENS. Findings showed that laser therapy was more effective than TENS (Casale et al., 2013). In a study comparing low-level laser therapy with magnetic field therapies, the effectiveness of the techniques in reducing pain was shown to be similar (Dakowicz et al., 2011).

Local corticosteroid injections are commonly used to treat CTS. The rationale for the use of this treatment is the ability of corticosteroids to reduce edema and improving the spatial relation between the carpal tunnel, median nerve and tendons. In a randomized trial of 111 patients (Atroshi et al., 2013) methylprednisolone (80 mg or 40 mg) injection into the carpal tunnel was more effective than placebo, reducing symptoms severity and rate of surgery at 1 year. However, the effectiveness of corticosteroid injections for halting disease progression was limited, because in this study three-quarters of patients had surgery within 1 year. The preferred site for local corticosteroid injection has been assessed. In a comparison of distal (palmar) with proximal (wrist) needle insertion, the palmar approach proved less painful. However, no differences were observed in nerve conduction studies (Kamanli et al., 2011). Furthermore, corticosteroid injection with the use of US guidance is better than blind administration and reduces the time to symptom resolution, even if it is more expensive (Lee et al., 2014; Makhlof et al., 2014; Ustün et al., 2013; Kim et al., 2013).

Regarding non-invasive methods of local corticosteroid administration, a comparison of phonophoresis and iontophoresis of dexamethasone sodium phosphate showed that phonophoresis was more effective in improving symptoms and hand function (Bakhtiary et al., 2013). Phonophoresis with dexamethasone combined with splint use has been shown to provide better symptom relief than iontophoresis with dexamethasone combined with splint use or splint use alone (Gurcay et al., 2012). Corticosteroid therapy has been compared with other drugs. By use of phonophoresis for local administration, corticosteroids led to greater reductions in nerve dimensions than did non-steroidal anti-inflammatory drugs and splint use alone in patients with CTS (Soyupek et al., 2012). Triamcinolone acetonide injection and procaine hydrochloride injection were more effective than placebo, and the effectiveness of procaine hydrochloride was similar to that of triamcinolone acetonide (Karadaş et al., 2011; Karadaş et al., 2012). A comparison between 17- α -hydroxyprogesterone caproate and corticosteroid showed that both treatments were efficacious, but only patients treated with 17- α -hydroxyprogesterone caproate continued to have symptom relief after 3 months (Ginanneschi et al., 2012). Corticosteroid injection has also been compared with extracorporeal shock wave therapy, which uses acoustic waves to produce transient pressure increases in tissues with no damaging effects. No difference in symptoms or electrophysiological outcomes was seen between the two treatments (Seok et al., 2013).

Non-steroidal drugs have been assessed as an alternative treatment for CTS. Palmitoylethanolamide improved median nerve motor latency, reduced the proportion of patients with positive Tinel's sign and reduced symptoms of discomfort compared with placebo. However, further studies are needed to substantiate these results (Conigliaro et al., 2011). Gabapentin was no more effective than placebo in reducing pain, numbness, paraesthesia, weakness or clumsiness or nocturnal awakening in a randomized controlled trial (Hui et al., 2011). Repetitive local injection of lidocaine resulted in symptom reduction and electrophysiological improvement compared with single injection (Akarsu et al., 2015).

Therapeutic US is a treatment approach based on the hypothesis that mechanical waves interacting with the tissues of the carpal tunnel (including the median nerve) will reduce inflammation. No clear evidence about the effectiveness of therapeutic US exists, but the reported results are similar to those

obtained with placebo and other non-surgical therapies (splint, exercises, oral pharmacotherapy) (Bakilan et al., 2014; Page et al., 2013; Page et al., 2012; Yildiz et al., 2011). However, findings from a randomized trial (Chang et al., 2014) suggest that US therapy is more effective than paraffin therapy.

Musculoskeletal manipulation is widely used. This approach includes massage, exercise and mobilization of the wrist joint. Another important non-surgical approach is the use of splints. These methods are designed to reduce the mechanical stress due to the contact between the median nerve and the surrounding tissues of the carpal tunnel. The possible mechanism of splinting and gliding exercises is edema reduction (Schmid et al., 2012). The use of a splint for 8 weeks combined with a formal education program improved hand function and reduced symptom severity compared with no intervention (Hall et al., 2013). However, a meta-analysis has shown that sufficient evidence does not yet exist to confirm the clinical usefulness of splints, its efficacy in comparison with other treatments or the efficacy of nocturnal splints (Page et al., 2012). A combination of splints and lumbrical stretches was shown to be more effective than splints and general stretches in terms of symptom severity and functional score, but further studies of this combination are needed (Baker et al., 2012). Although the use of stretches and splints might temporarily improve muscle deficits, residual strength impairments can remain 4 weeks later (Baker et al., 2013). The Madenci massage technique (30–60 s cycles of effleurage, friction, petrissage, shaking, and repeated effleurage) has been shown to be more effective than use of splints alone in a single study (Madenci et al., 2012). However, little evidence exists about the effectiveness of exercise and mobilization of carpal tunnel structures (Page et al., 2012). A combination of tendon gliding exercises and splint and paraffin therapy might be better than conventional treatments alone or in combination with median nerve gliding exercises (Hornig et al., 2011).

Other non-surgical treatments have been assessed in patients with CTS. Linseed oil might provide mild or moderate improvement in symptoms severity and functional scores and median nerve conduction velocity, as assessed in a randomised trial (Hashempur et al., 2014). Acupuncture was not better than placebo for treatment of CTS, but it has been shown to improve electrophysiological measures and reduce symptom severity compared with prednisolone (Yao et al., 2012; Yang et al., 2011). The effect of acupuncture was assessed in a systematic review that showed encouraging, but not convincing results (Sim et al., 2011). Eremostachys laciniata ointment, an extract of a plant in the Lamiaceae family used in Persian traditional medicine as an anti-inflammatory and analgesic was compared with placebo ointment. However, splints were used in both groups. Treatment with E laciniata ointment resulted in improvements in pain perception and palmar prehension. Further studies are needed to confirm these findings and to understand long-term effects of this treatment (Eftekharsadat et al., 2011). Interferential current therapy (i.e., a low frequency electrical nerve stimulation) resulted in function improvement, symptom severity and electrophysiological measures compared with TENS and splint use (Koca et al., 2014). Local microwave hyperthermia was more effective than sham therapy at providing short-term improvements in pain and function in patients with carpal tunnel syndrome (Frasca et al., 2011).

2.2. Perineural injection therapy with 5% dextrose

Wu et al. (2017) used a single ultrasound-guided perineural injection therapy (PIT) with 5 mL dextrose water (D5W) for treating mild-to-moderate CTS in a randomized, double-blind study. They located the injection site at the proximal inlet of the carpal tunnel via short-axis ulnar approach to simultaneously hydro dissect the subsynovial connective tissue (SSCT) and flexor retinaculum from the median nerve. The results showed significant improvement in symptoms, results of the electrophysiological study and CSA of the nerve persisting for at least 6 months compared to normal saline injection. Moreover, they also ratified PIT with 5 mL D5W being superior to corticosteroid injection (3 mL triamcinolone (10 mg/mL) mixed with 2 mL normal saline) at four to six months post injection to reduce symptoms and disability (Wu et al., 2018). Likewise, they retrospectively found that body height and sensory nerve conduction velocity of the median nerve were risk factors for poor outcomes after PIT with D5W in patients with mild-to-moderate CTS. Moreover, the sensory nerve conduction velocity of the median nerve was found to be an independent prognostic factor of poor outcome (Ho et al., 2021). Nevertheless, the electrophysiological study presented a limited diagnosis of CTS with varied sensitivity (56% to 85%) and specificity (94% to 96%) (Witt et al., 2004; Jablecki et

al., 2002). For example, Martin-Gruber anastomoses may lead to misinterpretation or erroneous results during routine nerve conduction studies in patients with CTS (Di Stefano et al., 2021). Hence, underestimation of CTS severity may be attributed to the failure in proficient diagnosis. This would also be reflected as conflicting results in clinical trials on CTS. Recently, Lin et al. (2020) designed a randomized, double-blinded, three-arm trial and ultrasound-guided PIT with D5W via short-axis radial approach to simultaneously dissect the median nerve from the SSCT and flexor retinaculum. The results showed that the 4 mL D5W group had superior efficacy to 1 and 2 mL D5W in symptom relief and functional improvement at the 1, 4 and 12 weeks post injection. Moreover, their extended study revealed that PIT with a higher volume of D5W also enhanced nerve mobility and reduced the CSA of the median nerve (Lin et al., 2021). The long-term effect of PIT with D5W on CTS was also satisfactory and safe, based on the latest study. Li et al. (2021) retrospectively traced 185 patients with all grades of CTS who underwent ultrasound-guided PIT with 10 mL D5W using an initial short-axis injection with subsequent long-axis injection with a follow-up period of at least 1 year after the last injection (mean 1–3 years follow-up). The results revealed that 89% of the patients showed an effective outcome (symptom relief > 50% compared to baseline), while 63% of patients showed an excellent outcome (symptom relief > 70% compared to baseline) after a mean of 2.2 injections, and there were no complications in any of the patients. Moreover, only two patients ultimately underwent surgery after the failure of injection therapy to cure the condition. In addition, 80% of the patients (12 of 15 patients) had a surgical failure or post-surgery recurrence and had an effective outcome. Additionally, the outcome is considerably related to severity grade because the severe grade is associated with poor outcome compared to mild-to-moderate grade. A mean of 1.7, 2.4, and 2.6 injections was required to achieve an effective outcome in mild, moderate, and severe CTS, respectively (Li et al., 2021).

3. Surgical treatment

Surgical decompression can be done by a traditional open technique (long longitudinal wrist incision and direct visualization of transverse carpal ligament), a minimally invasive approach (short wrist incision) or endoscopic technique. Studies have shown no significant difference between open and endoscopic release (Atroshi et al., 2015; Sayegh and Strauch, 2015; Michelotti et al., 2014; Vasiliadis et al., 2014; Chen et al., 2014; Larsen et al., 2013; Aslani et al., 2012). However, endoscopic technique shows a shorter postoperative recovery period, reduced scar tenderness and allows earlier return to work than the open technique (Atroshi et al., 2015; Sayegh and Strauch, 2015; Michelotti et al., 2014; Vasiliadis et al., 2014; Chen et al., 2014; Larsen et al., 2013; Aslani et al., 2012). However, endoscopic release is more expensive (Sabesan et al., 2012) and is associated with higher rates of transient and nerve damage (Sayegh and Strauch, 2015). Findings from a metaanalysis (Vasiliadis et al., 2014) suggest that endoscopic release is associated with fewer minor complications (such as scar pain and infection) compared with open carpal tunnel release, with similar rates of major complications (mainly complex regional pain syndrome) (Vasiliadis et al., 2014). Differences in outcome might be dependent on the expertise of the performing surgeon (Sabesan et al., 2012). In one study (Michelotti et al., 2014) the two techniques were compared in patients with bilateral CTS; each patient received both techniques, one in each hand. Although there were no differences between techniques in terms of functional outcomes, patients preferred the endoscopic approach, as shown by significantly higher overall satisfaction scores. Different open carpal tunnel release techniques are available and have shown similar effectiveness (Crnković et al., 2014). Z-type lengthening of the transverse carpal ligament results in significant improvement in function and satisfaction score compared with a standard open technique (Xu et al., 2011). Minimally invasive techniques, compared with the standard open approach seem to provide better outcomes (fewer complications, higher patient satisfaction, improvement in symptoms, results of Tinel's, Phalen's, and compression tests, electrodiagnostic assessment, grasp strength assessment, and time to recover ability to perform personal tasks) (Aslani et al., 2012; Elsharif et al., 2014; Taralo et al., 2014). A meta-analysis (Sanati et al., 2011) showed that the minimally invasive approach versus open carpal tunnel release allowed an early return to work. Further investigations are needed to recommend the best approach. Surgical complications of carpal tunnel release are reported to occur in 1–25% of patients (Soltani et al., 2014). The incidence of serious complications consisting of structural damage to nerves, arteries, or tendons is no more than 0.5% (0.5% for open carpal tunnel release and 0.19% for endoscopic methods) (Benson et al., 2006). One potential severe complication of carpal tunnel operations is complex regional pain syndrome, which

presents as hand pain, increased sweating and vasomotor instability. Complex regional pain syndrome complicates recovery, delays return to work, causes deterioration of health-related quality of life and increases the probability of poor outcomes and litigation. Its incidence after carpal tunnel release varies from 2.1% to 5%. Early diagnosis and treatment of complex regional pain syndrome is essential for optimizing patient outcomes. Other complications of carpal tunnel release are scar tenderness, pillar pain (tenderness close to the ligament release), transient neuropraxia, and reoperation (with little difference between open and endoscopic carpal tunnel release) (Sayegh et al., 2015). Regarding the postsurgical phase, there is limited low quality evidence for the benefit of postoperative rehabilitation interventions (Peters et al., 2016).

As previously described, the literature shows that both non-surgical therapies and surgical intervention have clinical benefit in CTS. In a randomised trial comparing local corticosteroid injection with surgical decompression, both treatments were similarly effective at alleviating symptoms, with corticosteroids being more effective in short-term follow up (3 months), and surgical release having additional benefit for symptom resolution in the long term (2-year follow-up) (Ly-Pen et al., 2012). There is evidence that clinical outcomes significantly improve after decompressive surgery and several non-surgical treatments (e.g., splints and low-level laser therapy), but decompressive surgery shows a higher long-term effectiveness (Shi Q et al., 2011). Surgery is also more effective than non-surgical treatment at improving electrophysiological measures (Andreu et al., 2014). Furthermore, a systematic review reported in 2011 that surgical carpal tunnel release is two times more likely to result in normal nerve conduction findings and resolution of symptoms after 6 and 12 months than non-surgical treatment (Shi Q et al., 2011).

3.1 US guided minimally invasive carpal tunnel release

Technological advances have led to the use of US guidance to improve CTS therapy. US determination of the relative position of the flexor retinaculum (FR) with the neural and vascular structures in relation to the bony landmarks correlates well with the actual anatomy (Chern et al., 2009). The location of a “safe-zone” between the median nerve and the ulnar artery for the transection has been clearly demonstrated (Chern et al., 2009). Echographic localization of the target structures facilitates safe and efficacious resection of the FR. Recent anatomical and clinical studies suggest that complete release of the nerve is possible by transection of only the deep fibers of the FR without cutting the superficial fibers (Stecco et al., 2010). The latter only amounts to a reinforcement that is well differentiable histologically. This superficial layer is more richly innervated, and sparing it by not transecting it -as a result of the use of US surgery should allow local postoperative pain to be avoided. Nakamichi (1997) proposed making use of US guidance during the conventional surgical intervention (Nakamichi and Tachibana, 1997). Over time, several teams were able to show that it is possible to perform the whole procedure with ultrasound guidance only (Osterman et al., 2012; Jain et al., 2014; Sayegh and Strauch, 2015; Nakamichi and Tachibana, 1997; Capa-Grasa et al., 2014; Rojo-Manaute et al., 2016). The foremost justification for the development of this new technique is its minimally invasive nature. An open surgery or limited approach requires an opening of more than 4-5 cm, mini-open surgery involves an incision of 2 cm, and endoscopic treatment employs an opening of 1 to 2 cm. US surgery allows for an incision that is up to ten times smaller, ranging from 0.1 to 0.5 cm. Comparison of surgical procedures in terms of the size of the approach route indicates that the smaller the incision the shorter the return to work time. Not surprisingly, the esthetic and functional consequences of the surgical scar are also more limited with a smaller incision. Indeed, Jugovac et al. (2002) observed that compared to a conventional open approach, a mini-open incision decreased the return to work time by half (i.e. 15 days versus 30 days). US surgery therefore appears to combine a decrease in size of the incision with an excellent surgical field of view by continuous image-based guidance.

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Review

Use of Insoles to Enhance Postural Control

Valenti F^{1,*}^{1.} University of Ljubljana, Ljubljana, Slovenija^{2.} Biotechnical Faculty, Ljubljana* Correspondence: Fabio Valenti; biofa76@gmail.com**Abstract:**

Postural control is a complex process involving sensory inputs from visual, vestibular, proprioceptive and tactile receptors, processed by the central nervous system (CNS). Sensory information provided by muscle and cutaneous afferents in the foot optimize the ability to stand upright and control the postural sway. The foot, as a direct and often only interface between the body and the ground, constitutes an essential functional whole participating in mechanisms of postural control and regulation, allowing the body to sense and interact with the surrounding environment. Among many somatosensory stimulations designed to improve balance, wearing shoe insoles presents one of the easiest and most cost-effective ways. This method can be used both amongst elderly population for fall prevention and amongst athletes to reach better performance and furthermore prevent injuries. With the growing interest in insole use, several prototypes have been developed to monitor movement during day to day use. For therapeutic purposes, the type of insoles used in the studies was often not clarified, and the term insole was used as a general term. The proposed theme of the discussion is to review already existing data on insole use for treatment of postural balance.

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Keywords: Postural control; Postural sway balance; Insoles; Foot stimulation



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1. Postural control

Postural control is a complex process involving sensory inputs from visual, vestibular, proprioceptive and tactile receptors, processed by the central nervous system (CNS). Afferent information received from these sources allows for an internal representation of the body's position and location in space. Postural control may be defined as the act of achieving, maintaining or restoring a state of balance during posture or activity (Pollock et al., 2000). It comprises of a combination of active mechanical control, characterized by a neural regulation of skeletal muscles responsible for sway detection and postural correction; and passive mechanical control, which refers to the stiffness and kinematic properties of the joints, as well as the effect exerted on them by gravity (Bauby and Kuo, 2000). When balance is threatened by either an external or internal perturbation, CNS initiates appropriate muscular response (Samuel et al., 2015). This is accomplished by comparing the collated afferent information to the internal model (expected state), thus eliciting a motor response, which maintains the center of mass within the limits of stability (Mancini and Horak, 2010). Due to a high center of mass over a relatively small base of support, maintaining an upright balance can be difficult for humans. Even while standing statically (often termed quiet standing), the body produces small amounts of sway (Peterka, 2002). These postural movements are an indicator of displacement and motor corrections to maintain the body segments in equilibrium. Consequently postural control requires constant conscious and subconscious regulation of postural sway to manage balance and achieve postural stability (Pruszynski and Johansson, 2014). These movements of regulation can be performed particularly by cutaneous plantar informations arising from the feet. (Inglis et al., 2002).

2. Cutaneous plantar feedback

The foot constitutes a functional whole that participates in the mechanisms of postural control and regulation. As a direct and often only interface between the body and the ground it allows the body to sense and interact with the surrounding environment (Kavounoudias et al., 1999). Sensory information provided by muscle and cutaneous afferents in the foot contribute to optimize the ability to stand upright and control the postural sway to detect both position and motion of the body in space. This ability results from coordination between the somatosensory afferent system and the motor system, which controls muscular activity and pressure exerted by the feet. Somatosensory feedback arises from a variety of sources, most notably the cutaneous mechanoreceptors in the skin and muscle spindle endings found within most of the skeletal muscles, together providing tactile and proprioceptive feedback. Plantar cutaneous afferents transmit spatial and temporal feedback concerning the pressure variations and skin stretch exerted on the soles of the feet (Vallbo and Johansson, 1984). This cutaneous feedback is provided by four classes of low threshold mechanoreceptors (Abraira and Ginty, 2013). They are highly sensitive to forces applied to the sole of the foot and demonstrate adaptive and receptive ground properties (Priplata et al., 2003). They are activated by moderate mechanical stimuli and encode reversible deformations of the skin induced by pressure, vibration or stretch (Zehr et al., 2001). Fast adapting type I (Meissner) and type II (Pacinian) afferent fibers are sensitive to dynamic stimuli; their activation evokes perception of fluttering and vibration. In contrast, slowly-adapting type I (Merkel discs) and type II (Ruffini endings) afferents respond to stretching of the skin. Their activation allows perception of pressure and movement of the skin (Macefield et al., 2009). The distribution, density and variety of cutaneous receptors indicates that the role of the feet is not only important for supporting the body, distributing plantar pressure and absorbing impacts from the ground but it also plays a vital role in controlling postural adjustments for maintaining an erect standing posture (Kavounoudias et al., 1999). It has in fact been shown that reducing information from receptors located in the skin through cooling foot sole, anesthesia, ischemia conditions and/or eliminating sensory information is associated with an increase in postural sway under perturbed postural responses (Palazzo et al., 2021). Controversially, stimulation of the foot sole, through the modulation of load of lower limbs and the positioning of feet, could lead to an improvement of balance (Zehr et al., 2014).

3. Somatosensory stimulations to improve postural control

Among various available interventions, one of the easiest and most cost-effective ways to solve problems foot disorders is wearing insoles (Iglesias et al., 2012). Insoles are commonly used to treat foot disorders. The use of foot orthotics is very old and insoles have (historically) been used either for

comfort or correction of deformities due to the feet characteristics of each individual, or to relieve pain caused by daily or sports activities (Laperiere et al., 2006; Sobel et al., 2001). Presently, prescription of these orthotics is no longer limited to correction of flat feet as it has been in the beginning. available literature describes the use of insoles in the elderly for fall prevention, the use of functional and proprioceptive insoles in patients with neurological disorders, and postural insoles for postural correction. In addition, insoles are often prescribed for patients with diabetic neuropathies or to athletes to either achieve better performances or prevent injuries (Elraiayah et al., 2016). With the growing interest in daily activity monitoring, several insole designs have been developed to identify postures, detect activities and count steps. However, the validity of these devices is not clearly established (Ngueleu et al., 2019). In recent years, studies have demonstrated that by using various stimulation methods, additional somatosensory input has positive effects on postural control. These findings confirm that mechanical stimulation of the plantar sole has an impact on the mechanoreceptors of the feet, which helps in upright standing. Nevertheless, the effect of mechanical stimulation on postural control by using insoles still remains unclear (Viseux et al., 2019). with a rapidly growing body of evidence in research literature defining the most effective insole type intervention is becoming more and more challenging. Insole types can be categorized as electrical and non electrical devices or also as (a) orthopedic, (b) vibrating, and (c) textured (Viseux et al., 2019).

3.1 Non electrical stimulation

Sensory feedback from the foot can be facilitated by non-electrical stimulation of plantar mechanoreceptors by using orthopedic devices, skin indentation, textured or different thickness material surfaces and by focal activation with additional thickness. Orthopedic insoles with arch supports, lateral wedge, metatarsal pads, and heel cups are conventionally prescribed to correct/compensate foot deformity and relieve pain (Watanabe and Okubo, 1981). They change the contact area between the foot and support surfaces and redistribute plantar loading. Such designs may provide supplemental information to users about the relative position of the foot on the ground during walking (Perry et al., 2008). In population with significant foot deformation, it may be difficult to wear a conventional shoe and customized insoles may be required. The skin stimulation by indentation produces delicate pressure changes under the feet, inducing a sensation of changing pressure under the feet with no sensation of body position change. an observed barely present postural response represents a protection response reducing pressure to the foot when the skin indentation increases. consequently, information deriving from cutaneous receptors of the foot could provide information about properties of the support surface and its contact with the foot (Takata et al., 2016). Textured insoles are another type of insole that often vary in shape and structure. they can be small and flexible or semi-rigid with different density shaped pyramids or spheric knots. The stiffness and textures of insoles influence mechanical stimulation of the plantar foot and affect postural stability. thus, adding various textures to insoles has been demonstrated to increase sensory afferent feedback via enhanced tactile stimulation of plantar cutaneous mechanoreceptors (Hatton et al., 2012). Specific focal sole stimulation, for example, showed that adding additional thickness (3 mm) coin-shaped piece of aluminum placed under the sole induced an increase in activation of the plantar flexor musculature and consequently confirmed predictable postural reactions (Kavonoundias et al., 2009; Hijamans et al., 2007).

3.2 Electrical stimulation

Stimulation of the plantar surface of the foot can be achieved by incorporating vibration devices. Stimulation of cutaneous receptors increases afferent feedback and may, therefore, decrease reaction time. Ideally, the CoP moves through plantar surface stimulating high sensitivity cutaneous receptors without excessive pressure, causing postural corrections to occur faster when balance is lost. (Wei et al., 2012). Application of vibration insoles is commonly used to activate low-threshold cutaneous afferents receptors. The subsensory noise signal amplifies tactile input, such as change in pressure distribution under the sole of the foot, resulting in earlier detection of pressure changes (Wang and Yang, 2001). In this field researchers have used different types of vibration inputs, at 0%, 85% and 90% of plantar sensory threshold values and have tested both static and dynamic balance (Wang and Yang, 2001). Collins et al., (1996) postulate that delivering subsensory noise (white noise) through vibratory factors (piezo-electric actuators) placed underneath the forefoot and heel regions, lead to earlier detection of changes in foot pressure and, therefore, earlier corrective postural responses. If

is not known yet, whether less precise noise levels between 70 and 90% of the sensory threshold would yield similar results, or whether the threshold changes throughout the day require repeated modifications of different noise setting amplitudes to remain effective during prolonged use (Wang and Yang, 2001). The phenomenon of Stochastic Resonance (SR) has been observed in a variety of physiological systems. It is also present in our body's somatosensory system where the presence of a sub-sensory threshold noise could enhance the system's response to weaker signals (Santhiranayagam et al., 2015). Imperceptible (subsensory) vibratory noise applied to the feet can improve balance especially in elderly subjects and patients with neuropathic diseases rather than in healthy young people. Mostly it depends of different foot sensory perception conditions. Another technological advancement is the ongoing development of digital gait analysis, which focuses on using body mounted inertial measurement units (IMU). These sensors mounted in the insole are able to measure angular velocity, linear acceleration, foot pressure etc. using Bluetooth, this data can be transmitted to any Android device, creating the potential for online data management, including mass data storage and automatic recording of personal gait data. Due to complex noise filtering, one of the most prominent difficulties in utilizing IMUs for gait monitoring remains deriving position-time data (Santhiranayagam et al., 2015).

4. Conclusions

The relationship between improvements of balance and facilitation of sensory feedback related to the activation of plantar cutaneous mechanoreceptors using insoles, due to a variety of therapeutic benefits, provides an interesting perspective for clinical application. In the studies reviewed, the type of insoles used as a therapeutic resource is often not clarified and a general term "insole" is used to describe insoles that can vary in type of material, form of evaluation or the form of customization used. The most commonly cited terms include "customized insoles", "prefabricated insoles" or "sham insoles.", resulting in magnetic insoles, vibrating insoles and all other forms of insoles all receiving the same interpretation, causing great confusion and doubts amongst clinicians and researchers (Mendes et al., 2020). Nevertheless, the future research goals are to investigate the effects of insole use step by step. The goal is to find and standardize the approach to proper evaluation of the insoles effects on specific tested populations. The importance of this lies in foot orthoses having both positive and negative effects on the detection of tactile input from the bottom of the foot. Soft soles can distribute pressure over the soles of the foot, which has a positive effect on pain, but it may also result in a deterioration of the detection of pressure changes, which can negatively affect balance. In contrast, firm inlays and inlays with tubing at the plantar surface boundaries may improve balance. Therefore an optimal insole design, texture geometry, material composition, new generation sensors and proper stimulation implemented can lead to the optimal effects of mechanoreceptors in isolated foot regions. All previous mentioned factors have to be considered for future footwear intervention designs.

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188 0722

Scientific contribution/Original research

Electrochemotherapy with a Reduced Dose of Bleomycin in the Treatment of Advanced Skin Cancer. A Case Study

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Abstract:

Squamous cutaneous skin cancer is common cancer with increasing incidence. Commonly is treated with surgery or radiotherapy, but a new technique has been emerging in recent years. Electrochemotherapy (ECT) is innovative and still developing treatment of malignant tumors, especially effective in treating cutaneous malignancy. Its feasibility and efficacy have been proven in elderly patients with comorbidities who cannot undergo standard treatments. Some recent studies have focused on reducing bleomycin dose in elderly patients to minimize side effects and concurrently obtain the same treatment outcome. This article presents a case study of an elder patient with advanced skin cancer, treated with electrochemotherapy with a reduced bleomycin dose, where a complete response was observed in a long-term follow-up period.

Keywords: Electrochemotherapy; Bleomycin; Reduced dose; Squamous cell carcinoma; Elderly patient

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1. Introduction

Besides basal cell carcinoma, cutaneous squamous cell carcinoma is the second most common non-melanoma skin cancer with increasing worldwide incidence rates (Muzic et al., 2017). The risk factors are UV exposure, skin type and immunosuppression (Schmitt et al., 2011). Most common cutaneous squamous cell carcinoma arise in the face region and are clinically presented as a form of a red scaly plaque with different degrees of infiltration (Waldman & Schmults, 2019).

Cutaneous squamous cell carcinoma rarely form metastases in the regional lymph nodes, but the metastases are associated with a poor prognosis. There is a higher risk for metastasis and local recurrence in tumors greater than 20mm and depth of invasion (DOI) more than 2 mm. Poor prognostic factors also include extensive perineural involvement and poor tumor differentiation (Burton et al., 2016).

Standard treatment for most patients is complete excision of the carcinoma, especially when a tumor is small and in a favorable location. Radiotherapy is an alternative treatment in circumstances where surgery could result in significant esthetic or functional deficit (nose, eyelids, lips, ear), in elderly patients with comorbidities and in patients reluctant to surgery (Burton et al., 2016). In recent years ECT is emerging as a novel, local ablative treatment with comparable results to other modalities (Clover et al., 2020). In the presented case study, we address the role of ECT in the treatment of elderly patients with advanced skin cancers.

2. Patients and Methods

2.1. Patient characteristics

The ninety-year-old patient with advanced dementia and severe comorbidities (severe heart failure, diabetes and chronic renal failure) was admitted to the Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre Ljubljana due to protruding mass from his left nostril. Two years before, an excision of cutaneous squamous cell carcinoma from the scalp region was performed. An ENT clinical exam revealed a solitary suspicious mass of size around 30 mm, arising from the left side of the nasal columella (**Figure 1**). Biopsy was taken from the mass and histopathological findings confirmed squamous cell carcinoma metastasis.

A detailed diagnostic workup, including CT scans, did not detect any other regional or distant metastases. The patient was presented on the multidisciplinary head and neck tumor board and the indication for ECT treatment was agreed upon by all members.



Figure 1. Two views on the patient with metastasis of squamous cell carcinoma to the nasal columella.

2.2. Electrochemotherapy procedure

The electrochemotherapy procedure was performed according to the recommendations of Update Standard Operating Procedure for electrochemotherapy under sedation anesthesia (Gehl et al., 2018). The reduced dose 18000 IU of bleomycin (BLM) (10 000 IU/m², Heinrich Mack Nachf. GmbH & CO. KG, Illertissen, Germany) was intravenously administered in bolus. After 8 minutes, metastasis was treated with linear electrodes. 5 electric pulses, each in duration of 100 μ s, were delivered by electric pulse generator Cliniporator Vitae® (IGEA, Carpi, Italy) (**Figure 2**). The procedure was finished 13 minutes after BLM administration.

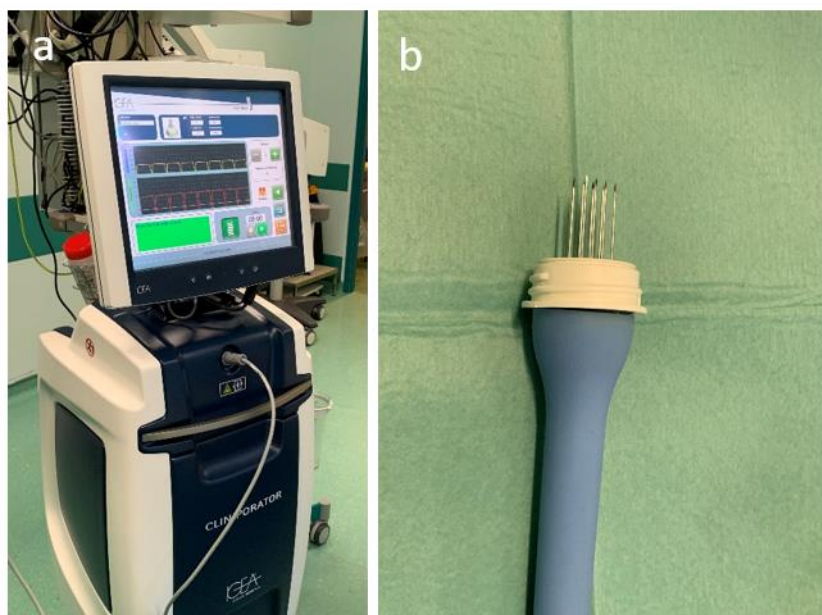


Figure 2. Electric pulse generator (a) and linear electrode (b) used in the treatment

3. Results

During the procedure and recovery time, we did not record any complications that would need additional medical interventions. Metamizole was used for the prevention of pain immediately after the treatment. The patient was discharged from the hospital the next day.

In the first follow-up visit two weeks after ECT, mild inflammation and ulceration were observed (grade I to II according to CTCAE ver. 4.0) (Chen et al., 2012). In the following weeks the wound healed spontaneously without any surgical intervention. After two months, the complete response was obtained and persisted more than one year after ECT (**Figure 3**).



Figure 3. Patient one year after treatment with electrochemotherapy.

4. Discussion

ECT is a local ablative tumor treatment with proven antitumor efficacy in preclinical and clinical studies (Bertino et al., 2016; Sersa et al., 1997). It has already been spread across more than 170 centers in Europe (Clover et al., 2020). In ECT, the electric field is used to increase membrane permeability and thus enhance the uptake of cytotoxic drugs into the tumor cells. Besides the direct antitumor effect, ECT also impacts tumor vascularization and provokes a local immune response, which both potentiate the effectiveness of treatment (Yarmush et al., 2014). Currently, two cytostatics are used in ECT – BLM and cisplatin (Gehl et al., 2018). Preclinical studies demonstrated that electroporation increases the effectiveness of BLM several 1000 times and of cisplatin up to 70 times (Miklavčič et al., 2014; Sersa et al., 2008).

The patient in our study had an advanced stage of cancer disease and several comorbidities. Consequently, surgery in general anesthesia could significantly hamper a patient's underlying diseases. Excision in local anesthesia was not possible due to the size and location of the tumor. Radiotherapy as an alternative treatment was not suitable because of the patient's lack of ability to keep still for the required time during irradiation. ECT was chosen as the only curative treatment option for the particular patient. According to our case study, we believe that ECT is not suitable only as a primary treatment for skin tumors (Clover et al., 2020), but it exceeds in use to other cases in which the standard modalities cannot be used due to different causes.

Another important conclusion of our study is that ECT can be safely performed in sedation. Since the whole procedure is done in a few minutes, the sedation is light and short; thus, the recovery time is faster. Our patient did not have any side effects during and after sedation, and he was discharged the following day.

ECT has a response rate between 60-80 %, comparable to other skin ablative techniques (Clover et al., 2020). Tumors with the highest responses are basal cell carcinoma. In addition to histological types, tumors differ in responses regarding tumor size (larger tumors are less responsive), and previous treatments, which decrease the rate of complete responses (Bertino et al., 2016; Clover et al., 2020; Marty et al., 2006). Despite the advanced stage of the cancer disease, a reduced dose (10.000 IU/m²) of BLM was used in our study. The decision to use the reduced BLM dose was based on the study of the pharmacokinetics of bleomycin in elderly cancer patients. This study demonstrated that a decline in lean body mass, total water content, and impaired renal function lead to decreased BLM elimination. Consequently, a prolonged higher serum concentration of BLM might be obtained when using a standard (15.000 IU/m²) BLM dose (Groselj et al., 2016). To the best of our knowledge, only a few clinical studies till now have shown that ECT with a reduced dose of BLM is equally efficient as ECT with a standard dose (Groselj et al., 2016; Jamsek et al., 2020; Rotunno et al., 2018).

In our case, two months after ECT, the complete response was recorded and persisted in the last follow-up visit more than a year after the treatment. Recently, Jamsek et al. (2020) published long-term results comparing reduced and standard BLM doses in ECT treatment, and they did not observe any statistically significant difference in long-term tumor control between both groups. It should be noted that tumors in their study were primary skin cancers; most of them were basal cell carcinomas with a median tumor diameter of 21mm. Our patients had unfavorable prognostic factors (regional metastasis and size more than 30 mm) that might worsen the probability of a complete response.

It is also important to emphasize that the healing process was favorable, with minor side effects that did not need additional wound dressing. The wound was healed in less than two months without any functional deficits and an excellent final cosmetic outcome. This is in concordance with the results of Groselj et al, who recorded a shorter healing time and better cosmetic results with a reduced dose of BLM in elderly patients (Strojan & Groselj et al., 2021).

5. Conclusions

ECT proved an efficient skin cancer treatment modality and was safely performed in sedation in old and comorbid patient. Specifically, in the elderly patient a reduced dose of BLM was efficient. More data is needed on optimal BLM dose. Our results warrant further investigations in the form of properly designed and well-conducted prospective clinical trials.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki.

Conflicts of Interest: The authors declare no conflict of interest.

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Invited lecture/Scientific contribution/Original research

Facial Nerve Reconstructive Surgery in Otorhinolaryngology and its Enhancement by Platelet- and Extracellular Vesicle-Rich Plasma Therapy

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Abstract: The facial nerve and its reconstructive surgical procedures are complex and challenging. The main function of facial nerve is namely motor innervation of facial muscles and its dysfunction presents as facial paralysis. Depending on the extent of facial nerve injury (neurapraxia, axonotmesis, neurotmesis) and consequently a physiological phenomenon of Wallerian degeneration, mechanism, location of the injury, time course of the paralysis and medical condition we decide about the type of the reconstructive surgery. Generally, possible surgical interventions to improve facial nerve functioning are mainly nerve decompression, neuroorrhaphy/end-to-end anastomosis, interposition (cable) grafts and nerve rerouting. Moreover, most commonly nerves undergoing facial reconstruction are great auricular and sural nerves. In addition, nerve rehabilitation can be improved by using platelet-rich plasma (PRP/PVRP), applied directly to nerve. There are many roles of PVRP, described in the literature such as neuroprotective, neurogenic, neuroinflammatory, angiogenic role and improving hemostasis. Also, its neoplastic and proliferative effects were not reported. Considering all these features implementing PVRP in the facial nerve regenerative treatment has strong potential in the future.

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1. Introduction

The facial nerve or seventh cranial nerve is special among twelve cranial nerves due to its predominant motor innervation, multiple functions, varied composition and long and variable pathway (Battelino, 2013; Seneviratne et al., 2022). The article aims to focus on facial nerve reconstructive procedures and options to reestablish nerve integrity. The outcome of surgical procedures can be improved by using platelet rich plasma in surgical field (Vozel et al., 2020). Therefore, the article also covers the therapeutic effect of PRP/PVRP on facial nerve through the clinical case.

2. Facial nerve segments, its fiber components and nerve injury types

The facial nerve emerges from the facial nerve nuclei in the brainstem, where the *intracranial/cisternal* pathway begins. After emerging the pons, nerve continues the *intrameatal/canalicular* pathway along the internal auditory canal (IAC) with the vestibulocochlear nerve to the fundus and leaves it through the meatal foramen. The bony canal of facial nerve from IAC to the stylomastoid foramen is called the Fallopian canal where the facial nerve is often compressed due to inflammations. There are three segments of the Fallopian canal, a labyrinthine, a horizontal tympanic and a vertical mastoid segment. At the stylomastoid foramen the facial nerve leaves the temporal bone and branches at the pes anserinus into temporal, zygomatic, buccal, marginal and cervical branch. All muscles of facial expression are innervated by general somatic efferent fibers of facial nerve as one of five fiber types (Seneviratne et al., 2022; Toulgoat et al., 2013; Cummings, 2005; Probst et al., 2006). Facial nerve also receives taste sensations from anterior two-thirds of the ipsilateral side of the tongue and the palatal mucosa from afferent gustatory fibers. Furthermore, it carries parasympathetic secretomotor fibers to the submandibular, sublingual salivary glands, to the lacrimal glands and to the glands in the oral cavity. Postauricular skin, auricular concha and wall of the external auditory canal are supplied by somatosensory afferent fibers and nasal and pharyngeal mucosa by visceral afferent fibers of the facial nerve (Battelino, 2013; Takezawa et al., 2018; Phillips et al., 2018).

In general, to understand the mechanisms of facial nerve injuries, it should be acknowledged that every nerve fiber consists of endoneurium, surrounding each nerve fiber and attaches to the Schwann cell layer. It is important for endoneurial tube regeneration and has poorer prognosis for regeneration when the layer is disrupted. Perineurium protects from spreading the infection and epineurium (nerve sheath) contains the vasa nervorum for nutrition (Pasha et al., 2014).

Depending on nerve injury cause (head trauma; parotid tumor compression; head and neck cancers; infectious diseases; peripheral nerve demyelinating lesions, etc.) we differentiate following types of nerve injury: Neuropraxia – presents compression of the nerve, causing loss of axoplasmic flow without axonal rupture, due to focal segmental demyelination. The conduction block is most often resolved with complete recovery. Axonotmesis represents damaged axon with intact endoneurium where Wallerian degeneration occurs distal to site of injury, but complete recovery is anticipated. In neurotmesis, neural tube is transected; myelin sheath, axon, endoneurium, perineurium and epineurium are disrupted. Without surgical treatment there is no possibility to restore nerve function (Seddon, 1943; Bhandari, 2019).

3. Facial nerve reconstructive surgery – general

Almost every disruption of the continuity of the facial nerve should be fixed regardless of the cause being traumatic, iatrogenic injury or tumor invasion (Cummings, 2005). Interventions are limited to restoring the function of the motor fibers of the facial nerve and not of the remaining four types of facial nerve fibers (Chu et al., 2008). We roughly distinguish three types of reconstructive procedures in case of facial paralysis: facial nerve procedures, procedures involving other tissues at the affected facial side and procedures at the opposite, healthy facial side (Battelino, 2013). A general rehabilitation for facial nerve repair covers: spontaneous facial nerve repairment after observation; facial nerve neurotomy (represents surgical suturing of divided nerve); facial nerve cable graft; nerve transposition; muscle transposition; microvascular transfer; static procedures (Cummings, 2005).

According to different facial nerve damage mechanisms and distinct involved pathways we distinguish between different reconstructive methods.

3.1. Facial nerve injury in temporal bone

In case of sudden facial paralysis, after imaging, we can make decompression of facial nerve which means relaxation of the facial nerve along the internal auditory canal and is possible in the first three extracranial segments (labyrinthine, tympanic and mastoid). The authors differentiate transmastoid (translabyrinthine) approach and middle fossa approach to decompress the nerve (Battelino, 2013; Cummings, 2005; Mehta, 2009). Retrolabyrinthine and retrosigmoid approaches can be used for intracranial segment of the facial nerve while preserving hearing (Cummings, 2005). The most demanding intervention of the facial nerve decompression is the area of the meatal foramen bony canal. Also transversely passing solid connective fibers compress the nerve (House et al., 1985; Fisch, 1974; Pulec, 1974; Darrouzet et al., 2001).

If facial nerve is transected, non-functioning facial nerve part should be removed and reconstructed by end-to-end anastomosis or by re-routing the nerve (Battelino, 2010) as described below.

3.2. Reconstruction of facial nerve in cerebellopontine angle (CPA)

Procedures in CPA with tumor that affects facial nerve functioning present a challenge. It is the goal of the surgery to ensure the stability of nerve stumps with sutures, thus using muscular fascia around them to achieve stability of necessary dendrites regrowth (Battelino et al., 2010). Approximation of the nerve ends using an acrylic glue has been described, and subsequent investigators have revealed that neural anastomosis with tissue adhesive yields results similar to nerve suture (Cummings, 2005; Ramos et al., 2015). Nevertheless, the literature reports about 21% to 35% failure rate of facial repair after reconstruction (Barfs et al., 1984; Pluchino et al., 1986). Using translabyrinthine approach accessed to the distal end of the facial nerve in the petrous bone, King et al. (1990) performed the nerve repair by end-to-end anastomosis. Also, nerve was repaired by a graft, using the sutured great auricular nerve or by making faciohypoglossal nerve anastomosis (King et al., 1990). Arriaga et al. (1992) described direct facial nerve neuroorrhaphy or anastomosis with a greater auricular nerve interposition graft as a successful repair option in cases after nerve transection in the CPA.

At the Department of Otorhinolaryngology and Cervicofacial Surgery professor Battelino and her team made end-to-end anastomosis of the facial nerve after middle fossa approach very close to pons where the lesion entered the IAC and destructed few millimeters of facial nerve. After removing the lesion, rerouting of the facial nerve and making facial nerve end-to-end anastomosis in IAC reconstructive procedures resulted in almost total facial expression symmetry at the rest.

3.3. Reconstruction in temporal segments

Reconstruction of transected facial nerve in facial bony canal presents three options: neuroorrhaphy, interposition grafts and rerouting of facial nerve. Neuroorrhaphy/ end-to-end anastomosis provides a tension-free anastomosis but is rarely used in practice (Mehta, 2009). End-to-side neuroorrhaphy is used as an alternative technique when the proximal stump of an injured nerve is unavailable or the nerve gap is too long to be bridged by a nerve graft (Lykissas, 2011). Interposition (cable) grafts are used when a tension-free primary nerve repair is not possible (Mehta, 2009). Option for graft source are: the great auricular, sural nerve, the medial lateral antebrachial cutaneous nerves or ansa cervicalis. Overall, motor nerve grafts were indicated to be better than sensory nerve grafts (Chu et al., 2008). Grafts allow reparation of the facial nerve with minimal nerve end handling, thus leading to lesser impact on nerve vascularization and preserving the anatomy of the middle ear. The disadvantages of grafting include the interposition of another nerve between the stumps of the facial nerve resulting in two junctions between the ends of the affected nerve and the graft, and loss or reduction at the donor site (Chu et al., 2008; Humprey et al., 2008). Thirdly, rerouting facial nerve approximates the stumps of the sectioned nerve. This is exclusively important for gaining nerve length. However, it is much more difficult to perform free of suturing (Battelino, 2013; Filho et al., 2013).

3.4. Reconstruction of injured facial nerve in extratemporal region

The main reconstructive procedure of facial nerve after leaving the temporal bone in stylomastoid foramen is primary neuroorrhaphy. It presents the best (but rare) possibility to regain the facial nerve function in case of tension-free closure with intact motor end plates. After the exit from the stylomastoid foramen, anastomosis with distally disrupted hypoglossal nerve can be made considering different modifications (Hadlock et al., 2005). Also, free nerve grafts can be connected to healthy facial nerve of the opposite side. In this case, nerve transplant is called “cross over” or “transposition nerve crossover”. Later on, it can be sutured with subsequent transplants of smaller muscle groups in place of atrophied facial muscles (House et al., 1985; Fisch, 1974; Stark, 1987; Bailey et al, 2001; Glasscock et al., 1979). In nerve transfer procedures a variety of donor nerves such as hypoglossal, spinal accessory, masseteric branch of the trigeminal nerve and motor branches of the cervical plexus can be used (Mehta, 2009).

4. Platelet- and extracellular vesicle-rich plasma (PVRP) therapy and its contribution to nerve repair

4.1. General

Blood plasma obtained in a specific procedure that yields a preparation rich with platelets is an autologous blood-derived product with immune, hemostatic and regenerative effects (Vozel et al., 2020; Wang et al., 2022; Brisson et al., 2017; Tao et al., 2017; Uršič et al., 2014). As it contains also extracellular vesicles, it is conveniently called platelet- and extracellular vesicle-rich plasma (PVRP) (Vozel et al., 2020; , Alves et al., 2018). PVRP is prepared according to a simple and low cost procedure and can be applied in different fields of medicine (Vozel et al., 2020). After fixing nerve injury, regeneration by itself is slow and unpredictable (Fowler et al., 2015). It depends on the state of the destructed surrounding soft tissue and its blood supply (Wang et al., 2022). The number of studies on PVRP application for treatment of peripheral nerve injury has increased in the past decade and most of the studies have revealed that patients with peripheral nerve injury receiving PVRP treatment have an accelerated recovery (Wang et al., 2022). According to animal studies many molecules, released from platelet derived extracellular vesicles (PDEV) in PRP and after platelet stimulation, are responsible for nerve and other tissue regeneration and responses (Wang et al., 2022; Guo et al., 2017; Koupenova et al., 2018).

4.2. PVRP preparation and composition

PVRP with or without buffy coat can be prepared from blood by centrifugation (Božič et al., 2021). After sedimentation of erythrocytes that form about 45% of the blood volume (hematocrit), the remaining 54% yellowish supernatant presents plasma. A thin whitish layer between the two (of about 1 % of the blood volume) is called “buffy coat” and consists mostly of leukocytes and platelets (Vozel et al., 2020).

PVRP preparation can be delivered to target tissue as a liquid or a gel. It was indicated that PVRP gel is favorable for Schwann cells (SCs) and can effectively promote nerve regeneration (Ye et al., 2012). SCs are responsible for nerve cell proliferation and formation of migration bridges to the nerve stump (Wang et al., 2022). PVRP contains various growth factors (for example platelet-derived growth factor - PDGF, transforming growth factor β -TGF β , vascular endothelial growth factor - VEGF, epidermal growth factor - EGF and insulin-like growth factor - IGF-1, special peptides, immune system messengers, enzyme inhibitors and other bioactive compounds (Arslan et al., 2022).

4.3. PVRP's potential of nerve repair and its enhancement

Nerve regeneration depends on lifetime, gender, patient's health condition, and associated diseases (Kuffler et al., 2020). Listed factors can be bypassed and the treatment improved with PVRP since it has neuroprotective, neurogenic and neuroinflammatory modulatory effects (Sánchez et al., 2018). Sánchez et al. (2018) described six levels of PVRP's potentials to promote nerve regeneration with biomolecules and different growth factors that act neuroprotectively: prevention of neuronal apoptosis; stimulation of vascular regeneration; promotion of axonal

regeneration; regulation of inflammatory response in the microenvironment; alleviation of nerve collateral muscle atrophy; improvement of human nervous system parameters (Sánchez et al., 2018). Moreover, Arslan et al. (2022) pointed to angiogenic role of PVRP and observed no neoplastic or proliferative effects of PVRP (Arslan et al., 2022).

Nerve injury closer to the cell body causes neuron death, therefore nerve fibers distally from the cell body start regenerating. The debris of nerve tissue is destroyed by macrophages recruited from peripheral blood or nerve tissue, which can provide a suitable environment for nerve regeneration. We know that released growth factors are mostly responsible for SCs proliferation, its migration and promotion of axon regeneration. Li et al. (2017) described PVRP's positive effects on facial nerve trauma by improving Schwann cell and axon recovery (Li et al., 2017). Moreover, platelet-derived growth factor-BB (PDGF-BB) and IGF-1 may be the main cytokines affecting SC proliferation and migration. Vascular endothelium growth factor (VEGF) as a powerful angiogenic factor, released by macrophages, that is specific to the vascular endothelium, was shown to have chemotaxis effects, to promote axonal growth and neuronal survival (Wang et al., 2022). Also, its neuroprotective role was indicated due to positive effects on motor neuron survival and reduction of ischemic conditions (Hillenbrand et al., 2015). Pelletier et al. (2015), found that application of VEGF gene therapy to nerve regeneration resulted in positive correlation between increased vascularization and enhanced nerve regeneration. Therefore, VEGF application may play a role in promoting the growth of degenerated nerve fibers through the combined effects of angiogenesis, neurotropy and neuroprotection (Pereira Lopes et al., 2011).

Protein 1 from Schwann cells promotes migration and recruitment of macrophages that manage regeneration. Injured axons release neuropeptides - substance P and calcitonin-gene-related peptide to cause neurovascular dilatation (Wang et al., 2022; Arslan et al., 2022).

Peripheral nerve injury repair involves various inflammatory cells, including macrophages which destroy debris of nerve tissue. Macrophages fall into two phenotypes-the classical activated macrophage M1 and selectively activated macrophage M2 (Delavary et al., 2011). In hypoxic condition, macrophages adapt to the microenvironment by secreting VEGF-A, which stimulates blood vessel formation in the direction of nerve regeneration. Schwann cells do not secrete high levels of macrophages M2, but act as inducers for promoting axonal outgrowth (Stratton et al., 2018).

In otorhinolaryngology, Ricci et al. (2019) have analyzed the efficiency of PVRP gel in superficial parotidectomy for benign tumors, applying it on the remaining parotid gland and the branches of the facial nerve. Their study revealed a positive effect of PVRP gel on clinical outcomes, postoperative observed facial palsies were reduced (Ricci et al., 2019). Scala et al. (2014) after using PVRP gel on patients who underwent superficial parotidectomy found a positive trend in the PVRP-group regarding facial nerve impairment (Scala et al., 2014).

4.4. Use of PVRP plasma in clinical practice regarding facial nerve

A 83-years old male patient with recidivant and advanced invasive squamous carcinoma of ear, external auditory canal and neck and parotid metastasis was planned for extensive surgical procedure to remove carcinoma (**Figure 1**). Because of endangered facial nerve it was controlled by neuromonitoring. After tumor excision and subtotal petrosectomy, facial nerve was preserved and PVRP gel was applied directly to the facial nerve. In our case and in general, PVRP gel application is important after relaxation of facial nerve from stylomastoid foramen as well as in case of compromised arterial supply, since PVRP induces neoangiogenesis. Immediately after the procedure, facial nerve was slightly affected due to tension of tissue removal manipulation, but at the later check-ups its facial motor skills were normalized.



Figure 1. Applying PVRP gel directly to facial nerve.

5. Conclusion

Facial nerve injury has strong negative impact on patient 's health and quality of life. Surgical interventions and management of facial nerve reconstructions are complex. Reconstructive and nerve repairing outcomes can be improved by using PVRP which has proved safe due to local applications and no observed side effects. We anticipate that wide using PVRP in nerve repair procedures will bring revolution in patients' nerve rehabilitation due to already indicated hemostatic, neuroprotective, neurogenic, neuroinflammatory and angiogenic effects and no record of neoplastic or proliferative effects.

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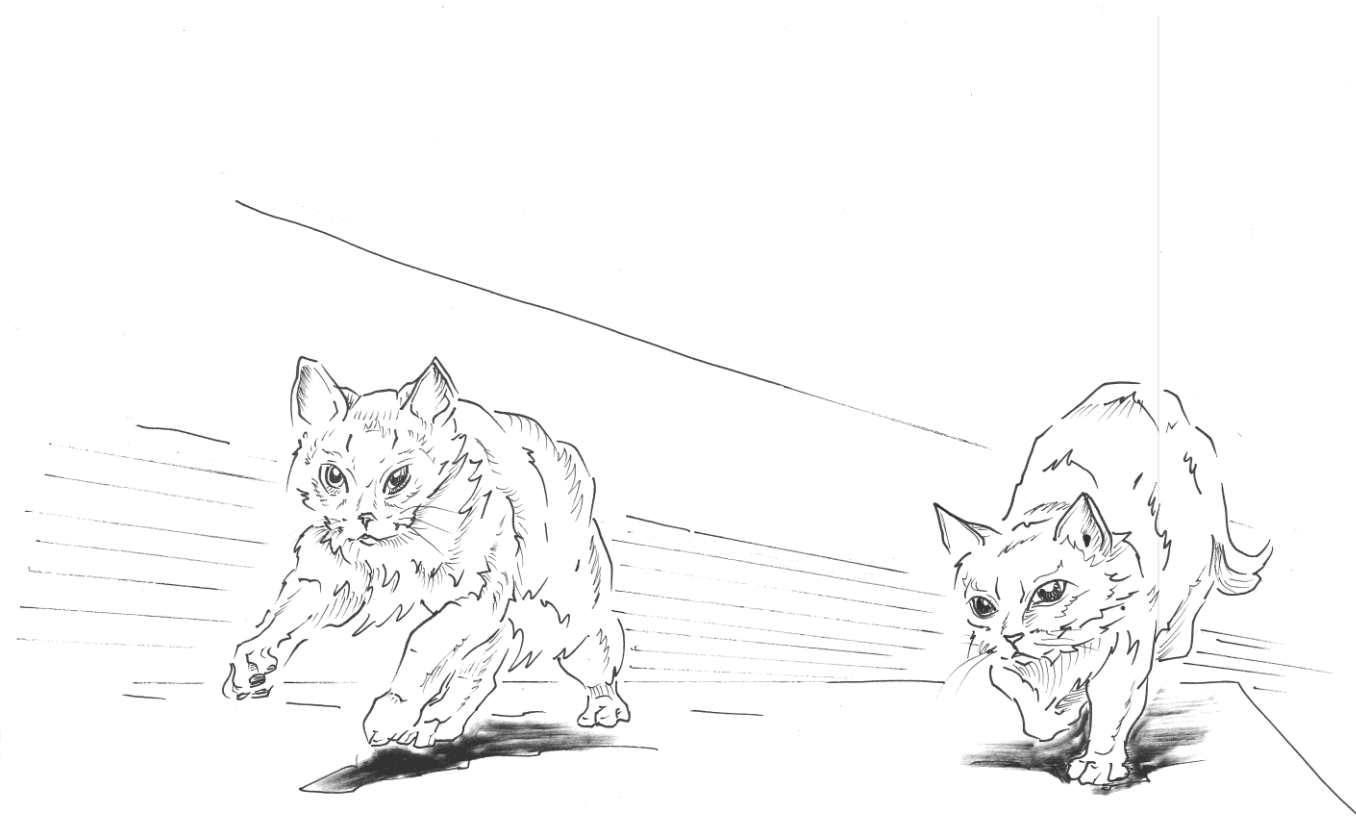
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Invited lecture/Scientific contribution/Original research

Surface-Based Total Blood Volume Calculation for Platelet and Extracellular Vesicle-Rich Plasma and Gel Preparation by Using a Mathematical Model

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Abstract: Platelet- and extracellular vesicle-rich plasma (PVRP) and platelet- and extracellular vesicle-rich gel (PVRG) are blood-derived products gaining attraction in regenerative medicine. However, despite their reported good efficacy, their preparation protocols are too time-consuming. Moreover, patient-tailored preparation protocols are desired to optimise platelet and extracellular vesicle (EV) count in PVRP and PVRG. This scientific contribution presents a clinical implementation of mathematical model for calculation of the desired total blood volume for PVRG preparation. PVRG was prepared according to previously derived mathematical model of blood cell and EV sedimentation during centrifugation based on the patient's erythrocyte sedimentation rate (ESR) (Božič et al., 2021). We estimated the volume of blood required for the treatment of the surface area of the individual patient, prepared the PVRG accordingly and applied it to the patient's wound. After six applications of 13 mL to 65 mL of PVRG, the osteoradionecrotic surface area decreased from 46 cm² to 18 cm² and infection was eradicated. The mathematical modelling of total blood volume needed to prepare PVRG proved useful to prepare the therapeutic amount of PVRG and also optimized time for the PVRG preparation protocol.

Keywords: Extracellular vesicles; Platelet-rich plasma; Regenerative medicine; Osteitis; Osteoradionecrosis; Osteomyelitis; Temporal bone

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1. Introduction

Significant concentrations of extracellular vesicles (EVs) have been measured and inspected in the autologous blood-derived platelet-rich liquid product, which was named platelet- and extracellular vesicle-rich plasma (PVRP) (Božič et al., 2021; Vozel et al., 2021). PVRP is defined a blood-derived product with concentrations of platelets and EVs higher than blood concentrations. Therefore, it presents an upgrade in autologous regenerative therapeutics (Vozel et al., 2020). PVRP, prepared by a two-step-plasma-based protocol, proved effective in treating chronic postoperative temporal bone cavity inflammation (Vozel et al., 2021). However, this time-consuming preparation protocol, which took on average 80 minutes, resulted in an approximately 10 % yield of PVRP from withdrawn blood (Vozel et al., 2021).

To overcome these obstacles, patient-tailored PVRP treatment has been proposed based on the mathematical model describing the movement of the cell constituents in the tubes during sedimentation in the centrifuge. Subjected to systemic centrifugal force, larger particles (i.e. erythrocytes) sediment faster and in such way to push smaller and less dense particles (platelets and EVs) in the opposite direction. As the latter accumulate above the erythrocyte boundary, they are enriched in plasma. However, after the erythrocyte boundary reaches the hematocrite level, platelets and EVs are sedimented from the plasma towards the bottom of the tube. It was indicated that the choice of optimal time for given centripetal acceleration of the centrifuge rotor is therefore key for preparation PVRP (Božič et al., 2021). Also, it should be taken into account that the concentration of platelets and EVs in PVRP strongly depend on the patient's erythrocyte sedimentation rate (ESR) (Božič D et al., 2021). Here, we used the model (Božič et al., 2021) to prepare PVRP according to two different protocols 1.) the "high platelet and EV" protocol aimed at creating a plasma with the highest possible platelet and EV concentration that can be obtained by a single spin preparation and EVs and 2.) a "half volume" protocol where we calculated the time needed for the upper border of erythrocytes to arrive down to one half of the length of the blood sample. We derived equations for exact calculations of blood volumes needed to treat the wound of the individual patient and provided a calculator to alleviate calculations (Steiner et al., 2022).

Then, PVRP has been successfully transformed to the semi-solid (i.e., gelatinous) state to create platelet- and extracellular vesicle-rich gel (PVRG) by the procedure previously described in a preclinical in-vitro pilot study (Vozel et al., 2020).

With the clinical implementation of described clinical protocols, we discovered that knowing the exact volume of blood needed to prepare PVRG proved beneficial. This scientific contribution shows the derivation of the novel equations for calculating the total volume of blood needed to prepare the desired volume of PVRG and reports on the effect of the applied preparation in a patient treated at the Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre Ljubljana, Ljubljana, Slovenia for osteoradionecrosis of the temporal bone.

2. Materials and Methods

2.1. The patient

A patient with osteoradionecrosis of the temporal bone was recruited in July 2021 at the Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre, Ljubljana, Slovenia, for the treatment with PVRG prepared by the novel patient-tailored ESR based preparation protocol. Patient signed written informed consent.

2.2. Autologous PVRG preparation protocol

2.2.1. Blood withdrawal prerequisites

Before blood withdrawal, a patient was ensured to meet the following criteria: the patient should not perform strenuous physical activity within 24 hours of treatment, the patient should be in a fasting

state at blood sampling (i.e., no food or beverages intake except water within 8 hours before treatment), the patient should not had high-fat dietary intake or alcohol consumption one day before the treatment.

2.2.2. Blood withdrawal procedure

The blood was withdrawn from a peripheral artery. Citrated 4.5 ml evacuated blood tubes (9 NC sodium citrate 0.105 M, BD Vacutainer, Becton Dickinson, San Jose, CA, United States) that were kept at room temperature were filled as indicated by the producer, to provide appropriate blood to anticoagulant ratio. Tubes were turned upside down 3-4 times immediately after sampling to achieve an adequate blood and sodium citrate mixture. The blood withdrawal volume was estimated as described by using mathematical model as described below. According to previous findings, blood yield from a citrated blood specimen yields is approximately 30-50 % of PVRP (e.g., from 10 ml of blood, about 3-5 ml of PVRP is produced in patients with normal ESR) (Božič et al., 2021). After withdrawal, blood was stored and transported to the centrifuge at room temperature (i.e., 22 °C) immediately taking into account the platelet concentrate storage guidelines (Božič et al., 2021).

2.2.3. Blood centrifugation

Before centrifugation, the blood tubes were again inverted 3-4 times to resuspend the samples. The blood was then centrifuged at the relative centrifugation force (i.e., RCF) of $300 \times g$ at 18 °C. The centrifugation time (t) was calculated individually from the patient's erythrocyte sedimentation rate (i.e., ESR) according to the mathematical model (Božič et al., 2021). The model estimates the volume of plasma and concentration of platelets and EVs as plasma forms during centrifugation. Following the possibilities considered in (Božič et al., 2021), we considered two options: The first option was the "high platelet and EV" protocol aimed at yielding plasma with the highest possible platelet and EV concentration that can be obtained by a single spin preparation without pelleting platelets and EVs for which the time $T_{\text{high platelet and EV}}$ is given by (Božič et al., 2021),

$$T_{\text{high platelet and EV}} = x_{\text{max}} \ln(x_{\text{max}}/x_{\text{min}})/(2\varepsilon \text{ ESR RCF}) \quad , \quad (1)$$

where x_{max} and x_{min} are the distances from the centrifuge rotor axis to the bottom and the level of the blood sample, respectively, ε is the adjustable constant, which was set to 0.13 (Božič et al., 2021) and RCF is the multiplicity of the gravitational constant that expresses the centrifugal force (in our case 300). The second option was the "half volume" protocol where we calculated the time needed for the upper border of erythrocytes to arrive down to one half of the length of the blood sample (Božič et al., 2021),

$$T_{\text{half volume}} = x_{\text{max}} \ln((x_{\text{max}} + x_{\text{min}})/2x_{\text{min}})/(\varepsilon \text{ ESR RCF}) \quad . \quad (2)$$

By inserting the respective times of centrifugation into the expression for the concentration of platelets and EVs, the calculated platelet- and EV - enrichment factor in PVRP is higher than 2 (in the case of high platelet and EV protocol) or equal to 2 (in the case of "half volume" protocol).

2.2.4. PVRP harvesting

After the centrifugation, blood was divided into two distinctive fractions: red, which contains mainly erythrocytes, and yellow, which is PVRP. Sometimes an opaque whitish layer (i.e., buffy coat) is present between layers. PVRP is gently and slowly aspirated with a pipette (ref.: 225-1S, Termofisher scientific, ZDA) above the red and white layer without disturbing them. PVRP extracts from all tubes were merged in another sterile plastic tube or container, and the total volume was measured. PVRP was then activated to form a PVRG immediately or as soon as possible after PVRP harvesting.

2.2.5. PVRP activation (i.e., PVRG formation)

PVRP, autologous serum (in the ratio of 1:5 to PVRP) and 1M CaCl₂ (in the ratio of 1:100 to PVRP) were mixed to transform PVRP to PVRG. Additional blood was withdrawn in a 4 ml plastic blood

tube without anticoagulant (e.g., Z Serum, Vacutube, LT Burnik, d.o.o., Slovenia) and stored at room temperature. This blood was used to prepare serum, i.e., activator, which transforms PVRP (i.e., liquid) into a gel (i.e., PVRG). After about 5-10 minutes, blood clot was formed within the tubes, and then the tubes were centrifuged for 10 min at 1260×g and 18°C. The supernatant above the red blood clot is serum, which was gently aspirated without mixing layers and transferred by a sterile pipette into a sterile polypropylene tube. The volume of autologous serum needed to prepare PVRG depends on the desired volume of PVRP that will be transformed to PVRG. A mixture in the ratio 1:5 with PVRP is needed (for activation of 5 ml of PVRP, 1 ml of serum is needed). The blood yield for serum harvesting was calculated from the haematocrit. For instance, in 35 % haematocrit, the serum presents 65 % of blood volume.

To start the PVRP activation process (i.e., transformation of PVRP to PVRG) 1M CaCl₂ (14.7%, Pharmacy of University Medical Centre Ljubljana, stored at room temperature) in the ratio 1: 100 with PVRP (i.e., 1%; e.g., 170 µL to activate 17 ml of PVRP) was applied to the petri dish with the sterile pipette. Then, an autologous serum in the ratio 1: 5 with PVRP (i.e., 20 %; e.g., 4 ml to activate 20 ml of PVRP) was applied over the CaCl₂. Finally, Autologous PVRP was administered with the sterile pipette over the mixture of CaCl₂ and autologous serum. At least 5 minutes elapsed for the activation process to occur and PVRG was formed. It was expected that some of the mixture would not turn into a gel.

3. Results

3.1. Total blood volume calculations for PVRG preparation

In our case, a higher PVRG volume was required due to higher wound surface area. For that reason, a "half volume" centrifugation protocol was used (Eq. (2)).

The required PVRG volume was calculated from the wound surface area,

$$V_{PVRG} = S * h \quad , \quad (3)$$

where V_{PVRG} is PVRG volume, S is wound surface area, and h is the thickness of the layer of PVRG. In external wounds, as in our case, $h = 1$ cm was used for the calculation to cover the wound thoroughly. However, some of the mixture (PVRP, calcium chloride and serum) remains untransformed (i.e., liquid), and PVRG height is expected to be less than 1 cm.

PVRG application can be repeated several times, depending on the healing process. The time between applications can vary 1 to 14 days according to previous blood-derived products therapy regimens (Hu et al., 2019). After applying PVRG, a glassy, water-resistant layer is formed over the wound. Wound healing (e.g., granulation tissue formation) can be observed through this layer. For that reason, we suggested at least 14 days interval between each application.

The total blood volume ($V_{T,blood}$) needed for the PVRG preparation was calculated as follows. The total volume of blood needed consisted of contributions used for obtaining plasma and serum,

$$V_{T,blood} = V_{blood \text{ for PVRP}} + V_{blood \text{ for serum}} \quad , \quad (4)$$

where

$$V_{PVRP} = \eta * V_{blood \text{ for PVRP}} \quad (5)$$

and η is the proportion of PVRP in blood. For the "half volume" protocol, $\eta = 0.5$ (Steiner et al., 2022). Volume of serum can be expressed by haematocrit Ht ,

$$V_{serum} = (1-Ht) * V_{blood \text{ for serum}} \quad , \quad (6)$$

whereas it is estimated that



$$V_{\text{serum}} = 1/5 * V_{\text{PVRP}} \quad . \quad (7)$$

Volume of PVRG is composed of contributions from PVRP and from serum,

$$V_{\text{PVRG}} = V_{\text{PVRP}} + V_{\text{SERUM}} \quad . \quad (8)$$

Volume of CaCl₂ is taken to be negligible and was excluded from the volume calculations. Blood for PVRP and for serum should be withdrawn into different tubes (citrate 4.5 ml blood tubes for PVRP and 4 ml plastic tubes without anticoagulant for serum), therefore patient-customized blood volume needed for PVRP ($V_{\text{blood for PVRP}}$) and for serum ($V_{\text{blood for serum}}$) should be calculated. It follows from Eqs. (7) and (8) that

$$V_{\text{PVRP}} = 5/6 * V_{\text{PVRG}} \quad , \quad (9)$$

from Eqs. (5) and (9) that

$$V_{\text{blood for PVRP}} = (5/6 \eta) * V_{\text{PVRG}} \quad (10)$$

and from Eqs. (6), (7) and (9) that

$$V_{\text{blood for serum}} = V_{\text{PVRG}} / (6 * (1 - Ht)) \quad . \quad (11)$$

However, the volume of blood taken from the patient according to Eq.(3) and Eqs.(4), (10) and (11) would be

$$V_{T,\text{blood}} = (5/6) * S * h * (1/\eta + 1/(5 * (1 - Ht))) \quad . \quad (12)$$

3.2. Calculation of the PVRG volume for patient with osteoradionecrosis of lateral skull-base

3.2.1. Patient profile and summary

84-year-old male with arterial hypertension, hyperlipidaemia, aortic stenosis and chronic atrial fibrillation on therapy with apixaban presented with a squamous cell cancer of the left auricle. The patient was primarily treated with tumour resection, suprafacial parotidectomy and left sided selective neck dissection. He received postoperative radiotherapy. One year postoperatively, he presented with a cancer recurrence in a left external ear canal and was treated surgically with complete microscopical cancer resection, including auricle amputation and subtotal petrosectomy. The tissue defect, which reached the superior part of the squama of the left temporal bone, could not be covered with a free flap due to the patient's age and vessel disease. Thus, the defect was entirely reconstructed with left extended musculocutaneous pectoralis major muscle flap. A few days postoperatively, the patient tested positive for COVID-19, which compromised the patient's general condition. The flap started to necrotise, and osteitis of the lateral-skull base developed. Moreover, the epithelialisation of exposed cortical bone was hampered due to osteoradionecrosis, and it was exposed on two areas of 0.4 cm² and 1 cm². The patient's general condition remained poor but stable.

3.2.2. Treatment regimen

Due to the patient's general healthcare status, which posed a high risk of general anaesthesia, surgical revision with flap reconstruction could not be performed. The patient was initially treated with intravenous antimicrobials and regular flap necrosis removal to prevent intracranial and systemic complications. Despite this treatment, the osteitis persisted, and epithelialisation did not occur. Therefore, outpatient salvage treatment, including multiple PVRG administration and necrosis removal, was offered to the patient. The wound was treated with six applications of PVRG and debridement on day 0 (baseline), 8, 17, 35, 56 and 108. The number of PVRG applications are strictly patient-specific and depend on the wound healing rate. It can be repeated as many times as needed until the wound reaches the desired degree of healing. Blood was withdrawn from the radial artery in citrated tubes due to the requirement of large PVRG volume and unsuccessful peripheral vein cannulation. Blood withdrawal volume was prepared with a "half-volume" centrifugation protocol.

Over the PVRG, a nonadhesive absorbent dressing, Mepilex® (Mölnlycke, Gothenborg, Sweden), was applied. Directed intravenous antimicrobial treatment for osteitis of six weeks duration was discontinued at the first PVRG application.

3.2.3. Determination of the PVRG volume

Table 1 shows the results on V_{PVRG} obtained during the treatment of the patient. Here the time of centrifugation was 5 minutes which yielded about the maximal possible volume of PVRG compatible with haematocrit (about half of the volume of the blood taken) (**Table 1**). It can be seen that the time of centrifugation should have been shorter than the one proposed by a general protocol. As the ESR of the patient changed decreased along the treatment, the centrifugation time should have been accordingly adjusted (**Table 1**). The best improvement in wound surface was achieved between day 35 and 56 (the wound surface decreased from 40 to 21 cm²).

Table 1. Regimen of the treatment of the osteoradionecrosis of the lateral skull base with PVRG (Steiner et al., 2022).

Day	Wound size (cm ²)	ESR (mm/h)	$V_{T,blood}$ (mL)	V_{PVRG} (mL)	$T_{half\ volume}$ (min)
0	46	56	125	65	1.54
8	46	55	30	13	1.57
17	43	40	96	50	2.16
35	40	40	80	45	2.95
56	21	42	65	33	2.05
108	18	38	57	30	2.27

Centrifugation was performed at 300 * g and 18 °C. PVRG – platelet and extracellular vesicle-rich gel; ESR – erythrocyte sedimentation rate.

3.2.4. Treatment outcome

PVRG formed a protective biofilm over the wound after each application (**Figure 1, Panel C**). The infection healed, i.e., the antimicrobial swab was negative, and there was no discharge—fibrin and granulation tissue formed under the PVRG biofilm. Later epithelialisation began from the wound edges and its central parts. However, the epithelialisation did not cover the exposed cortical bone, not even after the cortical bone was drilled out and PVRG applied over the cancellous bone. One of the exposed areas of the bone surfaces was reduced from 1 cm² to 0.4 cm². The second one remained non-epithelialised (0.4 cm²).



Figure 1. Photographs of a patient with an osteoradionecrosis and osteitis of left lateral skull-base treated with six applications of platelet- and extracellular vesicle-rich gel (PVRG). (A) pre-treatment wound in the area of necrosed (N) extended pectoral muscle flap (PM). Two areas of exposed bone (EW) are shown around the exposed mastoid cavity (MC) area. (B) PVRG is administered over the debrided wound. (C) on the 56th day after the first PVRG administration, NE is growing centripetal in the wound, which bleached due to inflammation regression. Dark areas are crusts formed after exposed cortical bone drilling. Fibrosis (F) is seen in centre.

4. Discussion

This clinical research presents for calculations of blood volume needed for PVRG preparation to cover a surface with targeted area. Using the equations helps to estimate the amount of blood needed to prepare 3-D model of PVRG that would cover the total wound surface. In our practice before the development of the model, it has happened that too much or too little blood was withdrawn. Repeated harvesting of too much blood from the patient can be harmful, and withdrawing too little results in an insufficient wound covering.

4.1. Patient-tailored, ESR-based PVRP and PVRG preparation protocols

The preparation protocol that is based on the mathematical model can be chosen according to the size and location of the target tissue and the patient's ESR (Božič et al., 2021). The model yields the time of centrifugation at chosen centripetal acceleration of the centrifuge rotor. In subjects with normal *Ht*, the "high platelet and EV protocol" (Eq. (1)) yields higher platelet and EVs concentrations in PVRP at the expense of lower PVRP volume (Božič et al., 2021). Thus, this protocol is appropriate in cases where the target tissue surface is small. The "half volume protocol" (Eq. (2)) yields higher PVRP volume (50% blood yield) at the expense of lower platelet and EVs concentrations in PVRP. However, these concentrations are in patients with normal *Ht* and ESR still above the baseline blood levels (Božič et al., 2021). The latter protocol was used to treat osteoradionecrosis of lateral skull-base, which was a sequela of radiotherapy of external ear canal cancer and the patient's overall health status post-COVID-19 was poor. It is known that severe and fatal complications of osteonecrosis in this area can develop due to drastic changes in bone tissue from post-radiation DNA damage and cell death (Lyons et al., 2008). This causes disorganisation of the extracellular matrix and prevents new vessel formation resulting in hypocellularity and tissue hypoxemia (Haubner et al., 2015). Irradiated bone becomes necrotic, exposes through the skin and may become infected (i.e., osteitic) (Beacher et al., 2018).

Since in the case presented the wound surface area decreased noticeably after multiple PVRG applications (from 46 cm² to 18 cm²) and bacterial osteitis was treated successfully, PVRG could be considered a treatment modality for scalp osteoradionecrosis in further research. Furthermore, we observed that the calculated volume of PVRG with novel equations adequately covered the wound.

In a previous study of the effect of PVRP on chronic wounds in the middle ear (Vozel et al., 2020) the same two-spin protocol for PVRP preparation was used for all patients. The first spin was performed at 300g for 5 minutes. According to the model (Božič et al., 2021). The patient considered in this work had an increased ESR and the centrifugation time 5 minutes would be sufficient for the erythrocytes to reach haematocrit even before the spin were completed. As the platelets and extracellular vesicles in the plasma above the haematocrit are no longer subjected to counterflow, they would be sedimenting and therefore become depleted in plasma. Besides yielding higher quality plasma, estimating the time of preparation also shortened the preparation procedure, which was favourable.

5. Conclusions

Calculations of blood volume needed to prepare PVRG in order to cover the desired wound surface are expected to improve the quality of PVRG and optimize the time of the PVRG preparation protocol.

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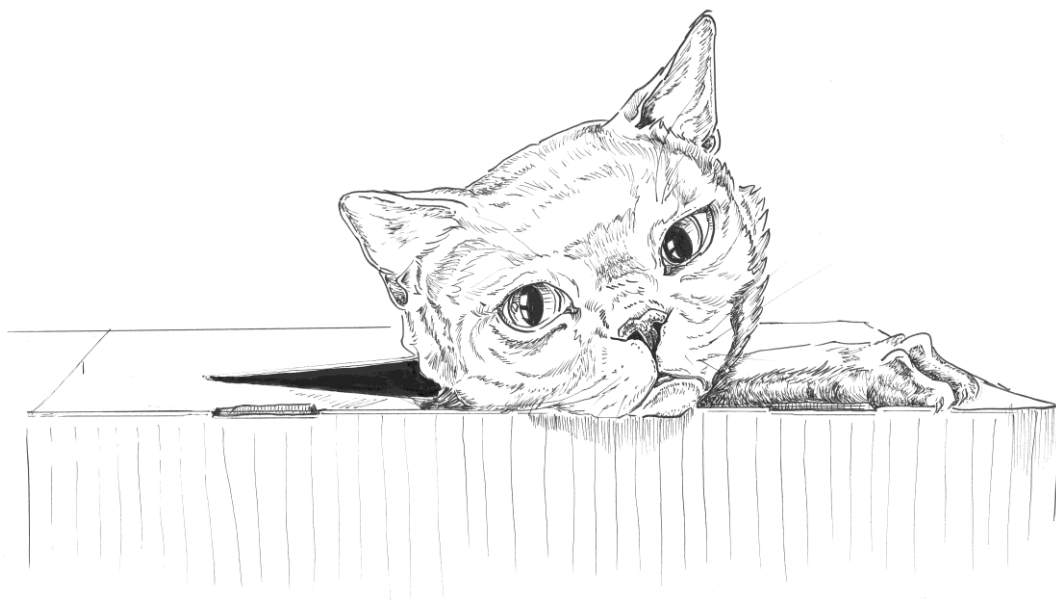
Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Medical Ethics Committee of the Republic of Slovenia (No. 0120-146/2019/5, April 17th, 2019 and No. 0120-498/2020-3, 13.1.2021).



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Review

Adverse Human Health Outcomes Associated with Psychological Trauma: A review

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Abstract:

Until 30 years ago it was believed that psychological stress increases cortisol secretion, but later studies gave contradictory results. Decrease in cortisol levels in post-traumatic stress disorder (PTSD) reflects a nonnormative and inadequate response to severe stressors, with its pathophysiology involving maladaptation or dysfunction in stress-regulatory systems. To have more insights in response of human body to physiological stress, inflammatory signals, oxidative stress parameters and other health parameters were measured. As for the cortisol level results, also inflammatory signals, including proinflammatory and anti-inflammatory cytokines and C-reactive protein (CRP), have been reported to increase and decrease in PTSD. Levels of interleukin (IL)-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, tumour necrosis factor (TNF)- α , interferon gamma (IFN- γ) and CRP were reported higher and lower in blood samples of individuals with PTSD. Some studies report that dysregulation of the stress axis could have direct effects on brain regions responsible for the regulation of fear and anxiety (such as the prefrontal cortex, insula, amygdala, and hippocampus). Early-life stress, such as childhood adversity (abuse, neglect, or family disfunction), is a potent risk factor for developing PTSD in response to later trauma, and elevated peripheral markers of inflammation are one of the best-replicated findings in children and adults with early-life stress. Those who develop PTSD may have an inability or failure to activate an innate immune response. PTSD can also result in other adverse outcomes, such as heightened oxidative stress (OXS), eating disorders, metabolic disorder, and cardiovascular disease (CVD). Since the results are very contradictory for PTSD and inflammation response of the human body, further research is important. Small cellular particles that can be isolated from body fluids present potential biomarkers of the clinical status and will be considered in planning the future research. This contribution presents perspectives in assessment of psychological stress by objective parameters.

Keywords: Cortisol; Post-traumatic stress disorder; Inflammatory response; Oxidative stress; Cytokines; Eating disorders; Metabolic disorder; Cardiovascular disease; Small cellular particles as stress markers, Extracellular vesicles as stress markers

1. How can Psychological Trauma affect human body

Post-traumatic stress disorder (PTSD) is a chronic disorder with dysregulated stress axis function (Michopoulos et al., 2015). It is associated with psychological, genomic and biological risk factors (Michopoulos et al., 2015) as well as other morbidities including major depression, substance and alcohol abuse, panic disorder, suicide, reduced life expectancy. It can also result in increased health care utilization and disability in daily activities (Coughlin, 2011). Studies also confirmed connection between PTSD and obesity, diabetes as well as cardio-vascular disease (Boscarino, 2004; Heppner et al., 2009). PTSD has also been linked to accelerated aging, reduced cortical thickness, and neurodegeneration (Miller and Sadeh, 2014; Yang et al., 2021). Developing PTSD is associated with psychological trauma in childhood (Edwards et al., 2003). Maltreated children with significant emotional problems showed higher average daily cortisol levels across 1 week (Cicchetti and Rogosch, 2001). Similarly, maltreated children with PTSD showed higher 24-h urinary free cortisol and daily salivary cortisol levels compared to a healthy comparison group with no maltreatment history (Carrion et al., 2002; De Bellis et al., 1999). Focus in the scientific literature has been on disturbance in the hypothalamic–pituitary–adrenal (HPA) axis in connection with PTSD. HPA axis is activated in acute stress conditions and the hypothalamus starts to secrete corticotropin-releasing hormone (CRH) under the influence of serotonin from the amygdala. Afterwards, the pituitary is stimulated by the CRH to release adrenocorticotrophic hormone (ACTH). This activation results in the production of glucocorticoids (cortisol) in the adrenal cortex. Cortisol serves to stop many metabolic, neuronal defensive and immune reactions (Meewisse et al., 2007). Described process with inflammation and oxidative stress (OXS) pathway can be seen in **Figure 1**. Over time and with continuous exposure to stressors, both HPA and immune function become dysregulated. Studies using psychological stress to stimulate the HPA axis have shown an exaggerated cortisol response in PTSD (de Kloet et al., 2006). However, studies from more than 30 years ago suggested that PTSD has been associated with lower levels of cortisol (Yehuda et al., 1990). This paradigm involved the acknowledgment that stress is not necessarily equal to high cortisol secretion, but indeed reflects a nonnormative and inadequate response to severe stressors, with its pathophysiology involving maladaptation or dysfunction in stress-regulatory systems (Yehuda and McFarlane, 1995). Contradictory results forced the researchers to rethink prevailing models of stress and disease and expand research.

One biological process that has been increasingly interrogated over the last decade is the inflammatory system, as it has a clear role in the pathophysiology of chronic mental and physical illness. Also, oxidative stress in human body as well as other health parameters such as eating disorder, metabolic disorder, and cardiovascular disease (CVD) are of interest.

Increased inflammation in PTSD is probably caused by the activation of the stress response in central and peripheral immune cells that then release cytokines (Michopoulos et al., 2020). Peripheral monocytes can infiltrate the brain and produce macrophages that secrete cytokines and promote neuroinflammation under stressful conditions. Peripherally produced inflammatory mediators as well as glucocorticoids can stimulate microglia which after activation produce cytokines and other substances that are important for adaptive regulation (Dantzer et al., 2008; Nair and Bonneau, 2006). Many blood-based immune mediators, including proinflammatory and anti-inflammatory cytokines as well as chemokines were measured within 3 hours after a traumatic exposure and after up to 12 months after a traumatic exposure (Michopoulos et al., 2020). Monitored individuals were classified in three groups: 1) those who were resilient and did not develop PTSD, 2) those who developed symptoms but recovered, and 3) those who developed chronic PTSD symptoms. The authors found that blood-based levels of all proinflammatory cytokines, in particular tumour necrosis factor (TNF)- α and interferon (IFN)- γ , immediately after the traumatic exposure are not the same for those individuals who went on to develop chronic PTSD symptoms from those who were resilient or had recovered from their symptoms at the time of follow-up.

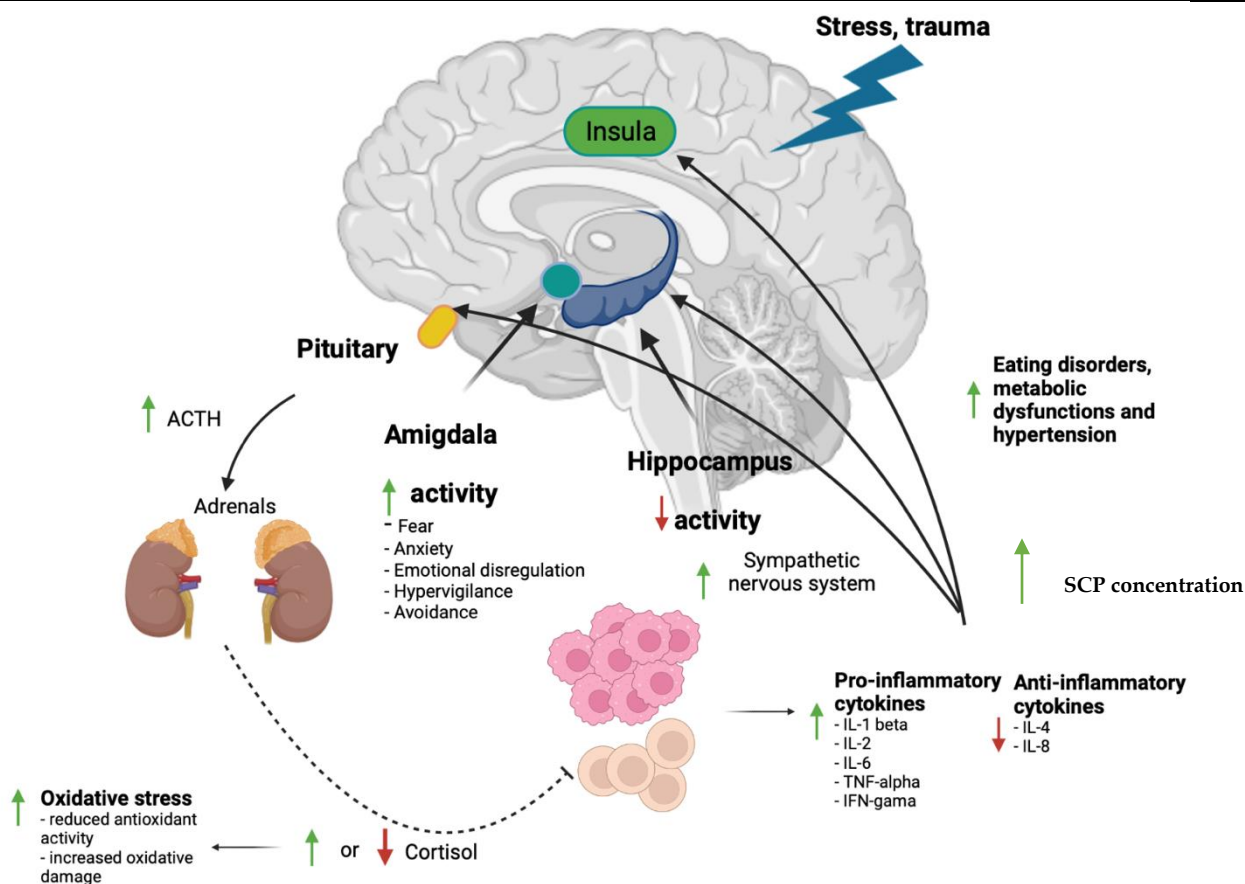


Figure 1. Inflammation process in post-traumatic stress disorder (PTSD). Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is present in PTSD. Gonadal steroid hormones and the HPA axis modulate neurotransmitter and neuropeptide systems, influence amygdala activity, influencing inflammatory and oxidative stress responses. Also, eating disorders (ED), metabolic disorder and cardiovascular diseases (CVD) increase. Some studies report that cortisol levels decrease in case of PTSD, on contrary, other studies report increasing of cortisol levels. Although results are also contradictory for PTSD association with inflammatory response, cross-sectional data linking PTSD to a pro-inflammatory state and associates PTSD with chronic inflammation. Increase in immune activity in both the periphery and the central nervous system via stress and trauma effects neuroendocrine systems and the sympathetic nervous system (SNS). The overactivity of the SNS and decreased activity of the parasympathetic nervous system increases the release of pro-inflammatory cytokines (Interleukin (IL)-1 β , IL-2, IL-6, tumour necrosis factor (TNF)- α and interferon (INF)- γ) and decrease anti-inflammatory cytokines (IL-4 and IL-8) that can influence neurotransmitter systems, neurocircuitry, and finally, affective behaviour. Cytokines may contribute to the maintenance of fear- and anxiety-based symptoms by affecting the activity and connections of regions of the brain implicated in the etiology of these disorders, including the amygdala, hippocampus, insula, medial prefrontal cortex, and the anterior cingulate (adapted from (Michopoulos, Norrholm, and Jovanovic 2015), (Meewisse et al. 2007) and (Michopoulos et al. 2017)).

2. Psychological Trauma and inflammation

Exposure to psychological trauma is associated with inflammatory activity. Inflammation is present during high PTSD symptom state, but it is not yet clear if inflammation plays a role in PTSD risk. A prospective study (Marine Resiliency Study) of PTSD development in service members deployed to combat zones reported that high levels of plasma CRP collected before deployment were associated with increased risk for developing PTSD after combat (Eraly et al., 2014). Similarly, those that developed PTSD after combat exhibited altered gene expression patterns in peripheral immune cells collected before combat compared to those that did not go on to develop PTSD after combat (Breen et al., 2015) and also, higher glucocorticoid-dependent cytokine production and T-cell proliferation before deployment is associated with increased PTSD symptoms after combat. These findings suggest that immune factors might not only be markers for symptom state, but also contribute to pre-existing risk for PTSD upon trauma exposure. It can be suggested that immune factors might be markers for symptom state of PTSD and can also contribute to pre-existing risk for PTSD upon trauma exposure (Deslauriers et al., 2017).

Some studies report increase in circulating concentrations of pro-inflammatory signals - interleukin (IL)-1 β (Hoge et al., 2009), IL-2 (Guo et al., 2012), IL-6 (Bersani et al., 2016), TNF- α (Bersani et al., 2016), INF- γ (Hoge et al., 2009) and the acute phase reactant C-reactive protein (CRP) (Tursich et al., 2014). IL-1 β was suggested as biomarker of illness duration, and IL-6 as a biomarker of PTSD severity (Passos et al., 2015). It is also reported that childhood maltreatment can result in increased inflammation in adulthood. Anti-inflammatory signals were reported to decrease - IL-4 (Smith et al., 2011) and IL-8 (Song et al., 2007). Authors reported increased levels of CRP in adulthood (Bertone-Johnson et al., 2012; Lin et al., 2016; Matthews et al., 2014; Tietjen et al., 2012), also concentrations of IL-6, IL-1 β , and TNF- α are increased (Tietjen et al., 2012; Gouin et al., 2012; Kiecolt-Glaser et al., 2011; Smith et al., 2011). Increase in TNF- α concentration in PTSD also positively correlate with total PTSD symptomatology, as well as all three Diagnostic and Statistical Manual of Mental Disorders, 4th Edition – text revision symptom sub-clusters (avoidance, re-experiencing, and hyperarousal) (von Känel et al., 2007). Elevated CRP is associated with impaired inhibition of fear-potentiated startle in the presence of a safety signal, a well-characterized biomarker of PTSD (Jovanovic et al., 2012). Perhaps it might be speculated that a certain degree of inflammation is necessary for adaptive plasticity.

Contradictory to results of increase pro-inflammatory signals, other authors report decreases or no relation between PTSD and circulating concentrations of IL-1 β (Tucker et al., 2004; Song et al., 2007), IL-2 (Smith et al. 2011; Hoge et al. 2009), IL-6 (von Känel et al., 2007) and CRP (McCanlies et al., 2011). In relation to that, also pro-inflammatory signals were reported to increase: IL-4 and IL-10 (Guo et al., 2012; Hoge et al., 2009; von Känel et al., 2007) as well as IL-8 (Guo et al., 2012; Hoge et al., 2009). IL-4 concentrations have been correlated negatively with total hyperarousal symptoms (von Känel et al., 2007) and in a small clinical trial in soldiers with PTSD, symptom improvement during psychotherapy was accompanied by increases in peripheral TNF- α (Himmerich et al., 2016).

3. Psychological Trauma and Oxidative stress

OXS is a biological process which triggers pro-inflammatory signalling pathways and can be activated by inflammation (Miller and Sadeh, 2014). It can cause cellular damage due to an imbalance between levels of antioxidants and free radicals. Variety of biomarkers can be measured for detecting OXS with the aim to quantify either antioxidant capacity or the degree of oxidative damage present in a bio-sample. It is connected with neurodegeneration, aging, accelerate cellular aging and pathogenesis of several chronic conditions (including diabetes, cardiovascular illnesses and neurodegenerative conditions) (Miller et al., 2018). OXS markers were suggested to be associated with PTSD. In most studies on patients with PTSD levels of specific OXS markers increased and activity of antioxidant enzymes reduced (Borovac Štefanović, et al. 2015; Zieker et al., 2007; Tylee et al., 2015; Atli et al., 2016). Possibly, the traumatic experience itself responds by increasing OXS. Potentially relevant process that links between PTSD and OXS is sleep disturbance—a common symptom of PTSD that manifests as recurrent nightmares, restless sleep and difficulty falling and staying asleep - sleep disturbance promotes OXS in the brain by interrupting elimination of free radicals, which, in turn, contributes to cognitive decline and neurodegeneration (Calhoun et al., 2007). In earthquake-exposed individuals with PTSD authors report significantly decreased paraoxonase-1 activity and elevated malondialdehyde concentration in comparison with healthy controls; in the same study, such differences were not significant between healthy controls and earthquake survivors who did not develop PTSD (Atli et al., 2016). Also, other authors reported that OXS parameters were associated with risk for PTSD development (Glatt et al., 2013; Tylee et al., 2015). Similarly, Stefanovic et al. (Borovac Štefanović et al., 2015) found decrease in blood levels of superoxide dismutase and glutathione transferase in Croatian war veterans with PTSD compared to controls. Studies have shown that clinically depressed patients show elevated levels of oxidative DNA damage and suppressed antioxidant activity (Forlenza and Miller, 2006). Patients with generalized anxiety disorders have shown evidence of elevated lipid peroxidation (Bulut et al., 2013) and suppressed antioxidant activity was found in bio-samples from patients with panic disorder (Ozdemir et al., 2012). There are reports for altered reactive oxygen species (ROS) and glutathione S-transferase (GST) in chronic PTSD (Neylan et al., 2011). Future studies with larger samples are needed to investigate the levels of OXS markers and the role of this biological process in PTSD (Peruzzolo et al., 2022).

4. Small cellular particles and extracellular vesicles as stress biomarkers

Already more than 20 years ago it was revealed that small cellular particles (SCPs) detected in isolates of human blood are connected with inflammation processes in human body (Beyer and Pitsetsky, 2010). Research in this field has been developing since and various mechanisms have been considered. Chiaradia et al. (2021) considered SCPs' involvement in the pathophysiology of oxidative stress-related diseases, as mediators of cell-to-cell communication. Direct effects and indirect effects on the regulation of oxidative stress through SCPs were distinguished; SCPs can deliver antioxidant substances or oxides to recipient cells, directly relieving or aggravating oxidative stress, or - they can deliver regulate factors of oxidative stress-related signaling pathways to recipient cells (Qi et al. 2021). In a recent study, it was reported that the quantity of oxidative stress markers correlated with concentration of SCPs isolated from blood (Jan et al., 2021). With all the different roles SCP can play in human body and with all the connection to oxidative stress and inflammation related processes, investigating SCP role in psychological stress is of interest.

5. Eating disorders, metabolic disorder and cardiovascular disease in psychological trauma

PTSD, especially the one caused by childhood maltreatment, especially sexual abuse, is commonly connected to eating disorders (EDs). Yet, EDs can also be triggered by many other forms of victimization, trauma, and neglect, including but not limited to sexual assault (rape and molestation) during adulthood, sexual harassment, physical abuse and assault, emotional abuse, emotional and physical neglect (including food deprivation), teasing, and bullying (Johnson et al., 2002). PTSD is more common in bulimia nervosa and binge-eating disorder and less common in anorexia nervosa (Mitchell et al., 2012).

Connection between PTSD and adverse health disorders and diseases, e.g., CVD (Boscarino 2004) metabolic disorder (including type 2 diabetes mellitus (T2DM)) and obesity (Michopoulos et al., 2016) has been reported. Obesity and metabolic disorder result in reduced sensitivity to the anorexic peptide leptin (secreted from adipose tissue) that results in hyperleptinemia and leptin resistance (Santoro et al., 2015). Effects of the HPA axis and stress on metabolism can provoke hyperglycaemia and insulin resistance already present in metabolic disorders (Rosmond, 2005). When inflammation alters HPA activity, it has adverse effects on cardiovascular function (Nijm and Jonasson, 2009). A longitudinal study of traumatic stress resulting from prolonged war in Beirut found that individuals with more war-related traumatic experiences were at greater risk for CVD-related mortality. Mortality was more evident in women who suffered personal traumas, such as injuries or family deaths, while it was more prominent in men who had suffered property loss, work-related problems, or displacement from home (Sibai et al., 2001). In a study of cardiovascular morbidity in World War II prisoners of war, increased risk of CVD was present in those who developed PTSD (Kang et al., 2006). 30 year follow-up study with Vietnam-era veterans noted that those veterans at PTSD were at increased risk of CVD-related mortality (Boscarino, 2006). In a study of Australian veterans, PTSD associated with increased risk of hypertension (O'Toole and Catts, 2008). Studies report that PTSD is connected with elevated baseline systolic and diastolic blood pressure (Kellner et al., 2003). PTSD was also associated with elevated high density lipoprotein (HDL) cholesterol, low density lipoproteins (LDL), and triglycerides, as well as decreased high density lipoproteins (Solter et al., 2002). Alleviating inflammation might be beneficial to individuals suffering from PTSD and metabolic disorders and could even help prevent the occurrence of metabolic syndrome in individuals with PTSD (Michopoulos et al., 2015).

6. Conclusions

Overall, the cross-sectional data linking PTSD to a pro-inflammatory state and associates PTSD with chronic inflammation, suggesting the inflammation may serve as a possible therapeutic target for alleviating PTSD symptoms et al., 2015). The inconsistencies between published reports on PTSD associated with cortisol level and with inflammation may be related to small sample sizes, distinct study and ethnic populations, the presence of uncontrolled confounders (medication usage, presence of infection, co-morbidity with depression, and other chronic illnesses), and the use of different control groups for comparison. Although this meta-analysis and coincident subgroup analysis move the field forward, future studies and analyses are necessary to determine how other factors (ie, smoking

status, alcohol use, obesity, infection, and pulmonary and CVD) influence the association between PTSD and inflammation (Michopoulos et al., 2017).

Several behaviours may contribute to the increased risk for poorer health in individuals with PTSD. Individuals with PTSD have tendency to engage in increased alcohol use, have higher BMI, and greater rates of smoking. Studies of both men and women report poorer health habits including less physical exercise, less self-care, and greater caloric intake (Dedert et al., 2010).

It would be interesting to investigate impact of Marital and Family Therapy on the health parameters that were listed and presented in this review paper.

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Invited lecture/Scientific contribution/Original research

Brachycephalic Dogs with Brachycephalic Obstructive Airway Syndrome Have Increased Variability in Red Blood Cell Size

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Abstract:

Brachycephalic obstructive airway syndrome (BOAS) is a conformation-related respiratory disorder of dog breeds with congenitally flattened facial and skull anatomy. BOAS is characterized by chronic shortness of breath and subsequent difficulty in exercising, a tendency to overheat, increased and abnormal respiratory noise, and low oxygen levels. The aim of our retrospective study was to investigate the level of red blood cell distribution width (RDW), a biomarker of chronic hypoxemia, in groups of BOAS patients with different degrees of BOAS and a group of healthy non-brachycephalic dogs. Red blood cell distribution width provides information on the variability in the red blood cell volume. It is a simple and inexpensive variable included in the complete blood count report. Seventy-two BOAS patients and 24 non-brachycephalic dogs were included in this retrospective study. Patients with BOAS were classified into grade 1 (13 dogs), grade 2 (27 dogs), and grade 3 (32 dogs) according to the severity of the disease. In our study, a significantly ($p < 0.05$) higher RDW was found in all groups of BOAS patients compared to the non-brachycephalic dog group. However, we found no significant difference in RDW between the groups of BOAS patients. Thus, we may conclude that BOAS patients have increased variability in the size of red blood cells compared with healthy non-brachycephalic dogs. Our results warrant further studies to determine the potential utility of RDW in BOAS and to clarify the role of RDW in BOAS patients in relation to the severity of BOAS and cardiovascular risk.

Keywords: Brachycephaly; Brachycephalic obstructive airway syndrome; Dogs; Haematology; Erythrocytes; Red blood cell distribution width

1. Introduction

In recent years, brachycephalic dogs, which exhibit various characteristics of young animals have become increasingly popular internationally. Their large, round, wide-set eyes and flat, rounded faces resemble those of human infants, making these dogs a pleasure to care for. Common breeds of brachycephalic dogs include English and French bulldogs, Boston terriers and pugs; these breeds are characterised by a severe shortening and widening of the skull that results in narrowing of the airway, making them susceptible to conformational respiratory condition known as brachycephalic obstructive airway syndrome (BOAS) (Fasanella et al., 2010; Meola, 2013; O'Neill et al., 2015; Packer et al., 2015; Dupre and Heidenreich, 2016; Liu et al., 2017). Artificial selective breeding for extreme brachycephaly has resulted in bony changes and upper airway deformation, leading to increased airway resistance caused by excessive soft tissues that has not decreased proportionally with bony changes. Affected dogs present with clinical signs of respiratory dysfunction that may include inspiratory dyspnea, snoring, stertor, stridor, and panting, gastrointestinal signs such as vomiting and regurgitation, and intolerance to stress, exercise, and heat, sleep disturbances, and in severe cases, cyanosis and even syncopal episodes (Fasanella et al., 2010; Roedler et al., 2013; Packer et al., 2015; Dupre and Heidenreich, 2016; Liu et al., 2017). Brachycephalic dog breeds may have additional systemic complications (Meola, 2013). Even when these dogs are systemically healthy, they have been shown to have hypertension, and significantly higher packed cell volume and arterial pCO₂ and significantly lower arterial pO₂, compared to non-brachycephalic dog breeds (Hoareau et al., 2012). Furthermore, hypomagnesemia (Mellema and Hoareau, 2014) and hypercoagulability (Hoareau and Mellema, 2015) have also been demonstrated in clinically healthy Bulldogs. In addition, the presence of a hypercoagulable state (Crane et al., 2017), elevated levels of inflammatory markers (Rancan et al., 2013), and higher levels of cardiac troponin I have been reported in canine patients with BOAS (Planellas et al., 2012).

Brachycephalic obstructive airway syndrome shares features of obstructive sleep apnea syndrome (OSAS) (Hendricks et al., 1987; Hendricks, 1992), which is a highly prevalent breathing disorder in humans, caused by the repetitive collapse of the narrow upper airway during sleep (Eisele, 2015; Lavie, 2015). Obstructive sleep apnea syndrome is characterized by intermittent hypoxia, leading to blood hypoxaemia, hypercapnia, sleep fragmentation, augmented respiratory efforts and increased sympathetic activity. In patients with OSAS, repeated episodes of cessation of breathing, lead to hypoxia and reoxygenation, which results in increased production of reactive oxygen species/reactive nitrogen species (ROS/RNS) and thus oxidative stress. The latter adversely affect the associated cardio-/cerebro-vascular disease in OSAS patients (Eisele, 2015; Lavie, 2015).

Increased values of red blood cell distribution width (RDW) (Ozsu et al., 2012; Leon Subias et al., 2017) and an association between RDW and OSAS severity have been reported not only in adult OSAS patients with and without cardiovascular disease (Sokucu et al., 2014; Leon Subias et al., 2017), but also in children with OSAS (Morell-Garcia et al., 2020). For many years, RDW was used for the differential diagnosis of anemias. Nowadays, RDW is a parameter with numerous clinical applications (Salvagno et al., 2015). It is also considered a biomarker of chronic hypoxemia (Yčas, 2017). Red blood cell distribution width, an index of circulating red blood cell volume heterogeneity (conventionally known as anisocytosis), is included in routine complete blood count reports, making it a simple and inexpensive parameter. The RDW is a coefficient of variation of red cell volume calculated by dividing the standard deviation of red cell volumes by the mean corpuscular volume (MCV) and multiplying by 100 to express the result as a percentage. The RDW thus provides information on the variability of the volume of circulating erythrocytes. In addition, RDW is associated with several serious diseases, including most of those that cause hypoxemia. It has been reported that RDW responded to applied hypoxia (Salvagno et al., 2015; Yčas, 2017). Intermittent hypoxia, which also occurs in OSAS and BOAS patients, stimulates the synthesis and secretion of erythropoietin, which is one of the factors with great impact on the increase of RDW (Salvagno et al., 2015; Yčas, 2017; Li et al., 2018). The RDW has not been reported in BOAS patients. Therefore, our retrospective study aimed to evaluate the RDW in canine patients with different grades of BOAS admitted to the Small Animal Clinic for surgical treatment of BOAS.

2. Methods

2.1. Dogs

The present study is based on a retrospective evaluation of complete blood count reports of 72 client-owned dogs diagnosed with BOAS and 24 healthy dogs of non-brachycephalic breeds that underwent elective surgery. The 24 healthy non-brachycephalic dogs served as controls. These dogs were considered healthy based on normal history, normal clinical examination, and results of hematological and biochemical analyzes. At inclusion to the study, the history of the BOAS patients was obtained using a questionnaire about behavior, health, and lifestyle. Additionally, the owners of BOAS patients completed a preoperative owner questionnaire in order to investigate a wide range of clinical signs (respiratory and gastrointestinal signs, exercise tolerance, and sleep disorders). All dogs that showed signs of concurrent disease or had received any type of therapy or vaccination within the last month were excluded from the evaluation. The diagnosis of BOAS was based on clinical signs of upper airway obstruction and anatomical anomalies, as described elsewhere (Dupre and Heidenreich, 2016; MacPhail and Fossum, 2019). The severity of the disease was classified based on the anatomical anomalies of the airway. Patients were classified as grade 1, grade 2, and grade 3 based on the decrease in the radius of the airway at the level of the nasopharynx, oropharynx, laryngopharynx, and larynx after soft palate surgery (Erjavec et al., 2021). The patients with BOAS were scheduled for surgical treatment under general anesthesia. The overall patients' health status was assessed by history, physical examination, and the results of haematological (complete blood count with white blood cell differential count; only results on RDW are reported in this manuscript), biochemical (data not shown), and venous blood gas analyzes (data not shown).

Written informed consent was obtained from the owners. All procedures complied with the relevant Slovenian governmental regulations (Animal Protection Act, the Official Gazette of the Republic of Slovenia, 43/2007). The study was evaluated and approved by the Ethical Committee on Animal Research of the Veterinary Faculty, University of Ljubljana.

Haematological analyzes were performed within one hour after collection of blood samples using an automated laser-based haematology analyzer (ADVIA 120, Siemens, Munich, Germany) and multispecies software. Data were analyzed using commercial software (IBM SPSS 25.0, Chicago, Illinois, USA). We used the Shapiro-Wilk test to check whether the data were normally distributed. Based on the results of normality tests, the Kruskal-Wallis test followed by multiple comparisons and Mann-Whitney test (non-parametric tests) were used to compare the parameters (RDW, weight, age) between the groups of dogs. A value of $p < 0.05$ was used to determine significance.

3. Results

Baseline data for patients with BOAS and healthy non-brachycephalic dogs are shown in Table 1. No significant difference in weight was found between the control dogs and the groups of BOAS patients. However, control dogs and grade 1 BOAS patients were significantly younger than grade 3 BOAS patients. Regardless of the severity of BOAS, French bulldogs were the most common breed (37/72). Statistical analysis revealed a significantly higher RDW in all groups of BOAS patients (Table 2) compared with the group of non-brachycephalic dogs. However, there was no significant difference in the level of RDW among the three groups of BOAS patients.

Table 1: Baseline data of patients with different grades of brachycephalic obstructive airway syndrome (BOAS) and healthy non-brachycephalic dogs (Control)

	Control	Grade 1	Grade 2	Grade 3	All BOAS patients
Number	24	13	27	32	72
Sex (F/M)	11/13	7/6	16/11	21/11	44/28
Age (months)	15.0	13	31.0	35.0*	30.5
Median (IQR)	11.0 – 40.5	8.5 – 28.5	16.0 – 55.0	19.3 – 67.5	16.4 – 55.8
Weight (kg)	10.8	8.5	10.1	10.0	9.84
Median (IQR)	7.8 – 22.7	6.5 – 12.1	8.8 – 12.6	8.4 – 11.6	8.4 – 11.8
Breeds	Non-brachy	7 FB, 4 ST, 1 EB, 1 BST	13 FB, 6 BST, 6 P, 1 EB, 1 ST	17 FB, 8 P, 6 BST, 1 ST	37 FB, 14 P, 13 BST, 6 ST, 2 EB

*grade 3 patients significantly were older than grade 1 patients ($p = 0.010$) and control dogs ($p = 0.016$). BST: Boston Terrier; EB: English Bulldog; F: female; FB: French Bulldog; IQR: interquartile (25th to 75th percentile) range; M: male; P: Pug; ST: Shih Tzu

Table 2: Red blood cell distribution width (RDW) of patients with different grades of brachycephalic obstructive airway syndrome (BOAS) and healthy non-brachycephalic dogs (Control)

Group	RDW (%) Median; IQR	p values ^a
Reference range ^a	11.9 – 14.5	
Control (n = 24)	12.5; 12.1 – 13.1	
Grade 1 (n = 13)	13.0; 12.9 – 14.0	$p = 0.037$
Grade 2 (n = 27)	13.4; 13.1 – 13.9	$p < 0.001$
Grade 3 (n = 32)	13.1; 12.7 – 13.6	$p = 0.027$
All BOAS patients	13.2; 12.9 – 13.8	$p < 0.001$

*p values indicating the significant difference in comparison to control dogs

^aHematology analyzer Advia 120 (Siemens, Munich, Germany)

IQR: interquartile (25th to 75th percentile) range; n: number of dogs

4. Discussion

In this study, RDW was investigated in brachycephalic dogs with different severity grades of BOAS. We could not compare our results with those of similar studies performed in BOAS patients because to our best knowledge no papers have been published on the subject. Because of the similarity of the two syndromes (Hendricks et al., 1987; Hendricks, 1992), we discussed our results with those obtained in OSAS patients. The results of our study showed that RDW was significantly higher in all groups of BOAS patients than in healthy non-brachycephalic dogs, although the median values were within the reference range. These results suggest that BOAS patients have greater variability in the size of red blood cells compared to controls. Similar results were obtained in OSAS patients (Ozsu et al., 2012; Leon Subias et al., 2017). The higher RDW values in BOAS patients may be due to increased erythropoietin synthesis, as a consequence of the hypoxemia present in these patients (Hendricks et al., 1987; Hendricks, 1992; Hoareau et al., 2012). In addition, a high RDW values in BOAS may be due to other factors, such as inflammation (Lippi et al., 2009; Leon Subias et al., 2017). Intermittent hypoxia is one of the important factors that cause systemic inflammation (Dewan et al., 2015). In BOAS patients, plasma concentrations of pro-inflammatory and anti-inflammatory cytokines and nitric oxide were significantly higher than in control dogs and appeared to be related to disease severity (Rancan et al., 2013).

Some limitations must be considered when interpreting our results. The first and the most important limitation of our study is the lack of erythropoietin measurements, which could help us in the

conclusion of our study. The second limitation is the fact that the the control dogs and patients in BOAS grade 1 were significantly younger than the patients in BOAS grade 3. The third limitation is the gender distribution of BOAS patients. We included more females than males, 44 and 28, respectively.

5. Conclusions

Based on our results, we may conclude that BOAS patients have increased variability in red blood cell size compared with healthy, non-brachycephalic dogs. Our results warrant further studies to determine the potential utility of RDW in BOAS and to clarify the role of RDW in BOAS patients in relation to the severity of BOAS and cardiovascular risk.

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Invited lecture/Review

Anthelmintic Resistance in Gastrointestinal Nematodes of Ruminants

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Abstract:

Gastrointestinal nematode (GIN) infections remain one of the most prevalent and important issue affecting ruminants worldwide. Until date, the majority of GIN control has relied on the administration of chemical anthelmintic medications on a regular basis, in recent years, the problem of anthelmintic resistance has reached new heights where it can no longer be ignored as a major issue in the control of parasites of livestock. Anthelmintics are generally used at farmers' discretion, with no restrictions to access to commercially available drugs and without any assistance from veterinarians. Thus, inadequate use of anthelmintics is not rare, animals are often treated excessively, interfering with production, accelerating selection of resistant parasites, and posing significant problems for the ruminant industry. The unusually high frequency of multi-drug resistance (MDR) in sheep and goat nematodes threatens the sustainability of small-ruminant enterprises in several parts of the world. Although resistance in horses and cattle nematodes has not yet reached the levels reported in small ruminants, data shows that resistance issues, particularly MDR worms, are rising in these hosts. Both innovative non-chemical parasite control methods and molecular tests capable of detecting resistant worms are urgently needed.

Keywords: Anthelmintics; Multidrug resistance; Gastrointestinal nematodes; Ruminants; Prevalence

1. Nematodes in ruminants and anthelmintic resistance

Infections with parasites, particularly gastrointestinal nematodes (GINs), continue to pose a severe threat to the health, welfare, productivity, and reproduction of grazing ruminants across the world (Morgan et al., 2005). At pasture, all grazing animals are susceptible to helminth infections, and any future intensification of livestock production will raise the risk of helminth infection.

GINs are important parasites that infect cattle and small ruminants in a variety of ways (Kaplan RM., 2004) and can cause anemia and other symptoms in due to the blood-sucking actions of specific nematode species, decrease reproductive performance, causing a low growth rate, weight loss, and poor food conversion (Mello et al., 2006). *Haemonchus placei*, *Cooperia* spp., and *Oesophagostomum radiatum* are the most common species in cattle (Neves et al. 2014), while *Haemonchus contortus*, *Trichostrongylus colubriformis*, and *Oesophagostomum columbianum* are the most commercially important GINs in small ruminants (Amarante, 2014). *Strongyloides* spp. and *Trichuris* spp. are the most frequent nematodes found in ruminants, but *Strongyloides* spp. having a global range. Other species found in cattle and small ruminants include *Ostertagia ostertagi* and *Teladorsagia circumcincta*, respectively (Bisset et al., 2014). The huge number of prevalence surveys and field epidemiology studies conducted in various locations give a qualitative picture of the distribution and relative relevance of various species throughout Europe. In warmer, southern latitudes, *H. contortus* is more frequent and poses a greater danger to sheep health and productivity, while *T. circumcincta* is the dominating nematode of sheep in temperate and northern areas (Kao et al., 2000). *Trichostrongylus* and *Nematodirus* spp. are widespread, with varying degrees of significance on a local basis. Only in northern Europe is *N. battus* a major source of illness in lambs (Morgan and van Dijk, 2012).

Factors influencing the occurrence of GINs include the link between crop adaptability and climatic circumstances such as pasture quantity and quality, temperature, humidity, and the host's grazing behavior (Pal and Qayyum 1993). Environmental circumstances are favorable for the growth of gastrointestinal parasites during the hot, rainy months of the year, and they proliferate quickly with a high intensity as a result. In the microclimate of the pasture, the best temperature range for larval development of many nematode species is 22 to 26°C, with an appropriate humidity level of close to 100%. The majority of larvae perish in unfavorable climates (Ramos et al., 2016).

Since the 1960s, when very effective broad spectrum anthelmintics with wide safety margins became available, producers have relied on these treatments to manage GIN (Rose et al., 2020). Anthelmintic products such as benzimidazoles (BZ; e.g. albendazole, triclabendazole), levamisoles (LEV), and macrocyclic lactones (ML; e.g. ivermectin, eprinomectin, moxidectin) are used heavily in ruminant helminth control to keep infections below levels that can cause clinical and sub-clinical disease (Rose et al., 2020).

The yearly cost of treating parasite infections in ruminants is estimated to be in the billions of dollars, from the sales of anthelmintic drugs by pharmaceutical companies, excluding production losses, also the economic impacts of anthelmintic resistance around the world are massive with global losses projected in the billions of dollars per year. In contrast to veterinary health, resistance in human parasites is not widely reported with only a few confirmed cases. The selective pressures put on human parasites by mass drug administration, will lead to an increase in resistance (Means et al., 2017).

1.1. Anthelmintic resistance

The term "resistance" is defined differently in different publications. Anthelmintic resistance is a heritable trait that occurs "when a higher frequency of individuals in a parasite population, who were previously affected by a dose or concentration of compound, are no longer affected, or a higher concentration of drug is required to achieve a certain level of efficacy." The World Association for the Advancement of Veterinary Parasitology (WAAVP) produced a guideline on anthelmintic combination products targeting nematode infections in ruminants and horses (Geary et al., 2012) that states: "parasites' ability to withstand medication dosages that would typically kill parasites of the same

species and stage." Resistant helminths avoid the effects of therapy and convey resistance to the following generation. Resistance genes that arise as a result of mutation are initially uncommon in the population, but as selection continues, their relative percentage in the population grows, and the number of resistant parasites grows as well. Chemical resistance is referred to as cross resistance.

Understanding the evolution and inheritance of anthelmintic resistance has been a global research focus for many years. Many veterinary parasitic nematodes have genetic characteristics that facilitate the development of anthelmintic resistance. Rapid nucleotide sequence evolution and extremely large effective population sizes are two of the most important, giving these worms an incredibly high degree of genetic diversity (Anderson et al., 1998, Blouin et al. 1995). Furthermore, the majority of nematode species analyzed have a population structure that is compatible with significant levels of gene flow, suggesting that host migration is a key factor of worm population genetic structure (Blouin et al., 1995). As a result, these worms not only have the genetic capability to successfully respond to chemical attack, but also the ability to ensure the spread of their resistant genes through host mobility.

A rise in resistance within a worm population to clinically detectable levels is usually a lengthy and gradual process that takes several generations of drug selection and many years. In practice, this means that the genetic phase of resistance grows slowly over time, making it hard to detect, but then rapidly rises in its later phase. When a large enough fraction of the worm population is resistant, the phenotype of diminished effectiveness will become clinically noticeable. Treatment failures due to medication resistance can develop almost quickly or over a relatively short period of time, depending on how many animals are acquired that house resistant worms and their worm loads, as well as other management and pasture characteristics.

In the early stages of resistance, certain medications within a class are more effective than others (e.g., moxidectin vs. ivermectin), but if resistance reaches high levels, no therapy in that class is likely to remain effective. There is also strong evidence that once resistance is diagnosed as a clinical problem for the benzimidazoles (BZ) and avermectin/ milbemycins (AM) classes, reversion to susceptibility is unlikely. With levamisole, there is some indication of reversion back to susceptibility, although any such return is likely to be short and of little long-term benefit.

2. Prevalence of anthelmintic resistance in ruminants

Over the past 20–25 years, we have witnessed a rapid increase in both the prevalence and magnitude of anthelmintic resistance, and this increase appears to be a worldwide phenomenon. *H. contortus*, *T. circumcincta*, *T. colubriformis*, *Ostertagia* spp., and *Cooperia* spp. have acquired resistance, which is confirmed in Australia, New Zealand, South Africa, several European nations, numerous Asian countries, and both American continents. The problem of anthelmintic resistance is clearly worse than current data suggests, as every nation that took part in an occurrence study revealed resistant gastrointestinal worm populations. Several scientific findings suggest that helminth resistance to earlier types of anthelmintics (benzimidazoles, tetrahydropyrimidines, imidazothiazoles, and macrocyclic lactones) is increasing in the Europe to varying degrees (Borgsteede et al., 2007, Sargison et al., 2007). After their introduction to the market, resistance to other anthelmintic classes, notably those employed in sheep and horses, has evolved quickly. Resistance to the imidazothiazole, tetrahydropyrimidine and avermectin-milbemycin classes, for example, evolved in sheep within 3-9 years of their debut to the market (Kaplan, 2004). Anthelmintic resistance is a serious limitation in the sheep sector in Australia today, but it is also present in Europe. Multidrug-resistant (benzimidazoles, imidazothiazoles, and macrocyclic lactones) populations of *Haemonchus contortus*, *Teladorsagia* spp., and *Trichostrongylus* spp. have been found in sheep across Europe.

3. Conclusion

Anthelmintic resistance is a natural evolutionary process that cannot be avoided when anthelmintics are employed on a farm. The frequency of MDR in small ruminant gastrointestinal nematodes is quite high, and many goat and sheep farms currently have resistance to all available anthelmintics. This circumstance poses major issues for parasite management, necessitating the incorporation of innovative nonchemical techniques into parasite control programs. Drug resistance issues in cattle

are less serious, but they are getting worse. Because cattle's gastrointestinal nematodes have lower degrees of resistance, it's possible to apply what we've learned from sheep to change how anthelmintics are administered to increase the long-term viability of chemical-based management.

Conflicts of Interest: The authors declare no conflict of interest.

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Invited lecture/Review

Canine Spaying-Potential Health Benefits and Risks

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Ovariectomy is the most popular and reliable method of reproductive control in dogs. There are many health benefits to spaying. If spayed early enough, the risk of mammary gland neoplasia decreases significantly, and ovariectomy also eliminates the possibility of pyometra. On the other hand, spaying and the absence of gonadal hormones is also associated with an increased prevalence of many health problems. Urinary incontinence, various orthopaedic disorders, endocrinologic disorders such as hypothyroidism, many neoplastic disorders (including hemangiosarcoma, osteosarcoma, lymphoma) and immune-mediated disorders are among the most common health problems associated with early spaying and spaying in general.

Keywords: Spaying; Health benefits; Health risks; Neoplasia; Gonadal hormones exposure; Urinary incontinence

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1. Introduction

Ovariectomy (i.e., removal of the ovaries in female dogs) remains the most reliable method of permanent spaying for reproductive control in dogs (Rooster and Porters, 2017). This method allows us to control pet overpopulation and thus reduce the number of animals in shelters (Kustritz, 2007). Dog owners are often advised to have their dogs spayed for health reasons. Removal of both gonads eliminates pregnancy and parturition related diseases as well as diseases of the ovaries such as cysts and, although rare, ovarian tumours (carcinoma, adenocarcinoma, and teratoma). Although spaying has many positive health effects, research shows not only the benefits associated with spaying, but also many long-term health risks. Some of the long-term risks could include urinary incontinence, a predisposition to various neoplasms, and orthopaedic disorders.

Whether spaying improves or decreases the chances of overall good health depends on the age of the dog at the time of spaying and the relative risk for various health conditions in different breeds. The purpose of this article is to summarise many of the potential health benefits and risks associated with spaying at different ages by reviewing epidemiologic research studies on dogs.

2. Positive effects of spaying

2.1. Mammary gland neoplasia

Ovariectomy is an important preventive measure against mammary gland neoplasia because it reduces the risk of their development, especially when it is performed before the first oestrus cycle. In a study of 599 patients with 160 bitches, that developed mammary gland neoplasia, only 2 tumours were detected in bitches that were spayed early. 13 cases of mammary gland tumours were noted in bitches spayed late and 145 in intact bitches. Spaying after the third oestrus cycle, or when the bitch is older than 4 years, does not significantly reduce the risk of developing a mammary tumour (Borrego, 2017; Beaudu-Lange et al., 2021). Ovariectomy before the first heat could be counterproductive because of other health effects. When deciding to spay early despite other risks, it is important to consider breeds that are predisposed to develop mammary gland neoplasia such as Brittany Spaniel, Dachshund, English Setter, English Springer Spaniel, German Shepherd, Maltese, Miniature Poodle, Pointer, Boxer, Cocker Spaniel and Yorkshire Terrier (Kustritz, 2012). In contrast, Beauvais et al., (2012) reported that there is no clear evidence that spaying reduces the incidence of mammary gland neoplasia.

2.2. Pyometra

Pyometra is bacterial infection of the uterus that frequently occurs in dioestrus in intact females. Pyometra is more commonly diagnosed in certain breeds, such as Bernese Mountain Dogs, Rottweilers, Golden Retrievers, Bernese Mountain Dogs, Cavalier King Charles Spaniels, Rough Collies and Beagles. Approximately 25% of all intact female dogs develop pyometra by the age of 10 under the influence of progesterone endometrial growth and glandular secretion increase. In addition, progesterone stimulates cervical closure and prevents drainage, decreases myometrial activity, and inhibits leukocyte activity in the endometrium. This creates an ideal environment for bacterial growth (Bergström, 2017). The origin of contamination in most cases is the normal vaginal flora. The most commonly isolated bacteria in pyometra are *E. coli*, followed by *Staphylococcus*, *Streptococcus*, *Pseudomonas* and *Proteus* spp. (Memon, 2013). Over the years, cystic endometrial hyperplasia develops with an increased number of cystic glands, resulting in thickening of the endometrium in all intact bitches. This is due to the repeated luteal phases. Cystic hyperplasia is often followed by pyometra, which is why older bitches are more commonly affected. After spaying progesterone secretion decreases and prevents the development of an ideal environment for bacterial growth (Bergström, 2017).

2.3. Diabetes mellitus

Diabetes mellitus is a metabolic disease, more commonly diagnosed in older animals with predisposition of females. Type II diabetes may be caused by increased progesterone levels. The mechanism of action is indirect by stimulating mammary glands to secrete growth hormone, which causes insulin resistance. Prevention of progesterone-induced diabetes mellitus in bitches can be achieved by ovariectomy (Bigliardi et al., 2014).

3. Adverse effects of spaying

3.1. Urinary incontinence

Urinary incontinence is defined as involuntary leakage of urine. It can be acquired or congenital (Pegram et al., 2019). The most common cause of acquired urinary incontinence is incompetence of the urethral sphincter mechanism. This spayed related side effect occurs in up to 20% of females due to decreased urethral muscle activity and periurethral tissue and urethral changes. This is probably caused by the decrease in oestrogen levels and increase in Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) levels after removal of the ovaries. In comparison, urinary incontinence occurs in less than 1% of intact bitches (Rooster and Porters, 2017). Predisposed breeds include Old English Sheepdog, Giant Schnauzer, Weimaraner, Doberman Pinscher, Rottweiler, and Boxer (Byron, 2017). Research on whether age of spaying affects the prevalence of urinary incontinence in female dogs found no relevant association (Bleser et al., 2011).

3.2. Orthopaedic disorders

Spaying increases the risk of orthopaedic diseases, especially in larger breeds, with the exception of Great Dane and Irish Wolfhound (Hart et al., 2020). Bitches spayed early (before 6 months of age) were found to have a 20% incidence of at least one of the orthopaedic disorders (hip dysplasia, elbow dysplasia, and cranial cruciate ligament rupture) was seen in a sample of 472 Golden Retriever females. In contrast, only a 5% prevalence was found in intact Golden Retriever females (Hart et al., 2014).

In 2008, Witsberger reported higher prevalence of cranial cruciate ligament insufficiency in spayed female dogs. An increased prevalence for cranial cruciate ligament injuries was also mentioned by Kutzler in 2020. The incidence in spayed dogs doubled compared to unaltered dogs. The possible mechanism for the higher incidence of cranial cruciate ligament insufficiency in spayed females is an altered angle of the tibial plateau, which affects the act of forces on ligament. Forces on the cranial part of the tibia are increased, which may result in injury to the cranial cruciate ligament. Altered steepness of the tibial plateau is thought to be associated with the expanded growth of the cranial portion of the tibia and early closure of the distal tibial growth plate. Overgrowth of the proximal tibia can be caused by early spaying. A study of 58 dogs with tibial plateau angle higher than 35 degrees plateau and 58 control dogs, found out that dogs from case group were more likely to have been spayed prior to six months of age (Duerr et al., 2007; Griffon et al., 2010). In addition, a study on Golden Retrievers found that spayed females were at substantially higher risk of cranial cruciate ligament rupture (Hart et al., 2014).

Susceptibility to canine hip dysplasia in castrated dogs was evidenced by Witsberger (2008), but not for female dogs. Furthermore, in a study that included 339 female Golden Retrievers with hip dysplasia no association was found between female sterilisation and hip dysplasia (Torres de la Riva et al., 2013). A report by Hart et al. in 2014 showed that the association between the incidence of hip dysplasia and spaying wasn't compelling.

Another orthopaedic disorder affected by spaying is intervertebral disc herniation. A study of the effects spaying on this condition was conducted on Dachshunds and concluded that the risk of intervertebral disc herniation was significantly higher in spayed bitches (Dorn and Seath, 2018).

The side effects of spaying on joint disorders were described primarily for larger breeds, whereas smaller breeds such as Cavalier King Charles Spaniel, Yorkshire Terrier, Pomeranian, Boston Terrier, Chihuahua, Corgi, and Maltese showed no negative effects of gonadectomy on joint disorders (Hart, et al., 2014).

3.3. Endocrinologic disorders

In spayed dogs, the LH blood level is thirty times normal due to the absence of gonadal hormones and decreased compensatory feedback to the hypothalamus and anterior pituitary gland. LH receptors are also present in tissues other than the reproductive system. In the thyroid gland LH receptors are located near TSH receptors. Constant stimulation of LH receptors could affect the normal function of TSH receptors, which would explain the decreased serum levels of thyroid hormones in spayed animals. Spaying could contribute to the development of hypothyroidism (Albright, 2020).

In a study of the epidemiologic feature of canine hypothyroidism it was found that spayed females were much more likely to develop hypothyroidism compared to intact females (Milne, et al. 1981).

Although it was mentioned above that spaying eliminates progesterone-induced diabetes-mellitus, neuter can also trigger the development of diabetes mellitus. This was confirmed for castrated males, which were twice as likely to develop diabetes mellitus compared with the overall male population. The same results weren't observed in female dogs, where there was no higher incidence of diabetes mellitus in spayed bitches (Mattin, et al. 2014).

3.4. Obesity

Obesity is a major health concern in dogs, and the success rate of treatment with food restriction and increased physical activity is often limited. In a study of 229 dogs, more than half were found to be overweight or obese (Sapowicz, et al. 2016). Prevention of obesity is a key to success. For this reason, it is important to know the impact of spaying on a dog's weight (Bjørnvad, et al. 2019). Gonadectomy is thought to increase appetite and stimulate food intake. One possible mechanism would be an increase in cholecystokinin and glucagon through stimulation of the LH receptors, as satiety results from food intake suppressing secretion of gastrointestinal hormones (Kutzler, 2020). This would explain the results of a study conducted in the United States that concluded that gonadectomy increased the risk of developing obesity in dogs (males and females) of all ages (Simpson, et al. 2019). In addition, research on 27627 dogs confirmed that spayed dogs have an increased risk of obesity, that is not influenced by the timing of gonadectomy (Lefebvre, et al. 2013). However, not all studies reach the same conclusion. For example, a study in Denmark did not identify sterilisation as an important risk factor for obesity in bitches (Bjørnvad et al., 2019).

3.5. Neoplastic disorders

3.5.1. Hemangiosarcoma

Hemangiosarcoma (HSA) is a highly malignant neoplasm with poor prognosis and is relatively commonly diagnosed in dogs, accounting for 2% of all neoplasms in dogs. Large breeds of dogs, such as German Shepherd and Golden Retriever, appear to be predisposed to HSA. Although the definitive aetiology of HSA in dogs remains unknown, many studies suggest that gonadectomy, among many other factors, is a risk factor for the development of HSA (Clifford, 2000). One study concluded that spayed females were four times more likely to be diagnosed with cardiac HSA than intact females (Ware and Hopper, 1999). Another study evaluated splenic HSA and found that spayed females were more than twice as likely to be diagnosed with splenic HSA when compared to sexually intact bitches (Prymak, et al. 1988). Finally, a study based on The Veterinary Medical Database was performed with a large population of both sexually intact and gonadectomized dogs diagnosed with HSA in all anatomical areas (spleen, heart, general, skin, etc.). Mentioned study concluded that spayed female dogs were significantly more likely to develop HSA in any anatomical site. Another piece of information to consider when deciding whether or not to spay is the breed factor; German Shepherd, Golden Retriever, and Boxer had the strongest association with a diagnosis of HSA regardless of the neuter status (Robinson, et al. 2020).

3.5.2. Osteosarcoma

Osteosarcoma is the most common type of bone cancer and is relatively common in dogs (Makielski, et al. 2019). In the United States approximately 10,000 dogs are diagnosed with bone sarcoma each year. There are many risk factors for osteosarcoma in dogs, with body size being the most important (Cooley, et al. 2002). A case related study done in 1998 supports this theory by finding that Irish Wolfhound, Saint Bernard, Great Dane, Rottweiler, and Irish Setter, all large dog breeds, are the most common dog breeds diagnosed with osteosarcoma. In the same study spayed female, and spayed male dogs were found to have a significantly higher risk of bone sarcoma. In the aforementioned study, it could not be determined whether the duration of exposure to gonadal hormones had an effect on the risk of developing osteosarcoma, because the information on the age at which the dog was spayed was not known (Ru, 1998).

In another cohort study of Rottweilers, a breed predisposed to osteosarcoma, information on age at spaying was considered. The aforementioned study found, that dogs spayed within the first year had approximately a one-in-four risk of developing osteosarcoma and were significantly more likely to develop osteosarcoma than sexually intact dogs. For each additional month of sexual intactness,

the probability of developing bone sarcoma decreased by 1.4% (Cooley, et al. 2002). Although these findings suggest that gonadal hormones have a protective effect against neoplastic proliferation in bone tissue, the possible mechanism is still largely unknown.

3.5.3. *Lymphoma*

Lymphoma is the most common hematopoietic neoplastic disorder in dogs (Vail, 2017). In humans, females have been found to have non-Hodgkin's lymphoma (NHL) less frequently than males. The incidence of NHL in females increases after the age of fifty, when menopause is regularly reached, suggesting that female gonadal hormones may have a protective effect against NHL. It has been hypothesized that the development of canine lymphoma in intact females is suppressed by endogenous gonadal hormones. The Veterinary Medical Database examined records of 14,000 cases of canine lymphoma registered over a thirty-eight year period and investigated whether sex was a risk factor for lymphoma. This cohort study found that intact female dogs were least likely to develop lymphoma and that spayed dogs of both sexes had a significantly higher risk. The same study also found that Bullmastiff, Boxer, Golden Setter, Scottish Terrier and Bernese Mountain dog had the highest risk of developing lymphoma, but did not investigate whether spaying affected these breeds differently than the general dog population. In the aforementioned study dogs were not differentiated by the age of gonadectomy (Villamil, et al. 2009).

3.6. *Other disorders*

Oestrogen has a trophic effect on the vaginal mucosa and muscular layers that decreases after sterilisation. In addition, anatomical changes of the lower genital tract have been noted in spayed bitches compared to intact females. Vulvar atrophy may also develop as a result of sterilisation, leading to perivulvar dermatitis. In addition, this change in local conditions could affect the composition of resident flora. In a study on the effect of hormone reduction on the vaginal mucosa, it was found that the vaginal mucosal layer in spayed bitches was fragile, as erythrocytes were found in 50% of the smears of intact bitches. In comparison, no erythrocytes were found in the smear samples of intact bitches. However, there were no significant differences between microbiota on the vaginal mucosa of spayed and intact bitches (Rota, et al. 2020).

Spaying is closely related to adverse effects of vaccines. Moore et al. 2005 described that spayed dogs are more likely to develop vaccine adverse reactions within three days of vaccination compared to intact dogs. Furthermore, gonadectomized dogs are more prone to develop immune-mediated thrombocytopenia, atopic dermatitis, inflammatory bowel disease and autoimmune haemolytic anaemia. Spayed females were also more likely to develop lupus erythematosus. Atopic dermatitis occurred in 83 of 9133 (0.91%) intact females and in 745 of 36574 (2.03%) spayed females. Autoimmune haemolytic anaemia was reported in 38 of 9133 (0.42%) intact females and 256 of 36574 (0.7%) spayed females (Sundburg, et al. 2016).

Spaying can result in coat changes. In 2008 a study of 15 spayed female dogs, spaying resulted in changes in hair follicles. In spayed dogs, more hair follicles were in the anagen phase and fewer in the telogen phase. However, coat changes were found in only 20% of the dogs in the form of increased woolly hair (Reichler, et al. 2008).

4. **Conclusions**

To conclude, although spaying is one of the most commonly performed surgical procedures in the veterinary field, the decision should not be straightforward. Spaying brings both positive and negative effects. The decision should take into account the breed, size and age of the dog on which the procedure will be performed, as the effects of spaying can vary for different breeds and age groups. Therefore, it is important that veterinarians are familiar with the latest research on this topic and can provide sound advice to dog owners on whether and when to spay their dog.

Conflicts of Interest: The authors declare no conflict of interest.

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Scientific contribution

Treating Burns in Cats and Dogs Using Medical Honey

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Abstract:

Common causes of burns in small animals are household accidents or iatrogenic burns in veterinary clinics (electric heating pads...). Burns are usually treated as open wounds, where honey can be used as a dressing, that creates a moist healing environment, promotes tissue debridement, eliminates infections, has a deodorizing effect, and reduces inflammation, edema, and exudation. Honey also stimulates angiogenesis, promotes granulation tissue and epithelialization, and reduces scarring. It is important to minimise the potential contamination of burns. The high viscosity of honey acts as a physical barrier against external contaminants and the effectiveness of the barrier is enhanced by the antibacterial properties of honey. In general, antibiotics are not necessary but they are indicated in septic animals. In early stages of burn healing excessive exudate occurs, requiring dressings to be changed up to twice daily. In later stages, they are usually changed every 2-3 days. For uneventful healing, the primary layer should not adhere to the wound, which is not achieved by the application of honey. Therefore, after the application of honey we covered wounds with low-adherent absorbent dressing. We describe the treatment of a chemical burn from an iodine dressing in a cat, a thermal burn from spilled boiling soup in a cat, and a thermal burn from a heating pad in a dog with medical honey. All wounds were treated as open wounds and healed completely by second intention healing.

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Keywords: Burns; Cats; Dogs; Medical honey dressing



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1. Introduction

Burns are painful injuries with partial or complete damage to the skin, and the degree of injury depends on the depth and size of the affected body surface (Tello LH, 2011). Burns are caused by heat (i.e., thermal and steam burns), mechanical friction (rope running across the skin), chemicals (acids, lye), radiation, and/or electricity. Common causes of burns in small animals are either **domestic accidents** (scalding water, caustic chemicals, stoves, fires) or **iatrogenic burns in veterinary clinics** (electric heating pads, dryers, microwaveable devices, hot-water bottles) (Tello LH, 2011; Lagutchik MS and Ford A, 2012).

The classification of burn depth in veterinary medicine differs from the classical classification in human medicine and is based on the layers involved. Superficial burns which correspond to first-degree human burns involve only the outer layer of the epidermis. Sunburns are examples of superficial burns and are common in animals with white coats and in animals with large nonpigmented scars. The skin is red, painful, and hyperaesthetic (Tello LH, 2011). Partial thickness burns correspond to second degree burns in humans and involve the epidermis and superficial layer of the dermis. Blisters, epidermal sloughing, and edema may be seen, and hair is well attached and mixed with yellow exudate. The animals show extreme pain. Full thickness burns, corresponding to third degree burns involve the epidermis, dermis, and subcutaneous tissue. The skin is black, dry, and bloodless, the hair coat is easily epilated, and there is no sensation of pain. Animals with extensive full thickness burns are presented in shock (Tello LH, 2011; Lagutchik MS and Ford A, 2012).

Depending on the body surface area affected, burns are classified as local when less than 20% of the total body surface area is affected and severe when more than 20% of the total body surface area is affected (Johnson RM and Richard R, 2003; Vaughn L and Beckel N, 2012; Vigani A and Culler CA, 2017).

Partial and full thickness burns are more severe due to their extent and their potential life-threatening nature from the development of shock and systemic imbalances (Tello LH, 2011, Lagutchik MS and Ford A, 2012, Gomes P, 2019).

Because of systemic imbalance in patients with severe burn injuries, immediate intervention with treatment of severe metabolic problems (i.e., hypovolemic shock, hyperkalemia, albumin losses, acidosis, anemia, renal failure, etc) and hospitalization is required (Doyle R, 2012). Initial emergency first aid is aimed at applying cold to the burn area with towels soaked in cold tap water or submersion in cold but not iced water. Ice is not appropriate because it causes vasoconstriction and impedes wound healing. Cooling helps limit pain and possibly progression of the burn (Lagutchik MS and Ford A, 2012). Superficial and partial thickness burns are treated either by open wound treatment or, in rare cases where closure is possible, by definitive closure (Tello LH, 2011, Lagutchik MS and Ford A, 2012).

It is important to treat patients with care and administer adequate analgesia to relieve pain, stress, and anxiety. Patients should be heavily sedated or anesthetized before wound treatment (Tello LH, 2011, Lagutchik MS and Ford A, 2012). Pain should be treated with strong opioids such as morphine, fentanyl and buprenorphine (Tello LH, 2011).

Hair must be clipped and lavaged with cold water or saline. Clearly devitalized areas in full thickness burns should be excised. The aim is to maintain a moist wound surface, remove necrotic tissue, and keep the wound clean to promote re-epithelialization from surviving adnexal structures (Doyle R, 2012; Lagutchik MS and Ford A, 2012). Honey has wound healing properties, anti-inflammatory and antioxidant properties, and the ability to scavenge free radical. Honey as a wound dressing provides a moist healing environment, promotes rapid tissue debridement, rapidly eliminates infection, deodorizes, and reduces inflammation, edema, and exudation. It increases the rate of healing by stimulating angiogenesis, promoting granulation tissue and epithelialization, and reducing excessive scarring (Molan PC, 2001; Pavletic MM, 2010), therefore honey is effective in healing burns in dogs and cats (Tashkandi H, 2021).

2. Methods

On the day of presentation of the animal to the clinic, the wound was protected with a sterile swab and the hair was clipped. The wound was irrigated with 500-1000 ml of cold isotonic 0.9% sterile saline. The wound was dried with sterile swabs. The dressing was applied once a day for 7 days, then every other day until the wound healed. The wound was rinsed again before each dressing. For the treatment of the wounds, we used L-Mesitran Soft, wound gel medical honey (Theo Manufacturing BV, Maastricht, The Netherlands), which was applied to the entire wound area and covered with a low-adherent absorbent dressing Melolin (Smith & Nephew Medical Limited, Hull, UK). Melolin was covered with sterile swabs, rolled cotton pad Soffban Natural (BSN Medical, Inc., Charlotte, USA), and a self-adherent elastic wrap Coban™ (3M, St. Paul, USA).

Case 1: A 4-year-old, neutered domestic shorthaired cat weighing 4 kg was presented to the Small Animal Clinic of the Veterinary Faculty, University of Ljubljana, with a wound on the right hind leg of unknown origin. The wound was infected and was treated with iodine dressing.

Case 2: A 3-year-old, neutered domestic shorthaired cat weighing 5.5 kg was presented with an infected and dirty burn in the dorsal thoracic region caused by spilling a boiling soup 3 days earlier.

Case 3: A 1-year-old whippet weighing 11.5 kg was presented for castration. During the procedure, the dog was in a dorsal position on a heating pad. A few days later, the dog was presented with a burn along the entire back.

The areas of wounds in cases 1 and 2 were determined using the Image J programme. All three cases were treated with dressings as described above.

3. Results

Case 1: Iodine dressings caused a severe burn on the entire lower part of the hind leg, most of the skin was sloughed off the following week (Figure 1A) and the cat became septic, which was the reason for not anaesthetizing it and amputating the leg. The wound was classified as a full thickness burn and was 53.4 cm² in size. Using medical honey, the wound healed completely within 108 days with minimal scarring (Figure 1B).



Figure 1: A: full thickness chemical burn in a cat 5 days after iodine dressing; B: leg after complete healing 108 days later.

Case 2: The wound was classified as a superficial burn, was 60.4 cm² in size and healed completely after 37 days without complication (Figure 2)

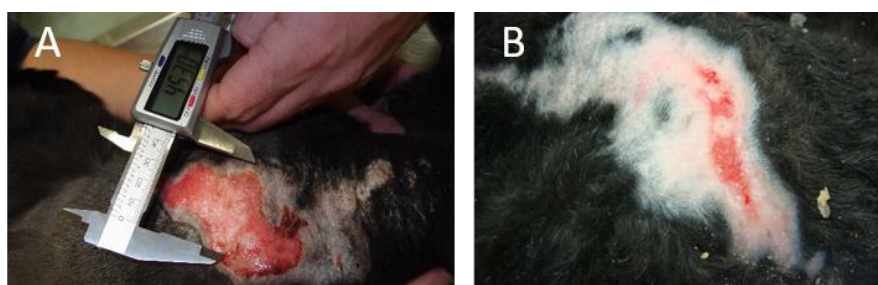


Figure 2: A: Superficial burn in a cat; B: the same area 27 days after presentation to the clinic.

Case 3: The wound was covered with black necrotic skin and was classified as a full thickness burn. The necrotic tissue sloughed off the wound in 13 days, and the wound healed in 31 days (Figure 3)



Figure 3. Full thickness burn in a dog, A: when the animal was presented to the clinic, B: 4 days later, C: 31 days later.

4. Discussion

Burns can cause in metabolic disturbances and secondary infections of the burn site and systemic infections can occur due to decreased neutrophil and immune system function (Tello LH, 2011, Lagutchik MS and Ford A, 2012, Gomes P, 2019). Honey is effective for the prevention burn site infections. Its antibacterial activity is attributed to the synergy of several factors, such as high sugar concentration, low pH, low water content, and the presence of methylglyoxal, hydrogen peroxide, flavonoids, phenolic acids and defensin 1 (Pereira RF and Bartolo PJ, 2013). Systemic antibiotics in burns are generally not recommended except in septic animals, which was the case in our cat with chemical burn.

In the early stages, burns produce a high amount of exudate. Honey applied to the wound does not cause maceration due to its ability to absorb excess exudate. However, the antibacterial efficacy of honey decreases significantly when diluted with wound exudate (Pereira RF and Bartolo PJ, 2013), which is why the dressing must be changed at least once a day in early stages of healing and every 2-3 days in older wounds (Zbuceha A, 2014). According to our previous experience with wound healing using honey and gauze, we found that the gauze adheres to the viable tissue and causes pain and irritation when removed (Lukanc B, et al., 2018; Lukanc B, et al., 2020). In the current study medical honey was applied to the wounds and covered with low-adherent absorbent dressing Melolin. When Melolin was used we observed no pain and no adherence of the dressing to the wound. This is due to the fact that the plastic film prevents the dressing from adhering and the perforations in the plastic film allow the exudate to pass from the wound to the body of the pad. The dressing must be applied correctly and not rub or slip mechanically which would cause additional injury to the burn (Lagutchik MS and Ford A, 2012).

5. Conclusion

Honey for the treatment of burns was successful in cleaning wounds, it inhibited bacterial growth and improved the rate of healing.

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Invited lecture/Scientific contribution

Automated Quantification of Microplastics – Challenges and Opportunities

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Abstract:

Plastics are an important material with widespread applications. However, their widespread use and poor end-of-life management have led to their extensive environmental pollution. They can be found in oceans, terrestrial ecosystems, and even remote corners of the Earth. Current methods for microplastic quantification and identification require big investments and highly trained personnel to operate the analytical equipment. In this paper, we propose an algorithm-based method for the quantification of microplastics in soil and organic fertilisers. The method is based on image analysis of a thinly spread sample that was heated until microplastics has visually melted. The algorithm-based method was validated with Focal plane array detector-based micro-Fourier-transform infrared imaging (FPA- μ FTIR), frequently used in microplastic characterisation. Herein, we present the preliminary results of an ongoing study. In a compost sample, five particles were detected with FPA- μ FTIR, whereas the algorithm detected eight. The algorithm has difficulties recognising elongated or oddly shaped particles. These were identified as several particles which led to overestimating the number of microplastic particles in the investigated sample. We will continue with further development of the computer algorithm by using a training set of images which will be quantified using different methods (visual detection by a human operator, FPA- μ FTIR). This growing training set will enable us to incorporate machine learning algorithms (neural networks) in the development of a more reliable particle detection algorithm. We expect that environmental monitoring of microplastics will be required under future legislation, therefore the development of cheap, user-friendly solutions is crucial.

Keywords: Machine learning; Algorithm; Infrared spectroscopy; Soil contamination; Organic fertilisers; Compost

1. Introduction

1.1. *The need for automation*

Plastics are a widespread material with critical applications across different sectors. However, their increasing production rates and poor end-of-life management are causing widespread pollution (PlasticsEurope, 2021; Geyer et al., 2017). Plastic contamination has now been found in every ecosystem, from oceans to land (Chae and An, 2017; Zhou et al., 2020), and even in the most remote corners of the world (Bergmann et al., 2019; Materić et al., 2022).

Currently, microplastics are isolated from environmental samples and quantified predominantly manually. Despite the numerous reports on the unsuitability of manual identification and sorting of microplastic particles due to human error and bias tendency, this is still the most widely used method, due to its simplicity and wide availability (Silva et al., 2018).

Due to the scale of plastic pollution and the public attention, this has received, we expect that microplastic monitoring will be mandatory under future legislation. For this, user-friendly automated quantification solutions will be of paramount importance.

1.2. *The current state-of-the-art of automated quantification*

In recent years, the development of commercial solutions for automated microplastic analysis, especially in the field of infrared spectroscopy has progressed. Instruments, such as Bruker Lumos II Fourier-transform infrared (FTIR) microscope and Agilent laser-direct infrared (LDIR) Chemical imaging system, are capable of scanning samples directly on a filter or a microscope slide and recording infrared spectra of (microplastic) particles. Spectra are then matched to reference libraries for identification. These instruments offer sophisticated analysis; however, they require a big investment and highly trained personnel to operate them.

Another solution for automated quantification includes custom-built hardware, e.g., fluorescent microscope attachment with blue LEDs for smartphones. Microplastic particles are stained on a filter with Nile Red, which is then excited by the blue LEDs which causes stained microplastics to fluoresce. Quantification is done using MATLAB algorithms (Leonard et al., 2022).

Other research focuses on quantification with near-infrared hyperspectral imaging (NIR-HIS). Hyperspectral cameras scan the sample and obtain a near-infrared spectrum of each pixel on the picture. This enables the recognition of microplastic particles directly on a filter or microscope slide. Quantification is then done manually or with computer algorithms. So far, this method has been demonstrated on artificially prepared (spiked) samples of water and soil (Piarulli et al., 2020; Shan et al., 2018).

1.3. *Our aim*

Timely quantification of microplastic numbers is critical for predicting the ecosystems' health. However, current methods for microplastic quantification are inadequate to handle high-frequency sample quantification. We, therefore, aim to develop an automated widely accessible analytical tool using image processing with a machine learning algorithm for the quantification of microplastic particles. In this method, there is no need for specialised hardware. A regular camera or smartphone camera can be used to record sample photos. The work is still in progress, and we, herein present preliminary results, challenges to overcome and opportunities for achieving our aim.

2. Methods

2.1. *Experimental design*

The objective of the study was to compare two methods for microplastic quantification: (1) Focal plane array detector-based micro-Fourier-transform infrared imaging (FPA- μ FTIR), frequently used

in microplastic characterisation, and (2) algorithm-based image processing, which showed the potential to be more time-efficient and user friendly.

Samples were extracted from soil and compost by density separation with saturated ZnCl₂ solution as reported by Prosenč et al., 2021. In short, 10 g of sample was placed in a 50 mL centrifuge tube, and ZnCl₂ (density 1.6 g cm⁻³) was added to the 50 mL label. The tube was then shaken vigorously for 30 sec. Samples were centrifuged for 15 min at 6000 rpm. The supernatant was filtered with a 45 µm glass fibre filter. The filters were dried at 60 °C and microplastics and the remaining debris were collected in a glass vial. A portion of the extract (1/10th of mass) was spread out to a 1 cm² area on a silicon wafer.

2.2. Infrared spectroscopy for detection of microplastics

First, the samples were analysed with a Hyperion 3000 FTIR microscope (Bruker Optics GmbH, Germany) equipped with a focal plane array (FPA) detector with 128 × 128 detector elements. Measurements were performed in reflection mode using a 15× IR objective with a 350 × 350 µm field of view. The signal coming from the 128 × 128 elements was averaged into 8 × 8 pixels, resulting in a final spatial resolution of 43.75 µm per pixel. All spectra were recorded using 16 accumulations at a resolution of 8 cm⁻¹ between 3850 and 900 cm⁻¹. Spectra processing was done in the OPUS 8.5 software (Bruker Optics).

2.3. Algorithm-based recognition of microplastics

After the FTIR analysis, photos of samples were recorded with a camera (Cyber-shot DSC-RX100 II, Sony, Japan). Samples were heated on a hotplate (Schott Ceran Top Line 2000, Rommelsbacher, Germany) while recording the temperature until microplastic particles were visibly melted. During the heating of the samples, a series of high-resolution snapshots were taken in continuous mode (1 per second).

The photos were then processed with a computer algorithm developed to streamline the detection of microplastic particles during the sample heating process. This algorithm uses snapshots of the melting process and calculates differences between subsequent snapshots. Small differences must be neglected due to the slight movement of particles during heating, but large differences indicate the presence of microplastic particles, since these particles melt during the heating process, changing both, shape and colour.

2. Results

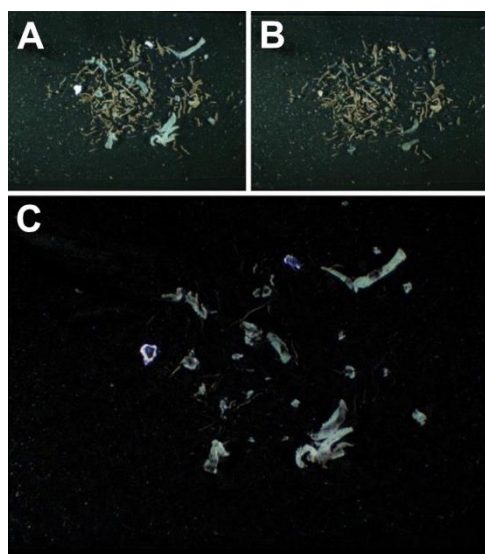


Figure 1. Processing of samples and creating a probability mask for potential microplastic particles. A – a photograph of a soil sample, containing microplastic particles; B – sample after heating; C – a probability mask with unchanged particles filtered out.

2.1. The principle

The computer algorithm developed for automated microplastic quantification first calculates differences between sample pictures before and after heating. When heated, thermoplastic microplastics melt and change shape and colour, while other particles remain unchanged. This creates a mask of the probability of microplastic particle presence. The probability mask is further processed to determine possible microplastic particle locations on the image and filters out particles that did not change shape and colour during the heating process (**Figure 1**). During the filtering step, certain assumptions on expected microplastic particle shape and size are made to minimise the number of false-positive identifications.

2.2. Quantification of microplastics in a compost sample

The computer algorithm was trialled for automated microplastic quantification in a compost sample that underwent microplastic extraction. It detected eight potential microplastic particles. The algorithm correctly located three microplastic particles (**Figure 2C**), however, the detection at two of these was multiple, e.g., three particles recognised in place of one. The algorithm also has difficulties with elongated or oddly shaped microplastic particles. These are usually identified as several particles which could lead to overestimating the number of microplastic particles in the investigated sample. Another detection was a non-plastic particle (**Figure 2**, red arrow). This particle moved during the heating process due to convection and was therefore recognised as a particle that changed shape by the algorithm. This could potentially be avoided by using a slower heating process and further development of the computer algorithm. One larger particle (bottom left) and several smaller ones (centre) in this sample were not recognised by the algorithm, as can be seen from the validation step (**Figure 3**).



Figure 2. Subsequent images of a compost sample, spread across a 1 cm² area. A – before heating, B – after heating, C – algorithm processed.

The same sample that was processed with the algorithm, was validated with FPA- μ FTIR, a frequently used analytical technique in microplastic identification and characterisation (Primpke et al., 2017). With this method, we mapped four larger particles (**Figure 3A, B, and D**) and possibly several smaller ones (**Figure 3C**).

Integration at different infrared (IR) regions (at different wavenumbers) reveals particles absorbing in those IR regions. In this sample, integrations in the following regions 1405-1490 cm⁻¹, 1455-1490 cm⁻¹, 2820-2970 cm⁻¹, gave a good signal for four larger particles that were identified as polyethylene (PE) (**Figure 4A, B, and D**). The several smaller particles (integrated between 1715 and 1760 cm⁻¹) were identified as polyester (PES) (**Figure 4C**). These could be several smaller particles or a cluster of fibres spanning across several focal planes, where only parts of fibres were in focus and gave a good enough IR signal. We found that the optimal IR signal is usually acquired at or just below the visual focus. This can be problematic with bigger particles because all particles in the sample might not be focused at the optimal focal plane and a compromise has to be made to acquire a signal for as many particles as possible.

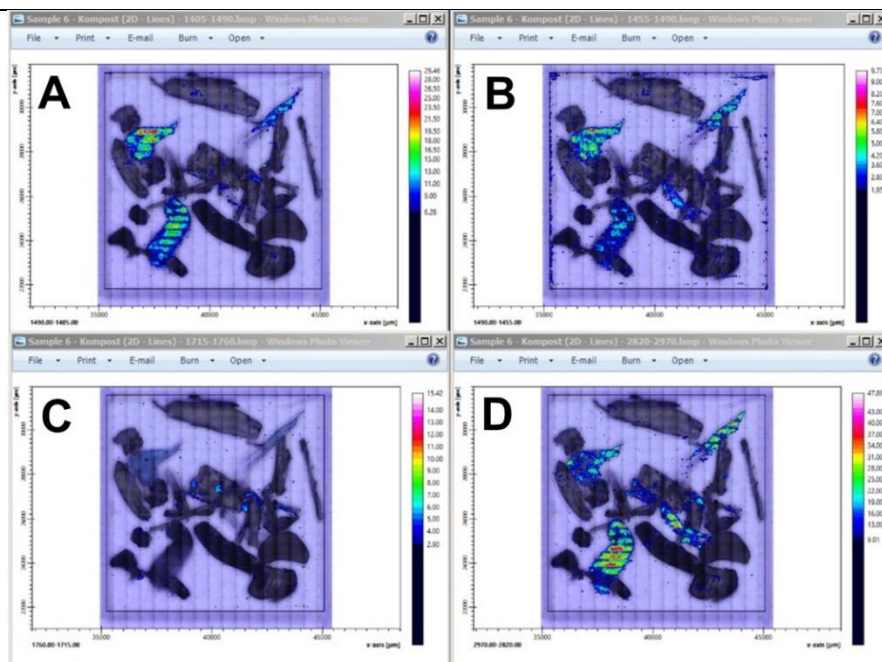


Figure 3. Infrared signal at different wavenumber integrations. Different plastic polymers give signals at different wavenumber integrations. A – integration at 1405-1490 cm^{-1} , B – integration at 1455-1490 cm^{-1} , C – integration at 1715 and 1760 cm^{-1} , and D – integration at 2820-2970 cm^{-1} .

The detection was matched in three out of five microplastic particles between the two methods (neglecting multiple detections of the same particle by the algorithm and assuming that the cluster of smaller PES particles detected by FPA- μ FTIR was one particle). Our future work will focus on the further development of the computer algorithm. Currently implemented detection based on calculating colour and shape differences does not give optimal results for elongated or oddly shaped particles and particles that are stained and subsequently do not change colour during the heating process. We are constantly building a base of samples, which are quantified using different methods (visual detection by a human operator, FPA- μ FTIR). This growing training set will enable us to incorporate machine learning algorithms (neural networks) in the development of a more reliable particle detection algorithm.

3. Discussion

This paper presents preliminary results of ongoing research, and the principles used in the development of a method for automated quantification of microplastics. This method has similar challenges to methods reported in the introduction. When working with solid matrices, especially rich in organic matter, such as soil and organic fertilisers, the presence of some residual debris is unavoidable. It can be significantly reduced by oxidation and digestion procedures (Hurley et al., 2018), but some remains in the extract. In methods relying on visual techniques, particles obscuring microplastic can be an issue, therefore, a lot of care should be taken when preparing the sample, e.g., spreading it out thinly, ensuring that particles are not obscuring other particles, etc.

Another issue with visual techniques is the challenges related to image capturing. This goes for the algorithm-based method as well as the FPA- μ FITR method. The big particles are spreading across several focal planes, which makes obtaining a good focus and therefore a strong and even infrared signal with FPA- μ FTIR, difficult. When capturing photographs for the algorithm processing, high-resolution images, and constant camera settings during the heating process (F-stop, exposure, ISO value) are crucial.

The limitation of this and other heating-related methods (e.g., pyrolysis GC-MS) is that they are not appropriate for thermosets. These are plastic polymers that become set in their physical and chemical properties after initial heat treatment but cannot be remoulded or heated after the initial forming.

Examples include epoxy, polyurethane (PU), and silicone, amongst others. However, the most commonly found microplastics in the environment are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and polyethylene terephthalate (PET), all belonging to the thermoplastics class (Nerland et al., 2014), therefore, this method could importantly contribute to the microplastic monitoring efforts.

Despite the challenges, this method also offers an opportunity to couple fast, automated, and user-friendly quantification of microplastics with potential identification of microplastic polymers. During the heating process, the temperature of the hotplate is recorded. We will couple the recorded temperature to the melted plastic particles in a time sequence. Due to the different melting temperatures of polymers, we will be able to estimate the polymer composition of different particles.

The challenges in automated, affordable, and user-friendly quantification of microplastics are vast. However, microplastics research, including their analysis, is a fast-paced field with new solutions emerging regularly. We expect that microplastic monitoring will be regulated in future legislation, therefore, the availability of high-throughput reliable methods is essential.

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Conflicts of Interest: The authors declare no conflict of interest.

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 Scientific contribution/Original research

Insights into Microplastics: from Physical and Chemical Characterisation to its Potential as a Vector.

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Abstract:

Particles with the largest dimension of less than 5 mm, also termed as microplastics (MPs), gained a lot of scientific and media attention in the last decade. MPs in the environment are of importance because of their potential for further fragmentation, accumulation, and impact on biota in the terrestrial and water environments. MPs research is challenging due to their diversity in size, shape, and chemical structure. For research purposes, MP particles can be purchased, but in terms of chemical structure, their properties might not correspond to the ones of MPs found in the environment. Compared to purchased MPs, plastic products in the environment can contain different additives, despite being the same polymer type as purchased MPs. Therefore, for environmental studies preparation of MP particles from plastic products is preferable. In this contribution two methods for laboratory preparation of MP particles, different sizes and polymer types, are presented. Method using ultrasound probe was found to be suitable for obtaining polyester fibres from thin sewing thread, while cryogenic milling was found to be preferable method for MPs preparation from larger and thicker plastic particles. In this way, MPs of other types of plastic (polyethylene terephthalate, polystyrene, polyvinyl chloride, polypropylene) were prepared from beforehand manually cut plastic particles, originating from everyday plastic products.

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Keywords: Microplastics; Cryomilling; Separation; Challenges of preparation; Surface topology

1. Introduction

Industrial development and increased usage of single-use plastic products in everyday life consequently result in increased plastics production. In 2018, the global plastics production was reported to be 359 million tonnes and it increased to 381 million tonnes in 2019, with China contributing the highest share (PlasticsEurope, 2020). In Europe, however, a slight decrease is observed in yearly plastics production from 2017. Data show that plastics production in Europe decreased from 61.8 million tonnes in 2018 to 57.9 million tonnes in 2019 (PlasticsEurope, 2020). Lately, due to the pandemic, a sharp rise in use of disposable plastics, for convenience or hygiene (e.g., pandemic) is observed all over the world. Inevitable issue of extensive plastics production and its use is the occurrence and formation of microplastics particles (MPs) in the environment.

MPs have gained a lot of attention in the past years, especially due to their ubiquitous presence around the world and in various environments, such as oceans (e.g. Great Pacific Garbage Patch), freshwater, wastewater, soil and air (Van A, et al. 2012; Hoellein TJ, et al. 2017; Magni S, et al. 2019; Huang Y, et al. 2020; Chen G, et al. 2020). Their presence has been linked to various risks and impacts on biota in the environment and indirectly to human health due to its fragmentation and leaching of additives, added during plastic production process, and other contaminants (Horodytska O, et al. 2020). In the future, even more attention could be given to the MPs, since their further fragmentation can result in nanoplastics, which are potentially one of the most hazardous type of debris in marine environment due to their possible uptake by marine animals into their bodies (Koelmans AA, et al. 2019).

MPs research is challenging due to their four characteristics: origin, shape, size, and polymer type. Origin of MPs is interconnected with the shape of MPs. Namely, based on their origin, MPs can be divided into primary MPs and secondary MPs. Primary MPs are intentionally produced in such small size for its use in e.g. personal hygiene products, pharmaceutical industry, and are mainly found in the shape of spheres and cylindrical pellets (Hartmann NB, et al. 2019; Cole M, et al. 2011; Hernandez LM, et al. 2017). Secondary MPs, however, are formed by fragmentation of larger plastic debris under the influence of mechanical, physicochemical, and biological factors, such as ultraviolet light, temperature, pH, abrasion forces and microbial degradation. Fragmented particles can be found in the shape of films, fibres and fragments (Hartmann NB, et al. 2019; Cole M, et al. 2011; Ngo PL, et al. 2019).

MPs are, as derived from marine MPs research, commonly known as particles or fragments with the size of less than 5 mm in diameter (GESAMP, 2016). Despite its widespread use in research studies, the uniform definition of MPs among scientific community is not yet established. Scientist have proposed different classifications, that differ on upper and/or lower size limits of particles to be termed as MPs. Some propose the lower size limit for MPs should be 1 mm (Ivleva NP, et al. 2017), while other suggest 100 nm (Rios Mendoza LM, et al. 2018). The latest scientific discussion from European researchers on the topic of terminology used in plastic debris research (Hartmann NB, et al. 2019) recommends classification of plastic debris to be based on the chemical composition, solid state, solubility, size, shape, colour and origin. According to the size, Hartmann et al. (2019) propose classification of particles into four groups: nanoplastics (1 to <1000 nm), microplastic (1 to < 1000 μ m), mesoplastics (1 to < 10 mm) and macroplastics (1 cm and larger).

MPs size is challenging from the analytical point of view, especially for quantification. In fact, for MPs identification, the most developed and verified methods so far, are the non-destructive infrared spectroscopy methods and the destructive ones, named thermochemical methods. In the group of non-destructive infrared spectroscopy methods belong the attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR), micro-FTIR (μ -FTIR) and Raman spectroscopy. Whereas in the group of destructive methods, the two main important are pyrolysis gas chromatography mass spectrometry (Py-GC-MS) and thermogravimetry with additional thermal desorption gas chromatography mass spectrometry analyses (TED-GC-MS) (Pico Y, et al. 2019; Krauskopf L-M, et al. 2020; Dümichen E, et al. 2017). With these methods polymer type of MPs can be determined.

Plastic products are made out of numerous polymer types as well as copolymers, to which many additives can be added (Hartmann NB, et al. 2019; Kusch P and Knupp G, 2004). The most abundant

polymer types, determined in plastic debris in the environment, are polyethylene (PE), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polyester (PES) and polypropylene (PP), and polyamides (PA) (Gatidou G, et al. 2018; Sang W, et al. 2021; Díaz-Jaramillo M, et al. 2021).

The overall issue regarding MPs is not only their presence as environmental micropollutants, rather their vector potential. Since their physical presence as particles and their mostly hydrophobic surface nature, they can act as an adsorption site for organic contaminants, such as pharmaceutical residues, pesticides, polychlorinated biphenyls and polycyclic aromatic hydrocarbons, as well as for inorganic contaminants, like heavy metals (Zhang H, et al. 2018; Dong Y, et al. 2020; Wang F, et al. 2020; Šunta U, et al. 2020; Bhattacharya A, et al. 2019).

To perform environmentally applicable adsorption studies for contaminants in laboratory settings, MPs of defined size and polymer type have to be purchased or prepared. Since purchasing of MP particles is not possible at all needed sizes and types, here it is a representation of two manners in which MPs of desired sizes can be prepared in laboratory, using ultrasound treatment and cryogenic milling.

2. Methods

MP particles (1 mm-100 μm) for laboratory research purposes were prepared with cryogenic milling (fragments) and ultrasound treatment (fibres). Cryogenic milling with liquid nitrogen (LN, Messer, Germany) and ball mill (MillMix 20, Tehnica, Slovenia) was used to prepare samples of PET, PS, PVC, PE and PP. 3 g of MPs of individual polymer type (size range 1 -5 mm) was placed in the grinding jar containing a stainless-steel milling ball. Before milling, grinding jar was submerged in LN for 6 min and afterwards milled in 4 series for 2 min at 35 Hz (PET) and at 25 Hz (PS, PVC, PP, PE) with intermediate cooling in LN for 1 min. Obtained particles were additionally sieved through 1 mm (Retsch, Germany) and 100 μm (Fipis, Slovenia) stainless steel sieve to obtain particles of desired size (1 mm - 100 μm). Scanning electron microscopy (SEM) was used to examine the morphology of particles. Original particles (5 mm - 1 mm) and cryogenically milled particles (1 mm - 100 μm) were coated with Au/Pd (PECS Gatan 682), placed on the double-sided adhesive carbon tape on the aluminum stubs, and analysed by JEOL JSM-6500F Field-Emission Scanning Electron Microscope (JEOL LTd, Japan) operated at 15.0 kV (Gniadek M and Dąbrowska A, 2019; Božič D, et al., 2021).

Ultrasound separation. The ultrasound probe (Labsonic M, Sartorius, Germany) was used to disperse the sewing thread into individual fibres. 50 mg of PES sewing thread (Moon, UK), that was beforehand manually cut into 1 mm pieces, was placed into a beaker, containing 25 mL of dichloromethane (Sigma Aldrich, USA). Dichloromethane was used because no attachment to the probe or to the glass walls of the beaker was observed, contrary to other media. The probe (\varnothing 3 mm) was then placed into the dichloromethane mixture and operated in continuous mode for 5 min (0.8 cycle, 60 % amplitude). Following the separation, mixture was filtered through 0.45 μm cellulose acetate (CA) filter (Sartorius, Germany). Obtained fibres were transferred from the CA filter into a glass Petri dish, half covered with glass lid, and dried in the oven at 65°C for 30 min. The dispersion of thread to fibres was examined under optical microscope Olympus CX21 (Japan).

3. Results and Discussion

Commercially available MPs and environmental plastics might seem similar in shape or/and size, however, they differ in the composition regarding additives and surfactant content, as well as morphology and density. Therefore, purchased MPs are not necessarily comparable to the MPs found in the environment (Eitzen L, et al. 2019). Laboratory pre-treatment enabling the preparation of MPs of defined size rely on cryomilling or cryogenic milling. This is a milling process performed near LN temperature (- 180 °C). It is achieved using cryomill or the combination of the immersion of grinding jar, containing the sample, in LN and high-energy milling (e.g., ball mill). Subsequent sieving can be used to obtain particles of the desired size range (Eitzen L, et al. 2019; Lagarde F, et al. 2016; Bai C, et al. 2000). Cryomilling has been used in numerous studies regarding MPs, either as a preparation technique for obtaining particles of specific size ranges (below 1 mm) (Eitzen L, et al. 2019; Bannick CG, et al. 2019; Capolupo M, et al. 2020) or as a technique for samples homogenisation before MPs determination (Dümichen E, et al. 2017).

In this study the cryogenic ball grinding process was used to obtain a fine fraction with PP, PS and PET particles in the size between 100 μm and 1 mm. However, there were few difficulties regarding the PVC and PE cryogenically milled particles. Since PVC fragments originated from tablet pharmaceutical packaging partially covered with aluminium, it was difficult to distinguish PVC particles from the aluminium residue. In case of PE grinding, the glass transition temperature (T_g) was crucial. PE has the lowest glass transition temperature (T_g) from all polymers, ranging from -100°C to -70°C (Eitzen L, et al. 2019). PE particles were not milled into finer particles (1 mm - 100 μm), rather a colour change was noticed, due to their heat-oxidation (Rugg FM, et al. 1954). For cryogenic milling of PE particles, therefore, the cryomilling procedure needs to be improved, implementing longer pre-cooling time and/or shorter grinding cycle.

An insight in the topological diversities in MPs' surfaces after cryogenic milling, was achieved with SEM microscopy (**Figure 1**). PET cryogenically grinded particles (1 mm - 100 μm) had more ragged surface compared to the manually cut particles from PET bottle (5 mm - 1 mm). This indicates that MPs particles of smaller sizes have more potential for interactions with organic pollutants, as well as for microorganisms to adhere and form biofilm. The effect of MPs size on arsenic adsorption onto PS MPs was confirmed in study by Dong et al. (2020), where the amount of adsorbed arsenic was decreasing with increasing MPs size.

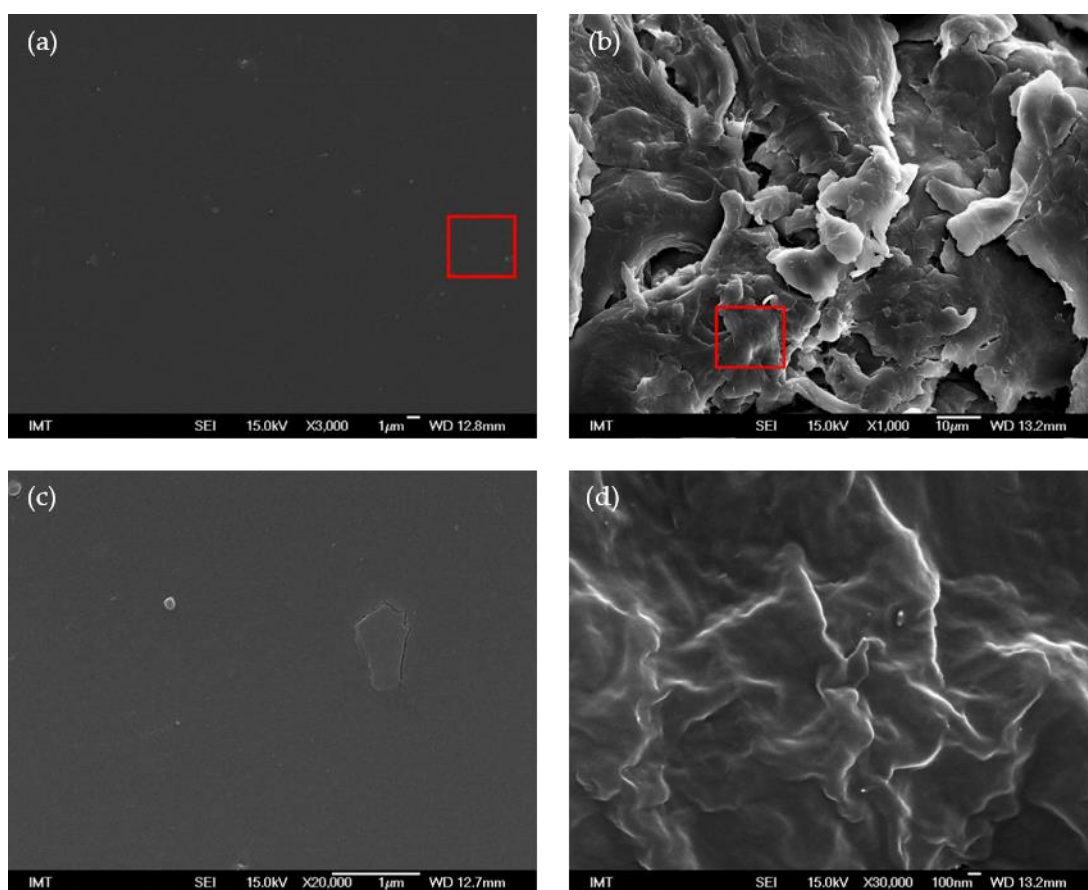


Figure 1: Pre-cut fragment (5 mm - 1 mm) and cryomilled particle (1 mm - 100 μm) of PET under SEM microscope at 3 000 x magnification (a), 1 000 x magnification (b), 20 000 x magnification (c) and 30 000 x magnification (d). Red rectangles in (a) and (b) indicate the areas containing the structures shown in (c) and (d).

The cryogenic milling has beside roughness also some other physical consequences, which influence MPs and the course of the experimental studies and its results. Density and surface charge are two of those. Eitzen et al. (2019) observed that some cryomilled PS particles did not completely disperse in water and were accumulating along the glass walls. Obtained cryomilled particles can become

charged and consequently float in the selected medium when according to their density they should sink or be submerged (von der Esch E, et al. 2020).

Therefore, von der Esch et al. (2020) developed and specified a method for secondary MPs generation in laboratory setting using ultrasonic bath. In this study fragmentation of PS, PET, PE, PP, PVC, PA and polylactic acid polymer particles was tested. Just before that study, we have tested the preparation of PES fibres from a sewing thread using ultrasound probe. Sewing thread was successfully dispersed to individual PES fibres (**Figure 2**), however, due to the chosen medium (dichloromethane), leaching of the colour from coloured sewing thread was observed. This indicates that the solvent might be too strong for PES fibres and damages of fibre's surface and structure can occur. Selection of medium for dispersion has to be taken into account when preparing the material for the experiment. In the method proposed by von der Esch et al. (2020), 0.25 M potassium hydroxide was used, which supposedly does not dissolve MPs.

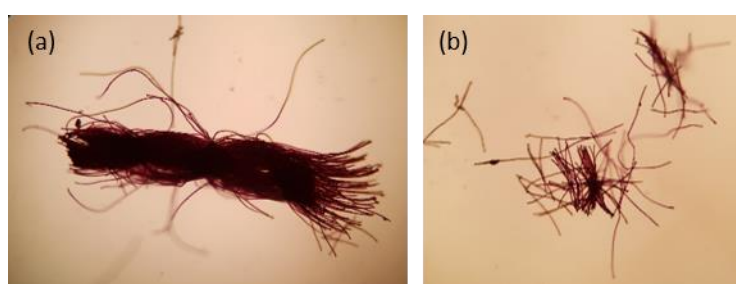


Figure 2: Polyester sewing thread before (a) and fibres after (b) dispersion with ultrasound probe treatment under 40x magnification.

4. Conclusions

MPs have gained a lot of scientific attention in the last decade in various scientific disciplines. For studying the effects of MPs in the environment, however, the purchased particles might not be representative material to use in research studies. They can differ in chemical structure and additive content, compared to the MP particles found in the environment. In this contribution two manners for successful MPs preparation in laboratory setting from plastic products we use in everyday life are presented, one using cryogenic milling and the other using ultrasound probe.

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Conflicts of Interest: The authors declare no conflict of interest.

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 Invited lecture/Scientific contribution/Original research

Scanning Electron Microscope Images of HUVEC Cells Treated with Materials Used for Processing of Orthopaedic and Dental Implants

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Abstract:

Use of orthopedic implants (OI) and dental implants (DI) is increasing due to obesity and ageing of the population. To increase the bio-functionality of metallic biomaterials, used for OI and DI, it is important to modify their surface composition, roughness, and structure without altering their mechanical properties. Different materials, such as minerals and inorganic compounds are used for coating OI and DI, however, they may cause response of the cells that are in contact with them in the body. To optimize the use of the materials in implant design, it is of interest to study the effect of the materials on cells. Here we present observations of micron-sized particles of milled Al₂O₃, TiO₂ and hydroxyapatite (HA) on human umbilical vein endothelial cells (HUVEC) by scanning electron microscope. We observed morphological changes of the cells – budding of the cell membrane. Comparing to the control, more cells were detached from the glass they were grown on, indicating possibility of increased cell death or inability of the cells to attach to the surface. Described changes can be due to oxidative stress and inflammatory response of the treated cells.

Keywords: Orthopedic implants; Inorganic coatings; Dental implants; in vitro cell lines; Inflammatory response; Oxidative stress

1. Introduction

1.1. Coating of orthopaedic and dental implants

With bio ceramic coatings and coatings made of minerals and inorganic compounds the life-time stability and biomineralization of metallic OI and DI with bone can be enhanced. Despite the progress made in fabrication of particles of different sizes, morphologies, and chemical properties, we do not fully understand how particle properties modulate immune responses in human body. Previous reports indicate that ceramics is not as bio-inert as suggested (Lee et al., 2017; Malem et al., 2013; Campbell et al., 2017). Materials used for coatings are called bioactive and are important for clinical use as bone-repairing materials (Dolinar et al., 2018). Implant surfaces achieve faster osseointegration and a stronger bone to implant interface, therefore it is important that used materials are being improved continuously (Ting et al., 2017). Bioactive ceramic coatings are utilised due to their chemical stability and ionic dissolution yet understanding of the interactions between implanted materials and host cells is of interest in recent decades. Small defects and injury in maxillo-facial region can heal on their own in healthy people, but DI are necessary to treat big defects in hard and soft tissues (Zeng et al., 2018). Hydroxyapatite (Ahn et al., 2018) and titanium and titanium alloys (for example TiO₂) are currently most used implant materials in clinical dentistry (Saito et al., 2021) and in orthopaedy of deteriorated hip and knee joints, due to their favourable mechanical properties and biocompatibility (Siebers et al., 2005). Hydroxyapatite spontaneously forms a bone-like apatite layer on its surface and bond with the bone once OI or DI is placed in human body (Kokubo et al., 2004). Survival rate of joint replacement is high yet risk for inflammation without fatal outcome and therefore need for revision surgeries is common. Prevalent reason for revision surgery is aseptic loosening which often occurs as collateral to osteolysis caused by immune-mediated inflammation responses to debris from materials, used in OI and DI (Tsaousi et al., 2010).

1.2. Implant debris associated inflammation, oxidative stress and cytotoxicity

Cells of the innate immune system (e.g. macrophages, dendritic cells, neutrophils) are believed to be the first to response to implantation of a biomaterial, with the phenotype of these cells modulated by the structure and composition of the implant (Anderson et al., 2008). Macrophages respond around implanted material (Sussman et al., 2014) and dendritic cells recognize response of other cells to implantation, when they are damaged, stressed or necrotic and produce danger-associated molecular patterns (Gallo and Gallucci, 2013). Also, the type of biomaterial implanted can impact the maturation of dendritic cells which activate the adaptive immune system (Carroll et al., 2016). Metal debris from COI has previously been linked to the development of inflammatory pseudotumours (Jamieson et al., 2021). Pseudotumours are made of soft tissue mass composed of different inflammatory cells such as macrophages and T cells which are localized near the COI (Hart et al., 2012). Because of the common use of COI, investigation of potential inflammatory responses to ceramics is becoming more and more important. Previous results indicate that needle-shaped and smaller HA particles significantly enhance cytokine secretion, while larger smooth spherical particles did not. These findings indicate that HA particles have the ability to regulate immune responses that are induced after biomaterial implantation (Lebre et al., 2017). Titanium oxide (TiO₂) is one of the most used materials in clinical dentistry. Osseointegration is achieved when implanted TiO₂ attaches to alveolar bone (Brånemark, 1983). It is chemically stable and is suggested to be bioinert – not causing inflammation and cytotoxicity in periodontal tissue (Saito et al. 2021). However, highly concentrated fluorides used for caries prevention (Schiff et al., 2002) can corrode TiO₂ and titanium ions can elute into the body (Rodrigues et al., 2013) which can induce peri-implantitis and allergic reaction (Delgado-Ruiz and Romanos, 2018). This means that titanium loses biocompatibility in acidic environments (Saito et al., 2021). TiO₂ nanoparticles (NPs) stimulate a wide array of oxidative stress related pathways. The use of TiO₂ nanotube-coated titanium implants is on the other hand suggested to reduce oxidative stress and promote osteogenesis in bone remodeling (Abdulhameed et al., 2022).

It is the aim of this work to contribute to better understanding of the physicochemical and biological effects taking place at the implant-tissue interface. Previous studies indicate that the surface properties of the implant including particle debris that forms in processing of the implants may have important impact on the adjacent cells when implanted in the body (Jenko et al., 2017, Feizpour et al., 2019). It is of particular interest to analyse the effect of small particles that are formed in the processing of the implants and particles found on the surface of retrieved implants that underwent fail-

ure (Avsec et al., 2019). We focused on the effect of micron-sized particles of Al₂O₃, TiO₂ and hydroxyapatite (HA) on inflammatory, oxidative stress-related features in two cultures of human umbilical vein endothelial cells - HUVEC cells.

2. Methods

2.1. Treatment of the Cells

HUVEC cells were placed in 6-well plate with glass disc on the bottom of each well. 20 × 10⁴ cells/well were in 6-well plate for 24 hours for cells to attach to the glass surface. Cells were then exposed to TiO₂ and hydroxyapatite (HA) particles and three different corundum ceramic particles: u-Al₂O₃ – used white fused alumina, Al₂O₃ – unused white fused alumina, Al₂O₃-SiZrO₄ nanocomposites: aluminium oxide and zirconium silicate for 24 hours.

Original-sized particles, provided by Institute of Metals and Technology (IMT) were milled in smaller particles using mill (Milimix 20, Domel, Slovenia). Cells were exposed to micron-sized particles at concentrations of 10, 50 and 100 µg/mL for 24 hours.

2.3. Scanning electron microscopy

Small cellular particles (SCPs) formation by cells is considered a physiological process (Hurley et al., 2010) that can be accelerated by oxidative stress (Borras, et al. 2020) and by inflammation process (Chaar et al., 2011). (Yarana and St Clair, 2017)) suggested that during oxidative stress, oxidized proteins are formed, and cells release SCPs as a compensatory mechanism to maintain homeostasis. To observe the processes leading to SCP release, after treatment cell samples were fixed using standard protocol. Cells were fixed in Karnovski fixative (1 mL of 25% glutaraldehyde, 0,5 mL of 8% paraformaldehyde, 8,5 mL of Na-P buffer) for 12 hours at 4 °C, washed 3 times for 10 minutes with Na-P buffer (36% of component A*, 14% of component B**, 50 % of dH₂O); *3,561g Na₂HPO₄×2H₂O + 100 ml dH₂O, **3,131g NaH₂PO₄×2H₂O + 100 ml dH₂O, incubated with added 1% OsO₄ for 1 hour, washed 3 times for 10 minutes with dH₂O, incubated with added TCH in dH₂O for 15 minutes, washed 3 times for 10 minutes with dH₂O, incubated with added 1% OsO₄ for 1 hour, washed 3 times for 10 minutes with dH₂O, dehydrated in graded ethanol (EtOH): 30%, 50%, 70%, 80% and 90% for 10 minutes at each concentration and in absolute EtOH 2-times for 10 minutes, incubated with added absolute EtOH and HMDS (ratio 3:7) for 10 minutes, incubated in added absolute EtOH and HMDS (ratio 1:1) for 10 minutes, incubated in added 100% HMDS for 10 minutes and depleted of HMDS by evaporation in exicator with silica gel for 12 hours. After fixation, samples were gold-sputtered and observed by the scanning electron microscope (SEM, JEOL JSM-6500F).

3. Results

SEM images revealed morphological features of the treated cells – budding of the cell membrane and detachment of the cells from the glass disk surface. No such features were observed in control – untreated cells (**Figure 1**). Untreated cells were better attached to the glass disk surface while treated cells were detached at several places. Also, budding of the membrane can be seen in treated cells (pointed to with white arrows in **Figure 1**).

4. Conclusions

Membrane budding of the cells that can be seen after treatment micron-sized particles, used for OI and DI is a key step in vesicular transport, multivesicular body and exosome biogenesis (Hurley et al., 2010). We noticed that untreated cells are more attached to the glass disk surface, on the other hand, treated cells are at many parts detached, indicating cell death or inability of cells adhesion to the surface. Cell adhesion is essential for cell integrity, cell growth, and communication with other cells, therefore detachment from the surface represents adverse effects of treatment to the HUVEC cells. Inflammation process and oxidative stress are two important factors that can contribute to reduction of cell adhesion. Cell inflammatory and oxidative stress response correlate with vesiculation process (Jan et al., 2021), indicated by budding of the membrane.

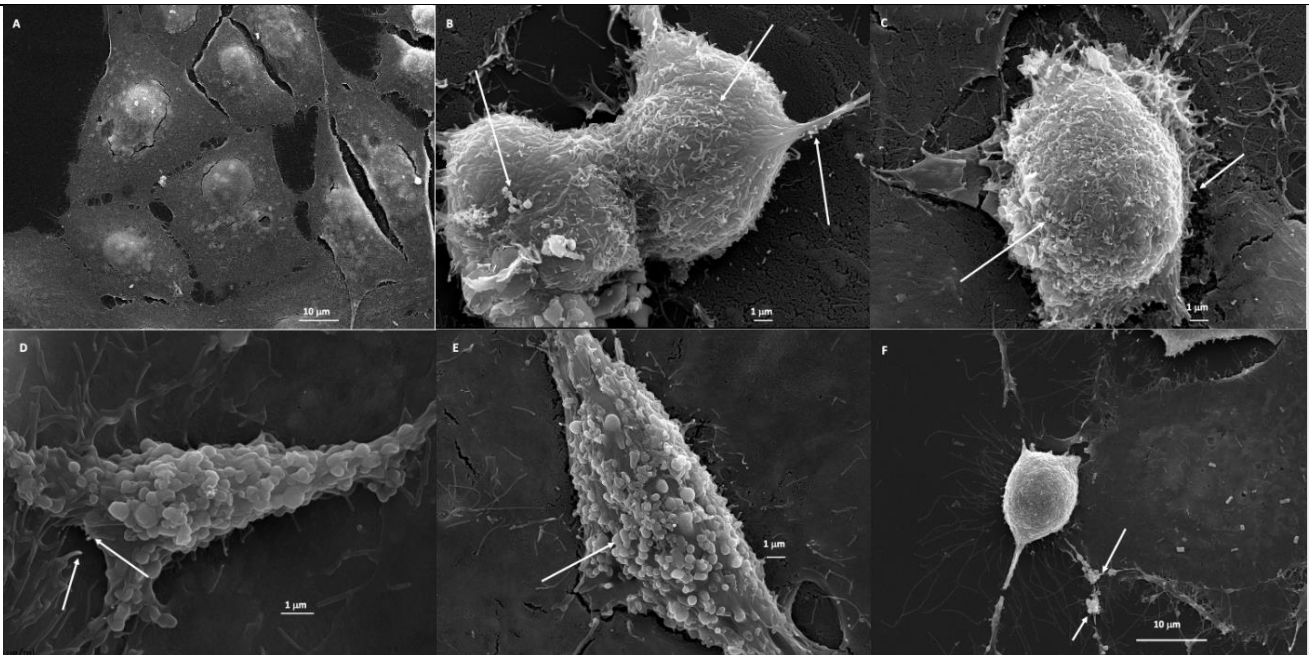


Figure 1. Scanning electron microscope image of untreated (A) and treated HUVEC *in vitro* cells with different particles with concentration of 100 µg/ml. **B:** TiO₂, **C:** Hydroxy apatite (HA), **D:** Al₂O₃-SiZrO₄, **E:** used Al₂O₃ and **F:** unused Al₂O₃.

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Conflicts of Interest: The authors declare no conflict of interest.

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Scientific contribution/Original research

Impact of a Saccharin Higher Homolog on *Saccharomyces cerevisiae*

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Abstract:

Saccharin is an organic compound, which is often used as a calorie-free artificial sweetener. It salts are being produced for the market for over 80 years. Saccharin and its derivatives are very applicatory oriented, therefore researchers synthesize more and more active ingredients, which could potentially show better performance.

This work considers the effect of biological activity of a newly synthesized saccharin derivative Methyl 4-hydroxy-1,1-dioxo-2H-1,2-benzothiazine-3-carboxylate (6Sac) on yeast *Saccharomyces cerevisiae*. Qualitative comparison of the studied activity with the activity of the saccharine sodium salt is presented. Our results were gained by two different ways of viability detection: counting dead/live cells dyed with methylene blue and counting colony-forming units (CFU). The study has shown that the saccharine derivative with an ester functional group has negative effect on growth and reproduction of yeast. The qualitative comparison of the activity of the tested substance with the already known activity of saccharine sodium salt is a convenient method for following the model organism *Saccharomyces cerevisiae*.

Keywords: Saccharin, sodium saccharinate, *Saccharomyces cerevisiae*, Viability, Methylene blue, Colony-forming units (CFU), Medicine

1. Introduction

Calorie-free synthetic sweeteners are commonplace in the food industry and kitchens. Nonetheless, health issues may arise following their frequent ingestion (Ager et al, 1998). In fact, carcinogenicity studies thereof, did not reveal any tangible deleterious impact on human health, by contrast to the bladder cancer which resulted in rats (Weihrauch et al., 2004). In particular, saccharin which means “similar to sugar” (labelled E954) (Božič, 2016) is a common zero-calorie organic sweetener (Namartha et al., 2016). It is 200–700 times sweeter than saccharose (table sugar) but with a mild bitter metallic taste. It has been commercially available for >80 years (Report on carcinogens, 1998) following its accidental discovery in 1878 by the chemist Constantin Fahlberg during his work on coal-tar (Namartha et al., 2016). Saccharin is thermally stable at food baking temperatures and is inert towards other ingredients at room temperature. Structurally, saccharin belongs to the benzosultam class of organics; notably *gamma*-sultam for this 5-membered ring compound (Mihali, 2012). Owing to its relative *N*-H acidity (pK_a 2.32; with low water solubility), it can readily form salts and is also available as its highly water-soluble sodium or calcium salt (as white powders) (Namartha et al., 2016).

Antibiotic-resistant bacteria present a serious general health problem inciting the research and study of new heterocyclic compounds – e.g. the ones containing sulfur and nitrogen atoms—which are known to offer a wide spectrum of bioactivity (Patel et al., 2016). In addition, the diversification of a given basic molecule by appending different functional groups onto it may fortuitously endow a new derivative with an interesting bioactivity (Mehta, 1961).

2. Literature studies on the bioactivity of saccharin and its derivatives

Studies have shown that saccharin may cause cancer in animals qualifying it in 1981 to the list of carcinogens (Report on carcinogens, 1998). Later, many researchers have investigated the health impact of saccharin and its derivatives.

In a study wherein lab rats were fed a high level of 5% of various saccharin compounds out of 20 screened showed a significant increase of neoplasia. In a two-generation study, parents and offsprings were put on a saccharin diet noticing that the latter had developed an increased risk of bladder cancer (Weihrauch et al., 2004). Similar results were obtained switching to sodium saccharinate. It was indicated that these compounds impact the urinary system physiology; this concerns urine pH, osmolarity, volume, and the appearance of a precipitate. In another study on mice, saccharin was found to affect the structure of the microbiome by impairing glucose homeostasis (Spanogiannopoulos et al., 2016). As saccharin impact on monkeys or humans did not show enough evidence for increase of cancer risk, saccharin was de-listed from the carcinogens list (Weihrauch et al., 2004). In the presence of *S. cerevisiae* saccharin was found to inhibit the sporulation process and slightly increase the occurrence of diploid and disomic meiotic products without affecting the frequency of recombination (Persic, 1986). At a concentrations of 2–20 mg/mL, sodium saccharinate did not significantly affect cell growth, but slightly reduced cells survival; cell survival decreased even more at a concentration of 100 mg/mL (Moore et al., 1979).

Various benzosultams to which class saccharin belongs have interesting bioactivity and are therefore used in general as pharmaceuticals (Mihali, 2012). For example, a series of Schiff's base derived from *N*-substituted saccharin showed activity against some microorganisms (Musa, 2016). These displayed an interesting inhibition of cholinesterase which is potentially applicable to treating Alzheimer's disease (Hebda et al., 2016). Moreover, it was indicated that 1,2-benzothiazines possess anti-inflammatory, antidepressant, antimicrobial, and anti-carcinogenic properties such as the saccharin-derived anti-inflammatory agents piroxicam, ampiroxicam, and meloxicam (Patel et al., 2016). Screening 45 compounds of 1,2-benzothiazine, none of them showed any activity against Gram-negative bacteria (i.e. *Proteus vulgaris* and *Salmonella typhimurium*), but 12 compounds showed activity against Gram-positive bacteria (i.e. *Bacillus subtilis* and *Staphylococcus aureus*) (Patel et al., 2016). Finally, in a study concerning the inhibitory activity of saccharin-based compounds on the interferon signaling pathway, a compound was identified for possible therapeutic use. It inhibited the lipopolysaccharide signaling in primary macrophages without showing any cytotoxicity at concentrations

up to 100 μM . This could be potentially useful for therapy in inflammatory diseases such as systemic lupus erythematosus and multiple sclerosis (Csakai et al., 2014).

3. The goal and hypothesis

This work concerns the bioactivity of saccharin higher homolog 6Sac (**Figure 1**) against *Saccharomyces cerevisiae* (*S. cerevisiae*). Methyl 4-hydroxy-1,1-dioxo-2H-1,2-benzothiazine-3-carboxylate (6Sac), a saccharin higher homolog, which possesses a functionalized 6-membered ring (*i.e.* a *delta*-sultam instead of a *gamma*-sultam). 6Sac has been obtained by synthesis starting from saccharin by cycle enlargement/extension (**Figure 1**) (Jeran et al., 2017).

To observe the saccharin higher homolog 6Sac (**Figure 1**), we used two different methods – measurements of colony forming units (CFU) and determination of viability with methylene blue dye. We anticipated that compound 6Sac will show fungistatic and fungicidal activity against *Saccharomyces cerevisiae* (*S. cerevisiae*).

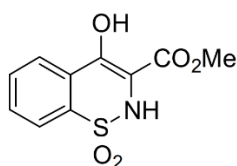


Figure 1: Structure of Methyl 4-hydroxy-1,1-dioxo-2H-1,2-benzothiazine-3-carboxylate (6Sac).

4. Methods

4.1. Culturing

To the liquid medium (*see below*) was added the inoculum (yeast containing active *S. cerevisiae*), and left undisturbed for 3 days. The suspension was diluted and divided into eight Erlenmeyer flasks. Two flasks served as controls: to one flask was added dimethyl sulfoxide (DMSO), and another one was kept unchanged. To the remaining six flasks, the test substance 6Sac was added. After 5 hours the cells were counted then recounted after every 20 hours for the next three days.

Two different methods for viability determination were applied: cells were grown on hard agar medium which permits colony-forming units (CFU) count, and viable cells were counted under Bürker-Türk chamber following addition of methylene blue dye.

The statistical analysis was performed by Microsoft Excel (Microsoft Corporation, version 2018). The mean values and the standard deviations of the two populations (treated and untreated samples) were calculated. The statistical significance of the differences between the two populations were calculated by the two-tailed *t*-test. The values of the probability *p* smaller than 0.05 were considered as statistically significant.

4.2. Preparation of the liquid medium (Biolife)

The reagents (5.00 g of yeast extract (Spar), anhydrous D-(+)-glucose (10.0 g) (*Sigma-Aldrich*), mixed powder extract (Torlak) (10.0 g)) were weighed into an Erlenmeyer flask and distilled water (200 mL) was added. The suspension was transferred to a 500 mL flask and distilled water added to reach the 500 mL level. The contents of the flask were mixed well and poured into the Erlenmeyer flask. The flask was heated on a burner to boiling till a clear solution was obtained, then covered with aluminium foil and kept for 20 min at 121 °C in an autoclave.

4.3. Preparation of the YGC agar solid medium

The powder medium contained 14.9 g/L YGC agar (*Sigma-Aldrich*, 9576), 0.1 g/L chloramphenicol, 20 g/L D-(+)-glucose, and 5 g/L yeast extract. To prepare 500 mL of a medium, Erlenmeyer flask was weighed with 20.0 g of YGC agar, and the contents to the 500 mL mark diluted with distilled water.

The flask was heated on a burner to boiling till a clear solution was obtained. It was then covered with aluminium foil and kept for 20 min at 121 °C in an autoclave. Then, the flask was placed at 45 °C in a water-bath for the mixture not to harden. When it cooled down, it was poured into petri dishes.

4.4. The growth curve

250 mL of liquid medium was inserted in an Erlenmeyer flask. After autoclaving and cooling the sample, 0.5 g of fresh yeast *S. cerevisiae* (Fala) was inoculated to the medium.

The procedure was performed under sterile conditions. The medium with *S. cerevisiae* was put on a shaker, so it was constantly mixing to obtain homogeneous broth with enough oxygen uptake. We took 1 mL of broth out of Erlenmeyer flask at 0, 22, 44, 66 and 144 hours and we made different dilutions of sample in 0.9% NaCl solution (countability, under microscope). Dilution of the sample was applied to the Bürker-Türk counting chamber, so we were able to count cells under a microscope and calculate the concentration of cells in broth. Concentration told us, when our cells are in stationary growth phase, what we used later in experiment.

4.5. Effect of compound 6Sac on *S. cerevisiae*

For first stage, we prepared a mixture of 125 mL liquid medium (described in chapter 5.1) in a 500 mL Erlenmeyer flask and then added 0.250 g of *S. cerevisiae*. The contents were stirred on a magnetic stirrer at room temperature for two days, so our culture came into the stationary phase of growth. After two days it was used as 25% inoculum for second stage, so we diluted our 125 mL of broth with 375 mL of the fresh liquid medium (the same one as before, described in chapter 5.1), stir it, so it was well homogenised and used it as fresh broth for second stage.

For second stage, we used eight 100 mL Erlenmeyer flasks. Firstly, we prepared empty Erlenmeyer flasks with compounds and then we added fresh broth for second stage. In first three Erlenmeyer flasks were added 50 mg of compound 6Sac and 500 µL of DMSO (1.0 mg/mL 6Sac + DMSO). The contents were mixed well to dissolve the 6Sac completely. We repeated the process for the next three Erlenmeyer flasks, where we added 500 mg of 6Sac and 500 µL of DMSO (10.0 mg/mL 6Sac + DMSO). In seventh Erlenmeyer flask we put just 500 µL of DMSO (Control) and eighth Erlenmeyer flask remained empty at that point (Control, blank). Then we divided our fresh broth for second stage in all eight Erlenmeyer flasks – we added 50 mL in each. All Erlenmeyer flasks were covered with aluminium foil and marked accordingly. They were placed on shaker and shaken constantly at room temperature for next three days.

After 5, 25, 45 and 65 hours, we aseptically took 1.00 mL of sample from all eight Erlenmeyer flasks and we prepared different dilutions in 0.9% NaCl solution. Hundred-fold diluted samples were mixed with methylene blue solution in a volume ratio of 1:1. Methylene blue was left to act for one minute. The contents were mixed well with the vortex mixer so that the cells were evenly distributed. The sample was cannulated onto the Bürker-Türk counting chamber, covered with a cover glass, and mounted to a microscope for counting the total number of cells and calculating the concentration of cells in broth. We also counted the number of living and the dead cells, because dead cells remained blue coloured, due to methylene blue.

All eight samples were also inoculated on plates with solid medium (described in chapter 5.2). We used a 10⁻⁵ and 10⁻⁶ dilution of a samples. 100 µL of the dilution was applied to the solid medium under sterile conditions and spread over by using a Drigalski spatula. Both dilutions of all eight samples were plated on 3 plates. All plates with solid medium were incubated for 24 hours at 37 °C. Due to the incubation process and potential infections (contamination), blank sample and solid medium without applicable solutions tests were also performed. The CFU formed were counted after 24 hours.



5. Results and Discussion

5.1. *S. cerevisiae* growth curve

The optimum time window for the growth and activity of the *S. cerevisiae* cells was between 44–66 h, which translates to *i.e.* 2–3 days (Figure 2).

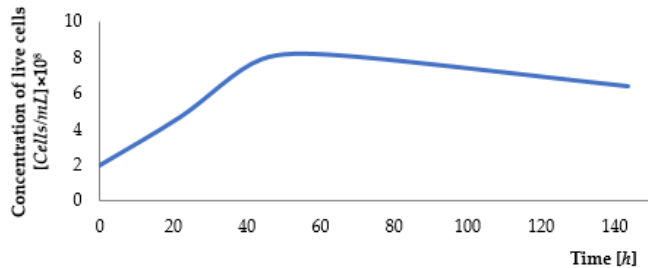


Figure 2: *S. cerevisiae* growth curve.

5.2. *S. cerevisiae* cultivability in presence of substance 6Sac

In the results of counting CFU on solid medium plates, we found that the addition of 6Sac to the yeast suspension successfully lowered the yeast growth as the cell concentration did not exceed 2 CFU/mL × 10⁷ (Figure 3).

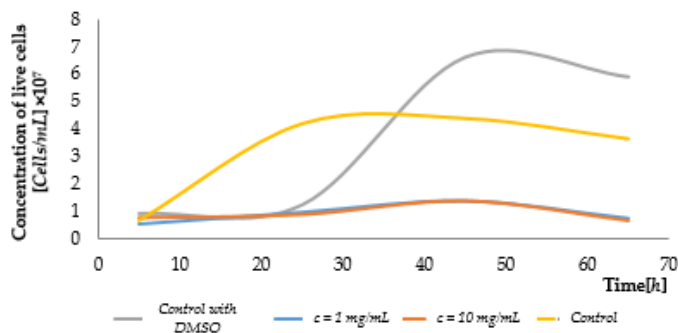


Figure 3: Concentration of live *S. cerevisiae* cells in presence of 6Sac from CFU results.

5.3. Viability of *S. cerevisiae* in presence of 6Sac.

We also got results about concentration of live cells with counting under microscope and we can see them in Figure 4. In the presence of 6Sac, concentration of live *S. cerevisiae* cells increased for about 45 h and then it started to decrease except for the control with DMSO (Figure 4). We can see that DMSO did not have a large impact on the concentration of live cells, if we compare control and control with DMSO. We can also see that there was no significant difference in the number of live cells between both concentrations of 6Sac.

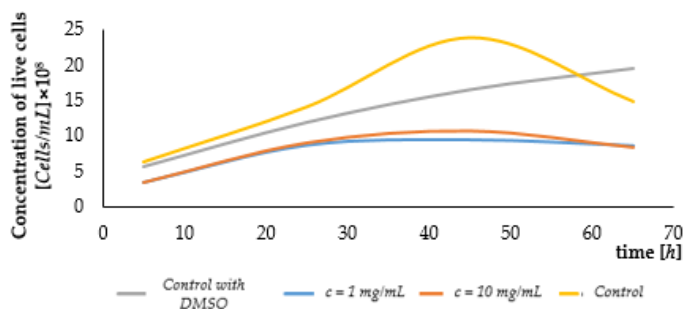


Figure 4: Concentration of live *S. cerevisiae* cells in presence of 6Sac from counting under microscope.

Under microscope we also saw that both 6Sac concentrations (1 mg/mL and 10 mg/mL) have impacted the *S. cerevisiae* viability (percentage of live cells out of all cells under microscope) (**Table 1**). The concentration of 1 mg/mL lowered the yeast cells viability in the first 5 h, then this was stable for some time. The greatest difference between viability in presence of 6Sac and viability of control (broth with DMSO) was observed at 65 h. On the other hand, the concentration of 10 mg/mL had the greatest impact in the first 5 h (**Table 1**). Later we can see higher viability, but at the end, at 65 hours, there is lower viability again, which is also very comparable with the viability of a 1 mg/mL (**Table 1**). The *p*-value also confirms what has been described. For most measurements (exception is viability with 1 mg/mL 6Sac at 5 hours), the *p*-values are less than 0.05, indicating that there is a greater than 95% chance that the difference in viability between culture treated with 6Sac and the control is statistically significant (**Table 1**).

Table 1: *S. cerevisiae* viability.

Time (h)	Viability (%) ± Standard deviation (<i>p</i> -value)		
	Control	1 mg/mL [6Sac]	10 mg/mL [6Sac]
5	95.74 ± 4.52	88.71 ± 9.54 (<i>p</i> = 0.266006)	80.30 ± 11.54 (<i>p</i> = 0.005470)
25	98.96 ± 1.79	86.71 ± 7.89 (<i>p</i> = 0.002355)	85.54 ± 14.61 (<i>p</i> = 0.016322)
45	97.78 ± 2.98	87.13 ± 3.42 (<i>p</i> = 0.001176)	89.89 ± 5.32 (<i>p</i> = 0.011357)
65	95.71 ± 4.06	80.95 ± 4.93 (<i>p</i> = 0.001942)	83.02 ± 6.39 (<i>p</i> = 0.002919)

5.4. Bioactivity of substance 6Sac versus sodium saccharinate (NaSac)

Table 2 compares our results (substance 6Sac) with the results (substance NaSac) from study by Moore and co-workers (1979) which reports on measurements after incubation at 30 °C after 60–65 h. Also, we obtained results after 65 hours, however, we used different concentrations of 6Sac (**Table 2**). We can predict that viability of yeast cells treated with 6Sac at a concentration of 1 mg/mL is comparable to those treated with sodium saccharine at the same concentration, because they observed viability at concentration of 0 and 2 mg/mL and they got results right around our result. However, we cannot say the same for a higher concentration of 6Sac (10 mg/mL). We can see that we got the same effect on viability at both concentration of 6Sac, but they found, that the viability depends on the concentration of NaSac.

Table 2: Bioactivity of 6Sac versus sodium saccharinate (NaSac).

Compound	Concentration of added compound [mg/mL]	Viability (%) ± standard deviation
NaSac (Moore et al., 1979)	0	100 ± 9
6Sac (Our study)	1	80.95 ± 4.93
NaSac (Moore et al., 1979)	2	74 ± 8
6Sac (Our study)	10	83.02 ± 6.39
NaSac (Moore et al., 1979)	20	61 ± 8
NaSac (Moore et al., 1979)	100	24 ± 10

6. Conclusion

The effect of Methyl 4-hydroxy-1,1-dioxo-2H-1,2-benzothiazine-3-carboxylate (6Sac) on the model organism *S. cerevisiae* was tested by two different methods. The first was to monitor the viability of *S. cerevisiae* cells with methylene blue, which enabled to differ between live and dead cells. We counted cells with the Bürker-Türk counting chamber. And the second was to monitor cultivability of yeast cells. We counted the number of colonies grown on solid media.

We showed that the compound 6Sac had an effect on *S. cerevisiae* by repressing their growth and increasing the percentage of unviable cells. With both tests, we confirmed a smaller amount of viable cells. In a concentration of 10 mg/mL, 6Sac showed the effect. After 65 h the results at both concentrations were the same. The research also showed that the viability of yeast treated with 1 mg/mL 6Sac is comparable to the one treated with sodium saccharinate, but we cannot say that for higher concentrations (**Table 2**).

Our results indicate that 6Sac is biologically active. Further research is needed to confirm the precise action of this compound. Preliminary tests are of importance due to the rising number of new active substances appearing on the market. We need to find out as much information about them as possible, to see whether they have an effect on human health. Any information from research work is a valuable resource for further studies, on which we build new knowledge.

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Conflicts of Interest: The authors declare no conflict of interest.

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Scientific contribution/Original research

Morphological Parameters of Erythrocyte Extracellular Vesicles at Hypoosmotic and Isoosmotic Conditions

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Abstract: Extracellular vesicles (EVs) are membrane-enclosed structures of nanometer dimensions. They are formed by cells and can be found in isolates from body fluids. It is indicated that they play important role in intercellular communication in health and disease. In this work we observed the morphological parameters of EVs isolated by differential centrifugation from washed erythrocytes, in which vesiculation was accelerated by addition of detergent (sodium dodecyl sulfate). Aliquots of the isolate were suspended medium of two different osmolarities. Isolates were imaged with a scanning electron microscope (SEM) and the images were analyzed by using the contours of EVs from which the volume V , surface area S , and relative volume $v = (36\pi S^3/V^2)^{1/2}$ were assessed by a geometrical model. EVs were considered as spheres or spheroids. The isolates were rich in erythrocyte EVs so that representative sets (86 vesicles at osmolarity of 50 mOsmol/L and 109 vesicles at osmolarity of 300 mOsmol/L) could be outlined. The EVs at osmolarity of 50 mOsmol/L had shapes close to a sphere, while at osmolarity of 300 mOsmol/L they had elongated shapes. The shapes were approximated by prolate spheroids. The average volume/surface area of EVs at osmolarity 50 mOsmol/L were $3.18 \times 10^5 \text{ nm}^3 / 2.20 \times 10^4 \text{ nm}^2$ and at osmolarity 300 mOsmol/L they were $4.3 \times 10^5 \text{ nm}^3 / 2.79 \times 10^4 \text{ nm}^2$. The respective differences in favor of the values at 300 mOsmol/L were statistically significant and of sufficient power. The relative volume of EVs at 50 mOsmol/L and 300 mOsmol/L were 1 and 0.96, respectively. While the difference in v suggests that, similarly to erythrocytes, water enters EVs in order to attain the Donnan equilibrium, the differences in V and A suggest that selective popping of (larger) EVs in the hypoosmolar sample took place.

Keywords: Extracellular vesicles; Erythrocytes; Osmolarity; Differential centrifugation; Scanning electron microscopy (SEM); Morphology; Interdisciplinary connection; Medicine

1. Introduction

Extracellular vesicles (EVs) are heterogenic group of nano-/micrometres particles, enclosed with a membrane, and found in samples of biological origin. They work as means of communication between cells and are involved in many physiological and pathological processes like embryogenesis, neuron communication, blood coagulation, inflammation, tumorigenesis, and horizontal gene transfer (Yáñez-Mó et al., 2015, van Niel et al., 2018). Procedures have been developed to harvest EVs from body fluids, blood being the one of particular interest. Extensive research has been put forward because of EVs' their potential implications as biomarkers for the observation the of development of different diseases, as medication, and as vectors for delivering active substances.

It is acknowledged that the erythrocyte shape depends on the composition of the intra and extracellular solution. The membrane of the erythrocyte is selectively permeable. The tendency of the species that can cross the membrane to equalize the (electro)chemical potential and osmotic pressure together with requirement that the outer and the inner solutions remain free or electric field constitutes the base of the Donnan equilibrium that determines the water content of the erythrocyte and therefore its volume (Loeb, 1921). It was of interest to investigate whether similar mechanisms are taking place also in sub-micron sized EVs that were shed off from erythrocyte plasma membrane and present from a physical view essentially similar membrane-enclosed system without internal structure.

In this work, we considered EVs isolated from samples of washed erythrocytes. Vesiculation was accelerated by addition of detergent. The isolate that we found rich in EVs was divided into two aliquots; one was suspended in a physiological isotonic solution (300 mOsm/L) and the other was suspended in a hypotonic solution (50 mOsm/L). The samples were imaged with scanning electron microscope, revealing the shape and size of the EVs. We approximated the contours of the EVs by known geometrical objects (sphere and prolate ellipsoids), for which the expressions for the three-dimensional volume and the surface area are available in an analytical form. As the micrographs contained very many images of EVs, statistical analysis could be made, and two populations pertaining to different osmolarities could be compared. To the best of our knowledge, this is the first report on the volume and surface area of erythrocyte extracellular vesicles, measured in populations of EVs of different osmolarities.

2. Methods

2.1 Blood sampling

Blood was donated by a female adult with no record of disease. The blood collection took place in the morning, the person was fasting for at least 12 hours before the collection. Blood was taken in evacuated Na-citrate tubes (4.5 mL; BD Vacutainers, 367714A, Becton Dickinson, USA).

2.2 Isolation of erythrocyte EVs

Isolation was performed as previously described in Božič, et al. (2021). Blood was centrifuged at $300 \times g$ for 10 min. Plasma was removed and erythrocytes were washed three times with 300 mOsmol/L PBS-citrate. Erythrocytes were then suspended in PBS-citrate with the addition of 10 mM sodium dodecyl sulfate (SDS) (1:10 per sample). The sample was then incubated for 2 hours at room temperature on a rotary stirrer, followed by isolation of EVs by differential centrifugation according to the procedure: $300 \times g$ 10 min, $2,000 \times g$ 10 min, and $4,000 \times g$ 10 min. The $4,000 \times g$ supernatant was filtered through a 0.8-micron filter, then the filtrate was centrifuged at $50,000 \times g$ and 4°C for 70 min. The resulting pellet was washed with PBS-citrate. To obtain hypoosmolar conditions, one aliquote of the the isolate was suspended in $d\text{H}_2\text{O}$ until a final osmolarity of 50 mOsm/L (for the sample 50 mOsmol/L) while the other aliquote was diluted to the same volume with the base buffer 300 mOsmol/L PBS-citrate.

2.3 Scanning electron microscopy (SEM) of EVs

A sample of EVs isolated from the fresh erythrocyte suspension and suspended in PBS-citrate buffer was diluted with the medium prepared with the appropriate combination of $d\text{H}_2\text{O}$ and 4 M NaCl. Samples of EVs at different osmolarities (50 and 300 mOsmol/L) in PBS-citrate were incubated according to the literature (Božič, et al. (2021)) in 2% aq. solution of OsO_4 for 2 hours and applied to a

0.05-micron MCE filter. The filters were taken from the holder and placed into wells of the microtiter plate filled with solutions as described below. After three stages of rinsing in dH_2O , the samples were dehydrated in a series of ethanol solutions – from the solution with the lowest ethanol content to the highest: 30%, 50%, 70%, 80%, 90%, and “absolute” ethanol. This was followed by treatment of the samples with 30%, 50% and “absolute” hexamethyldisilazane. The samples were left overnight to dry in air.

To observe the samples under a JSM-6500F scanning electron microscope (JEOL Ltd., Tokyo, Japan), the samples were coated with Au/Pd (PECS Gatan 682).

2.4 Determination of the volume and surface area of EVs

SEM micrographs (**Figures 1** and **2**) were imported into the *CorelDRAW* software program (Corel Corporation version 24.0.0.301, 2022). In each of the micrographs (**Figure 1**), we chose two rectangles of equal sizes within which we assessed the shapes of the contours of all elements that we recognized as EVs.

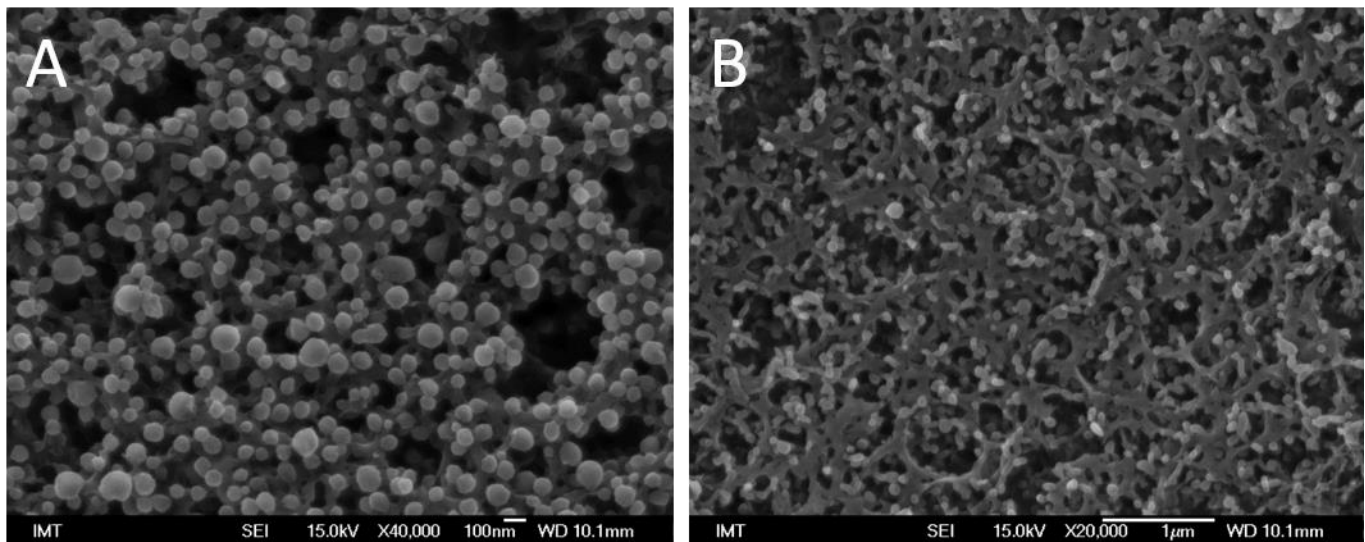


Figure 1. Micrograph of EVs at osmolarity 50 mOsmol/L, (A) and 300 mOsmol/L (B).

We assumed that the EVs had spheroid shapes (shape of rotational prolate ellipsoid, **Figure 2**). The contours of structures were fitted by ellipses with small semi-axes (marked a in **Figure 2**) and large semi-axes (marked b in **Figure 2**). To facilitate the determination of the semi-axes, the contours were rotated to present the larger semi-axis in the vertical direction (**Figure 2**).

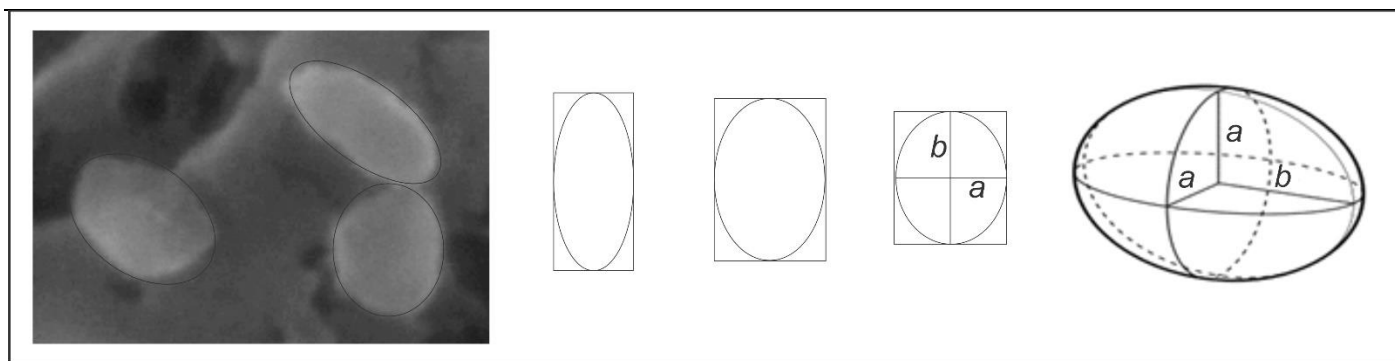


Figure 2. Fitting of the EV contours with prolate spheroid and determination of the semiaxes marked a and b .

The values of a and b were introduced into the expressions for volume and surface area of the prolate spheroid (Satterly, 1960),

$$V = \frac{4}{3} \pi a^2 \quad , \tag{1}$$

$$S = 2 \pi a^2 + \left(\frac{2 \pi a b^2}{\sqrt{b^2 - a^2}} \left(\arcsin \frac{\sqrt{b^2 - a^2}}{b} \right) \right) \quad . \tag{2}$$

Also we calculated the relative volume v representing the ratio of volume to the surface area,

$$v = \sqrt[2]{\frac{36 \pi V^2}{S^3}} \quad . \tag{3}$$

2.5 Statistical analysis

Figure 1 shows that the isolates contained a large number of EVs, which allowed statistical analysis of the samples. We captured 86 EVs from the sample at osmolarity 50 mOsm/L and 109 EVs from the sample at osmolarity 300 mOsm/L.

The geometric parameters (V , S and v) of both populations were compared by using descriptive statistical methods. The mean values and the standard deviations of the two populations were calculated. The statistical significance of the differences between the two populations were calculated by the two-tailed t -test. The values of the probability p smaller than 0.05 were considered as statistically significant. We used Microsoft Excel (Microsoft Corporation, version 2018). Also we determined statistical power of the difference by using free software: Brant R. Inference for means: comparing two independent samples (<https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html>). The values of P larger than 0.8 at alpha 0.05 were considered of sufficient power.

3. Results and Discussion

Statistical analysis showed that the average volume and surface area of EVs of erythrocytes at osmolarity 50 mOsmol/L were substantially and statistically significantly smaller than at osmolarity 300 mOsmol/L. The relative volume, which indicates the ratio between volume and area, was however substantially and statistically significantly larger at higher osmolarity (**Table 1**). This indicates that the population of EVs at higher osmolarity contains larger and more flaccid EVs.

Table 1. Geometrical parameters of EVs at two different osmolarities, the differences between the hypoosmolar and isosmolar samples Δ and statistical parameters of the differences: the probability p and statistical power P .

Average value (standard deviation)	50 mOsmol/L [86 EVs]	300 mOsmol/L [109 EVs]	Δ	p	P
V (nm ³)	3.18×10^5 (1.49×10^5)	4.32×10^5 (2.66×10^5)	-30%	$<10^{-3}$	0.82
S (nm ²)	2.20×10^4 (0.68×10^4)	2.79×10^4 (1.09×10^4)	-24%	$<10^{-6}$	0.94
v	1.00	0.94 (0.07)	6%	$<10^{-12}$	1.00

The difference between the values of both osmolarities, at 50 mOsmol/L and 300 mOsmol/L, was normalized by the mean value at both osmolarities.

It seems reasonable and in line with previous results on erythrocytes (Canham, 1970) that in hypoosmotic medium water enters the EVs to approach the Donnan equilibrium. It is therefore expected that the shapes of EVs would approach spherical shape with decrease of the osmolarity, which was actually observed in our results (**Figures 1,2**). In agreement with this, the relative volume was higher at smaller osmolarity (**Table 1**). However, as the two samples were aliquots deriving from the same isolate, a question can be posed why the average surface areas of EVs of both samples would be different. A possible explanation of this could be that some of EVs (e.g. the larger ones) that approached spherical shape popped, leaving in the population the smaller ones.

At an osmolarity of 300 mOsmol/L, where the shapes are not spherically symmetric, the two dimensional image is limited in revealing the three-dimensional shape of the particle although the brighter parts of the image are closer to the source of the electrons (**Figure 2**). Since we cannot identify a specific reason for the preferred orientation of EVs, it may be that the EV which seems spherical in the image is actually a prolate ellipsoid oriented perpendicular to the plane of imaging. Therefore the volume and the surface area of the EVs at 300 mosm/L is underestimated. This would however further increase the difference in V , S and v of the two samples.

4. Conclusions

Increase of the relative volume of EVs in hypoosmotic medium indicates that EVs are (like erythrocytes) subjected to Donnan equilibrium. The explanation of the difference in average surface areas of EVs pertaining to the two aliquots at different osmolarities remains indecisive.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, blood was donated voluntarily by the authors of the study.

Conflicts of Interest: The authors declare no conflict of interest.

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6/2022

Scientific contribution/Original research

Dehydrogenation of Hantzsch Dihydropyridines with Heterogeneous Cobalt Oxide Catalyst Supported in N-Doped Activated Carbon

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Abstract:

Hantzsch dihydropyridines represent an important source of hydrogen to be transferred to other unsaturated organic molecules, leading the formation of pyridine aromatic ring as driving force. The hydrogen transfer process was evaluated using 1,4-dihydropyridines and heterogeneous cobalt catalyst supported over N-doped activated carbon. The 4-position of the dihydropyridine ring was substituted with *H* (**4a**), *Me* (**4b**) and *Ph* (**4c**) groups, showing that only **1** reacted to yield the corresponding pyridine compound indicating that the presence of steric hindrance took place on the reaction. Additionally; three solvents –tetrahydrofuran (THF), acetone, and acetonitrile– were tested, showing reactivity only with unsaturated ones, but not with THF. This observation indicates that dihydropyridine works as hydrogen donor and solvent as hydrogen acceptor in the hydrogen transfer process.

Keywords: Hantzsch dihydropyridine; Heterogeneous cobalt oxide catalyst; Hydrogen transfer; Unsaturated solvents

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1. Introduction

NADPH bioorganic molecule (1) represents an important hydrogen donor agent in biological processes (Willner et al., 2021), moreover, when this molecule is used in synthetic transformations result as a too-expensive starting material (Lee et al., 2019). For this reason, dihydropyridine analogs are used instead of this biomolecule (Kraatz et al., 2019). In this regard, Hantzsch 1,4-dihydropyridines play an important role as bioactive molecules because some of this family compounds are used as drugs such as nifedipine (2) and amlodipine (3) used to treat high blood pressure and to control angina (chest pain), working as calcium-channel blockers (Chatterjee et al., 2022) (**Figure 1**).

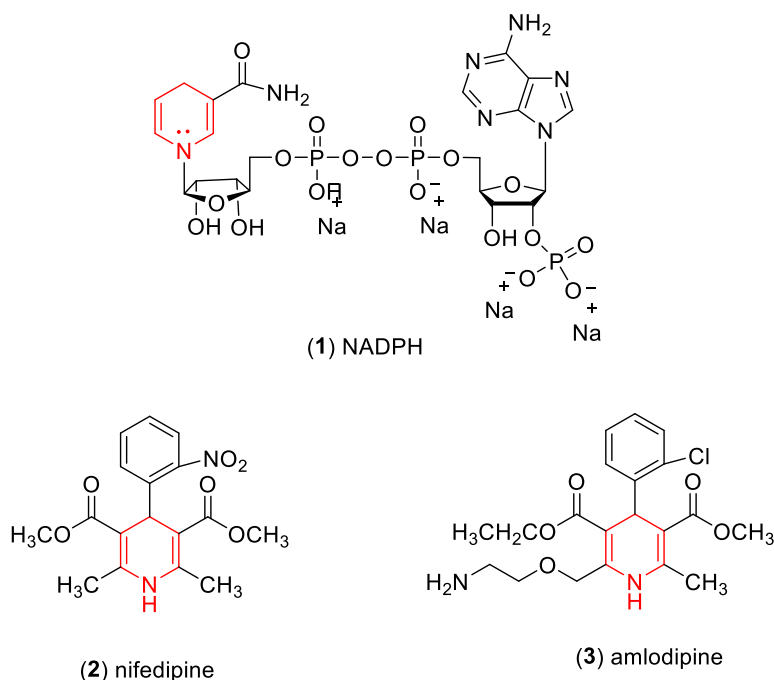


Figure 1. Structure of NADPH (1), and 1,4-dihydropyridine analogs nifedipine (2) and amlodipine (3). Dihydropyridine ring is shown in red.

On the other hand, catalysis represents one of the most powerful tools to transform molecules (Rawat et al., 2022), and usually, transition metals are used to build this kind of molecules because of their redox, optical and/or magnetic properties (Tsuji, 2002). In terms of green chemistry, the use of first row bioavailable transition metals as base of catalysts is preferred mainly because of their abundance and reincorporation into the biological processes (McCleverty, 1999). Beller et al., (2013) reported the preparation of a cobalt oxide catalyst supported in an *N*-doped activated carbon surface, starting with the synthesis of a homogeneous complex with pyridine-like ligands, followed by adsorption into activated carbon and finally, pyrolysis at 800°C to fix covalently the cobalt defined complex into the carbonaceous surface, and then, its application in the reduction of nitroarenes.

The current work describes the use of a heterogeneous cobalt oxide catalyst supported in *N*-doped activated carbon surface to dehydrogenate 1,4-dihydropyridines.

2. The Goal

The goal of the research was focused on the dehydrogenation of Hantzsch 1,4-dihydropyridines using a non-expensive heterogeneous cobalt oxide catalyst, testing several conditions such as temperature, load of catalyst, solvent and period of time, determining the optimal reaction conditions.

3. Methods

Catalyst was prepared according to the methodology described by Beller et al., (2013), and 1,4-dihydropyridines 4a-c were prepared according to a one-pot multicomponent modification of the methodology reported by Hantzsch (Bosica et al., 2020).

Cobalt oxide supported in *N*-doped activated carbon was prepared following the methodology described by Beller et al., (2013). A mixture of 1.0 equiv. of cobalt(II) acetate and 2.0 equiv. of 1,10-phenantroline to obtain the bisphenantrolinecobalt(II) acetate, following by the addition of 600 mg of vegetal activated carbon in ethanol and refluxing in ethanol for 2 hours. At the end of the time, solvent was removed by vacuum and dried at 80°C for 0.5 hours. Pyrolysis took place at 800°C during 2 hours, obtaining a compound containing 57.8 % of carbon, 14.31% of nitrogen, 21.76% of oxygen and 6.6% of cobalt according to the Energy-dispersive *X-ray* Spectroscopy.

1,4-Dihydropyridines (**4**) were prepared mixing 2.0 equiv. of ethylacetoacetate, 1.0 equiv. of aldehyde, an excess of NaOH and 2 drops of aqueous HCl (0.1 M) as catalyst in ethanol (**Figure 2**) in ethanol for 3 hours. At the end of the reaction, solvent was removed in vacuum and recrystallization of product took place in a mixture of methylenechloride/hexane solvents. Melting points **4a**: 182°C, **4b**: 189°C, **4c**: 270°C.

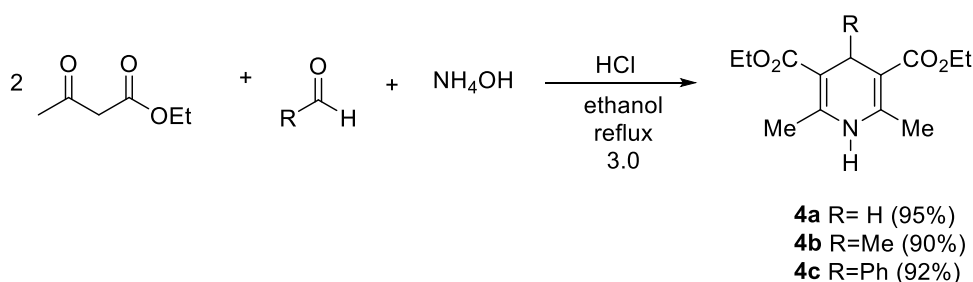


Figure 2. Synthesis of Hantzsch 1,4-dihydropyridines by one-pot multicomponent reaction.

Dehydrogenation of **4** was tested employing 1 equiv. of the corresponding Hantzsch dihydropyridine and 5mol% of cobalt oxide catalyst in a reflux of 20 mL of solvent (tetrahydrofuran (THF), acetone and acetonitrile). Reaction was followed by thin-layer chromatography (TLC) and products were isolated by silica column using a mixture 8:2, hexane/ ethyl acetate (**Figure 3**).

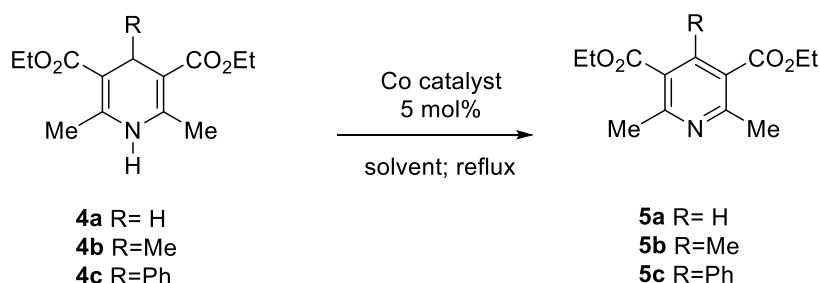


Figure 3. Dehydrogenation of 1,4-dihydropyridines using cobalt catalyst.

4. Results and Discussion

Three 1,4-dihydropyridines (**4a**, **4b** and **4c**) were tested, using three different solvents (THF, acetone and acetonitrile).

In the first attempt, **4a** reacted with 5 mol% of cobalt oxide catalyst in the selected (**Table 1**).

Table 1. Dehydrogenation of **4a** with 5 mol% of cobalt oxide catalyst in a reflux of solvent during 2 hours. Product **5a** was isolated by silica gel chromatography using an 8:2 mixture of hexane/ethyl acetate solvent.

Entry	Solvent	Yield
1	Tetrahydrofuran (THF)	Recovery of starting material
2	Acetone	63%
3	Acetonitrile	99%

According to **Table 1**, only unsaturated solvents allowed the formation of the product (**Table 1**, Rows 2 and 3), indicating the possible dependence of a hydrogen acceptor in the process. THF solvent does not contain double or triple bonds, and the possibility to transfer the hydrogen from **4a**

did not take place. On the other hand, the higher boiling point of acetonitrile (82 °C) compared to acetone (56 °C) could be associated to the almost quantitative yield in the reaction showed in (Table 1, Row 3). In this instance, the reaction affords the pyridine 4a only in the presence of unsaturated solvents according to Figure 4.

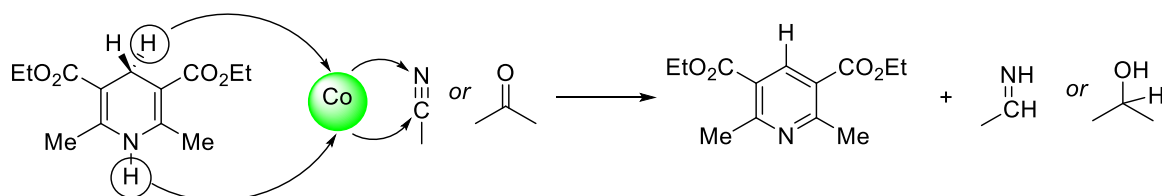
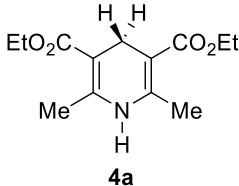
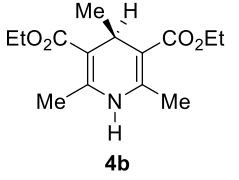
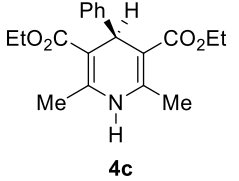


Figure 4. Hydrogen transfer from 4a to unsaturated solvents.

In a second attempt, the substitution of the 4-position of the dihydropyridine 3 ring was evaluated. Results are shown in Table 2.

Table 2. Dehydrogenation of 4 using 5 mol% of cobalt oxide catalyst in reflux of acetonitrile during 2 hours. Product was isolated by crystallization in methylene chloride/hexane mixture of solvents.

Entry	Substrate	Yield
1	 4a	99%
2	 4b	Recovery of starting material
3	 4c	Recovering of starting material

According to the results observed in Table 2, 4-position plays an important role in the dehydrogenation process exhibiting a high sensitive steric hindrance. Only 4a allowed the formation of product by means of the small substituent (H), instead the larger -Me, and -Ph substituents in 4b and 4c. This observation could show close interaction between the cobalt center and 4-position in the dihydropyridine ring (Figure 5).

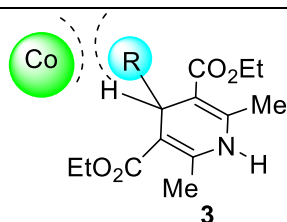


Figure 5. Steric hindrance in the approximation of cobalt catalyst to 4-position in 3.

In this way, the use of heterogeneous cobalt oxide catalyst was evaluated in the dehydrogenation process of Hantzsch 1,4-dihydropyridines.

5. Conclusions

The use of Hantzsch dihydropyridines as hydrogen donors can occur, but the substituent in the 4-position of the dihydropyridine ring plays an important role in the course of the reaction at using cobalt oxide catalyst. The structure of the solvent also resulted important because the requirement of an unsaturation as hydrogen acceptor for the reaction to proceed was essential. With these results, process to hydrogenate unsaturated organic molecules at using 4a as hydrogen donor and heterogeneous cobalt oxide supported in *N*-doped activated carbon as catalyst can be visualized.

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Conflicts of Interest: The authors declare no conflict of interest.

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122 0202

Scientific contribution/Original research

Informal Economic Activity in the Service Sector During the Pandemics of COVID-19

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Abstract:

In this contribution, we present the findings of our research on informal economic activity in the service sector during the pandemics of COVID-19. We found that approximately 28% of respondents were engaged in informal economic activity, while the most commonly purchased services were accommodation and food service activities and administrative and support service activities. Our results show that the primary reasons for such engagement were directly linked to the pandemics of COVID-19, which is an important finding for decision-makers. Based on the results of the research we believe that consumer habits have not shifted towards regular engagement in informal economic activities. Respondents also showed a general supportiveness of informal economic activities of service providers during the pandemics and lockdowns, which might indicate a dissatisfaction with the government restrictions on business activities. With the use of the Pearson chi-squared test, we found no association between the sex of the respondents and their engagement in the informal economic activity or their opinion on the legitimacy of service providers' activities during the pandemics and lockdowns.

Keywords: Informal economic activity; Grey economy; Service sector; Survey; Association

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1. Introduction

Several different definitions and terms, including grey economy, shadow economy, underground economy and so on, are used to describe the informal economic activity (Kaže V et al., 2011). Smith (1997) provides a broad explanation of the informal economy, defining it as the "market- and non-market-based production of goods and services, whether legal or illegal, that escapes detection or is intentionally excluded from the official estimates of GDP". In essence, they all denote an economic activity that is consciously hidden from the government regulation and tax authorities. More specifically, informal economic activity results in a revenue loss from value-added tax (or sales tax), tax on corporate income, customs duties revenue, social contributions, charges, and more. In addition, certain businesses that require government licences or permissions become unregulated in the grey economy. In Slovenia, the Financial administration of the Republic of Slovenia has a state authority to monitor and control informal economic activities (Republic of Slovenia, no date).

The service sector is a broad name for businesses that sell personal services rather than material commodities or tangible goods. There is no consensus on the classification of the service industry and different authors include different economic activities within the service sector. Stigler (1956), who has devoted much attention to the service industries, provides a very vague classification of the service sector. Among others, Stigler treats retail trade, government, insurance, private healthcare, and private education as a service industry. For our research we focused on the narrower classification that is used by the Statistical Office of the Republic of Slovenia (Table 1) and is based on the Regulation (EU) 2019/2152 of the European Parliament and of the Council of 27 November 2019 on European business statistics, repealing 10 legal acts in the field of business statistics.

Table 1. Statistical Classification of Economic Activities in the European Community (NACE) of service sector economic activities as used by the Statistical Office of the Republic of Slovenia (European Council, 2006).

Economic Activity	NACE code
H Transportation and Storage	H 49, 50, 51, 52, 53
Land transport and transport via pipelines	H 49
Water transport	H 50
Air transport	H 51
Warehousing and support activities for transportation	H 52
Postal and courier activities	H 53
I Accommodation and Food Service Activities	I 55, 56
Accommodation	I 55
Food and beverage service activities	I 56
J Information and Communication	J 58, 59, 60, 61, 62, 63
Publishing activities	J 58
Motion picture, video and television programme production, sound recording and music publishing activities	J 59
Programming and broadcasting activities	J 60
Telecommunications	J 61
Computer programming, consultancy and related activities	J 62
Information service activities	J 63
L Real Estate Activities	L 68
M Professional, Scientific and Technical Activities	M 69, 70.2, 71, 73, 74
Legal and accounting activities	M 69
Management consultancy activities	M 70.2
Architectural and engineering activities; technical testing and analysis	M 71
Advertising and market research	M 73
Other professional, scientific and technical activities	M 74
N Administrative and Support Service Activities	N 77, 78, 79, 80, 81, 82
Rental and leasing activities	N 77
Employment activities	N 78
Travel agency, tour operator reservation service and related activities	N 79
Security and investigation activities	N 80
Services to buildings and landscape activities	N 81
Office administrative, office support and other business support activities	N 82



2. Methods

We conducted a survey to obtain information about consumer behaviour and the degree of informal economic activity during the pandemics of COVID-19. The research was conducted in January 2022 and was retrospective – respondents were asked to answer a series of questions regarding their purchasing behaviour from the start of the pandemics in Slovenia (12.3.2020) onwards. The survey was shared among respondents via different online channels and no respondent was directly targeted. Regardless, this way of collecting data might bring statistical bias to the results. The survey began with an age-group question, which enabled us to obtain results only from the consumers with purchasing power. Respondents, whose age was lower than 18, finished the survey after this question and their answers were not recorded. The survey was divided into five topical parts, each assessing a different aspect of consumer behaviour. Each segment included an if statement that determined whether the respondent was engaged in given consumer behaviour or practice, followed by a different number of sub-questions.

To make the survey more understandable to an average consumer we defined engagement in the informal economic activity as receiving and paying for a service without getting an appropriate statement that acknowledges the payment (invoice or bill). In this sense, we do not incorporate all economic activities in the definition of grey economy, namely those that do not include payment (neighbour help and barter of legal services).

3. Results

Before we analyse consumer behaviour data, let us examine the demographic data of our respondent pool. The survey was solved by 213 respondents, of whom 72% were women and 28% were men. Approximately 72% of respondents were employed and 5% were retired, the remaining 23% were either unemployed, out of the labour force or still in the educational process. Nevertheless, we included the latter's results as such individuals still have purchasing power. In regards to the demographic data, we acknowledge a statistical bias in the education variable. The distribution of respondents based on the education level was different from for the Slovene population – around 61% of our respondents had higher degree education (bachelor's degree, master's degree, or a PhD), whereas only 25% of the Slovene population have a higher degree education. In addition, only 2% of respondents have primary education or no education, whereas 22% of the Slovene population falls into this category (Statistical Office of the Republic of Slovenia, 2021).

Our research showed that 28.2% of respondents engaged in informal economic activities within the service sector in the defined timeframe (12.3.2020 onwards). The most used services ($f = 50\%$) were (based on NACE classification) *Accommodation and Food Service Activities* (I55 and I56) and *Administrative and Support Service Activities* (N78 – N82), followed by *Transportation and Storage* (H49 – H53) (Figure 1).

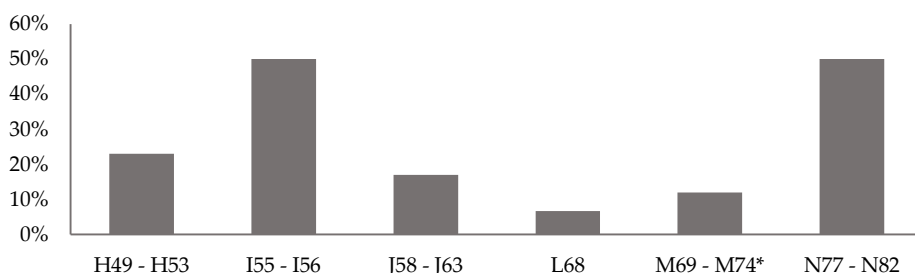


Figure 1. Frequency of informal economic activity in different service industries, based on NACE classification (Table 1). *Economic activities pertaining to M70.1 and M72 were not included in the research. The respondents' pool is described in Section 2.

Respondents that engaged in the informal economic activities were asked to provide reason(s) for such engagement (out of 213 respondents, 60 respondents engaged in the informal economic activity. All relative data in this paragraph refers to the latter respondents pool ($N = 60$)). The most common answer ($f = 47\%$) was the lower price of a service that results from a provider's tax evasion. The next answer with the highest frequency was directly linked to the consequence of COVID-19 – approximately 38% of respondents engaged in informal economic activity to support service providers in tough financial times. Similarly, 25% of respondents engaged in informal economic activity because

of the state restrictions on the business activity, therefore limiting the possibility to buy certain services. During these times, consumers could purchase such services solely with the engagement in the informal economic activity (receiving no invoice after payment). These two answers provide us with an insight into the consumer decision-making process and can offer a preliminary assessment that informal economic activity was higher during the pandemics of COVID-19 than before. As the legal supply of certain services was cut off by the state as a health measure, some providers started offering services off-the-books to meet the demand from customers. This claim is supported also by our findings of the frequency of engagement in the informal economic activity when purchasing services. We found that 18% of respondents participated in the grey economy only when state restrictions on the business activity were imposed, which meant that they could not buy the desired services legally. The majority of the respondent ($f = 65\%$) engaged in the informal economic activity when purchasing services only occasionally and 15% of respondents engaged every time, they purchased a service. Respondents were also asked to provide a self-assessment on the average amount of the purchase of services for which they did not receive an appropriate invoice. The most common average amount ($f = 33\%$) was 20–50 Eur, followed by <20 Eur ($f = 32\%$). Around 20% of respondents had an average amount of 50 – 100 Eur.

In the last segment of the survey, we checked for the respondents' sentiment on informal economic activity. We present an interesting finding – 55% of respondents believe that the informal economic activity of service providers during the pandemics of COVID-19 was legitimate. Furthermore, 52% of respondents did not oppose informal economic activity even during the lockdowns, when certain service businesses had to be closed.

We performed a *Pearson chi-squared* (χ^2) test of independence to assess the possible association between nominal variable *SEX* and three different nominal variables. We named these three variables *IEA* (nominal variable determining whether a respondent engaged in the informal economic activity), *OPINION* (nominal variable determining whether a respondent thinks informal economic activity of service providers during the pandemics of COVID-19 was legitimate) and *DISPUTE* (nominal variable determining whether a respondent opposes the informal economic activity of service providers during lockdowns). Our null hypotheses were the same for all three tests – H_0 : There is no association between variables ($f_{kg} = f'_{kg}$). All tests were made at $\alpha = 0.05$. We present basic findings in **Table 2**.

Table 2. Results of *Pearson chi-squared* test for different hypotheses at $\alpha = 0.05$.

	χ^2 -test value	<i>p</i> -value
<i>SEX-IEA</i>	2.425	0.1194
<i>SEX-OPINION</i>	0.31124	0.5769
<i>SEX-DISPUTE</i>	0.57417	0.4486

The critical value of the chi-square distribution at $\alpha = 0.05$ and degrees of freedom = 1 is 3.841. The *p*-value is greater than $\alpha = 0.05$ for all three tests, therefore there is insufficient evidence to reject the null hypothesis.

4. Discussion

Informal economic activity is negatively correlated with the health of public finance, tax revenues and quality of government policies. However, especially in times of economic downturn, positive impacts of informal economic activity can also be observed. Engaging in the grey economy can act as a social cushion that provides temporary monetary relief to the unemployed, thus improving their purchasing power which results in higher consumption. This simultaneously means higher value-added tax (or sales tax) revenue (Kaže et al., 2011). Our findings suggest that less than a third of respondents engaged in informal economic activities and the reasons for the majority of those were directly linked to the pandemics of COVID-19. This suggests that although the informal economy might have expanded during the pandemics, once restrictions are fully lifted and consumption habits normalise, the degree of the grey economy will return to the pre-pandemic level. Almost two-thirds of respondents engaged in the informal economic activity only occasionally, which indicates that such engagements have not become a habit for the majority of consumers. The majority of respondents supported (or did not oppose) the informal economic activities of service providers during the pandemics and the strict lockdowns. This shows that a lot of people opposed

government restrictions and supported small businesses' efforts to survive, despite their engagement in illegal activities. We found that sex is not associated with any of the three tested variables. Males were just as likely to engage in informal economic activity as females and their opinion on the legitimacy of service providers' activities during the pandemics and lockdowns was not associated with sex.

This research suits as a preliminary assessment of the informal economic activity that has occurred during the pandemics of COVID-19 and is a good starting point for our further quantitative study.

Conflicts of Interest: The authors declare no conflict of interest.

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Special Biomechanics at the 7th Socratic Lectures

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Abstract: 7th Socratic lectures featured plenary lecture of osteotomies for hip dysplasia, by prof. Duško Spasovski from Belgrade, Serbia and honorary lectures of Andreas Leithner, Graz, Austria and Marija Ipavec, Ljubljana, Slovenia. This was the final event for the students of the 1st year of Orthotics and Prosthetics at the Faculty of Health Sciences, University of Ljubljana, who during the curriculum of the subject Special biomechanics analyzed a series of X-ray images of hips before and after the operation that was supported by Erasmus student Honza Pluhar. In this way the students were actively participating in a multicenter research connecting four institutions (from Denmark, Serbia, Czech Republic and Slovenia). In the final event, the students provided their experiences with the HIPSTRESS method. Here we report on the teaching activities and the students' experiences.

Keywords: Teaching biomechanics; HIPSTRESS; Online teaching; Online examination; Open book examination

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1. Curriculum

In the academic year 2021/2022, the subject Special biomechanics took part of the spring semester. The lectures were organized in March and April and they were accompanied by analysis of the biomechanical parameters of hips. Socratic Lectures presented the final lectures with the examination. As the use of the computer is necessary and with availability of new technological tools to teach online, the curriculum was performed online (Bambič et al., 2021). Furthermore, we had a visitor student Honza Pluhar, who came for the Erasmus mobility and stayed 4 months in Ljubljana. Mr Pluhar analyzed series of about 150 hips and created tools for support of the learning the method HIPSTRESS (instructions, video). Students downloaded demo version of the program Corel to acquire the geometrical parameters of hips and pelvis that are needed for determination of biomechanical parameters. Biomechanical parameters were determined by using previously published mathematical model (Ursic et al., 2021). Each student analyzed 8 – 12 X ray images. During the lectures, all participants simultaneously analyzed the same X ray image and inserted the measurements as well as calculations in a common google drive Excel document.

2. Examination

The examination questions were sent to the students before the Symposium started. All students were to listen to the plenary lecture, then, they were distributed evenly to scientific sections. All tools and social networks were allowed to be used for answering the questions (Bambič et al., 2021). The plenary lecture was given by prof. Duško Spasovski, an orthopaedic surgeon from Belgrade, Serbia, on hip osteotomies. The honorary lectures was given by Marija Ipavec who besides being the first author of the HIPSTRESS model for hip stress (Ipavec et al., 1999) has a decades long experience of having an above-knee prosthesis. The scientific sections covered the fields of medicine, veterinary medicine, materials, ecology and small cellular particles. Students were encouraged to ask the lecturers to help them with the answers and to invite them to contribute to a Google drive document, however, this opportunity did not take place as the lecturers were delayed in lectures and the students did not get the chance to better organize the support. After the end of the scientific section the students have assembled the answers into one MS Word document and have submitted it to an official mail of the Socratic Lectures within the limited time. Below are the questions and students' answers. The first group of questions addressed analysis of the operation due to hip dysplasia, as shown below.

Q: What is hip dysplasia? What causes it and what can happen if left untreated? Explain it from a clinical point of view.

A: According to (Anon A, 2022), hip dysplasia is an abnormality in which the pelvis and the femur (thigh bone) do not fit together as they should. Symptoms include pain in the hip, limping and unequal leg lengths and we treat it with braces for babies, physical therapy and surgery. It is thought that most people were born with hip dysplasia. The reason is either because of the position of the baby in the womb where there is too much pressure on the hips or it's a genetic problem, where the issue is passed down in the family. If the hip dysplasia is not treated it can cause a lot of long-term problems such as hip labral tear, osteoarthritis, dislocated joint and unstable joint. According to the lecturer Borut Kovačič hip dysplasia from a clinical viewpoint means that the acetabulum can not contain the femoral joint head. It's referred to as the shallow acetabulum socket.

Q: The method HIPSTRESS enables quantitative assessment of hip dysplasia. What are the biomechanical parameters that can be obtained from this method?

A: In his presentation Honza Pluhar stated that the biomechanical parameters that can be obtained from HIPSTRESS method are peak stress on the weight bearing area p_{max} that can be given in dimensionless form $p_{max} r^2 / WB$, where r is the radius of the hip articular sphere and WB is the body weight, the position of the stress pole given by the inclination angle Θ , the hip stress gradient index G_p given in its dimensionless form $G_p r^3 / WB$ and functional angle of the load bearing φ .

Q: What are the surgeon's criteria leading to the decision that the operation will be performed?

A: The plenary lecturer Dusko Sapsovski stated that the gradient index to be positive/negative is an indication for any kind of hip surgery. There are other radiological criteria and clinical criteria for arthritis. If people do not have any complaints or not have significant complaints, the gradient index seems the main prognostic tool.

Q: What is the goal of the operative treatment of hip dysplasia?

A: The goal is to change the orientation of the socket so it can better cover the ball of the hip joint.



Q: There are different osteotomies that change the unfavorable geometry of the hip and pelvis. Why does the surgeon decide to choose the periacetabular osteotomy?

A: A periacetabular osteotomy (PAO) is a surgical treatment for hip dysplasia that is caused by a deformity in the acetabulum, the curved portion of the pelvis that forms the socket of the hip joint. This procedure is in comparison with other procedures much simpler.

Q: What happens to the patient during a periacetabular osteotomy (PAO)? How would you describe the process/changes from a medical and biomechanical perspective? Try to link the answers together.

A: PAO is a surgery used to correct a condition called hip dysplasia or acetabular dysplasia. Hip dysplasia is when there is insufficient coverage of the femoral head (ball) by an excessively shallow acetabulum (hip socket). During a PAO, the acetabulum is repositioned to cover more of the femoral head in order to improve the stability of the hip joint. The PAO surgery improves hip function, decreases hip pain, and stops the damage occurring inside of the joint that can lead to hip arthritis over time. After the surgery the patients have reduced pain, improved hip function and increased activity level scores in short-term. The 10-year outcomes for the PAO procedure show that 80-90% of patients are free of end-stage osteoarthritis (Anon B, 2022).

Q: You have performed biomechanical analysis of hips before and after periacetabular osteotomy by using HIPSTRESS mathematical models. Please give your experience. When you have already learned how to do the task, how long did it take to analyze one hip? Did you have difficulties measuring the center-edge angle? If so, please describe them and how you solved the problem. Were there any other decisions that you had to make that were not straightforward? Please describe them. Were the instructions that you have received (the lectures, the literature, the written instructions, the video) helpful to perform the task? Did you need help from each other by exchanging experience or you could perform the task alone with the suggested aids? Did you have problems with software? If so, how did you solve them? Please include the answers of all the students. The opinions are expected to be different.

A: The answers of individual students are given below.

Student 1: When I first saw our assignment (analysis of hips) I panicked. It was something I had no knowledge about and I thought that it would be impossible for me to complete my work. Then I said to myself that I need to try my best. We all know that the first step is the hardest one and that it usually takes the most of the time. Analyzing the first hip was the slowest - around 1 hour - because I really took time and did things rather slowly, step by step. At the same time I was listening and watching Honza Pluhar's tutorial on how to perform biomechanical analysis of hips before and after periacetabular osteotomy by using HIPSTRESS mathematical models, which was very helpful. I was able to learn so much from him. He explained every step in detail and guided us through all assignments. Because of his excellent guidance I started to enjoy doing my work, one of the main reasons was that I saw big improvement in understanding of what I am actually doing and also time improvement (from 1 hour to around 20 minutes for one hip). I had no difficulties measuring the center-edge angle and my decisions were quite straightforward (well not at the first but with time I was less scared and became more confident in what I was doing). I also did not have troubles with software or Corel in general (it is beginner friendly). I am thankful for all of the support and guidance I had through work, and also for the knowledge I will be able to use in my future career.

Student 2: The first hip I analyzed together with Veronika Kralj Igljč and Honza Pluhar who were explaining it in one of our lectures. It was a long process but after two lectures of them explaining how to use the HIPSTRESS method I finally understood. It took me around 15 to 20 minutes per X-ray. I did not have any additional issues with performing measurements. The software (Corel) was easy to use and very specific, which made it easier to gather the measurements for Excel. I had the most issues with the »Solve« function in Excel but after some help from one of my colleagues I had it covered.

Student 3:- At first I was scared, the work from Honza Pluhar looked complicated. Then I tried it myself. The first patient's analysis took me a while, but by the end I became better at the techniques and procedures to the point where I spent about 10 minutes on each picture. The video was a great help, as I was able to see how something was drawn or measured, and also how the measurements were put into the table. I had no problems with measuring, but it was a bit more difficult to draw accurate circles and points, as the images were a bit foggy. The most difficult part for me was defining the boundaries, so I had to make difficult decisions about where to end which line. I found it difficult to know which measurements to put in which column of the table, so I asked my classmates to help me. The software Corel seemed difficult at the beginning, but once I found out how to use it, it was quite easy.

Student 4. At first I was scared because the program Corel did not work. Then with help from Honza Pluhar and Veronika Kralj Igljč I finally succeeded in uploading the Corel program. Honza Pluhar's instruction and explanation was so helpful. He showed the procedure step by step and it was easy to understand. The first measurement took me one hour, then it was going faster. I had a little bit of a problem with Excel table, but then I watched the video explanation from Honza Pluhar and it was helpful. It was interesting and fun to learn something new.



Student 5. My experience with using HIPSTRESS mathematical models was quite interesting. At first, I was a little bit confused about how to use the program Corel as it was new to me. After listening to the lecture of Honza Pluhar on how to use this program I understood what to do. During the lecture I took notes and after that I helped other colleagues, so I refreshed my knowledge on how to analyze hips. On average it took me 10 minutes to measure one hip. Filling the data in Excel took me most of the time. Overall, I had fun during analysis of hips.

Student 6. The first time using the application, I stumbled upon many difficulties and problems, mainly because I had no prior experience on such work. Thanks to Honza Pluhar who showed us the basic functions of the program and how to analyze hips I managed to find a way to complete all the tasks. After adapting to the program, every hip analysis went faster, therefore it took about 45 minutes at first and later about 25 minutes. I had no problems with measuring the center-edge angle, after repeating the process a few times. The whole process was fairly straightforward after thoroughly watching the instruction video, which was very helpful and was a key part of understanding the software and completing the task. Of course, the task would be nearly impossible without the extra literature and lectures that we received. I sought no help from my colleagues, as I managed to finish everything by myself. The software is very useful and easy to use, the only difficulty I stumbled upon was downloading it without paying a subscription, as it only has a 15 day free period. The issue was solved by using a different computer.

Student 7. In my case the instructions for the HIPSTRESS method were not introduced to me by a professor or a lecturer but by my fellow colleague who has been present at the first lesson of Veronika Kralj Igljč and Honza Pluhar. She then gave me very clear instructions which I grasped pretty much immediately. I had no problem understanding the concept and no problems when using the program Corel. I found the program really easy to use despite never using it before. The X-rays were also pretty clear and fairly easy to read from so I had no problems by placing the points of measurement. It took only 5 to 10 minutes per image. The Excel program was also very useful and well prepared, the equations were pre prepared by Honza Pluhar so all you had to do was enter the measurements. Overall I found the experience very positive.

Student 8. At first I was a bit worried, because it was my first time using a program Corel. Veronika Kralj Igljč and Honza Pluhar did an amazing job explaining the whole process. I had no problems with the measurements, because the X-ray pictures were quite clear so it only took me about 10 minutes per image. The program in general was very easy and fun to use. The Excel table was well prepared, with clear instructions of where to insert each measurement. I also helped my colleagues with their measurements because they did not attend the classes. In the end I found the making of the HIPSTRESS models quite fun.

Student 9. My experience with Corel was quite nice. I did not have any problems with software, the only disadvantage of the program is that the free trial lasts only 14 days. I think Honza Pluhar and Veronika Kralj Igljč made a very clear explanation on how to draw lines and measure certain parts of the hip. The video where Honza Pluhar explains every single step was quite helpful, especially for those who couldn't attend every lecture of biomechanics. I did not have any difficulties with measuring the center-edge angle, however I had some problems determining the extreme point of each femoral head. The first hip took me around 20 minutes to analyze, because I was not used to working with Corel program. Overall it took me 2.5 hours to complete the task, including watching the video of Honza Pluhar and inserting the data into the Excel table. When it comes to the Excel table, this is where I had the most difficulties. I did not know how to calculate the angle θ . This part was a bit time consuming, but with help of my colleagues I managed to do it.

Student 10. My experiences with using HIPSTRESS mathematical models are positive. I was not present at the lectures and did not know the guidelines on how to use Corel or how to make the measurements. Luckily one of my colleagues was at the lectures and I asked her for help. She explained the procedures of using the program clearly and after doing one X-ray with her support I grasped the concept. After that I knew how to do the X-rays by myself. Most of the X-rays were clean but some of them were not and because of that it was a bit hard to do some parts of the measurements on them. It took me about 5 – 15 min per hip. In the Excel table prepared by Honza Pluhar, we had to write the measurements and then the Excel would calculate everything by itself. This type of taking measurements and drawing by using a program were new to me. Overall, it was fun and enjoyable.

Student 11. At first, the task seemed difficult to me, but while listening to the lectures, I realized that it's not. It took me around 40 minutes to complete the analysis of the first hip, but for the other analyses I only needed 20 minutes. At the beginning it was hard to start because I did not know how and I had some problems. But when I understood what I was doing it was easier. I didn't have any problems with Corel or with Excel tables. I think Honza Pluhar and Veronika Kralj Igljč gave us very clear instructions on how to draw and use the program Corel. Also the video of Honza Pluhar was very helpful. It was a great experience.

Student 12 - When I started, I was a little bit scared, because I did not know how to use the Corel program. The whole thing was new to me. I watched the video that Honza Pluhar sent to us. For the first picture I needed more time, because I was not sure if I was doing it right. I asked some classmates for advices and they helped me. I learned very quickly how to do it and at the end it was very



easy. For one picture I needed about 15 minutes. So all this didn't take me much of time. Actually it was fun. It was easy, because the X-ray pictures were really nice and clear. At the end I also had some problems with the excel table, but I learned how to do it right.

Student 13. My first impression of the assignment was that it looked very interesting, because the records were made for the first time. Help was available for both, X-ray pictures and analysis. With the help I was given it took me about 40 minutes to analyze the first couple of X-rays. For the next few it took me about 20 minutes. At first I was trying to figure out how to do things and when I understood, it took less time. In my opinion this type of lectures were very interesting and useful for the future because not many times do we get the chance to do something practical and to use technology (the program Corel). This can not be avoided in the modern world. So I am certain that the skills I have acquired will most certainly be useful in the future when we get to actually do something and take a better look into our field of profession. With the use of the program Corel I had no particular problems because it was easily downloaded and it proved beginner friendly. I missed a few of the lectures in the beginning where Honza Pluhar explained how we were supposed to do biomechanical analysis of the hips before and after periacetabular osteotomy by using HIP-STRESS mathematical models, so I had to ask for help from my fellow students. Also I used the video by Honza Pluhar.

Q: What is your opinion on the results of the biomechanical analysis of the operation?

A: Honza Pluhar stated that 50% of the hips on the contralateral side to the operation in the study got worse as regards biomechanical status. The plenary lecturer Dusko Sapovski agreed with that percentage. The overall opinion on the biomechanical analysis was that it is very important to identify changes in hips that were operated on, and to have accurate measurements of hips that will undergo surgery.

The second group of questions addressed the contents of the scientific sections.

Section 1, Medicine: Q: What is the value of population studies for treatment of a single patient?

A: Population studies are important because they provide the medical doctors and researchers with extensive data that helps them determine the reasons for different diseases and ways to treat them. The more information they have the easier it is for them to connect a specific type of disease to the single patient they are treating. The value of the population studies is immense when it comes to research data and result compilation.

Section 1, Medicine: Q: What are biomechanical features of carpal tunnel syndrome?

A: Previously presented evidence indicates that carpal tunnel syndrome is related to compression of the median nerve inside the carpal tunnel. Biomechanical arguments in which the extrinsic finger flexor tendons inside the carpal tunnel are characterized as a frictionless pulley-belt mechanism are useful to show quantitatively how the wrist size and position and the hand position affect forces on the tendons and their adjacent structures (Armstrong and Chaffin, 1979).

Section 1, Medicine: Q: Which biomechanical features are important in acquisition of platelet and extracellular vesicles-rich plasma? How can knowledge of physics help in optimization of the preparations from blood?

A: In centrifugation of blood, the time was calculated individually on the basis of patient's erythrocyte sedimentation rate (ESR) according to the mathematical model where two options were considered: The "high platelet and extracellular vesicle" protocol aimed at yielding plasma with the highest possible platelet and extracellular vesicle concentration that can be obtained by a single spin preparation without pelleting platelets and extracellular vesicles and the "half volume" protocol aiming to determine the time needed for the upper border of erythrocytes to arrive down to one half of the length of the blood sample (Božič et al., 2021).

Q: Honorary lecture by Andreas Leitzner: Briefly describe the novelties in technology recently introduced in orthopedics and traumatology.

A: The new technologies discussed in the symposium are deep learning machines that recognise patterns, next generation sequencing, particle therapy, robotics, virtual reality simulations, digitalisation e.g. speech recognition and hybrid operation theaters.

Q: Honorary lecture by Marija Ipavec: In your opinion, how can an engineer of orthotics and prosthetics best support a patient that needs an artificial limb?

A: The engineer of orthotics and prosthetics must listen to the patient, anticipate errors from experience. However, cooperation with others, for example physiotherapists, is also required. The engineer must envisage what is wrong with the prosthesis by observing the patient. When the patient starts walking the engineer must know how to select the prosthesis that will be comfortable for the patient.

Q: Please describe briefly (about half a page) the contents of the lectures of each of the sections of Socratic lectures.

A: Section 1: Medicine. The first presentation by Borut Kovačič, Klemen Stražar and Lenart Zore entitled Pelvic osteotomies: experience of University Medical Centre Ljubljana was mostly about pelvic osteotomies. An osteotomy is any surgery that cuts and reshapes your bones. The next topic was Biomechanical analysis of periacetabular osteotomy by Honza Pluhar where we learned that the aim of biomechanics is to create a simple model, based on which it can be decided whether the hip is dysplastic and needs to undergo a

surgery. We discussed the HIPSTRESS model that we also used on our individual X-ray assignments, what is the meaning of the parameters (peak stress, hip stress gradient) and how the results are useful to a surgeon.

Blaž Mavčič in his presentation The role of hip arthroplasty registries and cohort studies in orthopedic surgery explained the historical perspective and additional factors of arthroplasty. Nejc Steiner presented a lecture entitled Surface-based total blood volume calculation for platelet and extracellular vesicle-rich gel preparation, Domen Vozel presented a similar subject: Use of platelet and small cellular particles rich plasma for closure of skull base and Sara Bitenc Zore presented another lecture on plasma preparation: Facial nerve reconstructive surgery in otorhinolaryngology and its enhancement by platelet-rich plasma therapy. In these subjects, the main concept is the use of plasma that can be obtained from individual patients to treat their injuries and wounds. Platelet and extracellular vesicles rich plasma can be made into a platelet and extracellular vesicles rich gel that can later be applied on external or internal wounds and help them heal better and faster. We discussed which factors are important and how they differ, so that they can contribute to give us the optimal results. Some of the factors are patient preparation, blood withdrawal, blood centrifugation, plasma activation and total blood volume calculations. Domen Vozel described the basics of plasma and gel preparation. There are different autologous and heterologous preparations and plasma-based or buffy-coat-based protocols. He also explained the process of application and cryopreservation of preparations. It is believed that in the future these preparations will have more versatile use, mainly in the field of otorhinolaryngology. The presenters showed us a few videos and pictures of how the gel was used in skull-base surgery and how the plasma therapy works/enhances healing after a facial reconstructive surgery.

Section 2, Veterinary medicine: Pšenica Kovačič explained the activities to take care of stray cats and provide the behavior consulting and education of cat welfare. She presented evolution of cats and their changes throughout history and some of her cat clients with health problems (e.g. with low urine output and small ureter volume). Some breeds have more health problems than the others (Persian cats have more health issues than for example F1 savannah cats).

Section 4, Materials: The subjects of the section were the impact of corundum ceramic, TiO₂ and hydroxyapatite nanoparticles on cell line in vitro, fabrication of TiO₂ microflowers and their antibacterial effect against Escherichia coli, additive manufacturing in orthotics and prosthetics and transmission electron microscopy of biomaterials used in total hip arthroplasty. In failed hip prostheses the wear debris particles were successfully extracted from the tissue and were found to be of different composite, mainly Al₂O₃, Ti-Al-V alloy and additional elements such as Ca, Fe and P. Nanostructured Poly(D,L-lactide-co-glycolide)/titania composites may possess the ability to simulate surface and chemical properties of bone and cartilage, respectively, to allow for alternatives in the design of prostheses with greater efficacy. Pure TiO₂ has a high antibacterial effect on pathogenic samples of Escherichia coli from clinical isolates, which is further increased with the addition of increasing concentrations of silver (Mantravadi, 2017). The most interesting topic for many of students was additive manufacturing technology, which in comparison to the traditional one has many advantages that can solve problems such as material wasting and time consumption. On the other hand it also permits customization for special applications or consideration of individual characteristics, which are both very important when it comes to treatment and rehabilitation of patient.

Section 5, Small cellular particles: Nowadays there are improved methods of seeing cells, such as scanning electron microscopy - SEM and transmission electron microscopy - TEM. These methods are appropriate to observe also apoptotic bodies - small cellular particles that contain substances and information from cells that are undergoing programmed death, and other types of small cellular particles. To isolate small cellular particles that are shed into cell exterior, they should be separated from cells. Centrifugation is one of the methods for this purpose. However, at higher centripeta acceleration of the centrifuge rotor, the cells change shape or become destroyed, which is also one of the mechanisms to yield small cellular particles in the isolates.

SEM gives us the best records of three dimensional shape of small cellular particles. Also, this technique allows for observation of a large number of individual particles. However, the preparation of the sample is quite invasive and it can destroy or transform small cellular particles. In cryogenic TEM however, the sample is vitrified in ice of the thickness of around 100 nm. The preparation of the sample for imaging is less aggressive than in SEM, however, particles larger than about 500 nm are not appropriate to observe by cryogenic TEM because they could not be contained in thin ice. This imposes constraints in isolation of particles.

3. Discussion

It is evident from the answers of the students that they have entered a discussion with the speakers. Also, they have used internet to arrive to the answers to the questions. Their most exhaustive answers were on their experience with determination of the biomechanical parameters, which is most valuable. Almost all the students described that they were able to learn the HIPSTRESS method with the help that was provided by a teacher and a peer student Honza Pluhar. The students of orthotics and prosthetics were of the first year while Honza Pluhar is completing his masters in Prague. He is skilled with mathematics, physics and computer science. He performed the analysis of the majority of hips, checked the quality of the determination of geometrical parameters of the students, provided

tutorials and also presented the results in lectures to the students and to the colleagues in Socratic lectures symposium. As our aim was to analyze a great number of hips, the input of the students was important to be able to accomplish this aim.

In designing the students' activities during the symposia, it was our wish to introduce new possibilities into teaching; as it is now possible to share documents, more persons can write the document at the same time, so this document started to form at the symposium. Within Section 1 (Medicine) a 15 minutes interval was reserved to students to discuss their questions and it was suggested that the students can ask the presenters to help them improve the document. Unfortunately, this event could not take place because some presenters took more time than expected and the participants were then late for the joint section presenting honorary lectures. It is our wish that important aspect of reviewing the answers on spot would be taken into account. For example, the students' answer to the question: Why does the surgeon decide to choose the periacetabular osteotomy? was that this procedure is in comparison with other procedures much simpler. As great skills of the surgeon are necessary to perform this operation, an opinion of the surgeon that has ever performed this operation would be of much value.

4. Conclusions

The students have proved that they collaborated well to formulate the answers. They used the English language to communicate with the participants of the international symposium. They helped each other to complete the task on assessment of biomechanical parameters. One of the students wrote: »So I am certain that the skills I have acquired will most certainly be useful in the future when we get to actually do something«, not being aware that they already actually entered research with their interest in the subjects, providing the data for the biomechanical analysis and supporting the participants of the symposium.

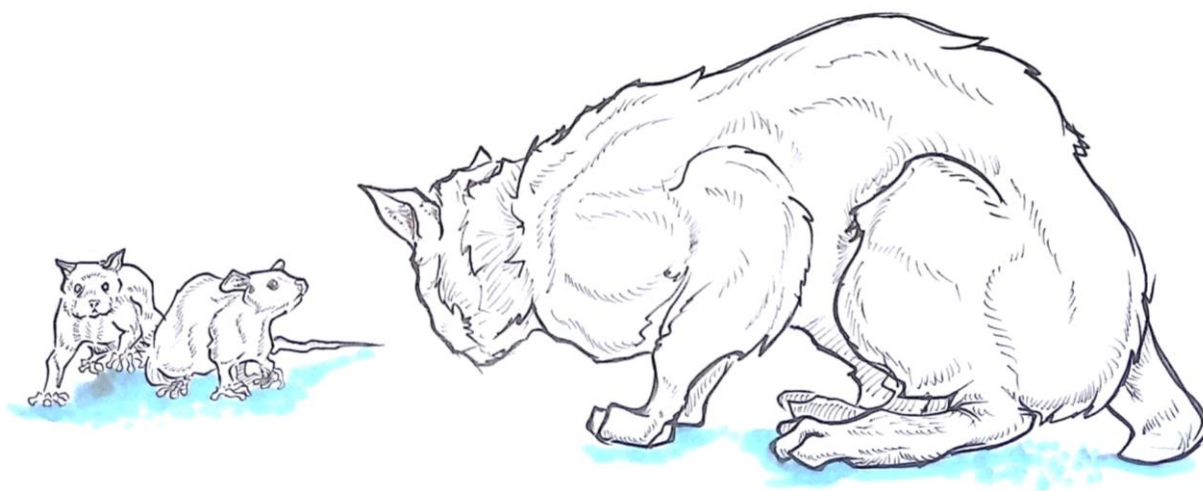
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Invited lecture/Reflection

A Reflection on Effect of Human on Evolution of Cats

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Abstract:

In this text we focus on the problems in human interference with evolution of domestic cats. Based on documented records on pedigree cats, their exhibitions, and photographs, a brief comparison is made with the popular culture, status and situation of cats in the 21st century. Problems arising from physiological and psychological transformation of cats living with human are discussed. In particular, art is exposed as a possibility to influence the understanding of cats and their relation with human.

Keywords: Persian cat; Exotic cat; Scottish lapwing cat; Munchkin cat; Sphynx cat; Cat breeds

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1. Introduction

Domestic cats have the status of a family pet or a domestic breed. Several breeds of brachycephalic cats are known with phenotypic characteristics such as large eyes, short muzzle (brachycephalic skull shape; e.g. Persian cat), long (dolichocephalic skull shape; e.g. Siamese cat) and small ears. Other physiological traits include decreased level of stress hormones, increased reproductive period and accelerated or slowed down somatic development. Besides Scottish fold, Munchkin and Sphynx breeds are brachycephalic, presumably because of the "cuteness" 'attributed to them. Persians are divided into two groups according to the degree of brachycephaly: the traditional doll face and the show face (**Figure 1**). They are categorised into four categories of brachycephaly: mild, moderate, severe and extreme. In modern times, Persian cats are no longer leading in popular culture; this role has been taken over by hybrid cats – crossbreeds between wild and domestic cats.

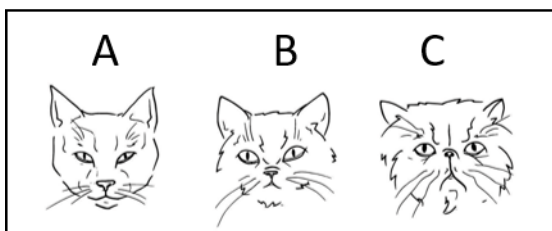


Figure 1: Mesocephalic shape, doll shape and show brachycephalic shape of the cat face.

However, brachycephaly causes respiratory, ophthalmological, dermatological, reproductive, endocrinological, and neurological health disorders that affect quality of cat's life. Brachycephaly and its systemic effects have led cats to have difficulty breathing, smelling, eating, defecating, and rolling (O'Neill DG., Romans C., Brodbelt DC, 2019). In particular, the sense of smell is the cat's primary sense of perception and communication. However, with a cranial deformity, perception is impaired and with it the ability to understand and order the stimuli of the world in an action-oriented manner. Brachycephaly affects motor, sensory and cognitive abilities. Health, cognitive and behavioural problems either make it difficult for cats to perform basic biological functions or render them dependent on humans (Hartwell S. 2019).

Domestication means human control over feeding, care and breeding of the animal. We prefer to speak of self-domestication in the case of cats and domestication in the case of dogs. In cats, we can speak of self-domestication over the last millennia and only recently of domestication. In the early days of agricultural culture (which began in some places 12,000 years ago in the Palaeolithic age and elsewhere 10,000 years ago in the Neolithic age), cats approached humans as predators that hunted prey in fields and stored grain (Budiansky, 2003). Cats can be characterised as free-ranging predators that can find and hunt their own food. However, over time, cats evolved from fierce, territorial and independent animals to animals that were more tolerant to human society.

2. Cat Shows

In July 1871 (the Year of the cat), the first cat show was held at the Crystal Palace in Hyde Park, London. Since then, the cat shows were increasing in popularity. Persian cats (a specimen is shown in **Figure 2**) were the most popular breeds, as were also Angoras, Siamese cats, tailless Manx and hybrids. The cats were different in sizes, weights, shapes, coat lengths, coat colours, patterns, eye colours, and also different degrees of disability, e.g., a white deaf cat with blue eyes which nevertheless won first prize. The show was a status symbol for aristocrats with the trend towards cat shows, pedigree cats and hybrids continuing to now. It was suggested that certain colours and coat patterns play a communicative role as well as a protective one (Jaroš, 2012). However, physical constitution and condition dictate a cat's level of fitness and thus its compassion, and that mesocephalic facial features influence normal perception and basic functions, especially eating.



Figure 2: Blue Persian "Gentian" owned by Lady Marcus Beresford, the prize-winner at Westminster in 1899. Courtesy of Landor, Public domain, via Wikimedia Commons.

3. Art

Art has always been a commentary or interpretation of reality. The depiction of cats in art history is an expression of observed characteristics that people symbolically idolise, which crosses over into mythology and religion. In that, the cat properties (including the facial features) are interpreted as to serve expressiveness and attractiveness to humans. This is, in turn, detrimental to understanding of a cat and living with it.

Have artistic representations contributed to the re-humanisation of the domestic cat? Could this motif be the cause of unethical breeding in impoverished, under-sized and cramped living conditions for those who were once acrobatic hunters and adventurers? Today's co-habitation and upbringing impose the need of human to make them "nice and cuddly". Is the desire for absolute control over a living creature hidden behind this in the name of love, care and safety? Can (popular) art put a stop to this or establish a critical distance from what it has so far presented as curiosity, cuteness and popularity? The artist is complicit in the corruption of his/her audience (Warner, 2018); the way he/she is portraying cats makes him/her partly responsible for their well-being.

3.1. *Naturalism and realism*

In the 19th century, in the age of industrialisation, especially with the advent of photography and new artistic movements, Naturalism and Realism (1830-1899) developed which no longer idealised and stylised motifs as in Neoclassicism (1750-1830) and Romanticism (1790-1880), but rather depicted them realistically or as they are. The motifs were everyday scenes, village genres, everyday objects and, of course, cats. The supposed cuteness and playfulness of the cats were in the foreground, but also their serenity and mysticism. While cats were depicted as companion animals, especially on the lap, there are many motifs of cats doing their business outdoors or indoors.

3.2. *Painting*

Henrietta Ronner-Knip, who came from a Dutch family of painters (1821-1909), mainly painted cats. She took in a stray cat which multiplied so much that the cats took over her whole studio and her motifs. She created more than 100 works featuring cats in drawing, watercolour on paper and oil on canvas. The subjects ranged from hunting and rustic to more playful genres. She depicted individual and group portraits of cats hunting, fighting, playing, resting, etc. She was interested in the poses, facial expressions, movements and behaviour of cats in different environments and situations. Not all her works are dated, but it is noticeable that the cats she painted in her home are more endearing than her earlier naturalistic works.

3.3. Photography

With the development of photography and its general availability in 1839, the photographic industry and the art of photography flourished (**Figure 3**). The photographer Harry Pointer is considered the most famous cat photographer. He started with ordinary subjects or cat subjects, but later moved to more anthropomorphised expressiveness for more appeal and humour. He photographed cats in various costumes, dresses, uniforms, ties, bow ties, in scenes on a tricycle, in shoes, at a tea party, etc. The photos were available as postcards with captions, e.g. "A happy new year!", at a tea party in "Five o' clock tea", at a dinner party in "Bring up the dinner Betsy". It could be said that this started the trend of humorous cards that are now known as memes.

The term meme is an abbreviation of mimeme from ancient Greece and means imitated thing. A meme is something that is spread, transmitted and exists among people as a cultural, social phenomenon. Today they are online messages, ideas, mainly in the form of a digital image with a caption. Memes spread, multiply, mutate, adapt...



Figure 3. "The Shah", a photo of a white Persian cat from "The Brighton Cats" series. Photo: Harry Pointer (Pointer, c1870).

The most popular memes are those of Grumpy Cat, a female Tardar Sauce (2012-2019) who was perceived as a grumpy and unhappy cat due to her peculiar appearance (dwarfism; large bulging blue eyes, sunken muzzle, drooping lips). She was anthropomorphised into a cynical and negativistic cat to achieve a deliberate misinterpretation that was amusing. Her defect turned her into a celebrity and earned the cat or (literally) its owner fame and fortune on the internet.

If we compare today's domestic Persian cat of 1871 with the exhibition Persian cat of 2022, we notice morphological changes in today's Persian cat, e.g., more concise facial features, larger, bulging round eyes, a smaller and suppressed muzzle, narrower nostrils and a shorter nasal bridge, a smaller and shorter muzzle and smaller ears, a lower and broader head, skin folds on the face, and a stockier body with lower and broader legs.

4. Neoteny

Neoteny means "stretching youth" (Kollmann, 1885) and is a "paedomorphosis" resulting from a slowing of somatic development (Gould, 1977). Heterochrony is observation of personal development or ontogenetic differences resulting from growth over time (Bavdek, 2015). It is divided into paedogenesis, in which the development of the body is arrested at sexual maturity, and paedomorphism, in which (some of the) juvenile characteristics are retained in the sexually mature individual. Part of the latter is neoteny. Neoteny is the retention of traits from youth in various forms, either phenotypic or developmental, at a later age. Biological neoteny leads to behavioural neoteny

and thus specific cognitive and emotional abilities and capacities. Neoteny is thought to, among other things, affect the brain and affect its plasticity. This can occur spontaneously, intentionally or through the development or neglect of certain neotenic traits.

Partial neoteny due to self-domestication could be evolutionarily possible, but it would likely occur spontaneously and slowly. An experiment by Russian scientist Dmitri K. Belyaev with Siberian foxes from a fur farm showed that the animals change morphologically, emotionally and behaviourally by selecting friendlier animals that show less fear and/or aggression towards humans. This is a domestication syndrome in which the animals spontaneously change their appearance to become more like human (Belyaev et al., 1983).

5. Owning and breeding

In nature, cats are capable of independent and self-reliant living and do not need humans to survive. In urban environment, domestic cats have developed survival strategies to coexist beside humans. If a cat is not neutered, uncontrolled breeding can be a major problem for the fitness and health of the cat itself and the cat colony, for the safety of other individuals, especially prey, and for hygiene. Too many cats in too small a space with too few resources is a recipe for feline disease and people's dislike of cats.

Owning and controlling is not the same as understanding and caring, and moral and ethical questions are raised about modern cat life. What causes humans to care for or "parent" cats?

It has been hypothesised that it is a neuroendocrinological link that regulates the emotional and motivational system of caring for others, especially children. When a person is young or dependent on parental help, this is reflected in development of the endocrine system and affects psychophysical state resulting in helping behaviour. In adolescence, this mechanism becomes inhibited by reduction of dependence on parents and care. This applies not only to biological parents, but also to owners, caregivers and guardians of cats (Herbeck et al., 2022). Problems arise when the issue moves from cooperative parenting to the level of ownership and control.

In preparing the mother cat to have kittens, sufficient quality food and a quiet place are sufficient. The gold standard of proper socialisation and habituation of the kittens is between 2 and 9 weeks of age. At that age they are gradually and appropriately exposed to external socio-physical stimuli according to their physiological and behavioural development, especially those with which they will be surrounded due to humans in the course of modern life. Excessive interference in the development and upbringing or unnecessary replacement of the mother cat may make cats more accustomed to human, but it also deprives them of their feline essence.

The moral and ethical dilemma is not only the breeding of abnormalities in the name of beauty (Ewer RF., 2018), but also of creating hybrids which were exhibited and popular at the above-mentioned cat show as early as 1871. Both trends are on the rise; breeders, owners and groomers consider grooming as a matter of daily routine rather than an indicator of disease and unsuitable conditions (Plitman et al., 2019). There are exemplary breeders who are committed to the health and beauty of cats.

Cats have natural instinctive needs and behaviours, natural-learned needs and behaviours and (un)learned needs and behaviours. Natural behaviours are primary and enable survival: seeking shelter, food, fluid (or eating and drinking), excretion, reproduction, avoidance of unpleasantness and retreat into pleasantness, or the need for safety. These are genetically determined and do not require associative learning. All this happens more or less instinctively or through non-associative learning, such as habituation- dishabituation, sensitiation- desensitisation. The Five Freedoms (Mellor and Reid, 1994, Mellor, 2017) concerns satisfying these needs. Since domesticated cats no longer need to strive for existence, its striving shifts to a new model of well-being: to satisfaction of motor, sensory, cognitive and social needs.

Due to modern lifestyles and selective breeding, Persian and domestic cats generally live an exclusively indoor life in an impoverished environment, are less athletic and acrobatic, and have untapped potential. In Persian cats, this potential has been genetically suppressed, making them slower to react, less interested and less active.

6. Consumer neoteny and anthropomorphism

Consumer neoteny, or infantilis ethos is an infantile form of consumer behaviour in adulthood in which people conflict with reality. It was suggested that anthropomorphised products or services allow them to escape from reality into phantasms (Olive, 2016). With products, in our case live cats or anthropomorphised representations of cats, instead of a responsible and sober relationship, a dependent and insatiable relationship is established for the sake of human satisfaction and well-being. It has been found that people with a higher level of education, especially those in animal science professions, have a better understanding of the welfare of cats with brachycephalic (and dolichocephalic) face shaped cats. Mesocephalic cats with medium length hair, orange colour, a striped pattern and green or blue eyes received the highest likability scores. Most owners have a preference for cats they own or have owned in the past (Plitman et al., 2019).

7. Conclusion

Why is demystifying the motifs for cat breeding important? Because it exposes attitudes towards cats and other animals, allows for better representations of cats and other animals and strives for their higher-quality of life.

Conflicts of Interest: The authors declare no conflict of interest.

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Invited lecture/Review

Monteverdi and Seconda Pratica: Music Should be at the Service of the Word

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Abstract:

This article provides insight into the music of the late Renaissance and early Baroque in Italy. Composer Claudio Monteverdi was one of the most important figures in the music of the early Italian Baroque. We consider the events that led to the creation of the new early Baroque style – Seconda pratica - (second practice) and describe the significant changes in vocal music that took place with the aim to depart from strict counterpoint at the turn of the 16th century.

Keywords: Claudio Monteverdi; Seconda pratica; Venetian school; Madrigalisms; Ornamentation

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1. Introduction

The Early Baroque period, which overlaps with the late Renaissance, is a period in which ancient Greek literature, philosophy and ideals revived throughout Europe. Empirical science was also developing and reaching its peak. In music, this was reflected in experimentation with new approaches to composition. This led to a number of treatises and reflections on the "new music". In 16th-century Italy, there were two leading schools of composition: the Roman School and the Venetian School.

1.1. Roman School

"The Roman School was founded at the Council of Trent, which set new directions in art and reformed music. In 1545, the Council of Trent, Italy, the Catholic Church adopted its position on the Reformation that was sweeping Europe at the time. All areas of the Church were discussed, including music. It was considered necessary to correct all the anomalies in the musical heritage that had accumulated over the last three centuries, such as the use of secular melodies, instruments, women's voices and virtuoso improvisations." (Reese, 1954) The main characteristics of the Roman school are: strictly ecclesiastical style, use of Gregorian chant and conservatism reflected in the equality of voices. Any excessive ornamentation was rejected, the texts should have been intelligible, homophonic declamation, no emotionality or drama in music, and the use of instruments in church and music was forbidden. It is a fusion of the Dutch school of composition with Italian melodicism, and it adopts a sea cappella style. Despite its conservative style, the Roman School represents the height of vocal polyphony. The main forms were the mass and the motet. An important representative of the Roman school is Giovanni Pierluigi da Palestrina, who cultivated the strict homophone of the contrapuntal style using imitation.

1.2. Venetian School

Venice, once Italy's most important trading city, has played an undisputed leading role in European history since the 10th century. It flourished in the 15th and 16th centuries. Their contribution to Italian humanism and to Renaissance art is extremely important. The Venetian school of composition is the complete opposite of the Roman school. The main characteristic of the Venetian school is the polyphony (Italian: *Cori spezzati*), the practice of performing a polyphonic composition with choirs separated in space. This technique is not new, but it nevertheless reached its peak in Renaissance Venice, with the architecture of St Mark's playing a particular role. High-impact effects were sought, which most often resulted in effective colour effects. In vocal compositions natural accentuation of words, chromaticism as a "colouring" effect and virtuoso improvisation were used. Instruments were often associated with the singing, and for the first time the prescription of a specific instrument can be found, being a characteristic of the later Baroque. The Venetian school contributed to the independence of instrumental music, the development of monody, and consequently to the development of opera. The culmination of the work of this school is represented by the composers Andrea Gabrieli (1510-1586) and his nephew Giovanni Gabrieli (1557-1613), who is considered to be the greatest representative of the Venetian school in the period of classical polyphony. Giovanni Gabrieli sought his musical expression in the colourfulness of the harmony and in the search for new sonorities in relation to the instruments. Giovanni Gabrieli is, so to speak, the herald of the early Baroque, in which Giovanni's musical tradition is summed up by Claudio Monteverdi.

2. Claudio Monteverdi

Claudio Monteverdi was born in Cremona in 1567, the first-born son of Baldassare Monteverdi (1542-1617) and Magdalena Zignani, his mother. Monteverdi's name appears in the baptismal register under the date 15 May 1567. It is known that he received his first musical training, which included studies in composition, singing and playing the viola, under the tutelage of Marcantonio Ingegneri. He soon revealed his exceptional musical abilities when he published his first collection *Sacrae cantiuiculae a tre voci* when he was only 15 years old. His most important works are operas *L'Orfeo* (1607), *L'incoronazione di Poppea*, Nine books of Madrigals, *Vespro della Beata Vergine*, and others. Claudio Monteverdi called his own style "Seconda pratica". It is characterised above all by a strong

expressiveness, which is strongly linked to the text. He used the term to distinguish himself from the rigid rules of counterpoint dictated by *Prima pratica*. Although he acknowledged the *Prima pratica*, he remained faithful to his own style and adhered to the principle: "Music should be at the service of the word". The first and guiding idea is the word, which begins to acquire a superior position over music. The meanings of the words dictate the music and thus the harmony. Composers began to use dissonance in ways that do not conform to the rules of counterpoint: preparation, performance, resolution. Their intention was to use unprepared dissonances to achieve the effect of colouring the words, which thus took on a completely different dramatic quality. "Linked to the theory of affects (*affetti*), this can be considered the first form of rhetoric to be adopted in the history of music. Its aim was to "mouvere gli affetti" - to move (the emotions of) the listener. The ancient Greeks were already aware of the ability of the art of sounds (*arte dei suoni*) to influence the emotions, so theorists and musicians began to use them in their music." (Tarling, 2004)

3. Madrigalisms

The end of the Renaissance is marked by a refined and extravagant style called Mannerism. It appeared simultaneously in music, painting, sculpture and literature. Mannerist painters no longer used regular shapes, pure and clear colours, or a linear representation of space in their works, as was typical of Renaissance painting, but their figures are elongated, twisted, as if caught in some inner cry. If we look for example at the pictures of Michelangelo Caravaggio: *The Conversion of Saint Paul*, or *Incredulity of Saint Thomas*, we can clearly see the characteristics of Mannerist painting. "The play with colours and shadows, the menacing faces, the introvertedness, the seriousness. Music bears the main characteristics of Mannerist painting too. Mannerism was manifested in music through the effects that composers used to emphasise their own feelings, the atmosphere of a piece. These effects are called madrigalisms (**Figure 1**) because they come from the modern expressive madrigal." (Busettini, 2015). Chromatic progressions, unprepared dissonance, ornaments, sudden pauses, harmonic contrasts and tempo changes were used to illustrate the lyrics. They served as means of expression and had the aim to move the listener (*muovere l'affetto del'animo*). Ornaments served as expressive devices, colouring the words and giving them a certain accent, an emphasis. Composers did not always write them down in musical notation, as they were part of free improvisation.

3.1. Interval of diminished quart

Diminished quart (**Figure 1A**) is an interval whose use has been prohibited in strict counterpoint. Composers love it and often use it to emphasise important passages that express deep restlessness. It is often used in isolation for this very reason. It is used in pieces with a slow character to emphasise bitterness and pain. The example (**Figure 1A**) is from Act 2 of Monteverdi's *Orfeo*. Here Orfeo learns that his beloved Eurydice has died and now mourns her death. Monteverdi condenses all Orfeo's pain by jumping down of the interval of diminished quart on the very important words "Tu se' morta" - You are dead (my life, and I live?) And he emphasises the pain once more with a semitone movement.

3.2. Descending semitone

In affect theory, the descending semitone (**Figure 1B**) is used to represent a sigh, a sob. Composers have used this effect to emphasize the drama of the text, the moment. In the aria "Messaggiera" from *Orfeo*, Monteverdi used the falling semitone on the word "sospiro"-meaning sigh-and added a dramatic note by using an eighth-note pause followed by another semitone progression.

3.3. Trillo

Ornaments served as expressive devices, colouring the words and giving them a certain accent, an emphasis. Usually, composers did not always write them down in musical notation, as they were part of free improvisation. Unlike instrumental music, the term *trillo* in 17th-century vocal music referred to the rapid repetition of the same note (**Figure 1C**).

Figure 1. Madrigalisms. A: diminished quart, B: descending semitone, C: trillo, D: groppo, E: ribattuta di gola, F: cascata, G: tirata.

3.4. Groppo

The groppo or gruppo (Figure 1D) is our modern trillo, used at the end of long phrases or cadences. It consists of an alternation between the downbeat and the main note and is started gradually. In vocal music, the "groppo" or "gruppo" is what we understand today as a trill. It consists of a second of alternating motion. It is performed gradually at first and then continues at an accelerated speed, most often ending in a decomposition. Trills are most often used at the end of pieces on cadenzas or as an intermediate figure during the piece itself.

3.5. Ribattuta di gola

Ribattuta di gola is a vocal ornament also found in literature for keyboard instruments. It is mainly created for doing *accelerando*, as it consists of an initial slow dotted movement (on the interval of a second) which gradually increases. Often the ribattuta results in a trill (Figure 1E).

3.6. Cascata

A cascata (Figure 1F) is an ornament characterised by a rapidly descending movement. Most often this fast passage consists of 16 notes. Giullio Caccini also calls it a "caduta" and in "Le nuove Musiche" he describes four types of "cascata".

3.7. Tirata

While the Renaissance style provided few leaps incorporated in lines of gradual movement, in the Baroque period the melodic leap gave emphasis to the notes that follow. This affect, which comes from the modern vocal madrigal, was a device for emphasising the text at a particular point with a stronger and more sonorous voice; as a contrast, it was usually followed by a softer and gentler passage (Figure 1G).

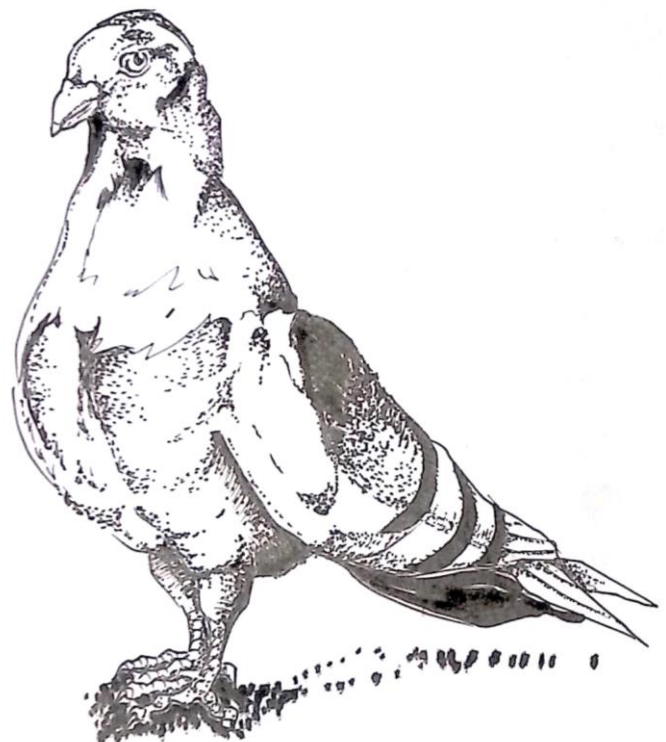


7. Conclusions

Claudio Monteverdi was one of the most important figures in the music of the early Italian Baroque. Vocal music in the 17th century dictated the way in which instrumental compositions were written and performed. The *Seconda prattica* and the parallel development of monody had a direct influence on the creation of instrumental music.

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Scientific contribution/Original research

Music in the life and works of Ivan Sergeyevich Turgenev

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Abstract: The article deals with the role of music in the life and literary works of Ivan Sergeyevich Turgenev. Music played an important part in his life, partly owing to his friendship with the mezzo-soprano Pauline García Viardot, who remained his muse for forty years, until his death. Turgenev was familiar with several musical genres, especially classical music, and knew personally many leading musicians and composers of the time, such as Liszt, Berlioz, Gounod and Saint-Saëns. Different musical impressions found an important place in the works of the writer, and some of the musical fragments of Turgenev's works are quoted and discussed in the article.

Keywords: Turgenev, Pauline García Viardot; Classical music; Italy; Opera; Rossini; Russian romance.

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1. Introduction

“But Misha, what a man! I too would immediately fall in love with him. A poet, talented, an aristocrat, handsome, rich, smart, educated, twenty-five years old - nature has given him everything. And on top of that, he is by nature very approachable, goodlooking and well-mannered.” (F. M. Dostoevsky in a letter to his brother Mikhail, 1846) (Saraskina, 2021, p. 135)

Ivan Sergeyevich Turgenev (1818-83) was one of the best stylists in Russian literature. All his life he was closely connected with music, and music also played an important role in his literary work. As a well educated personality, intellectual and aristocrat, Turgenev listened to music in salons and also attended music-making sessions both at home and at aristocratic dinners and parties, in concert halls and at the opera, as well as in pavilions in parks in resorts abroad. In addition to classical music, he was obviously familiar with Russian folk music and a special genre of song known as Russian romance. All these different musical genres and diverse musical venues are often mentioned by Turgenev in his work.

An extremely important feature of the writer's life was meetings with his muse, one of the greatest artists of that period, the mezzo-soprano Pauline (Polina) García Viardot, with whom he remained in contact for four decades until his death.

2. Pauline García Viardot – Turgenev's muse

Turgenev saw and heard Pauline García Viardot singing for the first time at performances of Rossini's Barber of Seville in St. Petersburg in 1843 and immediately fell in love with her. Between 1843 and 1845, the whole town was enthusing over the interpretation of the role of Rosina sung by this talented singer. The first tour of the famous mezzo-soprano in Russia in 1843 was followed by a second tour the following year and later a third. At that time, Pauline Viardot's husband, Louis, bought a house in Paris, where they began to organize private concerts and performances. The Thursday evening salon at the Viardots' house at 48 Douai Street in Paris was one of the most famous in the late 1840s and 1850s, being attended by the leading artists of the time. Among them were the painter Eugène Delacroix, the composers Gioachino Rossini, Hector Berlioz, Camille Saint-Saëns and Richard Wagner, and the writers George Sand, Gustave Flaubert and Ivan Turgenev. Musical evenings were also held at the Viardot family's rural residence in Courtavenel Castle (Landru-Chandès, 2021, 6/8). Although there can be no doubt about Turgenev's feelings for Pauline Viardot – they are documented in a long correspondence between them – it is difficult to determine clearly what Pauline's attitude towards Turgenev was. It was definitely a solid friendship and a great intellectual and artistic closeness. According to some speculations, Pauline's fourth child, Paul, was Turgenev's son (even Paul himself later mentioned this possibility) (Landru-Chandès, 2021, 8/8). In any case, it was an interesting "ménage à trois". Turgenev adored all of Pauline Viardot's children and became almost a member of the family. Wherever they travelled, he followed them. When the Viardot family was in exile in Baden-Baden (between 1863 and 1870), Turgenev also moved there, and the Viardots' villa there has since become the main musical centre of the city. Visitors to their salon even included the royal couple, the King of Belgium, the King of the Netherlands and Minister Bismarck, as well as important musicians such as Franz Liszt, Camille Saint-Saëns, the violinists **Henri Vieuxtemps, Pablo Sarasate and Joseph** Joachim, and the pianists Anton Rubinstein and Clara Schumann, a close friend of Pauline Viardot. In their salon, they also performed works by Verdi and the then revolutionary Wagner. Pauline taught singing and her students from all over the Europe included several Russian women whom Turgenev invited to participate during his travels (Landru-Chandès, 2021, 7/8).

At the outbreak of the Franco-Prussian War in 1870, the Viardot family emigrated to London, but when they finally returned to Paris in 1871, Turgenev officially settled to live with them so that he could hear his muse singing every day. Visitors to their salon included Saint-Saëns and Flaubert, as well as new faces such as the composers Jules Massenet and Gabriel Fauré and the writers Emile Zola and Guy de Maupassant (Landru-Chandès, 2021, 7/8). Since Pauline Viardot's family lost their residence in Courtavenel while away in exile, Turgenev bought a new villa in Bougival, where their

neighbours included the young composer Georges Bizet, whose opera *Carmen* greatly impressed Pauline Viardot (Landru-Chandès, 2021, 8/8).

Ivan Turgenev remained in contact with his muse Pauline for forty years, until his death in 1883. She remained with him throughout the last few weeks before his death, when he even dictated his last two short stories to her.

3. Music in Turgenev's novels

3.1 *Music in the salons*

Turgenev's most "musical work" is his novel *A House of Gentlefolk*, in which almost all the characters make music, compose, play the piano or sing; even if they don't play an instrument, they sincerely love music. Varvara Pavlovna was already considered the best musician in her boarding school, but her husband Lavretsky did not receive a musical education. "Though he played no instrument, he was passionately fond of music, real classical music" (Turgenev, 2001, p. 52). *A House of Gentlefolk* also features the old German, Christopher Fedorich Lemm, who was a music teacher and composer and taught music to the young noblewoman Lisa. Among other things, this man also set Schiller's ballad *Fridolin* to music. When he talks to Lavretsky about writing an opera, Lemm admits that he no longer feels capable of it, as opera requires "the liveliness, the play of the imagination, which is needed for an opera" (Turgenev, 2001, p. 53).

Franz Liszt even played twice in the salon of Varvara Pavlovna, who often sat at the piano and played for those present. Once, for example, "she sat down without ceremony at the piano, and very correctly played some of Chopin's mazurkas, which were then just coming into fashion" (Turgenev, 2001, p. 39). According to the novel, we can conclude that Turgenev was writing around 1829. Turgenev also mentions Chopin's popularity in high society in his novel *Smoke*: "the Princess Babette, she in whose arms Chopin died (the ladies in Europe in whose arms he expired are to be reckoned by thousands) [...]" (Turgenev, 2016, p. 3).

On another occasion, for example, Varvara Pavlovna "began suddenly playing a noisy waltz of Strauss, opening with such a loud and rapid trill that Gedeonovsky was quite startled. In the very middle of the waltz she suddenly passed into a pathetic motive, and finished up with an air from 'Lucia' Fra poco....¹ She reflected that lively music was not in keeping with her position. The air from 'Lucia,' with emphasis on the sentimental passages" (Turgenev, 2001, p. 94) moved one of the listeners. Once, Varvara Pavlovna played "a brilliant and difficult étude by Hertz very correctly. She had great power and execution."² (Turgenev, 2001, p. 93).

In one of the scenes in the novel, Varvara Pavlovna proposes that Panshin, a young man who also composed himself, should sing a duet, namely 'Son geloso' or 'La ci darem la mano' or 'Mira la bianca luna'. The first aria, 'Son geloso del zefiro errante', is a duet of Almina and Elvino from Bellini's opera *Sonnambula* (The Sleepwalker). 'La ci darem la mano' is sung by Don Giovanni and Zerlina in Mozart's *Don Giovanni*. 'Mira la bianca luna' is a duet - a serenade from Rossini's *Les soirées musicales*.

In Turgenev's "most German work" (as Dostoevsky described it in his *Writer's Diary* for the year 1873), *Rudin*, the piano is played in the salons, mainly music by Beethoven and Thalberg³ - more

¹ Fra poco a me ricovero' is a tenor aria, the pre-death aria of Edgard in Donizetti's *Lucia di Lammermoor*.

² Heinrich Herz was a Viennese-born pianist, composer and piano maker who studied and lived in Paris, performing as a virtuoso in Europe, Russia, North and South America and Mexico.

³ Sigismund Thalberg was born in Switzerland and was Franz Liszt's biggest rival in the 1840s. At that time, all of Paris was divided into two camps: Liszt fans and Thalberg fans. Thalberg's pianistic "secret" was a technique inspired by an English harpist, Parish Alvers. It was thought to be impossible to play the piano with one pair of hands and therefore to appear to be playing with three hands, so he was nicknamed the "harpist" among pianists (Schonberg, 1971, p. 141). His compositions, among them fantasies on themes from operas, are mostly intended to express exceptional virtuosity. The international career of this virtuoso, who was also one of the first great pianists to make a tour in America, ended voluntarily a few years before his death. Extremely rich, he moved to Naples and cultivated his vineyards (Schonberg, 1971, p. 144)

precisely, his études. Turgenev was able to listen to Beethoven at musical evenings with Pauline Viardot, especially at the Courtavenel residence, where they played all of Beethoven's sonatas and his symphonies in piano arrangements (Landru-Chandès, 2021, 8/8). Thalberg and Liszt, however, had been regular guests at the Paris salon of George Sand and Frédéric Chopin a few years earlier (Landru-Chandès, 2021, 3/8). Schubert's Lied 'Erlkönig' is also mentioned – this typical romantic music to Goethe's ballad of the same name is in perfect harmony with Turgenev's romantic bent.

In the novel *On the Eve*, a Russian of German descent, Zoya Nikitishina Müller, who was known to sing and play the piano beautifully, suggested 'La dernière pensée de Weber' when she was asked to play something sad. This is a composition by Carl Gottlieb Reissiger,⁴ extremely popular and often performed in mid-19th century salons, being used by some composers as a theme for variations and fantasies (such as Johann Peter Pixis for piano and Ferdinando Carulli for guitar), and orchestrated by Jacques Offenbach.

In the novel *Smoke* we learn that in the late evening hours those attending the salon discussed various topics, including the news about soprano Patti, who sang in *La Traviata* (Turgenev, 2016, p. 75). Adelina Patti (1843-1919) was one of the leading and also the highest paid singers of her time. She was an excellent coloratura soprano, and as a dramatic soprano she performed with great success, among other things, in Verdi's *La Traviata*. According to composer Verdi, Patti was the greatest singer he had ever heard (Britannica, 2022).

3.2 Music among the Russian aristocracy in general

In *On the Eve*, Turgenev vividly described the attitude of Russian nobles to music: "In Bersenyev's room there was a piano, small, and by no means new, but of a soft and sweet tone, though not perfectly in tune. Bersenyev sat down to it, and began to strike some chords. Like all Russians of good birth, he had studied music in his childhood, and like almost all Russian gentlemen, he played very badly; but he loved music passionately. Strictly speaking, he did not love the art, the forms in which music is expressed (symphonies and sonatas, even operas wearied him), but he loved the poetry of music: he loved those vague and sweet, shapeless, and all-embracing emotions which are stirred in the soul by the combinations and successions of sounds. For more than an hour, he did not move from the piano, repeating many times the same chords, awkwardly picking out new ones, pausing and melting over the minor sevenths. His heart ached, and his eyes more than once filled with tears." (Turgenev, 2016, p. 20).

Katia, the sister of Princess Odintsov in *Fathers and Sons*, was also an accomplished pianist (Turgenev, p. 94). For the novel's hero Arkady, she played Mozart's Sonata fantasy in C minor. "She played it well, but coldly, and not with any excess of precision. Likewise, she kept her lips compressed, her eyes upon the keys, and her form erect and motionless. Only towards the close of the piece did her face kindle at all, while at the same moment a tiny curl detached itself from her loosely-bound hair, and fell over her dusky forehead.

Otherwise, Princess Odintsov led an ordered everyday life, and we learn, for example, that "the rest of the evening would be devoted to a walk, to cards, or to music" (Turgenev, p. 104).

Strauss's music is also indispensable at the aristocracy's evening parties. To quote the novel *Smoke*:

"About midnight he walked under the windows of the Hall of Nobility. Countless lights of huge candelabra shone with brilliant radiance through the red curtains; and the whole square, blocked with carriages, was ringing with the insolent, festive, seductive strains of a waltz of Strauss." (Turgenev, 2016, p. 34).

⁴ Reissiger was a conductor and composer (he also wrote nine operas), succeeding Carl Maria von Weber as director of the Dresden Court Opera (Kennedy and Bourne, 1996, p. 599).

3.3 Music of wind orchestra in pavilions in the parks

At the beginning of the novel *Smoke* Turgenev describes the music played by a band in a pavilion in a park. »On the 10th of August 1862, at four o'clock in the afternoon, a great number of people were thronging before the well-known *Konversation* in Baden-Baden. [...] The orchestra in the Pavilion played first a medley from the *Traviata*, then one of Strauss's waltzes, then 'Tell her,'⁵ a Russian song, adapted for instruments by an obliging conductor." (Turgenev, 2016, p. 7). At 4 pm, the company began to gather in the park: "The blare of wind instruments floated up the avenue; it was the Prussian military band from Rastadt (in 1862 Rastadt was still an allied fortress), beginning its weekly concert in the pavilion." (Turgenev, 2016, p. 93).

3.4 Russian romance and popular foreign songs

Turgenev in his work also mentions Russian romance. An old Russian romance can be found, for example, as the epigraph of his novel *Torrents of Spring*: "Years of gladness, Days of joy, Like the torrents of spring They hurried away." Or, for example, in *Fathers and Sons*, while visiting the princess Kukshin, talking and drinking large amounts of champagne, the princess sits down at the piano and begins to "clattered her flat finger nails upon the keys, and essayed hoarsely to sing, first of all some gipsy ditties, and then the ballad "Dreaming Granada lies asleep"; while the third guest begins to sing the role of the longing lover joining her at the words "Your lips meet mine in a burning kiss". (Turgenev, p. 81).

Like Dostoevsky in *Crime and Punishment*, Turgenev also quotes poems in French, the language spoken by the nobility. For example, Russian nobles in Baden-Baden spoke French, and one of the generals remembers the French song 'Deux gendarmes, un beau dimanche' (Turgenev, 2016, pp. 41-42). This was a popular French military song from the 19th century, also called 'Pandore ou les deux gendarmes' written by Gustave Nadaud.

4. The role of music in Turgenev's novels

In *Rudin*, music also plays a calming role, much as "Orpheus soothed savage beasts" (Turgenev, 2016, p. 14). In order to use the sound of music in this calming role, one of the guests present in the salon, the hero of the novel, plays Thalberg's piano étude (Turgenev, 2016, p. 24). In the same novel, music is also resorted to as a means of evoking memories. In the scene depicting a summer's night at an open window, the protagonist, listening to Schubert's 'Erlkönig', recalls his student years in Germany:

"A fragrant mist lay like a soft shroud over the garden; a drowsy scent breathed from the trees near. The stars shed a mild radiance. The summer night was soft—and softened all. Rudin gazed into the dark garden, and looked round. 'That music and this night,' he began, 'reminded me of my student days in Germany; our meetings, our serenades.'" (Turgenev, 2016, p. 26).

In *On the Eve* music is harmoniously integrated into the environment and into the experience of the book's heroes. In one of the excerpts, the group rides a boat along the lake, and in a romantic description of nature, singing is heard: first the company unsuccessfully tries to sing a Russian song, then a young girl sings a "par excellence" romantic and thematically coordinated song, 'Le Lac' by Louis Niedermeyer, based on Lamartine's famous poem (Turgenev, 2016, pp. 51-52):

"Meanwhile the whole party went into the arbour, well known as Pleasant View arbour, and stopped to admire the view of the Tsaritsino lakes. They stretched one behind the other for several miles, overshadowed by thick woods. The bright green grass, which covered the hill sloping down to the largest lake, gave the water itself an extraordinarily vivid emerald colour. Even at the water's edge

⁵ The romance 'Tell her' is a love song composed by Princess Yelizaveta Vasilyevna Kochubey, a music lover who also composed music based on the works of the Russian poet Evdokiya Petrovna Rostopchina. This romance was quite popular in Russia in the second half of the 19th century and was also performed by the famous Italian tenor Enrico Tamberlick.

not a ripple stirred the smooth surface. One might fancy it a solid mass of glass lying heavy and shining in a huge font; the sky seemed to drop into its depths, while the leafy trees gazed motionless into its transparent bosom.

All were absorbed in long and silent admiration of the view; even Shubin was still; even Zoya was impressed. At last, all with one mind, began to wish to go upon the water. Shubin, Insarov, and Berseniyev raced each other over the grass. They succeeded in finding a large painted boat and two boatmen, and beckoned to the ladies. [...] The boat pushed off. [...] Shubin suggested that they should sing some Russian song in chorus, and struck up: 'Down the river Volga'... Berseniyev, Zoya, and even Anna Vassilyevna, joined in—Insarov could not sing—but they did not keep together; at the third verse the singers were all wrong. Only Berseniyev tried to go on in the bass, 'Nothing on the waves is seen,' but he, too, was soon in difficulties. The boatmen looked at one another and grinned in silence.

'Eh?' said Shubin, turning to them, 'the gentlefolks can't sing, you say?' [...] 'Wait a little snubnose,' retorted Shubin, 'we will show you. Zoya Nikitishna, sing us Le lac of Niedermeyer! [...] Zoya took off her hat and began to sing: 'O lac, l'année à peine a fini sa carrière!'

Her small but pure voice seemed to dart over the surface of the lake; every word echoed far off in the woods; it sounded as though some one were singing there, too, in a distinct, but mysterious and unearthly voice."

In Turgenev, the heroes can connect their emotions and experiences with music. When the hero Insarov in *On the Eve* dies, his beloved Elena reflects on the cruelty of fate and imminent death and recalls Violetta's words describing the heroine's internal struggle with impending tragedy – 'Morir si giovane' from *La Traviata*, an opera they had watched together. (Turgenev, 2016, pp. 117-118).

5. Italian opera, Italy and Rossini

As evidenced by his opinion of Verdi's *La Traviata* in *On the Eve*, Turgenev was not particularly impressed by this opera. Writing about a performance of *La Traviata* in Venice, he describes it as (Turgenev, 2016, pp. 115-116),

"an opera of Verdi's, which though, honestly speaking, rather vulgar, has already succeeded in making the round of all the European theatres, an opera, well-known among Russians". He writes about this opera in the Venetian theatre as follows: *"The season in Venice was over, and none of the singers rose above the level of mediocrity; every one shouted to the best of their abilities. The part of Violetta was performed by an artist, of no renown, and judging by the cool reception given her by the public, not a favourite, but she was not destitute of talent. She was a young, and not very pretty, black-eyed girl with an unequal and already overstrained voice. Her dress was ill-chosen and naively gaudy [...] Indeed, how could she, the daughter of some Bergamese shepherd, know how Parisian dames aux camélias dress! And she did not understand how to move on the stage; but there was much truth and artless simplicity in her acting, and she sang with that passion of expression and rhythm which is only vouchsafed to Italians."*

However, the protagonist of the novel, Elena, confesses to her fiancé Insarov (Turgenev, 2016, p. 116):

"They hardly clap that poor girl at all, but I like her a thousand times better than some conceited second-rate celebrity who would grimace and attitudinise all the while for effect".

The writer later admits that the singer's interpretation of Violetta *"became steadily better, and freer"* (Turgenev, 2016, p. 116) and at the end of the play she received a standing ovation. The writer calls the final duet of Alfredo and Violetta "the most beautiful pearl of opera". At the beginning of the last act of the opera, when she sees a scene with a hospital bed and medicines, Elena is overwhelmed by a foreboding of the future and the false cough of the singer on stage coincides with Insarov's coughing.

Mentions of other Verdi operas can also be found in the novel *Smoke*, when gentlemen in Baden-Baden talk about opera performances, and one of them exclaims (Turgenev, 2016, p. 7): *"It's the finale from Ernani they're playing. How delicious! ..."*. Verdi's 'Miserere' from *Il Trovatore* is also mentioned

(Turgenev, 2016, p. 16). Turgenev was enthusiastic about Germany. Many Germans appear in his novels, and some of his literary heroes have lived in Germany for some time, as he did himself. However, Turgenev also wrote an eulogy to Italy. In *On the Eve* he describes the beauty of Venice⁶ and the performance of an opera in a theatre. Varvara Pavlovna from *A House of Gentlefolk* also regularly went to the theatre, being particularly fascinated by Italian music (Turgenev, 2001, p. 41).

Italians also appear in *Torrents of Spring*: A young Russian nobleman, Sanin, meets an Italian family who own a pastry shop in Frankfurt. A mother and daughter in this Italian family sing Italian folk songs and opera duets, and a family friend was a former opera baritone who displays the beauty of his voice in Iago's aria from Rossini's *Otello*.

According to Turgenev, Italian singers are characterized by "passion in expression and rhythm", and the land of Italy is a source of inspiration for Russian artists. Thus, Shubin, a young sculptor in *On the Eve*, emphasizes that he needs "light, space...." to be able to create and to be inspired, and he does not find this light and space in cold Russia, where there is not enough sunlight. He says that he suffocates in Russia and wants to go to Italy, because "there is sunshine, there is beauty" (Turgenev, 2001, p. 9).



Figure 1. A: The building in Piazza Trieste and Trento, near the San Carlo Theatre and the Royal Palace in the centre of Naples, where the first Russian ambassador to Naples, A. K. Razumovsky (1779-84). B and C: Sorrento. D: A memorial plaque to Torquato Tasso in Sorrento, Commemorating the poet's return to his hometown in 1577.

The 22-year-old Turgenev's trip to Italy, to Sorrento, was reflected a decade later in the story *Three Encounters*. Here, Turgenev also mentions the house in Sorrento where the poet Torquato Tasso was born in 1544, and writes that he did not visit his home. As the epigraph to this story, Turgenev chose an Italian poem that he translated into Russian. In the Russian countryside, the narrator remembers his meeting with an unknown beauty in Sorrento who sang this song. It is a folk song from Tuscany »Passa que 'colli e vieni allegramente; non ti curar di tanta compagnia - Vieni pensando a me segretamente - Ch'io t'accompagni per tutta la vita ». The form of this song is the so-called "rispetto", that is, a form characterized by a verse of eleven. Its topic is love. "Rispetto" basically means respect, in this case a tribute to a beloved person. In the story the song acts as a kind of leitmotif. At the first meeting beside the home of the mysterious woman, the literary hero sings 'Ecco ridente', an aria sung by Count Almaviva in Rossini's *Barber of Seville*.

Inspiring, cheerful, passionate, expressive – "typically Italian" – Rossini's melodies quickly seduced Russian audiences and found their echo in Russian literature. As for other Russian writers of the nineteenth century (Pushkin, Dostoevsky, Goncharov), Rossini plays a very important part in the work of Turgenev, his compositions being frequently mentioned by the writer. Turgenev also saw Pauline García Viardot for the first time in the role of Rosina in Rossini's *Barber of Seville*.

⁶ In Nikolai Rimsky-Korsakov's opera *Sadko* (1898), a Venetian merchant sings of the beauty and glory of Venice, the city of all cities.

6. Turgenev on Russian music and Russian art in general

In the novel *Smoke* one of the heroes discusses Russian "untaught geniuses" (Turgenev, 2016, p. 65):

"The humblest German flute-player, modestly blowing his part in the humblest German orchestra, has twenty times as many ideas as all our untaught geniuses; only the flute-player keeps his ideas to himself, and doesn't trot them out with a flourish in the land of Mozarts and Haydns; while our friend the rough diamond has only to strum some little waltz or song, and at once you see him with his hands in his trouser pocket and a sneer of contempt on his lips: I'm a genius, he says. And in painting it's just the same, and in everything else. Oh, these natural geniuses, how I hate them!" (Turgenev, 2016, pp. 64-65). He wants to say that such "self-taught people" are praised only "it's only where there's no real science fully assimilated, and no real art, that there's this flaunting affectation of them." (Turgenev, 2016, p. 65). About Glinka he admits that the exceptions confirm the rules, but that this is also about Russian "bragging", namely the view that there are no comparable geniuses in other nations: "marvellous home-bred genius whose compositions are nothing but a poor imitation of second-rate foreign composers, yes, second-rate ones, for they're the easiest to imitate."

Turgenev's thesis is that there is no authentic, original or independent Russian art; all that is good has come to Russian art through foreign influences.

Turgenev did not achieve success in Russia with *Smoke*. Dostoevsky personally criticized Turgenev in 1867 in Baden-Baden, accusing him of not knowing what was going on in Russia while living abroad. He was most offended by Turgenev's contemptuous attitude towards his homeland and his compatriots. It was in this novel that Turgenev expressed his views on Russia: "If Russia were to disappear, there would be no harm for humanity and no one would be upset." Turgenev was even ashamed that he was Russian and considered himself almost to be a German (Saraskina, 2021, p. 417), while Dostoevsky had a rather negative opinion about the Germans. Dostoevsky was also disturbed by Turgenev's declaration of being a complete atheist, but he resented him most of all for not loving his homeland and even betraying it (Saraskina, 2021, pp. 416-417).

Turgenev also mentions what he regards as the ridiculous attitude of nihilists towards art and music. In *Fathers and Sons*, the couple (parents of the hero Arkady) spent their days on the farm, often "they read together, they play the piano together, and they sang duets" (Turgenev, p. 3). Arkady's father still played the cello after his wife's death. When Arkady's friend Bazarov, a nihilist who did not like music, heard about this, he was very surprised (Turgenev, p. 49):

"At this moment there came wafted to their ears the long-drawn strains of a violoncello, on which a sensitive, but inexperienced, hand was playing Schubert's Erwartung. Like honey did the voluptuous melody suffuse the air. "Who is the musician?" asked Bazarov in astonishment.

"My father."

"What? Your father plays the cello?"

"He does."

"At his age?"

"Yes—he is only forty-four."

Bazarov burst out laughing.

"Why do you laugh?" asked Arkady.

"Pardon me, but the idea that your father—a man of forty-four, a paterfamilias, and a notable in the county—should play the cello!"

7. Turgenev's literary work in music

Pauline García Viardot was also a composer and set some of Turgenev's songs to music; of her five operettas, three – *Trop de femmes* (1867), *L'ogre* (1868) and *Le dernier sorcier* (1869) – were written to libretti by Turgenev.

In November 1843, after Turgenev first saw Pauline Viardot at the opera in St. Petersburg, he wrote the poem 'Morning Misty, Morning Gray', also named 'On the Road'. It is an elegiac piece, full of nostalgia for the past, when the lyrical narrator, apparently somewhere along the way, remembers his first but also his last meeting. It was at this time that Turgenev broke up with his love, Tatiana

Alexandrovna Bakunina, the sister of Mikhail Bakunin, the philosopher and revolutionary. However, since he had already met Pauline Viardot, in this song he also looked wistfully to the future despite his nostalgia. This song has been set to music several times, the most famous setting being a romance by Viktor Abaza from 1897, and it was also performed by the famous baritone Dmitry Hvorostovsky.

8. Conclusion

Music played an extremely important role in Turgenev's life. The writer's muse, and one of the most influential female artists of the time, the mezzo-soprano Pauline García Viardot, made a determined contribution to this, and through her the writer was in constant contact with the world of music and with the leading musicians, performers and composers of his time. All these musical impressions were reflected in his literary work, where mention is frequently made of musical works of various genres. In Turgenev's works, music is used as a kind of accompaniment against a background of events, or it can describe the emotions and feelings of the heroes.

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Review

Role of Erythrocyte Sedimentation Rate (ESR) in preparation of Platelet and Extracellular Vesicles Rich Plasma

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Abstract:

Uses of platelet and extracellular vesicles rich plasma (PVRP) are in many fields of medicine as it was found that PVRP has regenerative properties. Preparation of PVRP is performed by separation of erythrocytes from the liquid they are immersed in. Erythrocytes are the most numerous blood cells; they are also relatively large and dense as they are filled with haemoglobin. Therefore they sediment due to gravitation or systemic centrifugation force thereby pushing plasma that carries smaller particles in the opposite direction. This mechanism that takes place during processing determines the composition of plasma. Its tuning is therefore key in acquiring a preparation with desired properties. In particular, it is of interest to study the effect of erythrocyte sedimentation rate (ESR) on composition and volume of acquired plasma. Different animals have different ranges of ESR values which may enable insight into mechanisms of plasma preparation. In this contribution we present the basic mechanisms of plasma preparation and properties of blood of different animals.

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1. Plasma rich with platelets and EVs

Application of blood derived products such as plasma (blood devoid of erythrocytes) to a damaged tissue area has hitherto proved to accelerate wound healing and help regenerate injured or damaged ligament and tendon fibers (Uršič et al., 2016, Vozel et al., 2020a). The preparation is made from patient's own blood following a procedure that is straightforward and easy to perform and can be injected directly to the damaged area. In this way, the healing substances can be delivered also to places that are not easily reached otherwise. With autologous application there is low risk of complications such as infections or immune rejection (De Pascale et al., 2015).

Initially, healing capacity of plasma preparations was attributed to platelets (**Figure 1 A,B**); it was found that activated platelets (**Figure 1B**) release different growth and inflammation factors to the extracellular milieu, which have been regarded as vectors of the healing process (Etulain et al, 2018). Later on, other features were considered, i.e. platelet membrane receptors, their effects on the immunomodulatory actions of the innate and adaptive immune system, effects of leukocytes and of mesenchymal stem cells (reviewed in Everts et al., 2020), indicating complex interactions between cells in the healing process.

Substances that were considered as possible promoters of regeneration in PVRP are growth factors and inflammation markers. Previous research outlined following growth factors: platelet-derived growth factor-BB (PDGF-BB), transforming growth factor β -1 (TGF- β 1) (Lee et al., 2013), insulin-like growth factor-1 (IGF-1) (Lubkowska et al., 2012), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) (Taniguchi et al., 2019). Inflammation factors Tumor necrosis factor alpha (TNF-alpha), interleukin 1 beta (IL-1 beta), Cholinesterase (ChE), erythrocyte Glutathione S-transferase (GST) and Interleukin 6 (IL-6) were already proved to be connected to concentration of EVs in blood-derived samples (Jan et al., 2021). TNF-alpha was originally described as a circulating factor that can cause necrosis of tumors, but has since been identified as a key regulator of the inflammatory response (Bradley, 2008). IL-6 has a role as an inflammatory mediator (Mendham, 2011). Potential marker of inflammation is activity of plasma Butyrylcholinesterase and erythrocyte Acetylcholinesterase. Both Cholinesterases may enhance inflammation. ChE is believed to modulate immune and anti-inflammatory response via the cholinergic system. Key factor in the anti-inflammatory cholinergic pathway is supposed to be oxidative stress (Villeda-Gonzalez, 2020). Erythrocyte GST activity has been proposed as the potential marker for oxidative stress (Neeffes, 1999) and EVs formation is considered to be provoked by oxidative stress (Borras, 2020) whereas inflammation processes in human body have impact on vesiculation (Chaar 2011).

Recent developments in the field of biology and medicine have outlined nanosized (ranging from 20 nm-1000 nm) membrane-enclosed cellular fragments (extracellular vesicles – (EVs)) which are abundant in preparations from processed plasma (Šuštar et al., 2011a,b, Božič et al., 2020) (**Figure 1G**) as important mediators of intercellular communication. It can be expected that these particles may be involved in the above features. EVs are formed by cells of all types during apoptosis, in the final stage of membrane budding (shown in platelets (**Figure 1B**), leukocytes (**Figure 1C**) and erythrocytes (**Figure 1F**)), by release from internal cell compartments or due to mechanical impact (Kralj-Iglič et al., 2020). Being free to move with body fluids, they can reach distant cells, interact with them and affect their biological function. Containing cell-type-specific signatures, EVs of selected cell types have been proposed as therapeutic agents in regenerative medicine, vaccination trials, and drug delivery (Fais et al., 2016, Lener et al., 2015). EV formation by cells is considered a physiological process (Su et al., 2018) and is accelerated as a result of oxidative stress (Borras et al., 2020) or inflammation process (Char et al., 2011).

Both platelets and EVs seem to be important bioactive substances in plasma. Importance of platelet-derived EVs was previously outlined and it was indicated that the mechanism by which they are formed is a critical determinant of their phenotype and function (Ferreira et al., 2020). A question can be posed about what would be the optimal composition of plasma preparation regarding platelets and EVs.

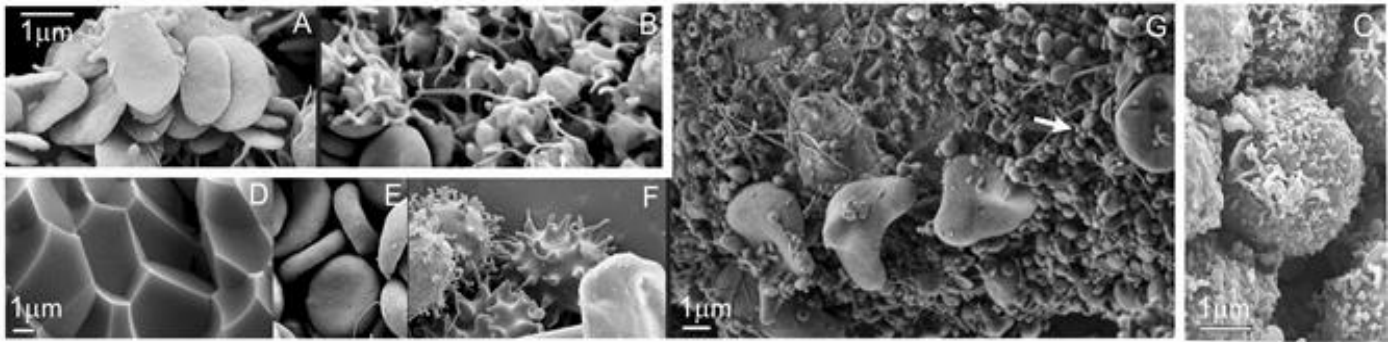


Figure 1. A: resting platelets, B: activated platelets, C: budding leukocytes, D: packed erythrocytes at the bottom of the centrifugation pellet, E: erythrocytes in physiologic in-vitro conditions, F: budding erythrocytes, G: isolate from blood showing numerous EVs (white arrow) and singular residual erythrocytes; there are no resting platelets and no leukocytes in the sample.

2. Preparation of plasma rich with platelets and EVs

The gold standard method for preparation of PVRP is centrifugation of blood samples. The procedure is simple to perform, it requires besides a centrifuge only few basic laboratory tools and contact with foreign materials is minimized as not to introduce corrosion particles into the preparation. The existing PVRP protocols aim at depletion of plasma in erythrocytes and enrichment in platelets. Different settings have been suggested, based on empirical findings. Widely used are two-spin protocols (Figure 2) where relatively low centrifugation pulls are used in both spins (up to 1000 times the acceleration due to gravity of Earth (g)). In Spin 1, blood is centrifuged to pellet erythrocytes. In Spin 2, the Spin 1-supernatant that is abundant in platelets and EVs is centrifuged again to pull the particles with densities higher than that of the surrounding solution towards the bottom of the centrifuge. PVRP is constituted from the lower portion of the sample (Figure 2). The recovery of platelets (the number of platelets in the PVRP sample divided by the number of platelets in the blood sample) reaches about 80% (Hsin et al., 2017) while platelet concentration can reach values above its baseline level in whole blood (Božič et al., 2021).

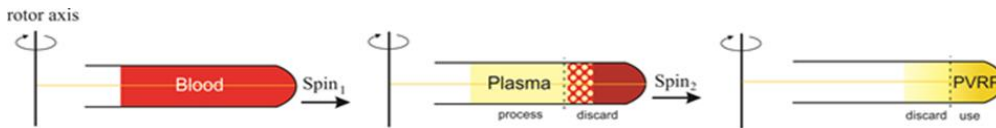


Figure 2. Two-spin protocol for preparation of PVRP. Blood is centrifuged (Spin 1) to separate erythrocytes (pellet) from plasma (supernatant). The acquired plasma is centrifuged again (Spin 2) to concentrate platelets and EVs that are denser than the surrounding liquid into the lower part of the tube (PVRP).

The forces on a particle are gravitational/systemic centrifugal force, the buoyancy and the resisting force that is described by the Stokes law. In equilibrium, the sum of the forces vanishes so that

$$\Delta\rho_{ERC} Xg - 4\pi r_{ERC}^3/3 = 6\pi r_{ERC} \eta u_{ERC} \quad (1)$$

where $\Delta\rho_{ERC}$ is the difference between the density of the erythrocytes and the density of the fluid, Xg is the respective acceleration that drives sedimentation expressed as a multiplicity of the Earth gravity acceleration g and X is the multiplication factor (generally referred to as relative centrifugal RCF in the centrifuge), r_{ERC} is the radius of the erythrocyte, η is the viscosity of plasma and u_{ERC} is the velocity of the erythrocyte. In gravitational field ($X = 1$), the velocity of sedimentation of erythrocytes is

$$u_{ERC} = 2\Delta\rho_{ERC}r_{ERC}^2g / (9\eta) \quad (2)$$

In the centrifuge, the force depends on the distance of the particle from the rotor axis x . The factor Xg is defined by the distance of the bottom of the tube from the rotor axis x_{max} and the angular velocity of the centrifuge (expressed also by its frequency in rotations per minute, $\omega = 2\pi\text{RPM}/60$), so that

$$Xg = \omega^2 x_{max} \quad (3)$$

It follows from Eqs. (1) and (3) that the velocity of sedimentation of erythrocytes in the centrifuge is

$$u_{\text{ERC}} = 2\Delta\rho_{\text{ERC}}r_{\text{ERC}}^2Xg_x / (9\eta x_{\text{max}}) \quad (4)$$

We can see that the velocity of the particle is proportional to the difference in particle density and the liquid density and to square of the size of the particle, meaning that larger and denser particles will sediment faster. This is the basis of the separation of erythrocytes from platelets in gravitational field and also in centrifuge. Erythrocytes are bigger than platelets and EVs. Moreover, they tend to form aggregates called rolleaux (**Figure 3**) that travel as unities and thereby promote the difference in sedimentation velocities between erythrocytes and smaller particles.

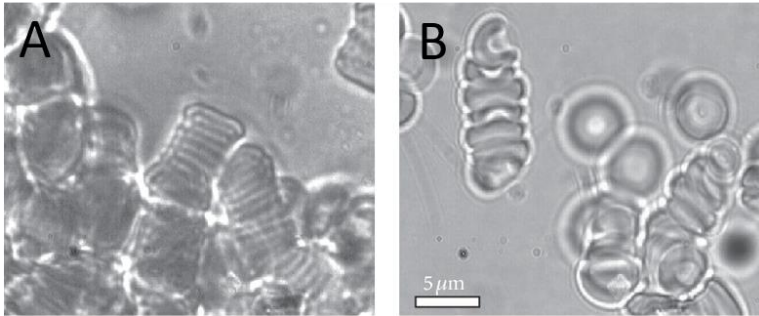


Figure 3. Rolleaux formation in normal *in vitro* conditions (A) and in the presence of anaesthetic (B).

It was found that the preparation procedure can significantly impact the blood cells and EVs (Šuštar et al., 2011, Božič et al., 2020) and therefore the volume and composition of PVRP (Everts, 2020). Besides EVs that are already present in plasma *in vivo*, the preparations contain EVs that are formed during the sample processing due to the shear and temperature stresses imposed upon the blood cells during processing of the sample (Šuštar et al., 2011, Božič et al., 2020). It is therefore of utmost interest to understand the effect of centrifugation on identity and formation mechanisms of EVs in plasma preparations as well as on their biological role.

3. Enrichment of plasma with platelets and EVs by centrifugation

Blood can be described as composed of a mixture of particle types differing in density, size and shape. It was previously observed that in concentrated multiparticle-type suspensions, the creeping flow considerably hinders sedimentation of particles (Major, 1978); if species of particles in suspension greatly differed in density and size, the lighter and smaller particles could be withdrawn into the flow opposite to the flow of the heavier and larger ones (Major, 1978). This principle was recently applied to blood where due to the large size and higher density of erythrocyte lumen, erythrocytes and leukocytes move faster than smaller platelets and EVs and push those against the centrifugation pull into the plasma zone (Božič et al., 2021). Furthermore, when an erythrocyte moves in the direction of the gravitational (or corresponding centrifugal) force, a following erythrocyte is dragged into the left behind “cavity”. As the concentration of erythrocytes in blood is high, the continuing action results in sequential ordering of the settling dispersed phase into erythrocyte channels and surrounding plasma zones (Pribush et al., 2010). The erythrocyte sedimentation is accelerated due to formation of the channels (Pribush et al., 2010), and is therefore faster than that expected from the relations considering movement of singular cells. Enrichment of plasma in smaller particles takes place as long as erythrocytes travel towards the bottom of the tube. When they pack in the pellet, smaller particles are no longer subjected to the drag of the erythrocyte counterflow and centrifugal pull reverses their direction toward the bottom of the tube. In this interval, plasma becomes depleted of smaller particles.

Distribution of different types of particles in the sample in dependence of time was modelled by applying laws of motion leading to a system of partial differential equations (Božič et al., 2021). Approximative simple and transparent solution was found. Model parameters were set by fitting to data from a cohort study including patients with chronic ear wounds and subjects without the record of disease (Božič et al., 2021) assuming that the movement of platelets and EVs is in the direction opposite to erythrocytes but with the proportional magnitude of velocity,

$$u_{P2} = -\varepsilon u_{ERC} \quad (5)$$

where P2 denotes platelets and EVs and ε is an adjustable constant.

It was found that Spin 1 (centrifugation of blood) was crucial in preparation of plasma. With appropriate choice of centripetal accelerations of the centrifuge rotor and appropriate centrifugation times, enrichment of plasma in the upper part of the centrifugation tube with platelets and EVs reached factor 2.3 (Božič et al., 2021).

4. Erythrocyte sedimentation rate (ESR)

ESR is a test that is routinely performed in clinical practice. It gives a rate (in millimetres per hour), at which red blood cells in the whole blood descend in a standardized Westergren tube in gravitational field during a period of one hour. With respect to model equations,

$$u_{ERC} = \text{ESR} \quad (6)$$

so that it follows from Eqs. (4)-(6) that

$$u_{P2} = -\varepsilon \text{ESR} X/x_{\max} \quad (7)$$

ESR is influenced by pro-sedimentation factors, mainly the presence of fibrinogen, and also physical properties of particles, e. g. the negative surface charge of erythrocytes. When an inflammatory process is present, the high level of fibrinogen enhances the formation of rouleaux. In humans, ESR was found connected to pathological states such as inflammation, infection, pregnancy, anemia, autoimmune disorders, some kidney diseases and some cancers. The rate of erythrocyte sedimentation is affected by both inflammatory and non-inflammatory conditions. In inflammatory conditions, fibrinogen, other clotting proteins and alpha globulin are positively charged, thus increasing the ESR. In non-inflammatory conditions, plasma albumin concentration, size, shape, and number of red blood cells and the concentration of immunoglobulin can affect the ESR. Non-inflammatory conditions that can cause raised ESR include anemia, kidney failure, obesity, ageing, and female sex. ESR is also higher in women during menstruation and pregnancy. An increased number of red blood cells (polycythemia) causes reduced ESR as blood viscosity increases. Hemoglobinopathy, such as sickle-cell disease, decrease ESR due to improper shape of red blood cells that impairs stacking. In animals like horses, cats and pigs, the rouleaux formation can be a normal physiological finding.

Westergren's original normal values (men 3 mm/h and women 7 mm/h) made no allowance for a person's age (Westergren, 1957). Later studies indicated that ESR tends to rise with age (normal values were found to be 1 to 2 mm/h at birth, rising to 4 mm/h 8 days after delivery, and then to 17 mm/h by day 14 (Böttiger et al., 1967)). In human, normal values are now thought to be 3-10 mm/hour, they were found to be slightly higher in normal populations of African Americans than Caucasians of both genders (Gillum, 1993) and in anaemic individuals (Kafner et al., 1997). ESR differs in different animal species and is especially high in the horse (120 mm/hour) while in the cow, goat and sheep it was found to be 1 mm/hour (Böttiger et al., 1967). It is proposed that ESR is higher in athletic mammalian species like pronghorn antelope (*Antilocapra americana*) (Popel et al., 1994).

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Scientific contribution/Original research

Overview of Wound Healing Differences between Dogs and Cats

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Abstract:

Wound healing is a crucial process in restoring skin integrity and is very important in all animal species. In veterinary medicine, clinicians often treat dogs and cats according to the same protocols. Yet, many studies have shown that there are differences in skin perfusion, macroscopic appearance of wounds, and even microscopic cell structure of wounds between these two species. It was found that prolonged wound healing and “pseudo-healing” are much more common in cats than in dogs. Understanding the differences in wound healing between these two species may provide clinicians with better outcomes in wound healing treatment. The use of medical honey, intradermal and skin sutures, and longer suture retention may all contribute to better wound healing in cats.

Keywords: Wound healing; Interspecies differences; Granulation tissue; Medical honey; Second intention healing; First intention healing

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1. Introduction

The skin is the largest organ of the body and due to its exposure to the outside, very vulnerable organ. Its integrity is of great importance, especially in protecting the deeper tissues from microorganisms and other external factors (Bohling and Henderson, 2006). Wound healing is a crucial process for restoring skin integrity. It is a dynamic process that begins with an inflammatory phase, followed by a phase of proliferation and then differentiation. Wounds heal according to the same principle in all vertebrates, but there are clinically important differences between species (Bohling and Henderson, 2006; Cornell, 2012).

Macroscopically it is often noted that large open wounds in cats heal slower than similar wounds in dogs and that less granulation tissue forms in cats. As a result of the comparatively slower healing processes, "pseudo-healing" occurs more frequently in cats than in dogs. In "pseudo-healing" the wound appears to be healed but dehiscence occurs after removal of the sutures (**Figure 1**). One of the reasons for this occurrence could be inappropriate collagen deposition beneath the epidermal surface resulting in lower tensile strength. For this reason, generalization of wound healing characteristics in cats and dogs may be detrimental to individual animals and expose them to stress due to delayed wound healing (Bohling et al., 2004; Pavletic, 2018).

Therefore, the aim of this paper is to highlight differences in cutaneous wound healing between dogs and cats.



Figure 1: "Pseudo- healing" in a cat.

2. Anatomical differences of the skin

The anatomy of the skin of cats is similar to that of dogs. Epidermis, dermis and subcutis are slightly thinner in cats than in dogs. Apart from the fact that skin in cats is looser, more pliable, and more mobile over most of the body surface than the skin of dogs, there are important differences in cutaneous perfusion (Bohling and Henderson, 2006).

Taylor and Minabe (1992) extensively studied angiosomes of mammals and other vertebrates. They concluded that dogs have a greater number of well-distributed cutaneous perforating vessels. Cats, on the other hand, have a smaller number of cutaneous perforators that are more widely distributed in two major lines along the trunk (Taylor and Minabe, 1992; Bohling and Henderson, 2004). They also have a relatively low density of tertiary and higher-order vessels, particularly on the trunk. This conclusion was also confirmed by Bohling et al. (2006) who used laser-Doppler perfusion imaging (LDPI) to assess cutaneous perfusion during wound healing. They found significantly higher baseline perfusion in dogs than in cats (Hartman et al., 1992; Bohling and Henderson, 2004). The lower density of vascular anastomoses, particularly in the trunk region, is reflected in the prolonged healing in cats (Hartman et al., 1992; Bohling and Henderson, 2006).

3. The role of subcutis

The subcutis plays an important role in wound healing. It is the supporting layer of the skin, serves as a flexible connection between the mobile skin and the underlying firm fascia, and supplies vessels and nerves to the overlying dermis (Scott et al., 2001). Studies have shown that removal of subcutis

has a negative effect on wound healing in dogs and cats, but when it comes to second intention healing, the negative effect is much greater in cats than in dogs (Bohling et al., 2006).

Subcutaneous tissue is also an important source of cellular precursors for granulation tissue; however, removal of subcutaneous tissue does not affect the first appearance of granulation tissue, but it slows granulation and wound contraction in both cats and dogs, with cats being more affected. Subcutis removal also impairs epithelialization in both cats and dogs (Bohling et al., 2006; Bohling, 2014).

4. Wound healing

Wound healing can be divided into healing per primam, or first intention healing and healing per secundam or second intention healing (Cornell, 2012).

Surgical wounds that are clean and fresh heal per primam, with suture bringing the edges of the wound closer together (Bohling, 2014). They heal quickly and do not leave large scars. Studies have shown that cats have 50% less tensile strength 7 days after wound closure per primam than dogs with the same wounds. This is thought to be due to significantly lower collagen production in wounds in cats compared to dogs (Bohling and Henderson, 2004). To reduce the risk of dehiscence due to “pseudo-healing”, sutures should be left in place for a longer period, at least for 3 weeks. Pavletic (2018) also suggests using intradermal sutures followed by skin sutures to further support incision healing and later reduce incision tension after suture removal.

Healing per secundam means that a wound heals on its own without being sutured. The veterinarian’s role in this case is to provide moisture, cleanliness, and protection of the wound. This can be accomplished through appropriate dressings, wound irrigation, and necrotomy (Cornell, 2012). There are many differences in wound healing per secundam between cats and dogs.

In the inflammatory phase, it can be macroscopically observed that canine wounds produce more fluid, are more swollen and erythematous than identical wounds in cats (**Figure 2**) (Bohling and Henderson, 2006; Bohling, 2014). This may be directly related to greater blood perfusion due to the higher capillary density of canine skin (Bohling and Henderson, 2004).

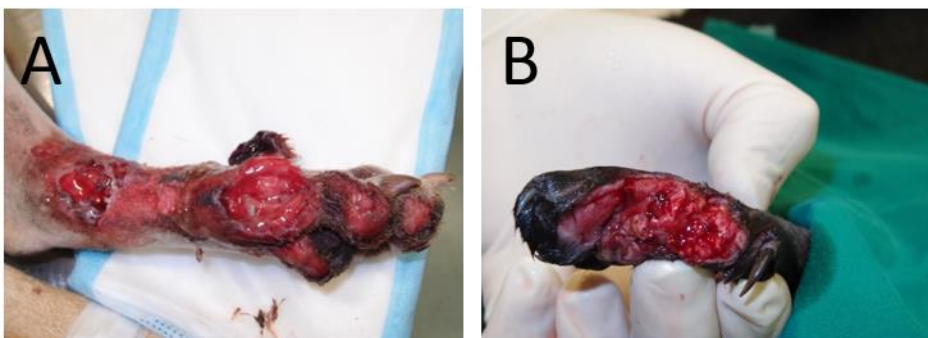


Figure 2: Wound in a dog (A) produces a lot of fluid, is swollen and erythematous compared to wound in a cat (B).

Histologically, there are no interspecies differences in cell infiltration at the beginning of the inflammatory phase, but over time it has been observed that the inflammatory phase is greatly prolonged in cats compared to healing in dogs (Bohling and Henderson, 2004; Bohling, 2014). Deviations in neutrophil granulocytes (indicators of acute inflammation) and mast cells were particularly noticeable, as their numbers were much higher in cats in single measurements from day 7 to day 21, and these cells remained in the wound longer and in higher numbers than in dogs. It was concluded that the inflammatory healing phase is more chronic in cats than in dogs, which may be the reason why the proliferation and differentiation phases occur later in cats (Bohling, 2014).

The appearance of granulation tissue is a definite indication that the wound is entering a proliferation phase (Bohling, 2014). Granulation tissue protects the wound from infection and desiccation (Cornell, 2012).

Bohling et al. (2004) found that the difference in the occurrence of granulation in cats and dogs is only 1-2 days, but in cats the wound granulates more slowly. In their study, they showed that cats take twice as long to fill a skin defect in similar wounds as dogs (Bohling and Henderson, 2004). Cats took an average of 19 days, while dogs completed granulation within 7.5 days (Bohling and Henderson, 2004; Bohling, 2014). However, in our previous study with wounds in cats treated with medical honey the granulation was completed in 9.7 days (Lukanc et al., 2018). Macroscopic differences in granulation tissue are also evident. In cats, the granulation tissue has a lighter pink appearance, whereas in dogs it is dark red (Figure 3). The reason for this could again be greater blood circulation in dogs (Bohling, 2014).

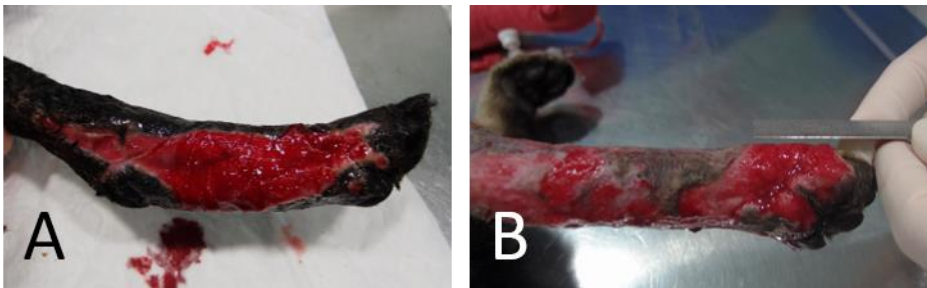


Figure 3: Granulation tissue in a dog (A) has darker colour than in a cat (B), where it is lighter.

In Bohling's study, it was observed that granulation in cats occurs only from the edges of the wound toward the centre, while in our previous study of wound healing with medical honey in cats (Lukanc et al., 2018, Lukanc et al., 2020), we observed the appearance of small granulation islands over the entire exposed surface of the wound, growing in all directions until the skin defect was filled, which is similar to that observed in dogs (Figure 4). The study also showed that medical honey promoted earlier appearance and faster coverage of the wound bed with granulation tissue, compared with other studies in which medical honey was not used (Bohling et al., 2004; Bohling et al., 2006). Therefore, we recommend the use of medical honey in wound healing in cats because it not only promotes faster healing but also provides protection for the wound and thereby decreases bacterial growth.



Figure 4: Small islands of granulation tissue along the entire exposed surface of the wound in a cat (Photo: Erjavec V.).

Since the epithelialization and contraction phases of the wound do not occur until the wound is filled with granulation tissue, these two phases are also significantly prolonged in cats (Bohling and Henderson, 2004). Bohling et al. (2004) found that open wounds contracted much slower in cats by day 7, but by day 14, the percentage of contraction was still greater in dogs, although, the differences between groups were not as significant. In cats it may take a longer for differentiated fibroblasts or myofibroblasts to appear in the wound, but once contraction begins, it is faster in cats than in dogs (Bohling and Henderson, 2004). This hypothesis is supported by studies that have shown that fibroblasts that appear early in wound contraction do not contract as much as those that appear later (Rudolph et al., 1992).

5. Other differences

Due to their unique skin characteristics, cats tolerate radiotherapy better in terms of overcoming the side effects that occur on the skin. It is also important to point out that skin grafts heal faster in cats and there are fewer rejections and complications associated with such procedures. Skin grafts are thinner than in dogs and are therefore relatively better nourished – in the initial stages by the process of diffusion from deeper tissues, and in later stages also by earlier revascularization. Because dogs



experience increased transudation and swelling of the wound during the inflammatory phase, this is not ideal for skin grafts and quickly leads to graft rejection (Bohling, 2014).

6. Conclusion

Cats and dogs should be treated separately when healing skin wounds. In cats, care should be taken not to remove sutures too quickly as they are slower to gain the tensile strength of the wound. It is also important to realize that the time during which the wound is protected only by the work of a veterinarian (with dressing, irrigation, etc.) is much longer in cats than in dogs, because the natural protection by the granulation tissue starts later.

Although dogs and cats are often treated by the same protocols, recent studies indicate a need to better understand the differences in physiology between species. It is up to veterinarians to use these findings to improve the quality of clinical practice, help the patients, and most importantly, do no harm.

Conflicts of Interest: The authors declare no conflict of interest.

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POSTERS

Removal process optimisation for emerging pollutants onto two biochars synthesised with classic and microwave induced pyrolysis

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OUTLINE

We investigated the removal efficiencies of emerging pollutants benzotriazole and its derivatives (4MBZ, 5MBZ, CIBZ and DMBZ) with the variation of different process OHBZ, parameters (e.g., pH, contact time, initial concentration) onto two functionalised biochars. Microwave induced and classic pyrolysis were used to synthesise the biochars from wild plum (WpOH) and apricot kernels (AsPhA), respectively.

EXPERIMENTAL METHODS

It was started with the examination of four low-cost lignocellulosic biochars, and after initial screening, AsPhA and WpOH were selected for further experiment. It was performed in batch experiments. The specified mass of biochar was mixed with 50 ml solution of mixed pollutants in a colonial flask and mixed on a mechanical shaker. The effect of initial pH (2-9), contact time (5-420 min) and initial compounds concentration (1-50 mg/L) were studied. Biochar dosage was 0.4 mg/L.

CONCLUSION

Removal efficiency was optimal at pH 4 for WpOH and at pH 6 for AsPhA and equilibrium was reached for both adsorbents after 240 minutes. Maximum adsorption capacity showed CIBZ (98.1 mg/g) for WpOH and DMBZ (120.41 mg/g) for AsPhA. It was shown that both adsorbents were efficient for benzotriazole and its derivatives removal. AsPhA was more efficient and suitable under neutral environment.

RESULTS

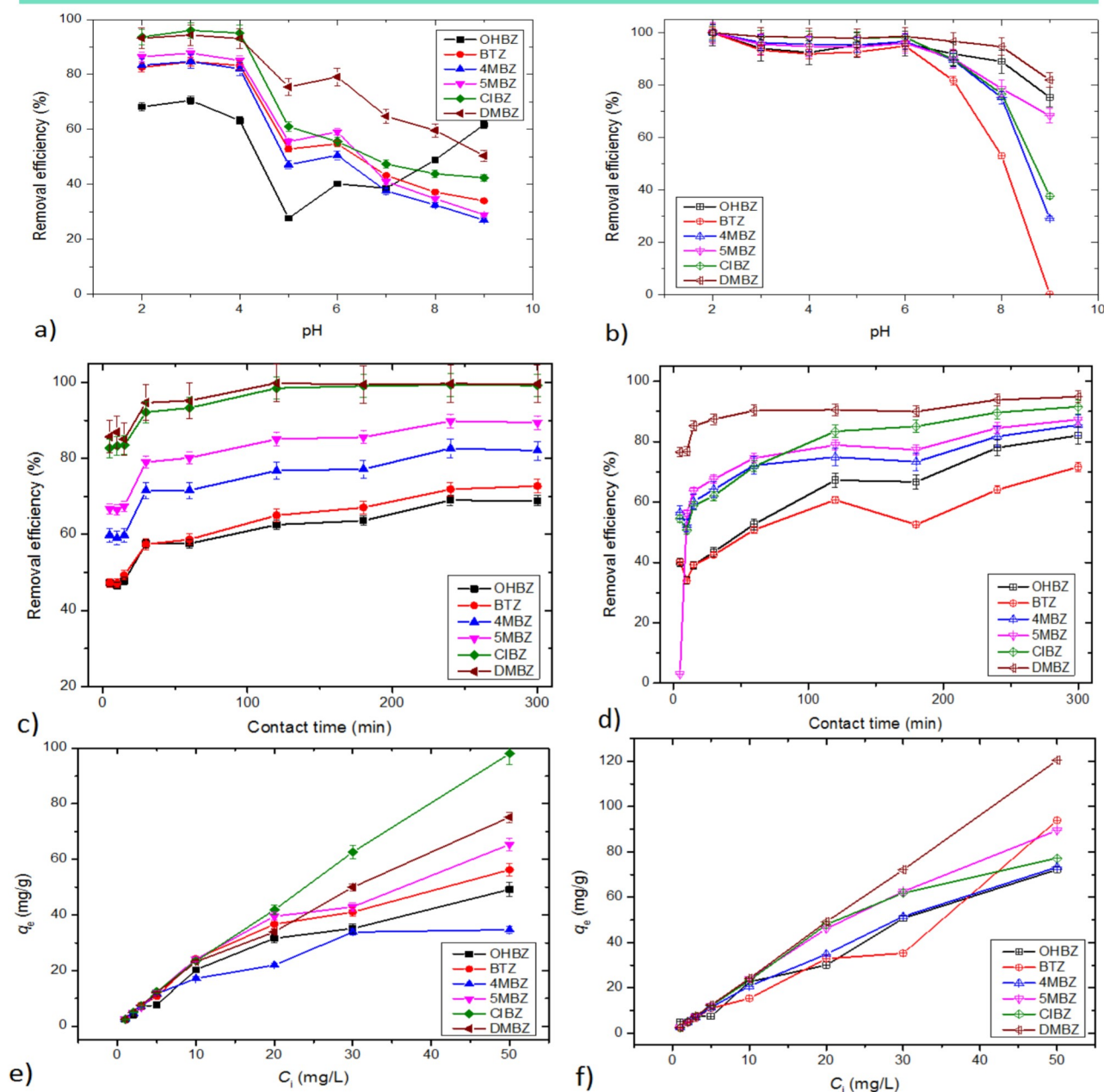


Figure 1: Influence of the pH value on removal efficiency on (a) WpOH; (b) AsPhA Influence of contact time on removal efficiency on (c) WpOH and (d) AsPhA Influence of initial concentration on adsorption capacity on (e) WpOH and (f) AsPhA



Simultaneous determination of formic acid dimethylhydrazide, 1,1- and 1,3-dimethylurea using SFC-APCI-MS/MS

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OUTLINE	EXPERIMENTAL METHODS	CONCLUSION
<p>The aim of this study is to develop an approach for the simultaneous determination of transformation products of unsymmetrical dimethylhydrazine: formic acid dimethylhydrazide and its isomers, 1,1- and 1,3-dimethylureas, by supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS). We investigated the retention of analytes on different stationary phases under SFC conditions and the influence of fluid parameters (mobile phase composition, back-pressure, temperature) on the separation. The ion source parameters that provide the highest sensitivity have also been selected. The developed approach was tested on real objects - samples of soil contaminated with rocket fuel.</p>	<p>Experiments were carried out using a SFC-MS/MS system consisting of an Acquity UPC² supercritical fluid chromatograph (Waters, USA), an additional make-up solvent pump (Thermo, USA) and a 3200 QTrap hybrid triple quadrupole mass analyser (ABSciex, Canada).</p> <p>Extraction of target analytes from soil samples was carried out using the ASE-350 accelerated solvent extraction system (Dionex, USA) according to a previously developed approach [Kosyakov, 2015].</p> <p>Sandy soil sampled at the site of the 2013 Proton rocket crash (Baikonur Cosmodrome) and peat soil sampled in the area of the Koida rocket crash were used as real objects.</p>	<p>A rapid and highly sensitive approach to the determination of formic acid dimethylhydrazide, 1,1- and 1,3-dimethylureas has been developed. The approach is based on a combination of SFC separation on a polar 2-ethylpyridine sorbent and tandem mass spectrometric detection in the multiple reaction monitoring mode with atmospheric pressure chemical ionisation.</p> <p>The developed approach allows isocratic separation in 2 minutes. The LOQs are in the range of 1.20-10.3 µg/l.</p> <p>The matrix effect was estimated by the spike recovery test, it was found that the determination error does not exceed 30%.</p>

RESULTS

Retention of the analytes (Fig. 1) on a number of stationary phases, both polar and non-polar, was studied. The studied compounds are characterized by weak retention regardless of the nature of the stationary phase. DMUs even in the absence of a dynamic modifier exhibit a good peak shape, whereas for FADMH a strong trailing is observed in most cases. On the basis of the data, the 2-ethylpyridine phase was selected as the most promising one providing an acceptable separation of all compounds in combination with the lowest tailing of FADMH.

The most common additives were introduced into the co-solvent: formic acid (0.10% vol.), ammonium formate (10 mM) and deionised water (5% vol.). These modifiers were found to have negligible effect on analyte retention and ionisation efficiency by APCI, therefore pure methanol was used as a co-solvent.

Influence of methanol content (5-15% vol.), column temperature (25-55°C) and back-pressure (110-190 bar) on analytes retention was also studied. The following analysis conditions were selected as optimal: BEH 2-EP, flow rate - 1.30 ml/min, methanol content - 10% v/v, temperature - 55°C, back-pressure - 130 bar, make-up solvent flow rate - 0.1 ml/min. The developed approach allows isocratic separation in less than 2 minutes (Fig. 2A).

Mass spectrometric detection was carried out in the positive mode using atmospheric pressure chemical ionisation (APCI). The source parameters were selected to provide the highest ion generation efficiency: needle current 4 µA, temperature 300°C, curtain, nebulizer and heater gases pressure: 20, 50 and 30 psi respectively. To improve the sensitivity and selectivity, a multiple reaction monitoring mode (MRM) was used and the ion transition conditions were optimised for each compound.

The study of the calibration solutions in the concentration ranges indicated in Table 1 showed a linearity of the dependence of the peak area on concentration with a correlation coefficient (R^2) greater than 0.999 for all components. Limits of detection (LOD) and limits of quantification (LOQ) were calculated based on 3σ and 10σ criteria respectively.

The matrix effect was estimated by the spike recovery test, known amounts of analytes were introduced into peat soil extracts. It was found that the determination error does not exceed 30% (Table 2).

The developed approach was tested on soil samples (peat and sand). The results obtained are presented in Table 3, chromatograms are shown in Fig. 2B and Fig. 2C, respectively.

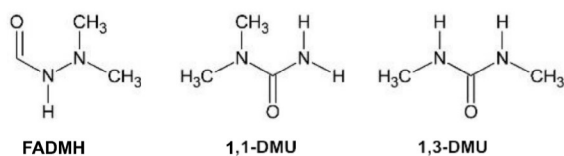


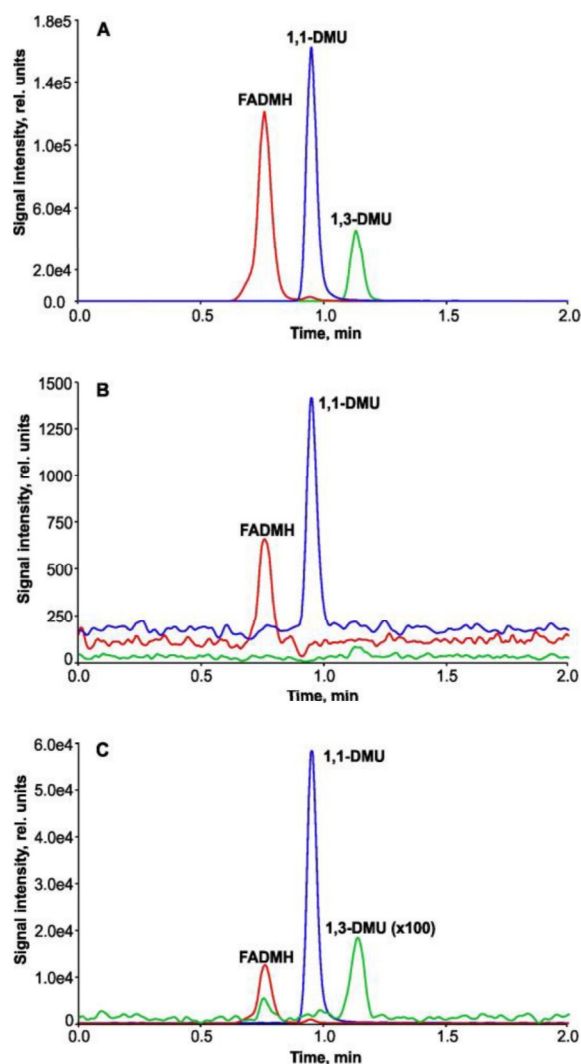
Figure 1: Chemical structures of formic acid dimethylhydrazide (FADMH), 1,1-dimethylurea (1,1-DMU) and 1,3-dimethylurea (1,3-DMU)

Table 1: Correlation coefficients (R^2), limits of detection (LOD) and quantification (LOQ) for FADMH and dimethylureas

Compound	Ion transition	R^2	Linear range, µg/L	LOD, µg/L	LOQ, µg/L
FADMH	89.1 → 45.1	0.9999	12.5 - 6250	3.08	10.3
1,1-DMU	89.1 → 72.1	0.9995	2.00 - 1000	0.36	1.20
1,3-DMU	89.1 → 58.1	0.9995	2.00 - 1000	0.49	1.65

Table 2: Matrix effect estimation (peat soil)

Compound	Added, µg/L	Found, µg/L	Found, %
FADMH	12.5	16.4	130
	312.5	314.4	101
	2500	2432	97.3
1,1-DMU	2.00	2.50	125
	50.0	58.0	116
	400	411.0	103
1,3-DMU	2.00	2.52	126
	50.0	49.0	97.9
	400	357.8	89.4



THE SCIENCE OF HONEY: BIOLOGICALLY ACTIVE COMPOUNDS AND PARAMETERS TO DETERMINE QUALITY

P3



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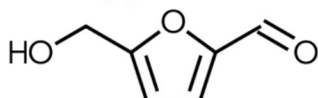
BEES AND HONEY

Bees live in highly developed social communities with 20.000 up to 80.000 members. There are different types of honey, based on the geographical and botanical source of nectar, collection method and season. Beekeeping is one of the oldest commercial activities.

HYDROXYMETHYLFURFURAL

Hydroxymethylfurfural (HMF), also 5-(hydroxymethyl)furfural, is an organic compound formed by the dehydration of certain sugars.

Maximal permissible concentration of HMF in honey is 40 mg/kg.



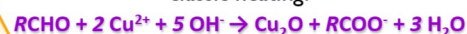
TOLLENS' TEST

The most intense colouring was observed in home-made flower honey and the smallest amount of aldehydes and ketones was present in synthetic honey from sucrose and lactic acid under classic heating conditions.



FEHLING'S TEST

Samples, which contained monosaccharides, were coloured intensely yellow, some even orange. Positive test results: natural honey and synthetic honey made from glucose and lactic acid under classic heating.



HONEY SAMPLES

- Purchased flower and forest honey
- Home-made flower and mixed honey
- Synthetic honey from: sucrose and lactic acid / glucose and lactic acid / glucose and citric acid under microwaves or conventional heating conditions



BIURET REACTION

Most samples gave a negative result, only a slight colour change was observed in the samples of natural honey.

XANTHOPROTEIC REACTION

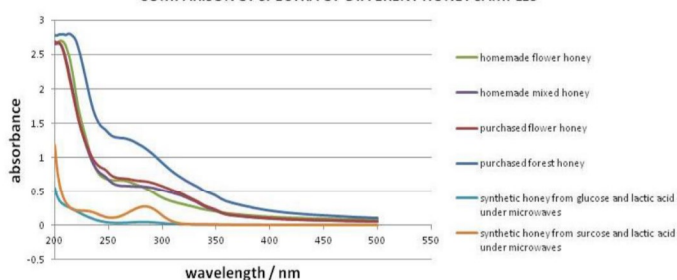
No colour change was observed in samples of synthetic honey, which don't contain proteins.

SPECTROPHOTOMETRIC HMF DETERMINATION

Sample	Content of HMF [mg/kg]
Bought flower honey	27,08
Homemade flower honey	13,80
Purchased forest honey	24,19
Homemade mixed honey	27,95
Synthetic honey from sucrose and lactic acid under microwaves	21,28
Synthetic honey from sucrose and lactic acid under conventional heating conditions	9,17
Synthetic honey from glucose and lactic acid under microwaves	11,57
Synthetic honey from glucose and lactic acid under conventional heating conditions	9,71
Synthetic honey from glucose and citric acid under conventional heating conditions	43,41
Synthetic honey from glucose and citric acid under microwaves	58,23

UV/VIS SPECTRA

COMPARISON OF SPECTRA OF DIFFERENT HONEY SAMPLES



SUMMARY

Our research work describes the properties of honey, which are the indicators of quality and possibly pollution of the environment, where the bees forage and therefore where the honey comes from. Absorption spectra of the analysed honey samples were decreasing from 200 to 700 nm, with a maximum between 250 and 280 nm. The results of quantitative hydroxymethylfurfural (HMF) determination in natural honey samples were below the legal limit (40 mg/kg), higher values are commonly associated with elevated temperatures and light exposure. Treating the sample with microwaves causes a significant increase in the HMF concentration, which is consistent with the principles of microwave activation. Qualitative tests for specific components are a useful tool for determining the main components in honey and differentiating between natural and synthetic honey samples and could be used as a basis for developing more accurate quantitative methods.

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Extraction process optimization and comparison for ambroxol, bromhexine and umifenovir by P4 pressurized solvent and ultrasonic extraction from bottom sediments

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OUTLINE

Ambroxol and Bromhexine are among the most generally used drugs worldwide. There are bromine in their chemical structure (Fig. 1). It makes possibility to determine these drugs by inductively coupled plasma mass spectrometry. Another drug containing bromine in chemical structure is umifenovir. The consumption umifenovir has increased in time the coronavirus pandemic. As a result, these drugs can discharge in urban wastewater in large quantities and if treatment facilities do not remove them completely, they are polluting into the environment. There are drugs can accumulate in the active sludge of treatment facilities and bottom sediments of natural reservoirs.

RESULTS

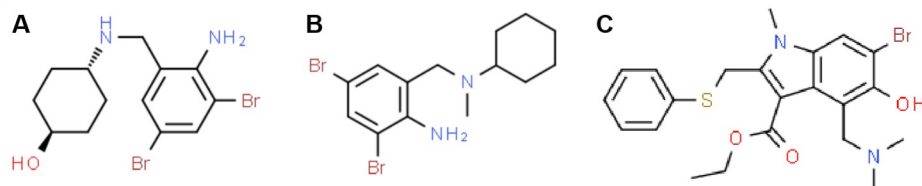


Figure 1: Ambroxol (a); Bromhexine (b); Umifenovir (c)

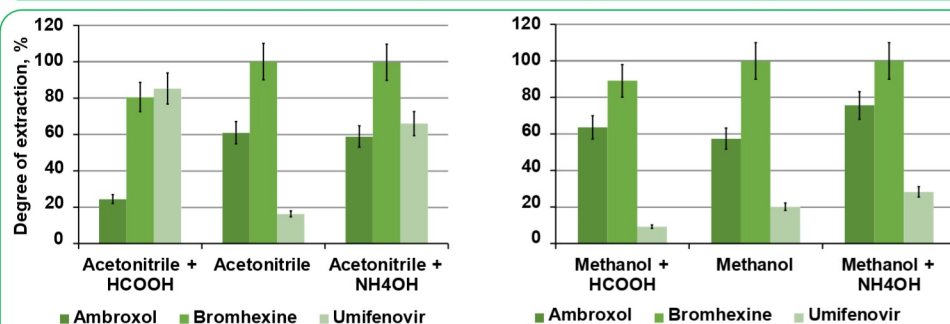


Figure 2: Eluent influence on recovery of drugs in PLE

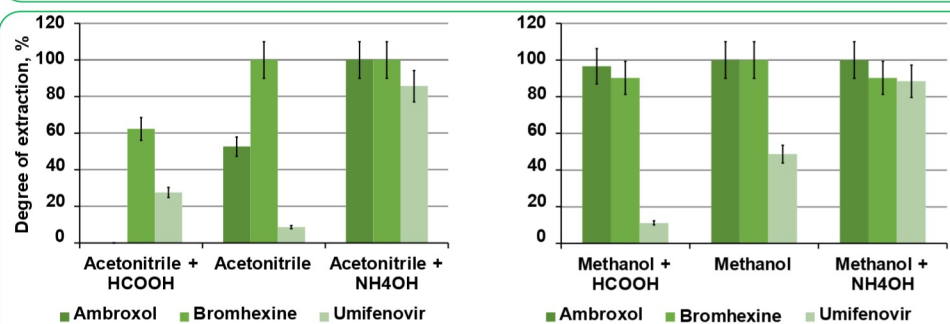


Figure 3: Eluent influence on recovery of drugs in USE

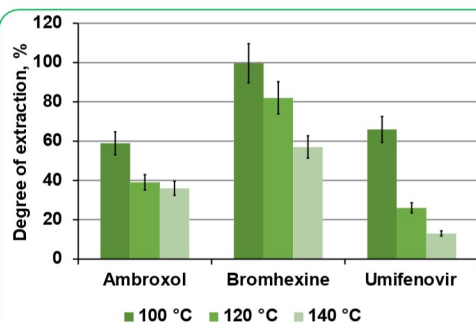


Figure 4: Temperature influence on recovery of drugs in PLE

Table 1: Concentrations ($\mu\text{g kg}^{-1}$) of ambroxol, bromhexine and umifenovir in environmental sample

	Bottom sediment	Active sludge
Ambroxol	2.3 ± 0.2	38 ± 2
Bromhexine	0.3 ± 0.1	6.0 ± 0.3
Umifenovir	15 ± 1	960 ± 50

EXPERIMENTAL METHODS

There was a study of the effect of various eluents on the degree of extraction of ambroxol, bromhexine and umifenovir from bottom sediments. The methods of pressurized liquid extraction (Fig. 2) and ultrasonic extraction (Fig. 3) have been studied. PLE parameters: sample weight 2 g, cell temperature 100°C (Fig. 4), static time 10 minutes, wash volume 60%, three repetitions. USE parameters: sample weight 2 g, processing time 15 minutes, solvent volume 10 ml, three repetitions. Solutions of acetonitrile and methanol modified with 0.5% formic acid or ammonium hydroxide were used for elution. Drugs were added to bottom sediments.

REAL SAMPLES ANALYSIS

The method was applied to real samples. Activated sludge samples were sampled from municipal wastewater treatment plants. Samples of bottom sediments were sampled from the river, where treated wastewater is discharged. The samples were dried, homogenized, and extracted by the pressurized solvent extraction. The extracts were analyzed by high performance liquid chromatography with inductively coupled plasma mass-spectrometry Aurora Elite (Bruker, Bremen, Germany). The following parameters were applied: RF power 1.60 kW, sampling depth 5.0 mm; plasma, auxiliary, sheath and nebulizer gas (Ar) flowrates 18.0, 1.65, 0.23, and 0.80 L min⁻¹, respectively; dwell time 500 ms. Detection was conducted in selected ion monitoring mode (m/z 79). High purity hydrogen (40 mL min⁻¹) was used as a CRI reaction gas suppressing interferences. The quantitative determination of bromine-containing compounds was carried out by external standard method. All drugs were found in large quantities (Table 1). This can lead to secondary pollution of water bodies.

CONCLUSION

For drug extraction, ultrasonic extraction with acetonitrile or methanol with 0.5% ammonium hydroxide was the most effective method. However, we preferred the PLE method. This method is faster and saves solvent. The best results for the sum of the three compounds were observed using acetonitrile with 0.5% ammonium hydroxide. The drugs recovery were 60% ambroxol, 100% bromhexine and 65% umifenovir. The influence of temperature was investigated. Increasing the temperature reduces the recovery of drugs. The levels of drugs in the activated sludge of urban wastewater treatment plants were determined: umifenovir 0.96 mg kg⁻¹, bromhexine 6.0 $\mu\text{g kg}^{-1}$ and ambroxol 38 $\mu\text{g kg}^{-1}$. This observation indicates the possibility of secondary pollution of ground and surface waters, which leads to some increase in the negative impact on aquatic ecosystems.

Examination of Existing Indicator Schemes for the Urban Water Circularity Assessment

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OUTLINE	EXPERIMENTAL METHODS	CONCLUSION
<p>In urban water management, we are currently facing increased demand for drinking water, adapting to the consequences of climate change, and protecting the environment and the population's health while aging existing infrastructure systems. Addressing problems at the source and monitoring the remaining impacts throughout the chain allows us to protect water resources, restore natural resources, reduce environmental pollution and optimize infrastructure systems that are not yet sustainably designed. Considering the concept of Circular Economy (CE), these problems in cities can be addressed through various measures that have already been developed and tested in many pilot projects but are not yet sufficiently implemented in practice.</p> <p>A key challenge preventing more effective use of the CE concept in various policies, planning, and governance is the establishment of an appropriate system of indicators to assess the current situation and measure the success of the measures taken. It is necessary to develop appropriate indicators that are sufficiently detailed to plan local actions.</p>	<p>A preliminary survey of existing indicator schemes and an analysis of the City Blueprint[®] scheme was conducted. Indicators were evaluated based on the criteria:</p> <ul style="list-style-type: none"> Whether an indicator address one of the urban circular water management challenges²: restoration and maintenance of the water cycle (1A), treatment and cleaning of wastewater with safe reuse (1B), processing and reuse of nutrients (1C). Can an existing or adapted indicator be used for: macro-level, e.g., city or municipality (2A); mezzo-level, e.g., part of the town, neighborhood (2B); micro-level, e.g., individual plot or building (2C). Whether the indicator measure the success of the implemented CE measures (3A). Is the indicator based on: results of other research, numeric or spatial data, or a predefined evaluation scale (4A). <p>We also determine whether there are publicly available data for indicators at different levels in Slovenia.</p>	<p>From a preliminary analysis of some existing indicator schemes, standards ISO 37123³, ASTM (E3136-18)⁴, European Environment Agency indicators⁵, the Green City Index⁶, the City Blueprint[®], and Water Sensitive Index⁷, it follows that existing indicator schemes for urban water management already address some of the CE's objectives. The indicators are based on surveys, data, or pre-prepared evaluation scales. The indicators under consideration generally do not allow analysis for the particular neighborhood (mezzo-level) or individual buildings (micro-level), as pointed out as research gap by CE researchers⁸.</p> <p>We can conclude that the CityBlueprint[®] indicators satisfy a substantial number of criteria, or some adjustments are needed. It is crucial to test available data for each proposed indicator and examine data quality. Only for some indicators, there are already publicly available data. Internal databases (e.g., public utility companies, ministry) will also need to be analyzed for the actual use of indicators. Based on the method presented in the poster, further research will include the consideration of additional already developed indicator schemes.</p>

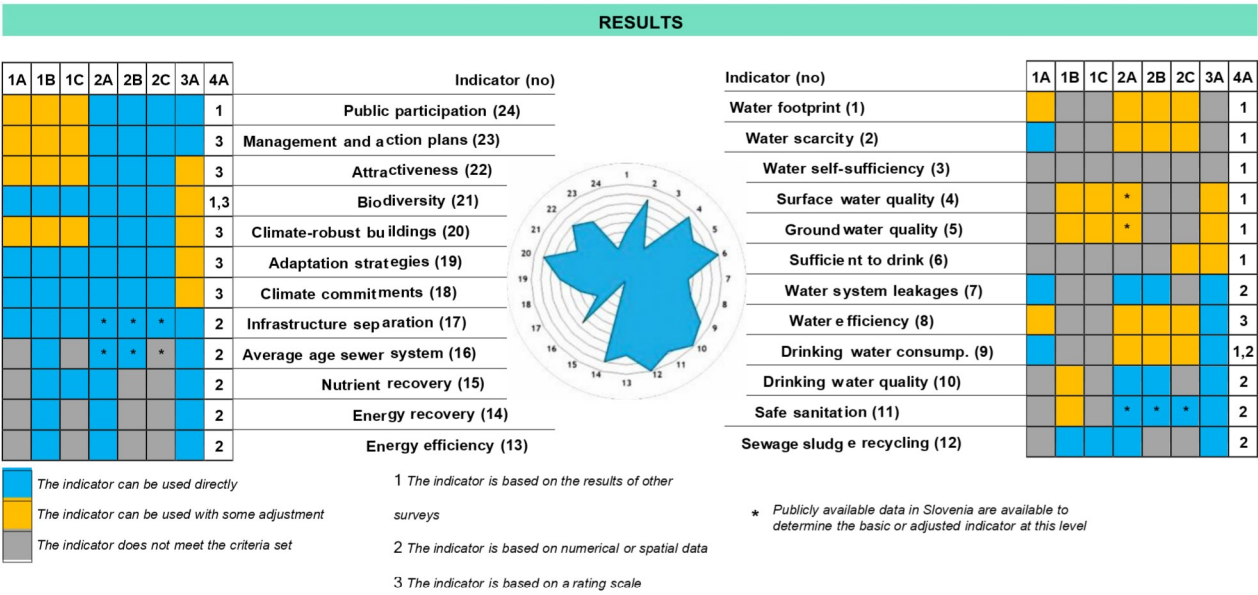


Figure 1: Results of the analysis of the City Blueprint[®] indicator scheme with the presentation of the original indicator determined for Ljubljana¹

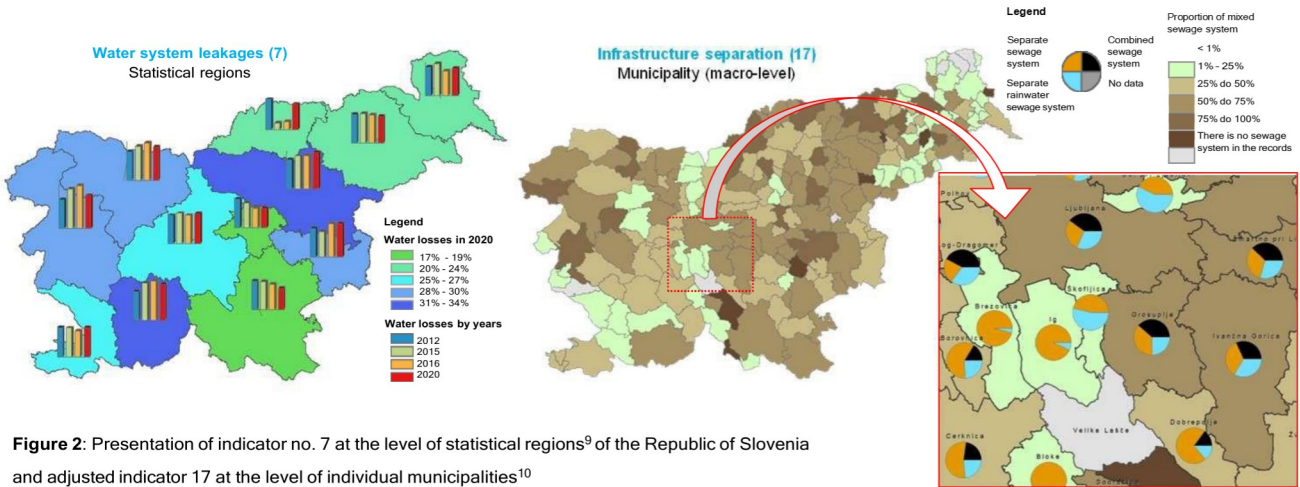


Figure 2: Presentation of indicator no. 7 at the level of statistical regions⁹ of the Republic of Slovenia and adjusted indicator 17 at the level of individual municipalities¹⁰

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