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8.00 Duško Spasovski, Klinika Banjica, Beograd: Human resting muscle tone and body deformation

8.45 Vesna Spasovski, Institut za molekularnu biologiju, Beograd: Uloga mikrobioma u zdravlju čoveka

9.30 Odmor/Break

9.45 Marija Ipavec, Vlada Republike Slovenije: 40 let izkušenj z nadkolensko protezo

10.30 Domen Vozel, Univerzitetni klinični center Ljubljana: Zdravljenje s plazmo, bogato s trombociti in zunajceličnimi vezikli

10.45 Bojana Uršič, Univerzitetni klinični center Ljubljana: Biomehanska analiza kolka pred in po trojni osteotomiji

11.00 Odmor/Break

11.15 – 15 Vsi udeleženci/All participants: Določanje biomehanskih parametrov/Determination of biomechanical parameters

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INTERDISCIPLINARY CONTRIBUTIONS

HUMAN RESTING MUSCLE TONE – ORIGIN AND IMPLICATIONS

Duško Spasovski

School of Medicine, University of Belgrade, Serbia, and Institute for Orthopaedic Surgery "Banjica", Belgrade, Serbia

Abstract

Human Resting Muscle Tone (HRMT) refers to the tension detected in skeletal muscles during electrically silent state. Hypotheses on the origin of HRMT include connective tissue viscoelasticity and trixotrophy, as well as the action of titin in muscle fibers.
Although low (1-2% of maximal isometric voluntary contraction –MIVC force), HRMT is present most of the time (96,53% on average) and produces significant pressure on bone and cartilage. It exceeds the force stimulation threshold at growth cartilages (0.3MPa), thus influencing bone growth. Disturbed HRMT magnitude or distribution is a mediator of bone orthopedic deformity.
HRMT intensity is dependent on various emotional and constitutional factors, the presence of muscle reflexex, amount of physical activity, hormonal impact on skeletal growth etc. HRMT can be measured directly by elastography (NMR or ultrasound), electromyiography, myotonometry or mechanomiography – expensive procedures used mostly in scientific setting. Instead we propose a clinical indirect assessment of HRMT by the analysis of HRMT effect – CoreFitMax method. It uses postural data (43 static and 40 dynamic) as input parameters. By discrete finite element analysis algorithm for myofascial chains-based kinesiological analysis, it calculates relative HRMT levels in a group of 62 skeletal muscles relevant for human posture and basic athletic performace. Analysis of HRMT distribution reflects the impact of structural and functional deformity of human body. Restitution of balanced HRMT improves body posture and correct locomotion. Methods include exercise, physical therapy, local infiltraction of medications, plaster casting and surgical procedures. Concept of structural integrity of connective tissue throughout the body and the discovery of myofascial chains places HRMT of indivicual muscles in the central spot of kinesiotherapy as well as sports performance assessment.

Keywords: human resting muscle tone, kinesiology analysis, muscle contraction, bone deformity, myofascial chains

1. Introduction

The term “human locomotor system” includes many tissues: bones of trunk and extremities, their articulations, and dense connective tissue elements that connect bony elements (tendons, ligaments, capsules, fascia). These elements are both innervated and controlled by various levels of CNS, under the influence of motor control routines and skills. Of course, biochemical processes that stand behind these tasks require adequate circulatory, metabolic and hormonal balance. If we look deeper, we can see that human body movement in general actually is one of most integrated and complex human functions. On a global scale, one can divide all human basic needs into ones that support or are supported by the need for movement, which is inherent to humans.

Several medical specialties deal with various aspects of human movement. Neurology investigates neuromuscular control, orthopaedics and traumatology analyse structural and functional integrity of locomotor system, physical medicine studies range and strength of movements while energy metabolism involved in locomotion is in the focus of internal medicine, sports medicine and pharmacology. Movement quality of (generally) healthy people, however, rarely comes into focus of medical professionals - it is usually passed on to sport professionals.

Rapid development of sport science with utilization of modern technology has generated large amount of information on human locomotion in various motoric conditions. Therefore we aim to combine the two closely related scientific fields in diagnostics and dosing of human movement by analysing human resting muscle tone.

2. Muscle tone

Skeletal muscle fibers contraction is triggered by nerve impulse and mediated by rise in intracellular concentration of Ca^{++} ions. Contraction changes the fiber shape but not the volume. Muscles get shorter and wider, generating concentric force alog contraction axis – the muscle tone (1).

Biomechanical measurements of muscle force during contraction-relaxation cycle showed that relation of tone intensity and muscle length is not linear (Figure 1).

Several biomechanical models have been used to describe these and other mechanical muscle properties. One of the most frequently adopted is the Hill’s model (Figure 2)

introducing active and passive components of the muscle force. The active compomemt is the result of muscle fiber contraction and the passive component comes from the elasticity of connective tissue that surrounds and permeates the muscle.

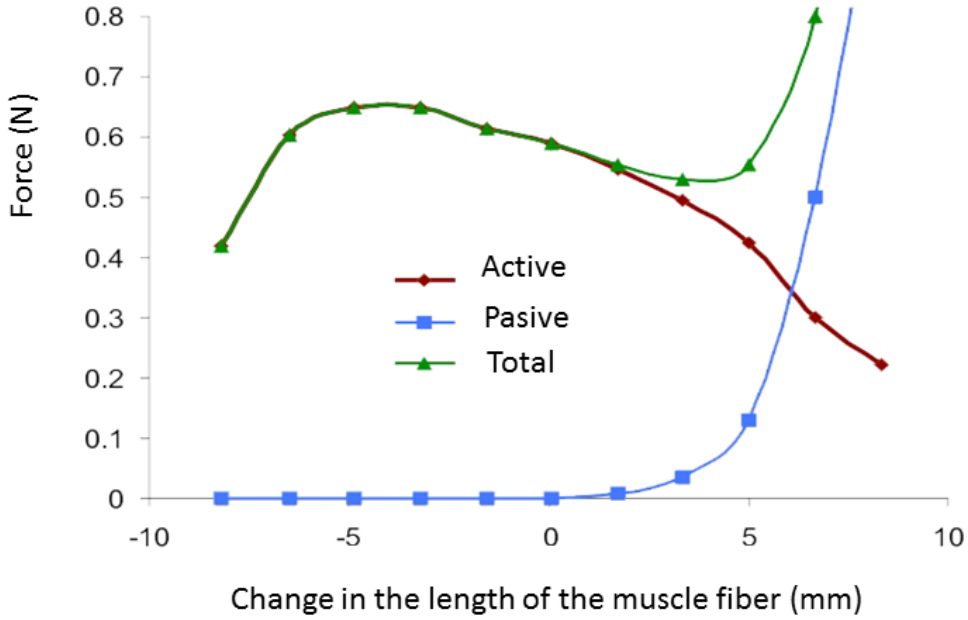

Figure 1. Muscle legth-force relation (2).

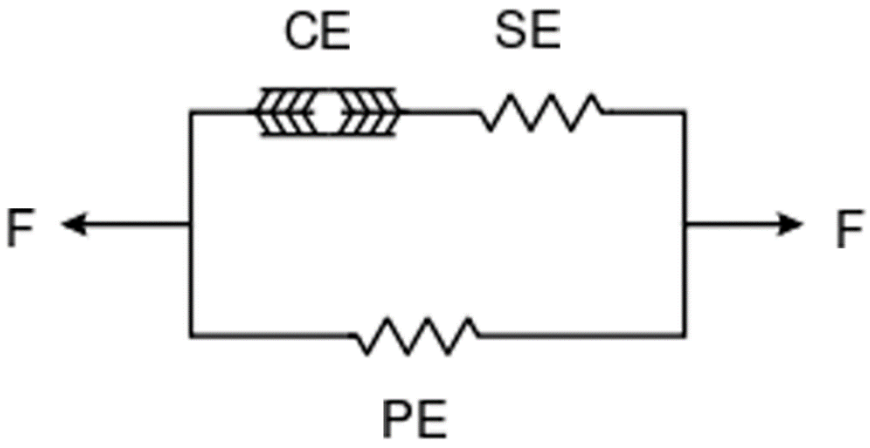

Figure 2. Hill's muscle model (2), F- force; CE- contractile element; SE- serial elastic element; PE- paralelel elastic element.

3. Human resting muscle tone (HRMT)

Electrophysiological measurements showed that in zero EMG state (without significant α- or γ-motor frequency) (3) there still is a basic level of muscle tension (4). This phenomenon is called Human Resting Muscle Tone - HRMT. Present theory behind HRMT is about connective tissue viscoelasticity (5), titin binding to contractile proteins (6-8) and trixotrophy (nonlinear viscosity-shear force relation) (9). HRMT is represented in Hill's model by parallel elastic component.

4. Impact of HRMT on musculoskeletal system

The impact of external and internal mechanical forces whose intensity is within physiological range on morphology and metabolism of all musculoskeletal tissues: bone (10), cartilage (11), muscle (12) and connective tissue (13) is well known. Of internal forces, HRMT has very low intensity: 1-2% of the force generated by Maximal Voluntary Isometric Contraction – MVIC, which ranges from 20 to 135 N/cm^2 of muscle physiological cross-sectional area, or up to 8.7 body weight (14,15).

Could such a tiny force as HRMT have a physiological effect on bone growth? Experiments show that the average pressure at the long bone physis ranges from 0.3 to 1.1 MPa, with an increase of 0.1 MPa slowing down physeal growth by 17,1% (16) while the pressure generated during MVIC exceeds 20 MPa (17,18). We conclude that HRMT-generated pressure is within physeal sensitivity range and that HRMT moderates physeal bone growth (19,20).

We can observe the influence of HRMT if we analyse cummulative force impact on locomotor system during long period of time. The data regarding urban population are very worrying: average cummulative daily medium and high intensity activity lasts for only 80 minutes for males and 72 minutes for females, with movements >50% MVIC adding up to only 5.1±6.4 minutes (21,22). This is equivalent to running for 31 minutes with speed of 10 km/h, or 65 minutes of walking (4 km/h) (23,24). We can count less than 12000 movements daily, resulting in an active tone present in only 3.47% of total 24 hrs. In other words, HRMT vastly dominates over the time. Since not all active movements engage fall body muscles in all possible directions, and the average force is less than MVIC, we can conclude that HRMT is actually the major mechanical stimulus that shapes our bones. Therefore, active muscle tone during exercise (muscle contraction, fatigue, injury or other acute mechanical changes) has a direct and immediate effect on musculoskeletal system, while slow and chronic effects

(structure and shape of bones and muscles) are dominantly indirect, delayed and mediated by exercise-induced residual changes of HRMT.

5. HRMT assessment

Manual testing, MRI or ultrasound elastography (25,26), piezoelectric mechanomyography (27) and dynamometry (28) are all not suitable for HRMT diagostics in clinical setting.

We propose reverse engineering method: assessment of HRMT by measuring its consequences on musculoskeletal system. One of such methods is CoreFitMax myofascial chains-based postural analysis algorithm (29-31). 83 postural parameters assessed by clinical examination and testing serve as an input, and CFM algorithm transforms them into output dataset: the relative HRMT value (in percentage) of the group of 62 major postural and locomotor muscles.

6. Conclusions

HRMT represents an important parameter in orthopaedics, describing force distribution along musculoskeletal system, which impacts physeal stimulation and may contribute to long bone deformity. HRMT can be influenced by exercise, physical therapy modalities, by immobilization devices or surgical interventions such as fasciotomy, tendon reinsertion or transfer.

HRMT assessment helps in establishing etiological diagnosis of structural and functional musculoskeletal impairments. Restitution of balanced HRMT leads to improved postural control.

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ARE WE A STEP CLOSER TO MOLECULAR THERAPY FOR DEGENERATIVE JOINT DISEASES?

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Abstract

Degenerative changes in cartilage due to aging and mechanical stress cause osteoarthritis to be one of the most prevalent diseases in population above the age of 60. Longevity and the growing prevalence of obesity in the western world point to requirement for more effective therapies for this chronic and progressive disease. Targeted therapies based on molecular pathways are a new and promising option in treatment of many diseases, and it seems that we are now a step closer to find the key players that could become target molecules in the therapy for osteoarthritis.

Keywords: Osteoarthritis, Stem cell therapy, Autophagy, Apoptosis, Blastema miRNA

Osteoarthritis (OA) is a degenerative joint disease that is common in elderly people, professional sportsmen and sportswomen, obese individuals and in persons with a respective genetic predisposition. Lack of an effective treatment which would have long lasting results as regards pain relief and cartilage regeneration caused OA to become the second most prevalent cause of work disability in men, right after heart diseases (1). The pathogenesis of OA is not yet fully understood. Eventhough the changes in cartilage are its main and obvious hallmark, OA is a disease that involves all joint tissues (2). Osteophytes on MRI images suggest involvement of subchondral bone in early stages of OA, and they may actually precede cartilage changes (3). Imbalances in bone turnover and mineralization, and a decreased bone volume result in altered density of bone, which in turn could affect biomechanical properties of the bone. Consequently, the affected overlying cartilage is predisposed to subsequent loss of integrity (3).

Accumulating knowledge on the mechanisms that maintain joint health and preserve cell and tissue homeostasis, leads to better understanding of the processes underlying degenerative changes in the joint due to ageing and OA. It was recently shown that two fundamental cell processes, autophagy and apoptosis, are compromised in OA (4). Reduction

of chondrocyte autophagy and elevation of chondrocyte apoptosis have been shown to profoundly contribute to the cartilage destruction (4). Subsequent synovial inflammation plays a role in worsening of symptoms and disease progression.

Autophagy is a basic cytoprotective pathway that ensures cellular integrity. It is a vital cellular mechanism for maintenance of cellular homeostasis by recycling of worn out proteins and organelles, including mitochondria (5). Decreased autophagy leads to aggregation of damaged intracellular material that further stimulates production of reactive oxygen radicals and alteration in function of autophagy-related genes. Recent studies showed decreased autophagy in aged joints and OA patients (4).

Cell loss by apoptosis is one of the earliest events that occur during ageing and exposure of the cartilage to mechanical stress, and this is followed by biomechanical softening of cartilage (6). Increased apoptosis stimulates macrophages to produce inflammatory mediators, further causing synovitis, edema and pain in affected joint.

Latest discoveries of innate cartilage's potential for self-healing and regeneration cast new light on targeted therapy for OA. Opposite to text-book knowledge, it was recently shown that cartilage possess intrinsic potential for regeneration, which is based on up-regulation of cartilage protein turnover via regenerative microRNA molecules (7). In addition, recent findings showed the presence of mesenchymal stem cells (MSCs) in adult cartilage (8-11), which is another significant discovery that reveals intrinsic potential of cartilage for self-repair.

Novel biological therapeutic strategies in treatment of OA, like stem cell injection and injection of diverse fractions of blood or tissue, represent new, effective, promising, and safe therapeutic options. The joints are closed structures, and it was shown in rat model that stem cells injected into the joint do not leak out (12). This enables that cells administrated into the joint remain in it and exert their full regenerative capacities within the joint niche. MSCs secrete various trophic and anti-inflammatory molecules and stimulate regenerative processes in the cartilage through the paracrine action. Stimulation of chondrocyte autophagy and attenuation of apoptosis are mechanisms of MSCs action (13). Moreover, MSCs directly and through their paracrine secretion significantly reduce production of pro-inflammatory cytokines (TNF, IL-1, IL-6), and stimulate production of anti-inflammatory cytokines (IL-10, IDO, IL-1Ra), contributing to a decrease of inflammation and pain relief (13,14). If they also stimulate blastema miRNAs (miR-21, miR-31, and miR-181c) remains yet to be clarified.

In years to come, knowledge on the molecular mechanisms underlying various processes of the body will change the paradigm how we look upon health and disease. Cell-based

therapies will certainly change therapeutic procedures that were used before, and bring new modalities into therapeutic choices. For osteoarthritis, cell based therapies are already in clinical protocols, eventhough there are still some issues to be clarified in this field. Development of molecular therapies, based on key regulatory pathways, will be designed to target chondrocyte apoptosis and autophagy and to stimulate endogenous cartilage repair processes. This exogenous regenerative molecules should provide new perspectives in treatment of this chronic disease.

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Amputation and rehabilitation of the lower limbs

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Abstract

The article describes the main causes of amputation of the lower limb, the types of amputation of the lower limb, the factors that affect the successful rehabilitation after such operation, different types of leg prostheses and the issues that the patients face after amputation.

1. Introduction

The causes of amputation of the lower limb in young people are mainly tumors and accidents, while in elderly, the causes are mainly vascular diseases and diabetes. Amputation is followed by rehabilitation and production of the prosthesis, which functionally and aesthetically replaces the amputated extremity or its part. The patient is suffering from various problems such as phantom pain, poor retention of the prosthesis by vacuum, blisters and lumps on the residual limb, allergy, limp and tilt at walking and damage to the residual limb due to falling. Since we live in the 21st century, during the rapid developing digital technology has been integrated also in prosthetics. Prosthesis with built-in microprocessors which carry out knee control allow for stable and efficient walking which seems similar to a normal walking pattern (1,2).

2. Causes of amputation of lower extremities

The causes of amputation of the lower extremities are: 1) diseases (tumors, infections, freeze, vascular disease, diabetes mellitus), 2) accidents (3,4).

In young people, the main causes of lower limb amputation are mainly tumors and accidents. Advance in medicine and pharmacy has brought changes in treatment protocols; amputation is no logner prerequisite in treatment of tumors. The indication for amputation depends on the type, position and size of the tumor. Typically, the tumor is located near the knee, so the amputation is performed above the knee. In the event of an accident, the lower limb amputation is performed only if the limb is extensively damaged and there is no other solution. In the elderly, however, the causes of amputation are mainly vascular disease and diabetes.

Leg amputations fall into two major categories (1): minor amputations such as finger amputations, partial amputations of the foot and ankle disarticulation and major amputations such as knee amputation, knee disarticulation, transfemoral knee amputation, rotacioplasty (tibia rotation and reattachment causing the ankle to function as a knee), hip disarticulation, hemipelvectomy.

3. Rehabilitation

Amputation is followed by rehabilitation. The main factors influencing a successful outcome of rehabilitation are: 1) patient's age, 2) the cause of the amputation and the patient's medical condition, 3) the length of the residual limb which must be neither too long nor too short, 4) muscle strength (should not lead to atrophy), 5) patients vital energy, 6) quality of the prosthesis, 7) weight of the prosthesis - the prosthesis should be light enough so that the patient is able to control the knee in the case of the above knee prosthesis, and firm enough to keep the gait stable, 8) type of the artificial knee - in the case of above knee prosthesis, 9) quality of rehabilitation (3,5-7).

In most cases the older the patient, the more difficult it is for her/him to get used to the orthopaedic device. Also an important factor affecting the outcome is the cause of the amputation. In the case of illness, the outcome depends on the progress of the disease. An important factor influencing the outcome of rehabilitation is the quality of the prosthesis that was made for the patient.

4. Kinds of prostheses and their parts

Lower limb prostheses are divided according to the amputation height into: 1) Below knee prostheses (which were made from wood 30 and more years ago but are now made are in plastic materials), 2) above knee prostheses (the socket of such prosthese was made of wood 30 and more years ago but after 1990, the material for the socket was replaced by plastic). 3) prostheses for patients without the residual limb.

There are generally two types of knees: mechanical and microprocessor-driven. Mechanical knees have an in-built mechanical joint that replaces the knee joint. The speed and facility of the swing can be controlled by friction in the joint, by a hydraulic system or by a locking mechanism. Microprocessors, however, perform the knee control. The microprocessor-driven knee gives a more stable and efficient gait, very similar to a normal gait pattern (2).

The artificial knee joint consists of the following parts: bed, valve, metal part from foot to knee, knee, part from knee to socket, foot and cosmetic parts (the metal parts are covered by a foam which is covered by the stocking.

The socket should be made comfortable to fit well with the residual limb, not too wide and not too narrow, and should conserve the vacuum. It should be designed so that the patient can wear the prosthesis for 16 hours/day. The patient should have no sensation of pain in the residual limb and no blisters should appear on the residual limb. The patient pulls the residual limb into a socket with a special sock and closes the socket with a valve. In case the patient feels that there is air in the socket, it should be released through the valve. The problem, however, arises in the summer when it is hot because the residual limb in the bed sweats. Sweat droplets can be absorbed by baby powder.

The metal part from foot to knee, the knee and the part from knee to socket are all covered by a foam which is shaped according to the contralateral leg and covered by a stocking. Knee mobility should be adapted to the patient's movement. When the patient steps on the prosthesis, the prosthesis knee should lock so that the movement to the contralateral leg is stable.

Considering that we live in the 21st century, in a period of rapid development of digital technology, the advances have been introduced also to the field of prosthetics. Intelligent prostheses (e.g. C-leg, Genium - Otto Bock, Germany) have a built-in computer system that senses the ground and adjusts the knee accordingly. Artificial knees with microprocessors have in-built sensors and are equipped by programs and a resistence system. An appropriate battery is required to supply the system with energy. A computer-aided system that includes sensors and internal computer controls the internal fluid in the knee (hydraulic or pneumatic) in all stages of walking. Continuous fluid monitoring and control allows the processor to regulate resistance and enables the patient to walk better at different speeds and move more safely up or down the stairs (1,2).

When making a prosthesis, the engineer must take care of the length of the prosthesis, which must not be too high nor too low. Both legs should be of the same length. The socket should be comfortable, it should not cause blisters and bumps on the residual limb. The patient should not feel pain in the residual limb when wearing a prosthesis. How the patient will walk with the knee prosthesis will depend on the type of the knee and adjustment of its mobility to the patient. The foot must correspond to the knee joint and should be adjusted to the height of the heel. Artificial feet exist that restore energy.

5. Problems after amputation

The patient has various problems after amputation of the limb and when wearing the prosthesis. These problems include: phantom pain, bad posture, blisters and lumps on the residual limb, allergy on the residual limb - reaction to the material from which the socket is made, limp and tilt when walking and injuries.

There are different ways to resolve the problem of phantom pain. Analgetics in combination with antidepressants are helpful if pain is unbearable. In some patients, pain is replaced by a sensation of warmth when hot water is placed on the residual limb. All other problems, such as a lump, allergy and blisters on the residual limb or poor maintenance of vacuum should be alerted to the engineer when the prosthesis is tested. Faster healing of blisters and aleviating allergies are promoted by using appropriate creams. A gauze or another convenient cloth can be placed on the bottom of the sicket in case of a lump on the residual limb.

Most patients tend to tilt when walking. Nollan et al. (8) studied walking speed and asymmetry, and distribution of weight to the prosthesis. They found that as the walking speed increases, the values of the time variables we observe decrease and the vertical force on the ground increases. A noticeably shorter time of standing on the prosthesis compared to the time of standing on the contralateral leg was observed (9).

A particular problem is trauma of the residual limb due to a fall. In cases of hardly visible fracture, conservative treatment is sufficient, meaning that the patient should not wear the prosthesis until the fracture is healed (e.g. for ahout 6 weeks). If the fracture is more severe, osteosynthesis is required (e. g. osteo-synthesis of pertrochanteric fracture with cannulated screws).

Several clinical studies have been performed with different types of prostheses. The number of falls turned out to be significantly lower in patients using C-leg compared to other types of prostheses. The C-leg was found to be one of the most reliable prostheses, offering precision and stability (1,2).

Walking up and down the stairs, walking backwards, walking over obstacles and standing in a relaxed position is made possible by the Genium Leg, which was manufactured in 2011. The movement of the patient with this prosthesis is safe because it provides very good support. The settings, however, are governed by the software applications that are accessible for Android, Apple (iOS) and other units. The movement is natural. Walking over obstacles is easy. The prosthesis can be adapted to the activity of the patient. Both Genium and C-leg prostheses provide a high level of safety so that a patient can prevent a fall (2).

The above problems can be elaborated by a physiotherapist, a specialist of physical medicine and rehabilitation specialist with advice from a prosthetics engineer. The medical doctor prescribes an optimal orthopedic device, checks if it is appropriate or not and suggests improvements if necessary, and immediately takes action if any of these problems occur. However, the prosthetics engineer should take into account the patient's opinion, collaborate with the patient and follow the technical novelties in the field of prosthetics.

6. Conclusion

Lower limb amputation is a severe event in life of each patient. However, as digitalization has also touched the field of prosthetics, creation of a prosthesis with a microprocessor brings hope for better solution in the future. Prostheses such as C-leg allows walking normally up and down the stairs, over obstacles or downhill. With additional features it is possible to take activities such as cycling, dancing and playing golf. Switching between different modes has been made easy. Although microprocessor prostheses have been on the market for over 20 years, their prices are still high. Nonetheless, the cost-effective management of the money that is brought to the health care fund should enable amputees who work full time to obtain modern intelligent prostheses.

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APPLICABILITY OF PLATELET- AND EXTRACELLULAR VESICLE-RICH PLASMA IN MEDICINE

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Abstract

Platelet-rich plasma is a blood-derived product with proven favourable effects after a local application in various healing disorders. It is also rich with extracellular vesicles - a heterogeneous group of nano- to micro-sized membranous structures -that are considered as the main mediators of regenerative effects. Hence, the prepared blood product can be suitably named »platelet- and extracellular vesicle-rich plasma«. Platelets and platelet-derived extracellular vesicles are not only important in haemostasis, but also in the immune response. Platelets are the most numerous blood immune cells. They are also the main source of blood-derived extracellular vesicles. Extracellular vesicles play an important role in intra- and intercellular communication, therefore they can be utilised in diagnosis and treatment. Platelet- and extracellular vesicle-rich plasma is being used for almost three decades in different fields of medicine, especially in surgery, due to its favourable regenerative properties. However, extracellular vesicles are seldom described in clinical studies that consider the platelet-rich plasma. Based on the molecular mechanisms of the healing process, functions of platelets and platelet-derived extracellular vesicles, platelet- and extracellular vesicle-rich plasma offers an important therapeutic solution in different diseases. Application of platelet- and extracellular vesicle-rich plasma is inexpensive and safe, however its preparation requires advanced laboratory skills. An article contains a description of this blood product and reported experiences on its use. We also present our recent advances which are a product of a collaboration of researchers from medical and biomedical fields. This collaboration leads to an advancement in the treatment modalities in different fields of medicine, also otorhinolaryngology and cervicofacial surgery.

1. Roles of platelets in immune response and tissue regeneration

Platelets are 2-5 µm sized blood cells without nuclei which are formed as a result of fragmentation of megakaryocytes in the bone marrow or lungs. Their lifespan is relatively short (7-10 days) due to the lack of the nucleus (1). According to the standards, their concentration in healthy human subjects is in the range of (1.5-4) x 10^{11} per litre of blood. Concentration in blood qualifies platelets in the second place, after red blood cells (2). Platelets have an essential role in haemostasis, that is why preparations with high platelet concentrations have been used (since approximately 1970) for the treatment of bleeding and for haemorrhagic diathesis. Back then the haematologists have named the preparation "platelet-rich plasma". Platelets are important not only in haemostasis but also in the immune response. They are the key cells of an innate and of an acquired immune responses and are therefore important in tissue regeneration (2). Due to a concentration greater than the blood concentration of leukocytes, platelets are the most numerous immune cells in blood. Platelet-derived extracellular vesicles (EVs) that are formed after platelet activation (1), are also important in providing haemostasis and immune responses. Knowledge on the roles of platelets and platelet-derived EVs has led to the rise of preparations with high platelet concentrations, especially platelet-rich plasma (PRP), which also contains high EV concentrations. This is why the preparation is called »platelet- and extracellular vesicle-rich plasma« (PEVRP) (2–5).

2. Extracellular vesicles

2.1. Description and classification

Extracellular vesicles (EVs) are a heterogeneous group of cell membrane structures that can arise from any cell, including plant cells and bacteria (6–8). EVs were isolated from various body fluids and cell culture media and subsequently examined by different microscopic techniques (9–11). EVs in blood isolates are a dynamic material derived from blood cell fragments (12) and surrounding solutions. Shear forces in the process of isolation are also important (13). Because most erythrocytes and leukocytes are removed from the blood sample in the first steps of EVs isolation, megakaryocyte- and platelet-derived molecules are often present in isolates (14). Standard laboratory tests do not currently cover the measurement of EVs in isolates, but the expected concentrations of EVs in isolates may be high (15).

Initially, the belief was that EVs are only carriers of cell waste (6), but later it became evident that they play an important role in intercellular signalling (8,16) and thus have a significant impact on the course of many diseases (17,18). Some divide EVs into two groups; microvesicles and exosomes, in regard to their size and composition, as well as to their

assumed origin (6). Microvesicles are described as membrane-bound particles of 50-500 nm in size, which are formed by the process of ectocytosis or plasmalemma budding (8). Exosomes are described as membrane-bound particles of 50-150 nm in size, which are formed in the inner compartments (endosomes) of the cell and released into the extracellular milieu by fusion of the compartment membrane with the cell membrane in the process of exocytosis. Several molecular mechanisms are involved in the formation and secretion of exosomes and microvesicles (6,16). When an EV reaches the target cell, it triggers a physiological or pathological response. The target cell may be remote, adjacent, or the cell of EV's origin. This points out an important role of EVs in autoregulation. The EV's activity is dependent on its contact with the plasmalemma of the target cell. EV can act in three possible ways: via binding to membrane receptors, via coupling with plasmalemma and consequent release of cargo (carried by the EV) into the cytoplasm of the target cell and/or through the uptake of the EV by endocytosis (6).

2.2. Applicability of extracellular vesicles in diagnosis and treatment

EVs may serve diagnostic or therapeutic purposes because of their roles in intercellular communication. Their use as biomarkers of infectious, neurodegenerative, autoimmune diseases and tumours is promising. For therapeutic purposes, they could be used as vaccines against infectious diseases or tumours, as immunosuppressive or regenerative therapy, as carriers of active substances and even in the cosmetic industry to regulate skin pigmentation (17). The use of EV isolates for systemic treatment of tumours is at least in the stage 2 clinical trials. Platelet EVs are on the other hand the main effectors of regenerative effects of platelet-rich plasma - an already established treatment for various healing disorders (17). The effect of platelet EVs is also confirmed by researches stating that platelet EVs alone have the same or even better regenerative effect than platelet- and extracellular vesicle-rich plasma (14). However, efficient and reproducible EV isolation protocols are required to initiate and implement their use for diagnostic and therapeutic purposes (19).

3. Platelet- and extracellular vesicle-rich plasma (PEVRP)

3.1. Description and preparation procedures

The literature describes platelet-rich plasma (PRP) as part of blood plasma fraction with a platelet concentration higher than in peripheral blood (20). The process of PRP preparation also retains particles smaller than platelets in PRP, which is why PRP is also rich in EVs (Figure 1).

After centrifugation of the blood sample, platelets are mostly found in the "buffy coat", i.e. layer between hematocrit and plasma. Due to their heterogeneous sizes and shapes, they are also present in plasma and hematocrit, which should be taken into account in the

preparation of PEVRP. Because centrifugation does not enable complete separation of different cell types from one another, leukocytes and erythrocytes are always present in the PEVRP, while their amounts and ratios depend on the sample preparation. Many procedures were developed and described, but they are rarely reproducible (22).

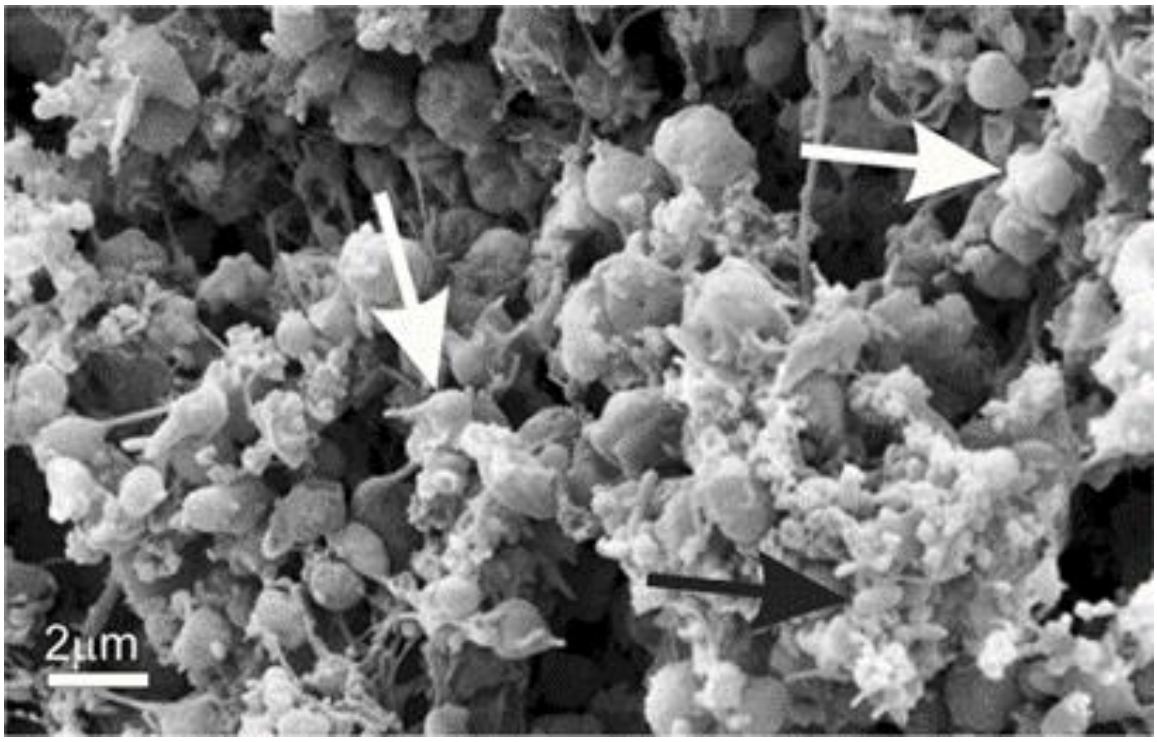

Figure 1: Electron microscopic image of activated platelets (white arrow) and extracellular vesicles (black arrow) in platelet-rich plasma. Adopted by Uršič et al. (21).

In general, procedures for PEVRP preparation by sequential centrifugation can be divided into blood plasma-based and buffy coat-based procedures, the blood plasma-based procedures being reported as the more effective ones (20).

The aim of the former is to separate blood plasma and platelets from leukocytes in the first centrifugation step. A leukocyte-poor preparation is formed at the expense of a lower platelet concentration because the buffy coat, which has a high platelet concentration, is discarded. The purpose of buffy coat-based procedures is to isolate "buffy coat", which results in PRP fraction with higher concentrations of platelets and leukocytes than obtained by other procedures.

The platelet concentration in PEVRP ideal for regenerative purposes has been determined in some studies and is approximately 5 times the average normal blood platelet count (i.e. baseline cocncentration: 150-350 x $10^3/\mu L$) (20). The preparation of PEVRP essentially depends on initial blood platelet count and on blood viscosity. As both of these parameters

are highly variable, it is complicated to prepare autologous preparations with the same concentrations for each patient. On the other hand, the concentrations in heterologous PEVRP are more easily adjusted. PEVRP may, according to DeLong et al. be divided into 4 types according to its platelet concentration; low, moderate, high and super. Low platelet concentration PEVRPs is considered when it is lower than the platelet concentration in blood, moderate when it is up to 4 times higher, high when it is 4-6 times higher and super when it exceeds 6 times baseline concentration in blood (20). PEVRP may contain different concentrations of leukocytes, depending on the preparation process. Buffy coat-based procedures yield a preparation with a high leukocyte concentration (higher than blood). Leukocytes in high concentrations, especially neutrophilic granulocytes, can inhibit healing, which is not preferred in the treatment of scars. In contrast, high leukocyte concentrations can have a beneficial effect in accelerating open wound healing and preventing infection. Blood plasma-based procedures result in PEVRP with a lower leukocyte concentration than that in blood (20).

Platelet activation in PEVRP can be triggered outside the body before administration (i.e. exogenously) or allowed to take place in the body after administration (i.e. endogenously). Endogenous activation is triggered by the type 1 collagen in tissues, which is a more effective activator than thrombin. The latter is used in exogenous activation. Exogenous activation creates a gel that results from the formation of a blood clot, allowing easier and more precise manual application for a more localized action on the damaged tissue. The gel is expected to have a longer duration of action, as it releases growth factors more gradually. On the other hand, endogenous activation supplies tissues with sufficient growth factors, while making their mechanism of action more physiological (20). In addition to the activation modes mentioned, platelets are also activated during centrifugation and later during the administration of PEVRP (20,23).

3.2. Applicability and adverse effects of platelet- and extracellular vesicle-rich plasma

Ten years after its first use in haematology in 1970, PEVRP started to be used in maxillofacial surgery and later in the treatment of musculoskeletal injuries in athletes (24). Its use has expanded to other fields of medicine for the treatment of various disorders (Table 1).

Adverse effects associated to the use of PEVRP and platelet gel are very rare, most of them are related to the process of drug administration or surgical procedure during which the preparations are used. The risk of transmission of infectious or malignant disease when applying autologous preparations is minimal. Heterologous preparation may provoke a rejection reaction (59).

Table 1: Examples of the use of platelet- and extracellular vesicle-rich plasma in different fields of medicine

FIELD	DISEASE OR INDICATION
orthopaedics and traumatology	lateral epicondylitis, knee osteoarthritis, achilles and patellar tendinopathy (21), anterior cruciate ligament reconstruction, knee arthroplasty, fracture healing (25)
plastic and cosmetic surgery	facial and neck rejuvenation (26,27), soft tissue reconstructions (28), facelift, reduction mammaplasty, abdominoplasty (29), breast reconstruction with lipofilling (30), alopecia (31,32)
wound care surgery	chronic wounds (25), venous leg ulcers, arterial ulcers, diabetic foot ulcers, traumatic wounds (33), pressure ulcers (34)
maxillofacial and oral surgery	odontogenic cysts of the mandible (35), tooth extraction wounds, periodontal disease (36), insertion of dental implants (36,37), maxillary sinus lift, bisphosphonate osteonecrosis of the mandible (35,36,38)
gynaecology	skin wounds after caesarean section and other procedures, cervical ectopia, vulvar dystrophy, cancer vulvectomy, vesicular, perianal, rectovaginal fistulae, urinary incontinence, premature ovarian failure, refractory endometrial thickening after artificial insemination, repeated vaginal infections, vaginal rejuvenation (30)
ophthalmology	corneal ulcers (39), dry eye syndrome after laser refractive surgery (40), Sjögren's syndrome (41)
cardiovascular surgery	prevention of sternotomy wounds infections (42)
otorhinolaryngology	acute eardrum perforation (43), reconstruction of the posterior wall of external auditory canal (44), auricular replantation (45), mastoid obliteration (46), chronic eardrum perforation (47–54), suprafacial parotidectomy (55,56), craniofacial reconstruction and frontal sinus obliteration (57), anterior cranial base fistulas (58).

4. Future applications of platelet- and extracellular vesicle-rich plasma

In the field of otorhinolaryngology, there are relatively few clinical studies on the use of PEVRP compared to the other areas, despite known disorders of healing in some diseases in this field. Chronic inflammation of the middle ear after surgical and standard conservative treatment presents a therapeutic challenge. Additionally, the use of PEVRP is promising in the treatment of pharyngocutaneous and orocutaneous fistulas (60), vocal cord diseases

(61,62) and facial nerve disorders (63). Based on ophthalmological experiences (40) the preparation could also be stored in appropriate containers and administered for the treatment of ear infections in the form of drops.

Before application, it is important to examine the blood cell concentrations in PEVRP as proposed by DeLong et al. (20). For further application of PEVRP in the sterile areas of human body it is also important to test PEVRP's sterility. Concentrations of EVs in PEVRP could be determined by flow-citometry, however the methods of isolaton and detection of EVs need improvements (61).

So far, we evaluated two different PEVRP preparation protocols during our preclinical research. Analyzes of blood cell concentrations using standard haematology tests and flow cytometry have resulted in our tailored protocol based on the protocol of Amable et al. (64). Flow citometry has also provided us concentrations of particles smaller than platelets, which also include extracellular vesicles. However, further sterility tests still need to be performed to validate the protocol of PEVRP preparation.

In the future, the PEVRP will be used for the treatment of chronic middle ear infections in the form of PEVRP-soaked ear wicks. PEVPR will be analyzed for the sterility, to use it for the treatment of sterile areas of the human body. In any case, adherence to the principles of asepsis and antisepsis in the preparation of PEVRP with experienced medical and biomedical staff is required. Knowing that that our product has an expectedly high concentration of extracellular vesicles and that EVs possess the main regenerative roles (14), our future goal is to produce isolates of extracellular vesicles from venous blood.

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Biomechanical analysis of the periacetabular hip osteotomy. A case report

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ABSTRACT

We determined biomechanical parameters (magnitude of the resultant hip force, its inclination with respect to the vertical, contact hip stress, position of the stress pole, size of the load bearing area, functional angle of the load bearing area and stress gradient index) in a single patient before and after the periacetabular osteotomy. The operation was performed due to hip dysplasia. We used two mathematical models to determine the resultant hip force in the one legged stance: the HIPSTRESS model (1) and the one-muscle model (2). Stress was calculated by using the HIPSTRESS model for contact stress (2). The geometrical parameters needed for calculation of biomechanical parameters were assessed from standard anteroposterior radiograms taken from the archive. Before operation, both hips were dysplastic according to the HIPSTRESS method criterion. We found considerable improvement of the contact stress distribution in the operated hip, however, contact hip stress became less favorable in the contralateral hip after the operation. After the operation, the operated hip became normal while at the contralateral side, the degree of the dysplasia has increased.

1. Introduction

The periacetabular osteotomy is supposed to reduce the disadvantageous distribution of pressure in the hip joint. It is a surgery designed to re-shape the hip joint for patients with hip dysplasia using a series of controlled breaks in the pelvic bone (3,4). Periacetabular osteotomy allows for extensive acetabular reorientation, including medial and lateral displacement of released elements (5). It was suggested that the Ganz osteotomy is indicated in patients suffering from constant pain related to the loading of the hip, provided that the range of motion allows correction without remarkable compromise of function (6). However, it requires highly skilled surgeon (7).

A study has shown that arthrosis is caused by hip dysplasia in nearly half of the treated hips (8). It is believed that hip dysplasia increases the joint contact pressures that cause degenerative changes and secondary arthrosis (9). Dysplasia of the hip is characterized by malpositioning of the proximal femur in a shallow acetabulum, providing deficient femoral head coverage (10). Early surgical treatment of hip dysplasia that preserves the joint is thought to prevent or defer the natural history of arthrosis (11).
The purpose of the periacetabular osteotomy is to improve hip and pelvis geometry as to alleviate unfavorably high contact stress in the hip joint and thereby slow down or prevent early degeneration of the hip cartilage (8,11). Namely, clinical studies have provided evidence in favor of the hypothesis that long lasting too high stress in the hip causes degeneration of the cartilage.
In order to describe the status of the hip and better understand the mechanisms of different pathologies in this region, various mathematical models were constructed. The mathematical model HIPSTRESS for calculation of the resultant hip force (1) was validated by several clinical studies (12-17) and has been proven useful in many different pathologies. HIPSTRESS model for stress (18,19) was used to study the effect of the periacetabular osteotomy (18). It was shown that hips with poor coverage of the femoral head by the acetabulum have unfavorable contact hip stress distribution (18). In these hips, stress attains its highest value at the edge of the acetabluar roof and falls off rapidly in the radial direction (18). A clinical study (20) showed that the theoretical predicitions were in agreement with the result of the biomechanical analysis.

Here we focus on a single subject. We calculated biomechanical parameters before the operation and after the operation to describe the change of the biomechanical status in a particular patient treated by periacetabular osteotomy for hip dysplasia.

2. Material and methods

We calculated biomechanical parameters (magnitude of the resultant hip force, its inclination with respect to the vertical, contact hip stress, position of the stress pole, size of the load bearing area, functional angle of the load bearing area and stress gradient index) by using two mathematical models (the acknowledged HIPSTRESS model and a simple model with one effective muscle force). Both models have previously been described in detail elsewhere (2,21,22).

Briefly, in both models for resultant hip force in the one-legged stance the body is imagined as composed of two segments: the loaded leg and the rest of the body. The equilibrium equations for forces and for torques of both segments are taken into account. The HIPSTRESS model includes nine effective muscles (gluteus minimus anterior, middle and

posterior, gluteus medius anterior, middle and posterior, tenskor fasciae latae, rectus femoris and piriformis) while the simple model includes only one effective muscle.

In the model for stress, the validity of the Hook's law implies that stress is proportional to strain within the cartilage. Consequently, stress is a cosine function of the space angle between a radius vector to the chosen point and a radius vector to the stress pole (a point at the articular sphere where stress is the highest),

$$p = p_0 \cos \gamma \quad , \qquad (1)$$

where p_0 is stress at the stress pole (P) and γ is the space angle. The position of the stress pole is given in the frontal plane by the inclination angle with respect to vertical (Θ) which is considered positive in the lateral direction from the vertical axis and negative in the medial direction from the vertical axis (22). The sum of the contact stresses over the contact surface is equal to the force **R**,

$$\mathbf{R} = \int p \, \mathbf{dA} \quad , \qquad (2)$$

where **dA** is the area element of the load bearing area. Integration is performed over the entire load bearing area which is defined as a part of the articular sphere bounded by two planes: a plane through the center of the femoral head which is inclined for ϑ_{CE} (center-edge angle) with respect to vertical and a plane which is inclined for $\pi/2$ from the position of the pole Θ (22).

The the load bearing area is a part of an articular sphere bounded by the cuts of the sphere with two planes through the center of the sphere: one inclined as to include the most lateral point of the acetabular roof and the other defined by the condition that the cosine function reaches the value 0. The solution of the component equations for the vector of resultant hip force as an integral of stress over the load bearing area yields a system of three equations which are solved for two angle coordinates of the stress pole (polar angle Θ and azymuth angle Φ) and the value of stress at the pole p_0. If the pole lies within the weight bearing area, the value of stress at the pole is also the maximal stress that is attained on the weight bearing area p_{max}. If the pole lies outside the load bearing area, the maximal stress it attained at the point of the load bearing area that is closest to the pole. All models use as an input data geometrical parameters of pelvis and proximal femora in order to estimate the positions of the muscle attachment points, half interhip distance x_{CM}, radius of the articular sphere r and coverage of the femoral head by the acetabulum ϑ_{CE} (Figure 1).

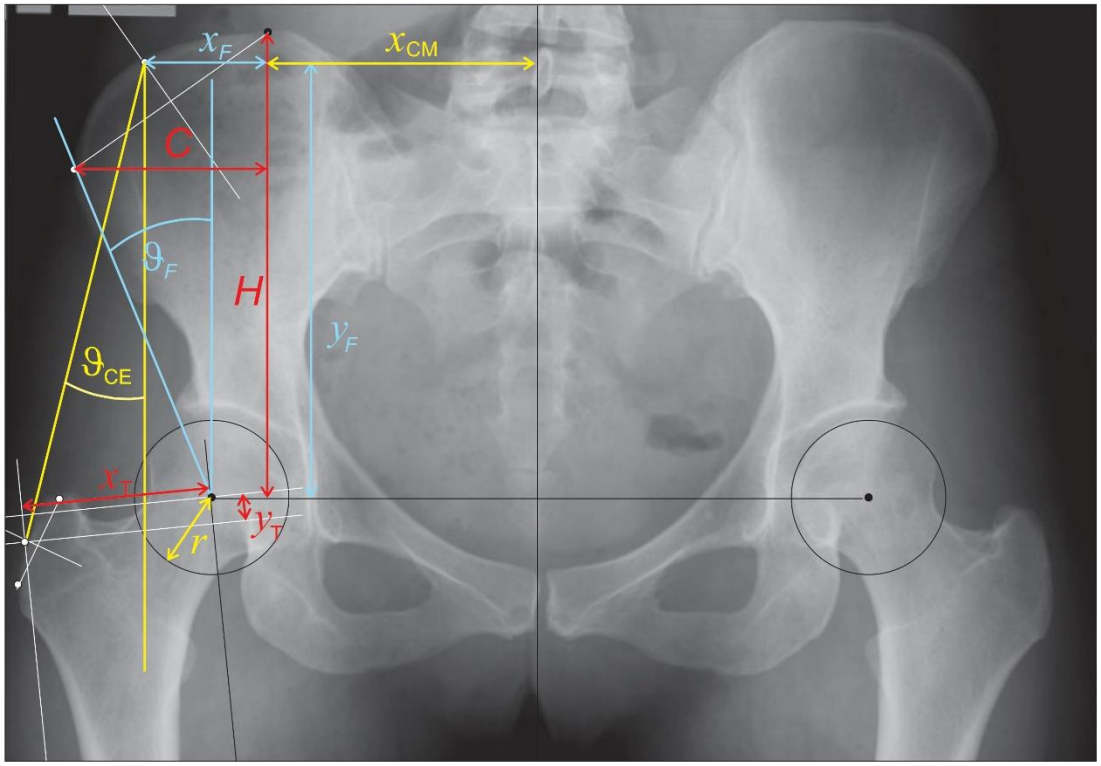

Figure 1. Geometrical parameters of the HIPSTRESS model for resultant hip force. Parameters used in the HIPSTRESS model for the resultant hip force are marked by red color, parameters used in the one-muscle model for the resultant hip force are markey by the blue color and parameters used to calculate stress are marked by yellow color.

Aside from the interhip distance, the geometrical parameters of the force models are specific to the model (Figure 1). As there were no data available on the body weight of the patient and there was also no unit length in the X ray picture, we determined dimensionless quantities: forces normalized to the body weight W_B (F/W_B and R/W_B, respectively), stresses and stress gradient index normalized with respect to the body weight and with respect to the radius of the articular sphere (p_0r^2/W_B, $p_{max}r^2/W_B$ and G_pr^3/W_B, respectively). These parameters expose the importance of hip and pelvis geometry.

X ray pictures of a patient that underwent a pelvic osteotomy were retrieved from the archive of the Department of the Orthopaedic Surgery, University Medical Centre Ljubljana, in an electronic form. The first picture was taken within a month before the surgery and the second was taken within a week after the surgery. The pictures were printed on a A4 paper and the geometrical parameters were measured manually by using a plastic ruler (estimating 5% of error).

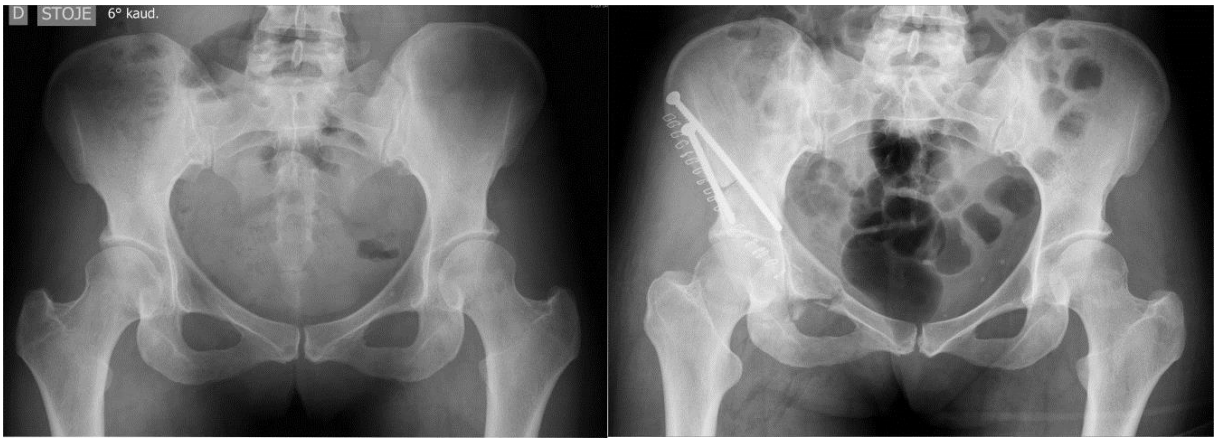

Figure 2. Standard anteroposterior radiograms of pelvis and both proximal femora of a patient that was treated by periacetabular osteotomy due to hip dysplasia. The image on the left was taken before the surgery and the image on the right was taken after the surgery.

3. Results

Scaling of the pictures (Fig. 2) was appropriate since the difference in measured femoral heads size was estimated 5% and no additional scaling was needed. We noticed a considerable (65%) increase of the centre-edge angle in the right hip after the operation (Table 1). The centre-edge angle before the operation was 12 degrees which indicates that the hip was dysplastic, while after the operation it was 27 degrees which can be considered as normal. As the increase of the centre-edge angle was intended by the surgeon it can be concluded that the operation was successful.

However, in the contralateral hip, the centre-edge angle has decreased after the operation, albeit this decrease was smaller (it decreased from 21 degrees to 15 degrees (33%)) (Table 2). Another notable change in both hips was a change of the parameter y_T (the vertical position of the greater trochanter) (Tables 1 and 2), however, change in this parameter does not crucially affect the biomechanical parameters (22).

A considerable decrease of normalized stress in the pole and of normalized peak stress on the load bearing surface took place after the operation in the operated hip (Table 3). Both models indicate more than 50% decrease in the respective parameters (Table 3). Concomitantly, the effective angle of the load bearing area has considerably increased (for more than 40%). Moreover, the sign of the stress gradient has become negative, as pertaining to normal hips (12). The biomechanical analysis clearly shows beneficial effect of the operation on the operated hip as stress is relieved and the load bearing area increased.

Table 1: Geometrical parameters of the hip that was operated, before and after the operation.

Operated hip	HIPSTRESS model			One-muscle model		
Parameter	Before operation	After operation	Difference (%)	Before operation	After operation	Difference (%)
x_{CM} (cm)	8.4	8.3	-1%	8.4	8.3	-1%
H (cm)	11.7	12.0	-3%			
C (cm)	3.7	3.5	-5%			
x_T (cm)	4.9	4.4	-10%			
y_T (cm)	0.9	0.5	-55%			
x_F (cm)				2.7	2.6	-4%
y_F (cm)				10.5	10.9	3%
r (cm)	1.9	1.8	-5%	1.9	1.8	-5%
ϑ_F (degrees)				10	8.5	-17%
ϑ_{CE} (degrees)	12	27	65%	12	27	65%

Table 2: Geometrical parameters of the contralateral hip before and after the operation.

Contralateral hip	HIPSTRESS model			One-muscle model		
Parameter	Before operation	After operation	Difference (%)	Before operation	After operation	Difference (%)
x_{CM} (cm)	8.4	8.3	-1	8.4	8.3	-1
H (cm)	12.1	12.0	-3			
C (cm)	3.1	3.7	18			
x_T (cm)	4.9	4.9	0			
y_T (cm)	1.0	1.2	18			
x_F (cm)				2.4	2.9	19
y_F (cm)				10.9	10.6	3
r (cm)	1.9	1.8	-5	1.9	1.8	-5
ϑ_F (degrees)				9	7	-25
ϑ_{CE} (degrees)	21	15	-33	21	15	-33

Table 3: Biomechanical parameters of the operated hip before and after the operation.

Operated hip	HIPSTRESS model			One-muscle model		
Parameter	Before operation	After operation	Difference (%)	Before operation	After operation	Difference (%)
F/W_B				1.6	1.7	6
ϑ_R (degrees)	9.5	7	-31	6.5	5.6	-15
R/W_B	2.7	2.8	4	2.4	2.6	8
Θ (degrees)	41.2	20	n.a.	42.8	22	n.a.
p_0r^2/W_B	4.12	1.93	-73	3.77	1.77	-72
$p_{max}r^2/W_B$	3.59	1.93	-55	3.56	1.77	-69
ϑ_f (degrees)	60.8	97	45	59.2	95	46
G_pr^3/W_B	2.013	-0.24	n.a.	1.93	-0.15	n.a.

Table 4: Biomechanical parameters of the contralateral hip before and after the operation.

Contralateral hip	HIPSTRESS model			One-muscle model		
Parameter	Before operation	After operation	Difference (%)	Before operation	After operation	Difference (%)
F/W_B				1.77	1.71	-3
ϑ_R (degrees)	10.0	9.0	-11.0	6.0	4.6	-26
R/W_B	2.7	2.6	-4	2.6	2.56	-2
Θ (degrees)	25.5	33.0	n.a.	33.2	33	n.a.
p_0r^2/W_B	2.40	2.96	21	2.4	2.6	8
$p_{max}r^2/W_B$	2.39	2.81	15	2.35	2.47	5
ϑ_f (degrees)	85.5	72	-15	78	72	-8
G_pr^3/W_B	0.19	0.92	n.a.	0.50	0.80	n.a.

In the contralateral hip, the increase of the centre edge angle caused an increase of the stress in the pole and on the load bearing area and concomittant decrease of the load bearing area (Table 4), however, to a smaller extent (not larger than about 20%).

Discussion

The biomechanical analysis has shown that after the operation, stress distribution has considerably improved in the operated hip, however, it has become less favorable in the contralateral hip. As the parameter that is the most important in dysplastic hips is the centre edge angle, an error in determination of the centre edge angle could partly contribute to the outcome of the analysis. Positioning the image (determining horizontal and vertical axes) is crucial in determination of the geometrical parameters.

Performing the analysis with two models has shown that their predictions were qualitatively equivalent. An advantage of the one-muscle model in the form as used in this work is that it is independent of the size of the picture. As we did not have an unit length present in the pictures it was favorable for us to use the model which in not burdened with the size effect. The HIPSTRESS model for the resultant hip force however, includes reference coordinates of the muscle attachment points in 3 dimensions. Therefore, in the cases when the actual sizes are not known, this may present a bias.
The periacetabular osteotomy is used in dysplastic hips to increase the load-bearing area of the hip and to prevent osteoarthritis (11). Many articles showed that the periacetabular osteotomy is very successful to prevent arthrosis. But some of them indicate considerable arthrosis progression after the surgery in the operated hip (9, 20). Our results however indicate that the contralateral hip should also be monitored as the surgery affects both hips.

Conclusions

Our findings indicate that determination of biomechanical parameters is valuable for monitoring the stress distribution and the load-bearing area in the hip joint of patients before and after periacetabular osteotomy because it may show us the disguised malformations of both hips hip before they could be detected by deformations on X ray images. We will research more on the matter in our future work.

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SYNTHESIS OF A WATER-SOLUBLE FLUORESCENT ACTIVE COMPOUND AND ITS POTENTIAL USE FOR LABELING OF CANCEROUS UROTHELIAL BLADDER CELLS

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Abstract

Bladder cancer is the ninth most common cancerous disease in the world, whose incidence in Slovenia is expected to increase by 45% in men and 105% in women by the year 2020. Therefore, new methods of early diagnosis of bladder cancer are essential. Fluorescent dyes represent an appropriate tool because they emit cold light and may be used for labelling the cells in tumours.

Our research was aimed at differentiating between healthy (normal) and cancerous bladder urothelial cells. We developed a water-soluble fluorescence derivative of fluorescein, sodium disulfonate (SUF), which is an organic compound and emits intense fluorescence. Next, we tested its influence on the viability of normal and cancerous urothelial cells and tried to distinguish between the normal and cancerous urothelial cells using a fluorescence microscope. We analysed if SUF can be used in cancer cell diagnostic and examined its potential for targeted therapy as an alternative to the currently used fluorescent dyes, which are more expensive and harder to obtain, and with this approach make such research more accessible.

The results of the study have shown that SUF is not toxic to urothelial cells and that it may be used to distinguish between healthy and cancerous urothelial cells, based on the way of labelling. SUF was proven to be a useful fluorescent marker. Importantly, its structure also allows other functional groups or anti-cancerous substances to be added through advanced synthesis strategies.

1. Introduction

Bladder cancer is the ninth most common cancer in the world. Its detection is difficult, as the clinical symptoms can be very similar to other not so severe bladder diseases. Often it is detected too late, and can consequently prove fatal. In addition to the latter, bladder cancer cells appear multifocally, i.e. in many separate spots in the bladder, so it is difficult to completely remove them by operation. The described fact indicates a high probability of a further recurrence of the disease (1).

In order to improve the detection and treatment of bladder cancer, different diagnostic methods have been developed. A very interesting and important direction of research is based on fluorescent active dyes that emit cold light (light emitted at low temperatures from a source that is not incandescent). These dyes can be used for labeling cells. The applicative potential of this kind of labeling material has already been described in the literature (2-5). Such labeling is intended for medical applications, in particular in monitoring the development of various degenerative diseases (6-8).

One of the most important and widely used dyes is the green fluorescent protein (GFP). In the field of cell biology research, GFP has an advantage, since it is extremely small, water-soluble, emits a large amount of light and is not toxic (9). However, large amount of this protein is inaccessible due to a high price, which indicates development of new forms of fluorescent dyes. The idea in this field is to prepare an active substance (probe), which can be quickly elaborated in a simple and low cost procedure following synthetic preparation strategies, and will have equivalent or improved properties comparing to the previous preparations of GFP.

The aim of this study was therefore to prepare the fluorescence probe with the above properties and to study some of its chemical parameters (characterization data and solubility data). The prepared ingredients were also tested for toxicity and usefulness in the field of cell biology, in particular for detection of healthy and cancerous urothelial cells of the urinary bladder.

2. The urinary bladder and bladder cancer

The urinary bladder is a part of the urinary system. Its task in the body is temporary storage of urine, which is constantly produced by the kidneys and enters the bladder through the ureters (10).

The organ has the shape of a hollow ball, which is divided into three parts: the central part (*Corpus vesicae*), the upper vault (*Apex vesicae*) and the lower vault (*Fundus vesicae*). The ureter enters the vault posteriorly and the urethra emerge from it anteriorly (10). The

bladder consists of four distinct layers: urothelium, submucosa, detrusor muscle and adventitia (11).

In the urinary bladder, urea is only temporarily stored until being conveniently excreted. Since its composition should not change during rest, the bladder has a specifically built epithelium – urothelium. Urothelium covers most of the lower part of the urinary tract, i.e. proximal urethra, urinary bladder, ureter and kidney bladder (12). Its task is to form a blood-urine barrier that prevents the returning of toxins, water, ammonia and ions from the urine into the blood (13).

Bladder cancer is a common malignant disease. Due to differences in the urinary system in men and women, the specificity of the disease is highly dependent on the gender of the patient (14). In the period from 2010 and 2014, bladder cancer was the 9th most common cancer in Slovenia in men, while in women, its detection was less often (15th most common). Currently, according to the data from Cancer Registry of the Republic of Slovenia, bladder cancer is the 8th most common cancer in men and the 13th most common when both genders are considered. Indicators of cancer burden (especially its incidence) should be compared carefully in each country since registration of bladder cancer is not uniform worldwide. Some cases include in situ and non-invasive papillary carcinoma (the Ta and Tis stages). The Register of Cancer of the Republic of Slovenia (RRRS) for all years follows a rule that T1-T4 bladder cancers are included in the incidence of cancer, especially in situ cases with non-invasive papillary carcinoma (1).

3. Urothelium

The urothelium is the most impermeable epithelium in the human body and surrounds the inside of the renal pelvis, ureter, urinary bladder and urethra. It consists of several layers of cells. They are interconnected by tight junctions that prevent the transfer of molecules into the tissue below and thus provide the blood-urine barrier. In the apical plasma membrane of superficial urothelial cells there are transmembrane proteins called uroplakins, which form urothelial plaques (15-17).

Urothelium functions as a blood-urine barrier by means of three mechanisms. 1) prevention of passive paracellular diffusion by tight intercellular contacts, 2) by presenting a specialized apical plasma of urothelial superficial cells built by uroplakin proteins, 3) by very low exocytosis/endocytosis activity, thereby reducing transcellular transport (18-21). The degree of permeability of urothelial cells depends on their degree of differentiation. Poorly differentiated cells have a higher endocytosis/exocytosis activity than terminally differentiated urothelial cells (19). Despite extremely low permeability, some substances pass through the urothelium, since they must be connected to their environment for the

proper functioning of the cells and their survival (viability). Substances can reach individual cells via two pathways, the paracellular pathway (between cells) and the transcellular pathway (through cells) (19).

4. Objective, purpose and hypothesis

The aim of this study was to synthesize the fluorescent active substance SUF (sodium 3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-4',5'-disulfonate (based on reference (22)) and test its applicative use in medicine. The purpose of the work is to find new methods and reagents, which could replace expensive and sophisticated procedures for obtaining fluorescent dyes in the field of cell biology. According to the innovative synthetic approach, SUF is suitable for preparation on a larger scale, since the preparation path is quick, simple and highly efficient (high yield). We believe that the proposed method could improve efficiency in tumor detection and advance cell biology in general.

We hypothesised that with the help of the compound SUF, the cancerous urothelial cells could be distinguished from the healthy (normal) ones by differences in the fluorescence intensity and/or distribution.

Research work with this approach requires knowledge of *in vitro* cell culture techniques, the use of microscopy techniques with an inverted and fluorescence microscope and work in a chemical laboratory.

5. Material and Methods

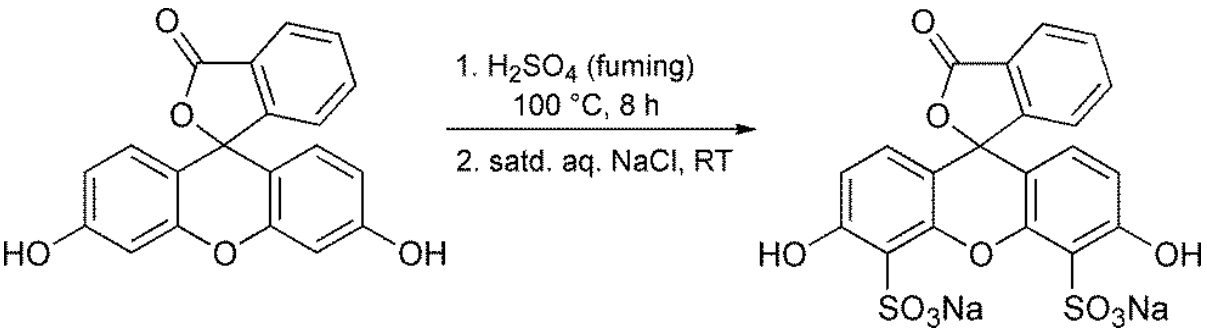

Figure 1: Scheme of SUF synthesis.

5.1. Synthesis and characterisation of fluorescence activity compound, sodium 3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-4',5'-disulfonate (SUF)

In a 250 mL flask 10.00 g of fluorescein (15.09 mmol, 1 eq) and 20 mL of oleum were mixed under an inert atmosphere. The reaction mixture was heated in an oil bath at 100° C for 8 h. Then, the resulting reaction mixture was cooled to the room temperature and stirred overnight at room temperature. The mixture was poured into a 140 mL mixture of ice/water in small portions. 200 mL of saturated NaCl solution was added to the mixture. The resulting product was then filtered and washed with ice-cold water (3 × 50 mL). The crude product was recrystallized (purificated) from water (90 mL). Further elaboration by filtration, washing and drying in an evacuated chamber gives 15.34 g of orange-brown product (95.0 % yield).

The compound was characterized by analytical methods and its chemical parameters and characteristics were determined. Knowing these properties improves understanding of the function of the molecule, its binding ability and uses. Preliminary tests of solubility in polar Fluorescence dye SUF was caracterised with fluorescence wavelength and intensity, ^{1}H and ^{13}C-NMR, UV/Vis and TLC (R_f). In addition, the fluorescence analysis under UV light of different wavelengths (366 and 254 nm) was performed. We found that SUF dissolve in both, polar and non-polar liquids (therefore it can be dissolved in an aqueous medium and can pass through the lipid bilayer) and better fluoresces at lower concentrations (0.1 mol/L) at UV light of 366 nm.

We measured: ^{1}H-NMR (300 MHz, DMSO-d_6): δ 12.17 (s, 2H), 8.00 (d, J = 7.5 Hz, 1H), 7.89 – 7.78(m, 1H), 7.73 (t, J = 7.4 Hz, 1H), 7.32 (d, J = 7.6 Hz, 1H), 6.62 (app q, J = 8.8 Hz, 4H). ^{13}C-NMR (300 MHz, DMSO-d_6): δ 168.53, 156.72, 152.22, 149.13, 135.88, 130.40, 130.18, 126.44, 124.90, 124.16, 116.86, 114.51, 109.28. λ_{max} (UV/Vis, 10^{-4} M in EtOH) = 485 nm, fluorescence at 504 nm and TLC (CH_2Cl_2 / MeOH, 9:1), R_f = 0.72. Here, ^{1}H-NMR (300 MHz, DMSO-d_6) is proton nuclear magnetic resonance, ^{13}C-NMR (300 MHz, DMSO-d_6) is carbon NMR (nuclear magnetic resonance), measured at 300 MHz in deuterated solvent dimethyl sulfoxide (DMSO), δ is chemical shift (for the resonance) of nucleus of element X (positive when the sample resonates to high frequency of the reference), J is indirect coupling tensor, s is singled, d is doublet, t is triplet, appq is apparent quartet, m is multiplet, λ is wavelength, TLC is thin layer chromatography, R_f is retardation factor which is equal to the distance migrated over the total distance covered by the solvent, CH_2Cl_2 is dichlorometane and EtOH is Ethanol.

5.2. Biological applications

The biological part of the study consists of the preparation of culture media for the cell cultures, seeding, and maintaining cell cultures in appropriate *in vitro* conditions as

described previously (23-25). In our case, the research involved testing the viability of urothelial cells in a 0.1 mol/L SUF solution.

Cell cultures of normal porcine urothelial (NPU) cells were established from normal porcine urinary bladders. The experiments were approved by the Veterinary Administration of the Slovenian Ministry of Agriculture and Forestry in compliance with the Animal Health Protection Act and the Instructions for Granting Permits for Animal Experimentation for Scientific Purposes. Urinary bladders were cut into 5 cm long and 2 cm wide strips and urothelial cells were gently scraped from the urothelium with a scalpel and collected in UroM medium as described previously (26,27). After collection of urothelial cells, the cells were centrifuged at 200 *× g* for 5 min, washed in UroM medium and filtered through a 40 µm Cell strainer (BD FalconTM, BD Biosciences, New Jersey, USA) to obtain a single-cell suspension. Primary and subsequent subcultures were plated with a seeding density of 2×10^5 cells/cm^2. They were grown in UroM with 0.9 mM calcium and 2.5 % fetal bovine serum (FBS) (Gibco) until confluence and were then transferred to UroM with 2.7 mM calcium and without FBS. For experiments in this study, urothelial cells from the V-XII passages were used. To obtain highly differentiated urothelial cells, the cells were cultured for 2 months (15).

T24 cell line originated from human invasive urothelial neoplasm (ATTC, Manassas, VA, USA). They were cultured in Advanced-Dulbecco's modified essential medium and medium F12 (1:1), 5 % FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin as described in 27 28. The seeding densities of cancer T24 urothelial cells were 5×10^4 cells/cm^2. All cells were grown on plastic dishes at 37 °C in a humidified atmosphere and 5 % CO_2.

5.3. Cell viability

Due to the multilayer nature of urothelial models, Trypan blue viability assay was used to determine the viability of urothelial cells after SUF exposure. Cells were grown in plastic dishes and then incubated with 100 µg/mL of SUF for a week. After one week of incubation, cells were washed to remove non-bound SUF, trypsinized until all cells were detached and immediately stained with Trypan blue dye (following manufacturer's instructions), which labels only dead cells. Live and dead cells were then counted manually under inverted light microscope (Leica DM IL).

The percentage of viable cells (% Viability) in a given sample was determined as the ratio between the number of viable cells in the sample (NS) and the number of all cells in the non-treated control (*N0*) for each cell model: *% Viability = 100 × (NS/N0)*. Two independent experiments were conducted, each in three technical repeats for each urothelial model.

5.4. Fluorescence microscopy

We established normal and cancer urothelial cell models in 4-well chamber slides and treated them with 100 µg/mL of SUF dye for 2 days. Then, the medium was removed with a vacuum pump and cells were washed with fresh medium several times in order to rinse all unbound SUF. After washing, a 4 % formaldehyde fixative was added and left therein for 15 min at room temperature. After 2 minutes, cells were washed with PBS solution for 10 times. Vectashield (a fluorescence-preserving compound) and DAPI (for staining DNA) were added. The samples were then observed under fluorescence microscope (Nikon Eclipse TE 300).

6. Results and discussion

6.1. Chemical part

Synthesis of the fluorescence active substance SUF was carried out as expected, with a high yield of the prepared product. The prepared product was successfully assessed by the characterization parameters that indicated a mechanism of its formation. With preliminary solubility tests of the fluorescent dye SUF in nutrient (culture) media, it has been shown that the SUF dye is very soluble in all the culture media used. Since nutrients are prepared on a water basis (polar agent), it has been shown that the active substance is very soluble in polar solvent systems. This kind of property was also used to measure the spectroscopic and separation properties of the active ingredient.
If we examine the entire structure from the point of view of substituents bound to the basic fluorescein skeleton, polar and nonpolar structural fragments are observed. It was assumed that the SUF could also dissolve in non-polar solvents, so hexane was used for the model solvent. An attempt to dissolve the SUF in hexane has shown that SUF is also partially soluble in non-polar solvents. From this, we concluded below that it will be able to pass by diffusion through a nonpolar phospholipid bilayer of the cell membrane.
An experiment with UV light has shown that SUF in polar solvents intensively fluoresces, while in nonpolar ones, it does not fluoresce. A preliminary study showed that the supplements in culture media are fluoresce better at lower concentrations (100 µg/mL) than at higher doses, which is of key importance for further studies on cell cultures. It is more difficult for cells to withstand higher levels of dye concentration (29).
The fluorescence of the SUF in a culture medium was qualitatively tested by excitation light of wavelengths 366 and 254 nm. It was found that under the light of the wavelength of 254 nm the fluorescence strength was negligible (barely noticeable). Under the light of the wavelength of 366 nm, however, the solution fluoresced the most. The culture medium did not dampen the fluorescence, the latter being more dependent on the wavelength of UV light.

6.2. Biological part

Viability

Cells were counted using a hemocytometer and their viability and number in each sample were calculated as described in the Methods. The obtained results are presented in the form of tables and graphs. Also we calculated averages of viability parameters.

We repeated counting and analysis sixty times, as NPU cells grow slowly and are more demanding for cultivation. Due to relatively small number of cells, the deviation of the results can be very large, which in our case was solved by calculating the averages of several repetitions.

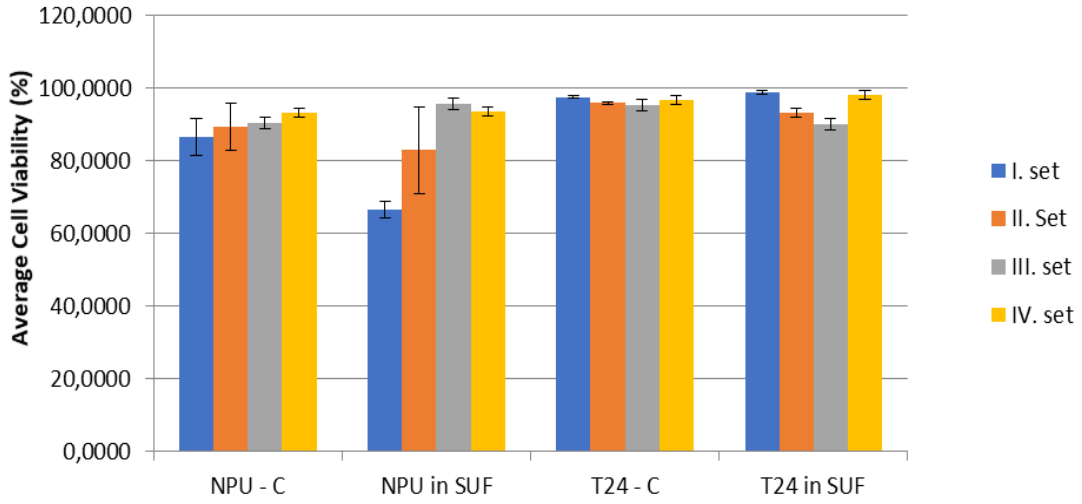

Figure 1. Comparison of the viability of SUF-treated normal cells (NPU in SUF), SUF-treated cancer cells (T24 in SUF), control normal cells (NPU-C) and control cancer cells (T24-C) . Each set represents one independent experiment.

Table 1: Average viability of NPU cells. From the average values of all four cell viability analyses, it was found that the SUF dye with a concentration of 100 µg/mL did not affect the viability of urothelial cells and could be suitable for use in further *in vitro* and *in vivo* studies.

Set	Average viability			
	NPU K	NPU and SUF	T24 K	T24 K and SUF
I	86.6667	66.6667	97.6667	98.8667
II	89.4600	83.0000	96.0000	93.4333
III	90.5100	95.8767	95.3567	90.0533
IV	93.3200	93.5717	96.9700	98.1483

5.3. Microscope visualisation

We observed samples under the fluorescence microscope. In the first sample, T24 p26 cancer cells with a setting density of 5×10^4 cells/cm^2 were fixed. In the second sample, the NPU P5U12a (the code represent the biological sample P5 and 12 represents the passage) cells with a density 2×10^5 cells/cm^2 and the T24 33p cells with a density of 5×10^4 cells/cm^2 were fixed.

The samples were examined and photographed under the green and blue light, and the subsequent photographs of the same field of vision were merged. The analysis of the photographs gave us an insight into the functioning and binding of the fluorescent dye SUF to the urothelial cells.

We observed, in particular, the sites that were more fluorescing, meaning that more fluorescent dye had passed into the cells. The source of this effect could be damaged or dead cells (17). To find the decisive answer, we determined also the state of the nuclei. Analysis of the images (Figure 2) revealed that a certain number of nuclei of NPU and T24 cells were fragmented (Figure 2A, yellow thin arrows). SUF dyes accumulated in the vicinity of the damaged nuclei, resulting in a more intense fluorescence in these areas (Figure 2B, red thin arrow). Further, we assumed that the fluorescent dye accumulated in the intracellular membrane compartments (Figure 2B, red thin arrows) or in the plasma membrane (Figure 2C and E, white arrows), which was perceived as a stronger fluorescence. We can not say with certainty that the fragmentation of the nuclei was due to the accumulation of the SUF dye, as the results of the cell viability analysis contradict the described ones. In addition to this, many cells with intact nucleus exhibited SUF in their internal membrane compartments.

In the sample, there were differences in the degree of differentiation between cells. We found that the SUF staining intensity was influenced by the degree of cell differentiation; less differentiated cells were more strongly labelled since they were not yet able to perform urothelial barrier tasks.

It is also evident from the fluorescence images that the labelling with SUF dye is non-specific. For comparison; the DAPI dye selectively binds to DNA molecules and consequently labels only the nucleus of the cell. The SUF dye is located around the nucleus and differs strongly in the fluorescence labelling of the intracellular membrane compartments in cancer cells.

The last analysis included also differentiated normal urothelial cells. The analysis was carried out in order to show that *in vitro* the distinction between differentiated and cancerous cells can be achieved.

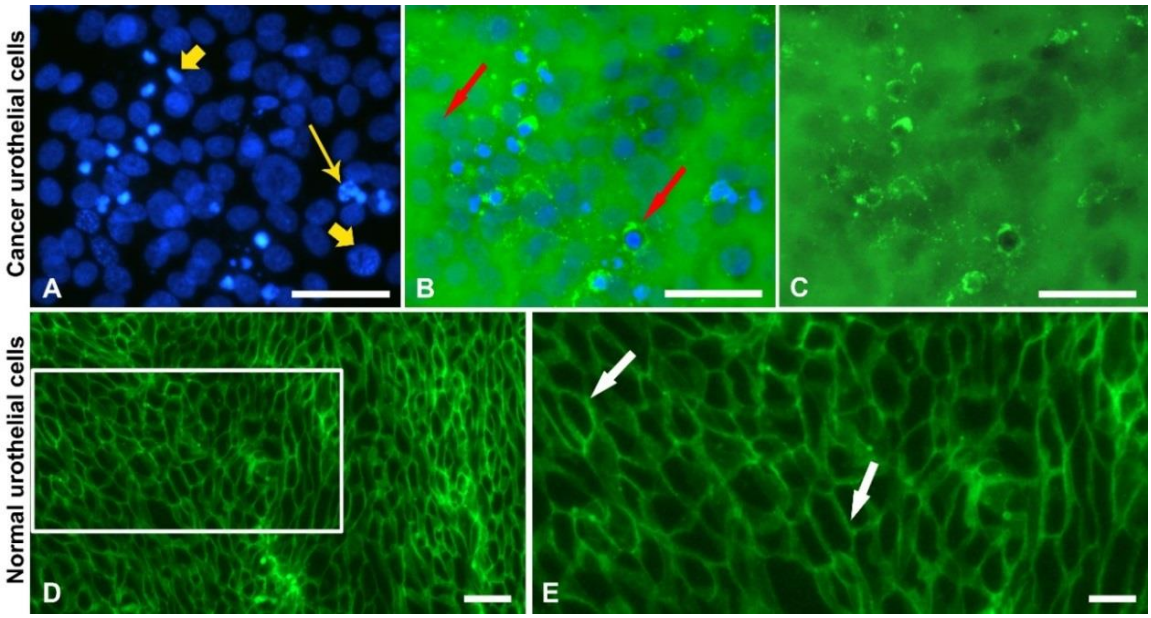

Figure 2: Cancer and normal urothelial cells labelled with SUF. A: cells labelled with DAPI (blue nuclei), fragmented nuclei (yellow thin arrows; chromosomes of cells in mitosis (yellow thick arrows) B: cells labelled with DAPI (blue nuclei) and SUF (green). Cancer urothelial cells with fragmented nuclei (yellow thin arrows) and membrane compartments with the compound SUF (red arrows), a cancer urothelial cell with a normal nucleus and diffuse labeling with SUF (orange thin arrow). C) The same field of view as in A and B, however only SUF is seen. D and E: Differentiated normal urothelial cells labeled with SUF (green) In E, the area inside the white box is enlarged. White arrows show SUF labelling of the apico-lateral part of the cells. Scale bars 50 µm in A, B, C and E, and 100 µm in D.

The results showed that SUF-labelled differentiated normal urothelial cells appear different from SUF-labelled cancer cells. Differentiated normal urothelial cells have special proteins on their membranes – uroplakins that act as a transport barrier. Because of this, SUF almost never entered the cells, but a lot of it accumulated in the areas between the apical and the basolateral cell membranes, resulting in the contrast in fluorescence light emitted by the cell inside and its outside. Since SUF does not fluoresce in non-polar media, we have ruled out the possibility of its binding to the lateral cell membranes. In cancer cells we assumed that the dye passes into their interior by endocytosis and induces labelling of endocytotic compartments. From the above we concluded that in *in vitro* conditions it is possible to distinguish between cancerous and normal urothelial cells.

Conclusions

The main aim of this research work was i) to synthetize the flurescein derivative, which is chemically referred to as sodium 3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene] -4',5'-disulfonate – SUF, and ii) to test SUF on normal urinary bladder cells and cancerous urothelial cells.

The compound demonstrated application potential in the field of medical applications. Based on its chemical structure, it was indicated that it is not toxic and that it can be used as a cellular marker. We showed experimentally that the active ingredient did not harm the cells and could show a difference between the normal and cancerous urothelial cells.

Moreover, from the viability analyses it can be seen that the SUF does not affect urothelial cells growth and viability. The results of viability analyses showed no differences between controls (cells grown without SUF) and samples (cells grown in the presence of SUF). In some cases, cell viability was even greater in the SUF dye medium. These results were also supported by the examination of cells with a fluorescence microscope, where cells were detected in different stages of cell division, which confirms that SUF does not inhibit cell division. Using the results obtained, we expect that the compound SUF is not cytotoxic. But further studies are needed to give a decisive answer.

Acknowledgements

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THE IMPACT OF DIFFERENT TIO_2 PARTICLES ON THE ACTIVITY OF ACETYLCHOLINESTERASE

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Abstract

Over the last decades, the production of nanoparticles (NPs) has been rapidly growing on the global scale. The data on biological activity of NPs is crucial in evaluating their safe use and in the development of new products containing NPs. Different tests are available for the purposes of determining interactions between NPs and biological systems. In this study, a modified Ellman's method, adapted to work with microtiter plate, was used. This study aims to evaluate the biological reactivity of 10 different titanium dioxide (TiO_2) particles with different primary sizes, crystallinity and different coatings. Biological reactivity was assessed by testing their adsorption to acetylcholinesterase (AChE) and to inhibit its activity. It was assumed that the tested particles inhibit AChE depending on physiochemical properties of particles, with crucial importance of their crystal structure. It was also assumed that a higher NP adsorption to AChE and a formation of so-called corona have no direct correlation to stronger AChE inhibition. TiO_2 particles sized between 5 and 100 nm showed the highest rate of inhibition and adsorption to AChE, whereas the particles with size between 100 and 500 nm showed a lower rate of both. In our study the crystallinity of TiO_2 particles did not have a significant effect on their ability to inhibit and adsorb AChE. Observed NP inhibition and adsorption to AChE was dependant on NP concentration. We observed higher NP effect on inhibition than on adsorption.

1. Introduction

AChE belong to a group of hydrolases which split neurotransmitter acetylcholine (ACh) and terminates cholinergic neurotransmission (1). AChE is mainly found in cholinergic neurons (2), but it can also be found on the erythrocyte membranes (3). It has an important role in proper functioning of central and peripheral nervous system (4). There are two different types of AChE inhibitors – reversible and irreversible inhibitors. Reversible inhibitors are mainly used as drugs in Alzheimer's disease treatment (5).

Measurement of AChE inhibition can serve us as a biological marker in human biomonitoring: it is easy to evaluate; the method is sensitive and the results indicate possible adverse effects on human health (4). Specific inhibitors for AChE are organophosphates and carbamates (6).

In the last decades, the TiO_2 NPs production has been rapidly growing, which leads to increased exposure. Annual production of TiO_2 NPs is up to 10.000 tonnes (7). TiO_2 is white, thermally stable and non-flammable inorganic substance with poor water-solubility. (8). There are three known crystal structures of TiO_2: anatase, rutile and brookite, most commonly used are anatase and rutile (9). In primary production the average size of TiO_2 NPs is less than 100 nm. It is used as UV absorber and photo-catalyst (10). TiO_2 NPs are used in sunscreens, due to their good capability of UVA- and UVB-light absorption (11). Besides TiO_2 NPs, macro TiO_2 with size greater than 100 nm is also commonly used in industry. TiO_2 is most frequently used in the form of pigment particles and food supplements (E 171) (12).

NPs have tendency to interact with biomolecules and form so called protein corona (13). Corona is also formed when NPs come in interaction with AChE, which can be used as a model system (14). By measuring and evaluating the effect of NPs on adsorption and inhibition properties of AChE, information about biological reactivity of NPs is provided. Inhibition and adsorption of AChE are related to NPs physio-chemical properties: form, size, crystal structure and properties of NPs coating (15).

2. Methods

2.1. Activity of AChE from electric eel (*Electrophorus electricus*)

Activity of AChE from electric eel was measured by the Ellman's method adapted for microtiter plates. When AChE hydrolyses substrate acetylthiocholine chloride (AChCl), thiocholine and acetate are produced. Ellman's reagent contains colour indicator DTNB (5,5'dithio-bis-(2-nitrobenzoic acid)) which is reduced by AChCl and forms 5-thio-2-

nitrobenzoic acid. Colour intensity is measured at 405 nm and relates to the activity of AChE. Higher colour intensity indicates higher AChE activity.
When assessing inhibition; the total AChE (AChE bound to NPs and unbound AChE) activity is measured. In this case, AChCl and Ellman's reagent are added prior to the centrifugation - before AChE-particle complex is separated. To assess adsorption, the activity of unbound AChE is measured and deducted from total AChE activity.

2.2. Inhibition experiments

The ingredients: 165 μL AChE (0,06 U/mL; dissolved in 100 mM potassium phosphate buffer (P-P buffer), pH 8.0) and 33 μL of particle suspension prepared in the same buffer (concentrations of 60, 600, 6.000 and 10.000 μg/mL)* /or 33 μL of 12 μM eserine prepared in same buffer, used as positive control OR and 33 μL of 100 mM P-P buffer, used as negative control were gently mixed and first incubated for 10 minutes at room temperature (~ 20 °C). Then, 330 μL of Ellman's reagent and 165 μL of 2 mM AChCl were added to the mixture. The mixture was gently mixed and incubated for additional 5 minutes. AChE-particle complexes were separated by centrifugation (5 minutes at 14500 × g and 20 °C).
3 x 200 μL of each sample's supernatant were transfered into a single well of the microtitter plate. Absorbance at 405 nm was measured every minute for 20 minutes, using microplate reader Infinite 200 PRO, Tecan.

2.3. Adsorption experiments

165 μL AChE (dissolved in 100 mM P-P buffer, pH 8.0; 0,06 U/ml) and

- 33 μL of particle suspension prepared in same buffer (concentrations of 60, 600, 6.000 and 10.000 μg/mL)* OR
- 33 μL of 12 μM eserine prepared in same buffer, used as positive control OR
- 33 μL of 100 mM P-P buffer, used as negative control

was gently mixed and incubated for 2 minutes at room temperature (~ 20 °C) followed by centrifugation for 4 minutes at 20 °C at 14.500 × g. Three times 50 μL/well of each sample's supernatant with unbound AChE was transferred onto the microtiter plate. 100 μL/well of Ellman's reagent and 50 μL/well of 2 mM AChCl was added to the supernatant. After adding reagents, absorbance at 405 nm was measured every minute for 20 minutes.
For every sample with NPs, blanks were prepared to evaluate interference of NPs with the reaction product. 165 μL of AChE was replaced with 165 μL of P-P buffer. The absorbance at 405 nm was read to verify the changes in colour. We observed no interference of NPs with the assay. Final concentrations of particles in the sample were 10, 100, 1.000 and 1.666,7 μg/mL since there was a 6-fold dilution in the well.

Table 1: Evaluation of average rate of inhibition and adsorption of acetylcholinesterase (AChE) for analyzed TiO_2 particles

Particle label	Particle size (nm)	Purity (%)	Coating	Crystal structure		Inhibition of AChE at various concentrations of the particles				Adsorption of AChE at various concentrations of the particles			
				Anatase	Rutile	µg/mL							
						10	100	1000	1667	10	100	1000	1667
G2–5	5–7	>80	Untreated	Contain	Do not contain	+				+	+	+	+
G6–3	10	>70	Al_2O_3, stearic acid	Do not contain	Contain		+	+++	++++		+	++	++
G8–2	20	>80	SiO_2	Do not contain	Contain			+	++			+	++
G10–4	15	>90	Tungsten-W	Contain	Do not contain		+	+++	++++			++	+++
G1–1	<100	>99	Untreated	85 %	15 %				+				
G4–19	>100	>99	Al_2O_3	Do not contain	Contain								
G7–5	~170	>80	Al_2O_3, SiO_2	Do not contain	Contain								
G9–5	~170	>80	Al_2O_3, P_2O_5	Do not contain	Contain								
G3–1	300	>99	Untreated	Do not contain	Contain								
G5–4	500	>90	Al_2O_3, ZrO_2	Do not contain	Contain								

Without +: Inhibition/adsorption < 5 %; +: Inhibition/adsorption between 5 and 20 %; ++: Inhibition/adsorption between 20.1 and 35 %; +++: Inhibition/adsorption between 35.1 and 50 %; ++++: Inhibition/adsorption > 50 %

3. Results and Discussion

In our study, we assessed biological reactivity of different TiO_2 particle suspensions. Our results showed that impact on inhibition and adsorption of AChE is dependent on TiO_2 particles concentration. It was previously reported that bigger particles interact less strongly with enzyme (16). Also our results indicate that particle size has important effect on AChE activity. NPs sized between 5 and 100 nm had higher impact on AChE due to their greater surface area, in comparison to bigger particles. Coating of particles can have a significant impact on how the particles react with biological systems. The results showed different impact according to different particle coatings and coating properties. However, we did not find significant differences between TiO_2 particles with different crystallinity.
Inhibition and adsorption rates of TiO_2 particles are shown in Table 1.

Conclusions

Due to an increased production and use of NPs in the last decades, it is important to know possible interactions between NPs and biological systems. For that purpose, analyses of NP impact on inhibition and adsorption of AChE should be implemented.
Results of AChE inhibition and adsorption show complex relationship between particle properties and their interactions with enzymes. Particle size and their coating have the greatest impact on inhibition and adsorption of AChE. Concentration of particles is also important. Higher concentration had a greater impact on inhibition and adsorption in most cases.
Knowledge about the impact of NPs on AChE can be improved with further research work. Bovine serum albumin could be used for coating of particles. It aims to reduce biological reactivity of NPs (17). Further work with human cell lines is also of interest, as it can help us to better understand the response of the human body to NPs.

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CAN SOME OF THE MODERN WORLD PROBLEMS CAUSED BY HUMAN BE SOLVED BY MICROORGANISMS THAT ENABLED HIS EXISTENCE? A REFLECTION

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Abstract

In modern times mother nature is often neglected and put aside for the sake of interests such as profits, security, economic growth, and threatening politics. These causes inevitably lead to alienation of man from nature, from which he originates and, ironically, will always be a part of, as long as he will exist at least. Disconnection of human from nature is one of the better covered-up causes for rising anxiety and frustration that a modern man faces daily even though all his needs are met. The purpose of this article is to encourage reflection and raise awareness about the topic of preserving and taking care of nature and its wellbeing. Modern times have endowed us with the knowledge and different possibilities to start solving global environmental problems that have begun to threaten our existence. This is still easier said than done, but let us start by getting to know the origins of life on Earth, since they are also the beginnings of our evolution, and continue with some of the uncounted possibilities we have to spare our planet further contamination. Some of this potential lies in bacteria and algae, the microorganisms that were here long before *Homo sapiens* took his first upright step.

1. In the beginning, there was dust and gravitation

Earth formed some 5,54 billion years ago and had a very different atmosphere in the early stages from the one we know today. It contained very little oxygen, which we recognise as particularly important for the existence of humankind. Not to mention, no liquid water was available because the solar system region was still too hot. The oceans formed 4,41 billion years ago, soon followed by first microorganisms, earliest known life forms on earth, estimated to have developed as early as 4,28 years ago. The very early forms of life were discovered in fossilized remains, precipitates in extreme environments such as hydrothermal vents, and stromatolites, formed by layered growth of microbes. Simple procaryotic

microbes, the first forms of life on Earth, were archaea and cyanobacteria. The later are photosynthetic organisms responsible for the occurrence of oxygen in the atmosphere and the development of more complex oxygen-dependent organisms (1, 2).

2. Great oxygen event, its cause, and its consequences

About 2,4 billion years ago, cyanobacterial photosynthesis led to a rapid rise in the oxygen levels. Oxygen first accumulated in the oceans and only 0,82 billion years ago its concentration raised in the atmosphere. When biologically induced molecular oxygen accumulated in Earth's atmosphere, it changed it from a weakly reducing to an oxidizing atmosphere (3). Free oxygen has been an essential constituent of the atmosphere ever since but despite the abundance of the oxygen we know and live in nowadays, local rise in oxygen levels was highly toxic to the surrounding biota in ancient microenvironments. Furthermore, it has been proved that oxygen-producing cyanobacteria can form a thin layer of oxygenated water in an otherwise anoxic environment; thus, the cyanobacteria had the time to adapt to oxygenated environment beforehand (4). Additionally, the selective pressure of rising oxygen concentration drove the evolutionary transformation of an archaeal lineage into the first eukaryotes (5).

3. Cyanobacteria and algae

Research shows that all plastids in algae, a diverse group of photosynthetic eukaryotes, origin from cyanobacteria. This is particularly astonishing detail because, together with the previously described evolvement of events, it leads to a logical thought that without cyanobacteria no oxygen, no oxygen-adapted organisms and also no algae would exist. Especially because algae are a polyphyletic group, meaning they have no common ancestor; however, their organelles (plastides) do have a common ancestor- cyanobacteria (6). In addition, algal chloroplasts are surrounded by two membranes and probably developed through a single endosymbiotic event, where endosymbiont was cyanobacteria. Algae are divided into three main groups: green algae (Chlorophyta), red algae (Rhodophyta) and Heterokontophyta. Land plants' pigments are similar to green algae pigments (chlorophyll a and b) and plants probably origin from green algae. Cyanobacteria, algae, and plants all use light and water for the production of energy and oxygen via a well-known process, photosynthesis. We can name this process »The mother of oxygen« on Earth and the gradient for the evolvement of many species, including mammals and, among them, human. To give the information a little more perspective- first members of the genus *Homo*, *Homo habilis*, evolved 2,8 million years ago.
Human, due to his intelligence and vision, managed to use the laws of nature to shift the odds of existence to his advantage. The invention of the internal combustion engine is such

an example. Or better said, Was such an example, arguably of course. However, quite a few current problems of this world source from the shift of energy utilization enabled by the internal combustion engine and similar inventions. So we can, arguably, claim that human is the cause or at least the catalyst of challenges the Earth is facing today: ecological crisis, energy insecurity, water scarcity, and pollution, etc. The purpose of this article is not to criticize, because us, humans, should be thankful for the life our ancestors have built for us via their discoveries, innovation, and knowledge, it is rather to acknowledge the fact that us, modern humans, should also strive for meaningful changes. We should aspire and work for positive changes, considering what we know now. This is similar to what our inventive ancestors did when they built a bicycle, an internal combustion engine, or a light bulb. Our brain has always been our solution.

4. Who else can help the humans besides our brain?

Doing some research about algae utilization for my doctoral studies and meanwhile thinking about how I could contribute to a better world, a thought anchored into my mind: Could it be that the same organisms that, through history, provided the vital oxygen for the evolvement and rise of human beings are the same organisms that have a potential to help us solve an ecological crisis we have caused? Below I will try to answer this question by explaining some examples of how early developed organisms, algae, most commonly in symbiosis with bacteria, can be used for significant ecological shifts and improvements.

5. Pollution control

Algae can be used as indicator organisms to monitor pollution in various aquatic systems (7). Additionally, wastewater from various sources can be treated with algae in order to cleanse it of organic and inorganic pollutants, simultaneously reducing the use of toxic chemicals that would otherwise be needed to clean the contaminated water (8). Algae can also be used to capture fertilizers in runoff from farms, moreover gained algal biomass can even be used as a fertiliser. This seems like an excellent example of circular economy benefits, and so does the fact that nitrogen and phosphorus can be efficiently removed from domestic and agricultural wastewater because algae reduce the nutrient runoff from agricultural fields. When subsequently harvested, the nutrient-rich algae can be used as an organic fertilizer. Research shows that 60–90% of nitrogen runoff and 70–100% of phosphorus runoff can be captured from manure effluents by algae, in addition scientists demonstrated that microalgae can recover all nitrogen and phosphorus from anaerobically treated black water (toilet wastewater), thus contributing to the quality of water flowing into groundwater, rivers, streams, and oceans (9).

Pharmaceuticals are another case of emerging contaminants that microalgae can efficiently remove from water. Pharmaceutical contaminants are a great environmental concern because their rise is connected with health issues and ecotoxicity. Most promising process for the removal of pharmaceutical contaminants is microalgal bioremediation, a solar-driven and sustainable process. Mechanisms of contaminants' removal include bioadsorption, bioaccumulation, and intracellular and extracellular biodegradation (11). The mechanisms listed are explained in more detail below.

6. Bioadsorption by Microalgae

Microalgae have a negatively charged cell wall because of the dominant presence of functional groups such as carboxyl, phosphoryl, and amine on the surface. Electrostatic interactions attract pollutants with cationic groups towards the cell wall, resulting in effective bioadsorption (11).

7. Bioaccumulation

Bioaccumulation is active uptake of pollutants. Those can, later on, induce reactive oxygen species (ROS) generation, that induce resistance to pharmaceutical contaminants at low concentration in microalgal species. In natural environments, antibiotics triclosan, trimethoprim, and sulfamethoxazole are removed by algae through bioaccumulation essentially (12).

8. Intracellular biodegradation

Microalgae catalytically degrade complex organic compounds in an aqueous medium. Examples of such contaminants effectively degraded by microalgal species are progesterone, ibuprofen, caffeine, carbamazepine, and tris(2-chloroethyl) phosphate (11). Intracellular biodegradation is the most effective path for contaminants removal due to microalgal efficient and complex enzyme system that consists of phase I and phase II enzyme families. Phase I enzyme is Cytochrome 450, which makes the internalized compound more hydrophilic by adding or unmasking hydroxyl group. Phase II enzymes, for example, glutathione-S-transferases, catalyze a conjugation between the electrophilic compound and glutathione. Various microalgal intracellular enzymes play an active role in biotransformation and detoxification of endogenous organic compounds (11).

9. Extracellular degradation

Microalgae can excrete different extracellular polymeric substances (EPS) to their surrounding environment. The EPS form a biofilm matrix that acts as an external digestive

system by keeping extracellular digestive enzymes close to the cells and enabling them to metabolize organic compounds, among them also xenobiotics and nutrients from the environment, contributing to its detoxification (13).
Consortia of microalgae and bacteria or cyanobacteria proved to be even more efficient than individual microorganisms for organic and inorganic pollutants removal and using nutrients from wastewater for own growth because synergistic interactions can be established, enhancing the overall uptake efficiency. Underlying mechanisms are the beneficial oxygen and carbon dioxide cycles, metabolites from bacterial degradation that can act as promoters for algal cell growth, algal exudates that are primary carbon source for bacteria, cell surfaces of algae that can provide a stable habitat for bacteria and metabolites such as auxins and vitamin B12 excreted by bacteria, which are essential for microalgal growth (14).

10. Possibility for algae usage

Fuels produced from algae, such as biodiesel and biogas, hold great promise because of algal potential to produce more biomass per unit area in a year than any other form of biomass (10). Additionally, algae do not take up space dedicated to crops; therefore, they do not interfere with food production and supply chain. Furthermore, algal biofuels usage would also reduce fossil fuels consumption and climate change impacts.
The main nutritional requirements for algal growth are nitrogen, phosphorus, and several microelements. Algae take up these nutrients along with carbon dioxide and produce biomass via photosynthesis. A convenient source of nutrients for algal growth is wastewater, which can be municipal, industrial, and agricultural wastewater. Nutrient removal is also an essential step in the process of cleaning these wastewaters because abundant nutrient streams discharged into natural water bodies can result in eutrophication, causing significant environmental damage (15). Additionally, the approach of using wastewaters as a resource of nutrients efficiently reduces the cost of algal production, while mitigating carbon dioxide emissions and benefiting the environment.
For biofuel production algae need to contain at least 20 % of lipids, some suggest even 40 %. The lipid content and composition can be regulated to a great extent since it depends on the algal species, the light conditions, and the nutrient availability (16). Even though there are still challenges associated with algal harvesting, species control and fuel conversion for large-scale production, a future potential exists that renewable energy derived from algae will play a significant role in providing energy security, while wastewater can be synergistically cleaned of otherwise damaging nutrients (15).
Today, algae are used by humans in many ways; for example, as fertilizers, soil conditioners, and livestock feed (17). Different algal species are cultured in clear tanks or ponds and are either harvested or used to treat effluents pumped through the ponds. Naturally growing

seaweeds are an important source of food, especially in Asia. They provide many essential vitamins, including: A, B1, B2, B6, niacin, and vitamin C, and are rich in iodine, potassium, iron, magnesium, and calcium (18). Commercially cultivated microalgae, including both algae and cyanobacteria, such as Spirulina, Chlorella, and the vitamin-C supplement from Dunaliella, high in beta-carotene, are therefore available as nutritional supplements (19). As previously described, algae on the one hand efficiently remove pharmaceutical contaminants from water, clean agricultural and domestic wastewaters and on the other hand, a valuable algal product can be manufactured subsequently. The examples of algal usage in the field of pharmacy and agriculture described here seem complementary and could be put to good use and manufacturing practice by pharmaceutical and agricultural industries.

Various polymers can be produced from algae, which is especially useful in the creation of bioplastics. These include hybrid plastics, cellulose-based plastics, poly-lactic acid, and bio-polyethylene. Several companies have begun to produce algae polymers commercially, including for use in flip-flops and surf boards (20, 21).

The synergy between algae and bacteria enhances the efficiency of wastewater utilisation for algal biomass production, contributing to better production of the desired nutrient-cleansed water and valuable final products, for example, biofuel, fertiliser, food supplement, livestock food, bioplastics, etc. The described operational principles for the algal production integration with water treatment are presented in figure 1.

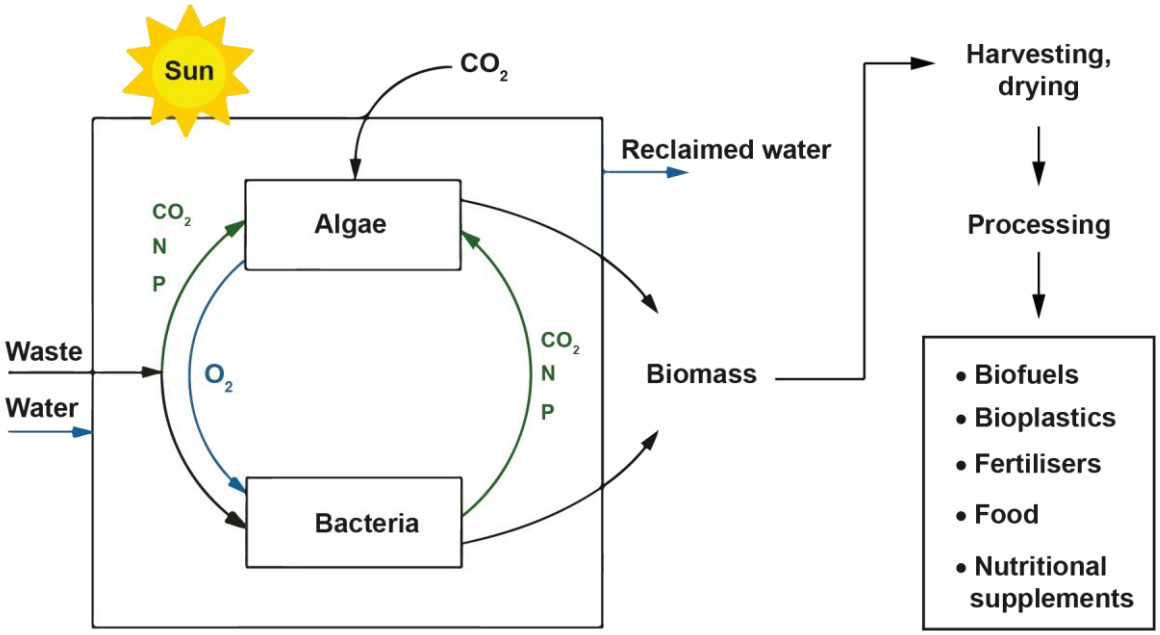

Figure 1: Basic operational principles for algal production integration with water treatment (15, 22).

Conclusions

Planet Earth is our only home and a home of all other forms of life we know of so far. For more than 4 billion years it managed to exist in its native state without significant interferences by its inhabitants. Only in the last 150 years or so human has irreparably damaged natural habitats by his irresponsible exploitation of natural resources. Nowadays, with a large quantity of available information and knowledge, it is time for a change. Change of attitude must come first, and actions must follow. Here, a possibility for a different approach to traditional industrial practices is proposed. It was shown that by microalgae and bacteria consortia, we can fight contamination caused by industrial and household wastewaters and simultaneously produce a valuable algal final product. This is a cost-effective, energy-saving and at the same time environmentally beneficial approach to the disposal of ecologically damaging wastes by putting microorganisms to work. Interestingly, those microorganisms paved the road for *Homo sapiens* development through billions of years by providing oxygen and afterward enabling the development and existence of oxygen-dependent organisms. Now, in modern times, those microorganisms could contribute to our survival once again only this time by cleaning up some of our wastes.

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EXTRACORPOREAL SHOCK WAVE THERAPY – WHAT IS WHAT?

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Abstract

Extracorporeal shockwave therapy (ESWT) has been used in the treatment of musculo-skeletal disorders like plantar fasciitis, calcific tendinopathies, greater trochanteric pain syndrome, Achilles tendinopathies etc., due to mechanotransduction; a biological pathway, where biomechanical forces are converted in biochemical responses that induce healing. Along with rapidly growing body of evidence in the field of ESWT interpreting the literature is becoming more and more challenging. To begin with, the researcher is confronted with various terminology. Besides an umbrella term ESWT, the terms: focused extracorporeal shock wave therapy (fESWT), radial extracorporeal shock wave therapy (rESWT), radial pulse therapy (RPT), radial pressure wave treatment (RPWT), extracorporeal pulsed-activated therapy (EPAT), low-energy ESWT and high-energy ESWT are used in a non-consistent and rather confusing way.

The proposed theme of the discussion is clarifying these terms.

1. Extracorporeal shockwave therapy (ESWT)

Shock waves (SW) are created by explosive events in nature like lightning stroke, earthquakes, volcanic eruption etc., and in technics (e.g., airplanes breaking through the sound barrier). They transmit energy from the point of generation to remote regions. The principle of this natural phenomenon has been transferred to medical application; since the 1980s extracorporeal shock wave lithotripsy (ESWL) has been used in medicine to destroy kidney stones in a humans. As a byproduct of ESWL, ESWT devices have been devloped in order to treat musculoskeletal disorders and have been used increasingly since the early 1990s.

2. Focused extracorporeal shock wave therapy (fESWT) versus radial extracorporeal shock wave therapy (rESWT)

fESWs are generated extracorporeally by means of electrohydraulic, piezoelectric, or electromagnetic mechanisms as a consequence of a rapid increase in pressure into the water. The resulting energy is focused by concentrating reflectors (lenses) and is transmitted to a small target area (2–8 mm diameter) in a noninvasive manner in order to induce therapeutic effects. One of the most descriptive parameters of fESWs is the energy flux density (EFD, in mJ/mm^2), the concentrated SW energy per unit area, which reflects the flow of SW energy in a perpendicular direction to the direction of propagation.
rESW are produced mechanically by a compressed air-driven metallic projectile which hits the applicator. The produced kinetic energy is directly transferred to the skin on the area of treatment through the tip of the applicator. They are acoustic waves as well, but have a different propagation mode (radial), and act through a ballistic mechanism. Energy source is a pneumatic system, that uses compressed air to transmit and control energy, much like pneumatic jack-hammers used in construction.
Parameters describing rESWT are the number of waves, their frequency and pressure (in bars). Unlike fESWT, rESWT has a more superficial effect (Figure 1 – Point of maximum energy flux density).

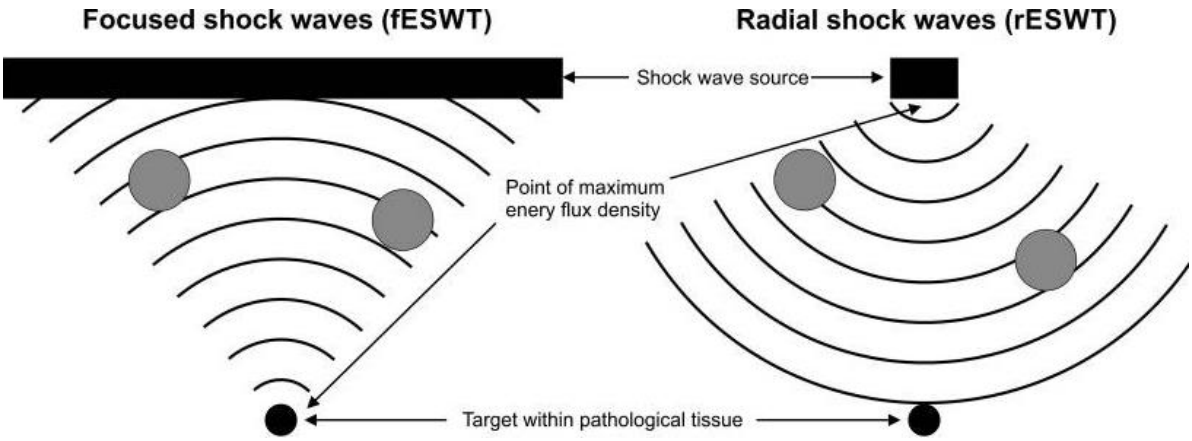

Figure 1. Working principle of focused and radial extracorporeal shock wave technology (1).

3. Radial extracorporeal shock wave therapy (rESWT) versus radial pulse therapy (RPT) versus radial pressure wave treatment (RPWT) versus extracorporeal pulsed-activated therapy (ETAP)

Cleveland et al. (2) preformed measurements of the acoustic field of a rESWT device that uses a ballistic source in an attempt to address nomencalture issues; »it has been shown that pressure waves generated by RSWT cannot be called shockwaves from a fundamental point of view because they lack the characteristic physical features of shockwaves such as a short rise time, a high peak pressure and nonlinearity«.
Based on these facts, rESWT a better term radial pulse therapy (RPT) was suggested (3). Though, some authors adopted the term RPWT (4) and others adopted the term ETAP (5). Nevertheless, the term rESWT is still the most widely used term.
Buizza et al. (7) concluded, that at low-amplitude settings, the piezoelectric and electromagnetic sources in ESWL, which are used in fESWT, do not produce shock waves. The nomenclature of SWT for treatment protocols therefore mismatches mainly the technical definition of a shock wave.

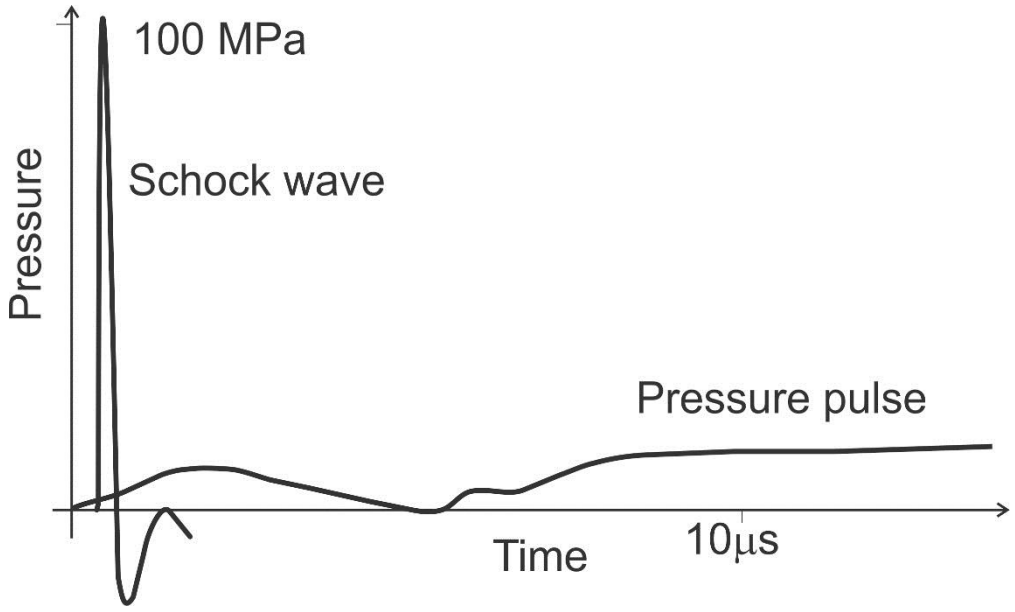

Figure 2. The difference between a shock wave and a ballistic pressure pulse is illustrated Adapted from (6).

4. Low-energy ESWT versus high-energy ESWT

Rompe et al. (8) defined an EFD of 0.2 mJ/mm^2 as the margin between low- and high-energy shock wave treatments, but other definitions were published too, so this distinction appears

arbitrary. For this reason, we suggest that it is not appropriate to define rESWT as a low-energy shock wave treatment and fESWT as high-energy shock wave treatment.

5. Which method is better?

There are not many studies comparing the effects of rESWT and fESWT. Because of the differences between the technologies, a difference in effectiveness would not be surprising. However, authors found no difference in effectiveness between rESWT and fESWT for patients with patellar tendinopathy in addition to eccentric training (9). The result of this study remains debatable, since there was no placebo or control group, and the improvement in both groups might not be clinically relevant. Krol et al. (10) also concluded that the therapeutic effects of rESWT an fESWT applied to patients with symptomatic heel spur are statistically significant and comparable.
Lohrere et al. (11) had better results with fESWT than with rESWT for treating patients with plantar fasciopathy, but the reason might have been the fact, that plantar fascia is a thick tissue located deeper into the body and fESWT penetrates deeper into the tissues then rESWT.
Study conducted by Wu et al. (12), suggested that rESWT and fESWT have similar effects on the spasticity of gastrocnemius muscle in stroke patients; improvement was significant in both groups. However, rESWT was superior to fESWT in terms of improving the ankle passive range of motion and plantar contact area during gait in patients with stroke.
At the moment there is not enough evidence of clinically relevant differences in effectiveness between rESWT and fESWT, so it is impossible to recommend one over another, but from the economic point of view, the cost of rESWT is significantly lower. This is probably the main reason why in practice, the use of rESWT is prevalent.

6. Conclusions

Both rESW and fESW are mechanical waves, yet they differ in the mode of the propagation. However, they do share certain healing effects through mechanotransduction in some soft tissue disorders.
The use of the term rESWT is not appropriate as regards the definition of schock waves, but is still the most widely used.

Acknowledgements
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THE ASSOCIATION BETWEEN DEGENERATIVE SPONDYLOLISTHESIS AND MUSCLE PATHOLOGY

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Abstract

The lumbar spine is subject to the degenerative process, which always begins first in the intervertebral disc. Loss of physiological preload decreases disc height resulting in excessive movements of the motion segment, slipping of the vertebrae or even instability. Degenerative spondylolisthesis is characterized by forward slip of cranial vertebra over caudal one. Prevalence of degenerative spondylolisthesis is up to four times greater in females than in males, the ratio not seen in any other degenerative skeletal pathology.

The aim of our work was to review articles describing association between muscle pathology and degenerative spondylolisthesis. The literature review was carried out using the Scopus database. Eight relevant articles were found in English language and they were reviewed. These studies show the important role that the abdominal musculature plays in stabilization of the spine. Reduced force of abdominal and global back muscles might lead to degenerative spondylolisthesis. Exercise therapy may improve the spinal biomechanical environment and should be designed to improve both, muscle strength and balance between abdominal and back muscles.

Key words: degenerative spondylolisthesis, muscles in degenerative spondylolisthesis

1. Introduction

Lumbar segmental instability with degenerative spondylolisthesis constitutes a subgroup of patients with chronic nonspecific low back pain (11). A model of spinal stability that seems to be a result of coordination among 3 major systems (active, passive, and neural) was proposed (12). Further, it was proposed that in lumbar segmental instability, stiffness of lumbar motion segment decreases and results in an increased range of motion, leading to the painful condition (13).

In this degenerative condition of the skeleton the prevalence is up to four times greater in females than in males (15) which is exceptional. In view of this disparity, surprisingly little attention has been directed at the aetiology of degenerative spondylolisthesis (DS), although hormonal influences and pregnancy have been implicated (2, 8, 15).

DS is a complex multifactorial problem. It is interlinked with other pathologies, such as, for example, disk degeneration, facet joint osteoarthritis and spinal stenosis. Without a thorough understanding of the natural history and factors associated with development of DS it is impossible to understand completely the etiology, and therefore not feasible to develop prevention and treatment methods for this condition (9).

Other suggested aetiological factors for DS include race, soft tissue abnormalities, the influence of the lumbosacral angle, sagittal spinopelvic alignment, a lower intercrestal line, lumbosacral bony anomalies, facet tropism and alignment and diabetes (1, 3, 4, 6, 17, 18).

The aim of our work was to review articles describing association between muscles and DS.

2. Methods

Scopus database was searched for the key words “spondylolisthesis and muscles” and “degenerative spondylolisthesis and muscles“ in title. We critically analysed all published material. The literature review was carried out using the Scopus database. Eight relevant articles in English language were reviewed.

3. Results

3.1. Fraser et al., (5) examined in a prospective cross-sectional cohort study the important role of abdominal muscles in stabilization of the spine. They found 205 patients completing a questionnaire. The investigator assessed integrity of the linea alba. MRI or CT scan of the lumbar spine was examined for DS. The study showed the important role that the abdominal musculature plays in stabilization of the spine and highlights its potential role as a factor in the development of DS.

3.2. Hiyama et al., (7) in a retrospective study evaluated 140 patients hospitalized for surgery to treat lumbar spinal stenosis (LSS) and/or DS. Spinal alignment, cross-sectional area (CSA) of spinal muscles, and body composition parameters were measured from full-length standing whole-spine radiography, MRI, and BIA before surgery. The following standard measurements were obtained from radiographs: sagittal balance (C7-SVA), cervical lordosis (CL; C2–C7), lumbar lordosis (LL; L1–S1), thoracic kyphosis (TK; T5–T12), pelvic incidence (PI), pelvic tilt (PT), and sacral slope (SS). Results of the study suggest that the posterior inclination of the pelvis may be correlated with paraspinal muscle area rather than age.

3.3. Shadani et al., (16) in nonexperimental, analytic case-control study investigated abdominal and lumbar mulifidus muscle size in 25 healthy subjects and 25 patints with DS, using ultasonography. According to the results of the study, it seems that the size of the abdominal and lumbar multifidus muscles at rest and during contraction was reduced in patients with spondylolisthesis.

3.4. Zhu et al., (21) constructed nonlinear 3-D finite model of L3-L5. They applied forces represented in global back muscles and global abdominal muscles along with upper body weight. Different degenerative conditions were simulated by lowering the forces of global back muscles. An additional boundary condition, which represented the loads from other muscles after exercise therapy, was set up to keep the spine in a neutral standing position. They concluded that reducing the force of global back muscles might lead to, or aggravate DS with forward slipping from biomechanical point of view. Exercise therapy may improve the spinal biomechanical environment.

3.5. Wang G. et al., (19) in a retrospective study measured (with MRI and X-ray) disc space and signal intensity ratio of muscles in 149 DS and 149 control group patients. Authors assumed that paraspinal muscles atrophy is also a risk factor for LDS. They found a decreased anterior disc height and multifidus muscle atrophy in the LDS patients and suggested that this could be the cause of LDS. The presence of erector spine hypertrophy could be a compensatory mechanism to compensate for the instability.

3.6. Nava-Bringeas et al., (10) performed a transversal, descriptive and observational study that included visual-analogue scale (VAS), Oswestry Disability Index (ODI) and isokinetic trunk testing. Assessent of multifidus atrophy and spinal stenosis was performed by MRI in 26 patients. Authors concluded that muscle trunk imbalance with predominance of extensor over flexors muscles is associated with functional disability. Therefore they proposed that rehabilitation programs should be designed to improve muscle balance rather than muscle strength alone.

3.7. Zhu et al., (20) in a retrospective study measured cross-sectional areas of bilateral erector spine and psoas major muscle on the L3-5 vertebral endplate levels using T2-weighted MRI in 80 LDS patients and 80 healthy persons. Results indicated that the changes in cross-sectional area of paraspinal muscle could induce LDS, and could be considered as a diagnostic standard for LDS.

3.8. Ramsbacher et al., (14) performed paravertebral muscle biopsies in 30 patients with monosegmental DS undergoing posterior lumbar interbody fusion. The tissue samples were submitted to histologic analysis including immune and enzyme histochemistry and electron microscopy. In addition, the muscle fibers were submitted to morphometry. They detected increased numbers of polyglucosan bodies,which has not

been described in patients with otherwise normal muscles. The importance of the finding for occurrence of DS is not clear.

4. Discussion

The studies included in the present literature review highlighted the importance of muscles, specifically abdominal and back ones, in development of DS. Back muscles are constituted of different muscle groups and of these, the pathology of Mulifidi seems to play a central role in occurrence of DS. In addition, eventhough perhaps counterintuitive, the association of abdominal muscles insufficiency and DS was clearly demonstrated. On top of that, it is the ratio of abdominal/back muscle strength rather than the condition of individual muscle group alone that affects the development of DS.
What is not clear form this literature review, though, is the association between underlying condition (e.g. parity, sagittal parameters), muscle insufficiency and DS. Further studies are therefore needed to elucidate the connection between, for example, parity and long term insufficiency of specific muscle group which would in turn lead to development of DS.
The limitation of current literature review is the traditional approach with its inherent bias. It is widely agreed that superior results could be anticipated with systematic literature review or meta-analyses.

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BRIDGING LEADERSHIP AND ECONOMICS STUDIES

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Abstract

The proposed theme of the discussion on bridging Leadership and Economics studies to better understand the mechanisms of distribution of power and wealth within and among the developed and developing countries, and the equality/inequality division within societies and among individuals, places the concepts of leadership and debt into a relationship that offers profound understanding of social relations and contribute to the growth of theory and practice in complex political environments. Thus, better understanding of the role of debt and money in societies, should make a substantial contribution to better policymaking to the benefit of all citizens.

1. On how economics believes that it studies the phenomenon of the economy debate

Economics is preoccupied with trying to understand the relations between symptoms of the economy without ever being capable to predict a future cyclical downturn or explain the last one (1). Crazy Economic Rationalizations for aNomalies (CERN), an economic research lab at Massachusetts Technical Institute (MIT), discovered a “new particle” called “Full employment real interest rate” (FERIR). Economists herewith refer to it (maybe rather mockingly) as a phenomenon in the economy that was not previously theoretically predicted. CERN at MIT was mooted by Professor Larry Summers at the 2013 International Monetary Fund (IMF) Research Conference (2). Symptoms of debt creation in models of economics which are based on questionable and simplistic assumptions: 1) ‘invisible hand’ of the market, 2) rational behavior of individual agents, 3) symmetry of information, indicate assumptions on which theories are built and policies proposed.

Analogously, physicists in the European Organization for Nuclear Research (Switzerland), known as CERN are discovering new particles that were not predicted by previous theories. Thereby they search support for an improved description of micro and macro universe.

2. On how money is a moral category

If there is a single word that appears most frequently in discussions of the economic problems now afflicting both the United States and Europe, that word is surely “debt” (3).

The strength of the statement "Surely one has to pay one's debts." lies in the fact that it is actually not a statement concerning economy but rather a statement within the moral category (4).

3. On how power/money is distributed by the leaders

Dealing with debt overdue among companies and nonperforming bank loans of companies to the banks is studied in economics on the aggregate level. The policies are confined to:

Monetary measures like setting the interest rates and quantitative easing, are adjusting the money supply in an economy and cause further development of social inequality.

Fiscal side measures, bankruptcy legislation and regulation are tightened thereby causing more companies, individuals and banks to go bankrupt even if their receivables are plenty. They are just not serviced on time.

Classical measures to fight bad debt in a sovereign economy are basically emitting more money by the central bank and the government, causing looser crediting policies in the banks, longer payment terms to settle an invoice, increasing the government/public sector deficit and loosening bankruptcy policies.

Austerity policies function in the opposite direction. They increase bad debt in an economy, redistribute goods unevenly and to dramatic proportions in regimes where monetary and fiscal policies are not politically in balance (as for example presently in Euro-zone).

4. On entropy in the economy

Entropy in a financial system is increased trough debt overdue among companies and nonperforming bank loans. Without external intervention, entropy (in the financial system) increases and leads to economic downturns and crisis. Here, the financial system refers to the monetized economy, the part of the economy in which invoices/loan contracts are issued for the products and services delivered.

5. Bank credit and trade credit

Money is generated by a bank credit and a trade credit. The bank credit is generated by issuing bank loans to companies and individuals. The trade credit generates money by issuing invoices to buyers of products and services. The phenomenon of a monetized economy could thus be described by one big consolidated balance sheet, i.e. linearly. Would there be a difference if banks and companies as actors of credit generators were robots instead of human? We would be most likely able to find the equilibrium predicted by neo-classic and neo-Keynesian economists. But human minds and emotions of the decision-makers are not linear and that is where unpredictability and nonlinearity that are experienced and observed in the phenomenon of the economy comes from.

We suggest a thesis that economic downturns and crisis are exogenously driven by the actors that cannot settle their outstanding obligations on time as agreed upon. Psychology (5) explains that such actors are deprived of their existential means of existence and are thus consequentially in economic terms in no position to invest or consume.

6. On clarity what is the phenomenon under research

Leadership and Economics as a meta-science should address its own weaknesses and must find means of understanding the phenomenon of a society and economy much deeper by simply considering what is it that is going on in the phenomenon itself.

Leadership is a network of social influences.

Money creation forms a network of invoices and credit contracts, in short “I Owe You” (IOU) (Fig. 1).

Ultimately, if there are 7 billion people in the world , there are at most 7 billion companies, which amounts to 21 billion financial statements (incomes, balances, cash) that we argue about. Availability of data to provide insight into the depths of the phenomenon of an economy is already available in the Americas and soon it will be available in Europe, to allow the shift of multilateral netting towards ever smaller transactions. This is predicted by the Bank for International Settlements (BIS) in their paper on Core Principles for Systemically Important Payment System (6). Technological processing is a miniscule against what many social networks do every day. It is up to scholars and politicians to use it ethically and morally for the benefit of all; this would allow Economics combined with Psychology, Sociology and Humanities to build coherent theories that could serve as grounds for policy development.

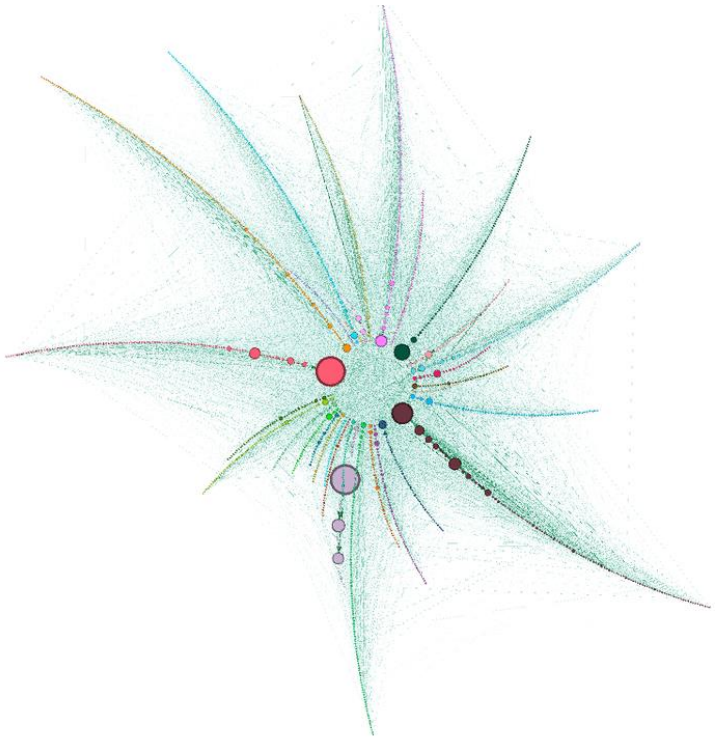

Figure 1. A snapshot in time of an economy, where the dots represent companies while the size of the dots represent how much do they owe overdue to other companies. The links indicate to whom they owe, while the width of the links is the amount owed overdue.

7. On why heterodox economics is needed

Heterodox economics view on the role of debt/money is needed (7-9) on monetary circuits and further on Euro from the Italian perspective (10) and governing by debt (11). Laws of entropy in economy and in nature are relevant in micro and macroeconomics (12). It was suggested that everything goes back to the individual (13). “Ergosophy” which regards economics, sociology, and history with the monetary system must fulfil its proper distributive mechanism of society (14).

Heterodox monetary economic theories of sovereigns and their monetary and fiscal systems (15) provide insights into an economy and provide policy actions in the public interest. Creation and destruction of money is a consequence of deliberate actions of individual leaders of governments and central banks on level one, leaders of banks on level two and leaders of companies on level three of the legal frame of a monetized economy (Fig. 2).

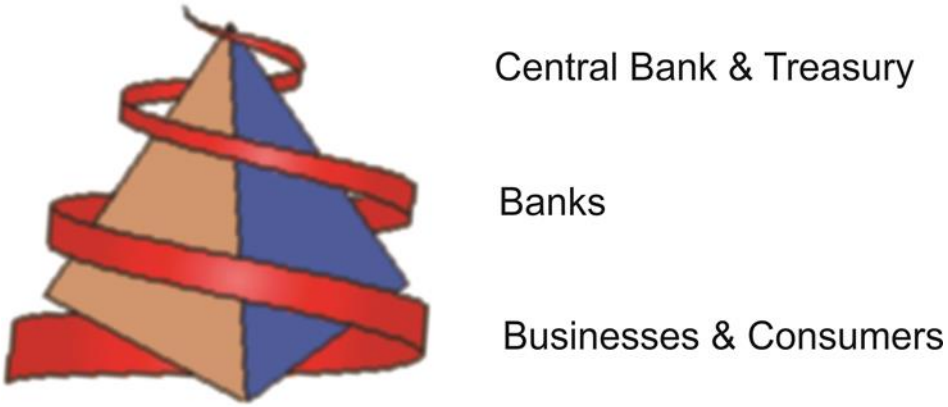

Figure 2. A scheme of the model refering to creation and destruction of money in an economy.

The agreed-upon division between the private and the public sector is the budget proposed by the government and approved by the parliament.

“States with sovereign currency control (i.e. that do not operate under the restrictions of gold standard, fixed exchange rates, dollarization, monetary unions or currency boards) are not limited by financial constrains (although they may face political constrains)”.(15)

8. On what makes the field of Heterodox Economics relevant to the field of Leadership

What makes the field of heterodox monetary Economics relevant to the field of Leadership research is that it starts with a very fundamental philosophical understanding of money, or better, debt.

9. The material form of debt is older older than money

The concept of debt is as old as mankind, like leadership. Parallels between leadership and debt, the latter represented by today's monetary and fiscal policies, can be better understood in how they stir societies, governments, banks, businesses and individuals that are the constituents of the sovereign model (15).

On the basis of the above we suggest that the level of entropy in the economy is in the debts overdue. The debt overdue or bad debt can be and should be empirically measured and analyzed to be resolved by the redistribution of power by the state or by the markets. Our thesis is that the lead indicator of economic downturns must be defined theoretically and measured empirically.

10. On where to focus future research

We suggest that the research should be focused on multilateral set-off of debts in an economy, bank credit and trade credit, nonperforming loans (NPL) and debt overdue.

Methodology and algorithms have been developed in the past 25 years as Tetris Core Technologies (16).

The work based on analysis of data and the potential actual deleverage of the economy is currently in preparation by the authors of this paper, for the governments of Italy and Brazil as two top ten economies with GDP in the range of 2.5 trillion EUR.

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ART AND SCIENCE – TWO HALVES OF THE WHOLE

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Abstract

The paper describes the musical involvement of the scientists as creators, producers and listeners of the music. Music gives scientists fun and aesthetic pleasure, inspires them and helps them to work better while their scientific work promotes their musical creativity and the emergence of new compositions.

The paper describes the events organized and performed by scientists and artists in Napoli and Ljubljana in 2019, initiated by the collaboration within the EU Commission project Extracellular vesicles from a natural source for tailor-made nanomaterials (Ves4us). The main purpose of the events was a support to the collaboration between the scientists involved in the projects and between artists and scientists of the cities of Napoli and Ljubljana. The events were characterized by integration of performers of different nationalities and the style diversity of programs.

1. Introduction

In this report we will present the concert activity of scientists working at the "Consiglio Nazionale delle Ricerche" institute (CNR) and at University of Ljubljana, and musicians from both cities. The activities took place from January to March 2019 in Napoli and at the accompanying concert of the Socratic Lectures Symposium in Ljubljana in April 2019.

2. Concerts in Napoli

In the period between January 2019 and March 2019, 3 musical concerts were organized in Napoli, Italy; they were intended to support the research. Two of them were open also for general public.

The first concert took place January 23 at the church Parrocchia Santa Maria della Rotonda. It was performed by a renown organ player Roberta Schmid, while Veronika Kralj-Iglič (who visited Napoli due to collaboration with the group of prof. Gabriella Pocsfalvi at CNR) and

Roberta Schmid played together a piece entitled “Si dolce è tormento” by C. Monteverdi and a piece entitled “Bist du bei mir” by J.S. Bach, as transcribed for flute and organ.

The second concert took place at the CNR Institute on February 21 to accompany a scientific meeting within the Ves4us project. The project is coordinated by prof. Antonella Bongiovanni from CNR of Palermo. The colleagues from Palermo visited Napoli in order to discuss the progress of the project with the colleagues from Napoli and from Ljubljana. The concept of the concert was original and unique. In the context of the Institute CNR there is also a small detached building near the main part of the institute's premises. This is the abandoned playroom (Ludoteca) , which had been originally used for the daily care of children of employees. It ceased to serve the original purpose, however, it was left full of children's toys and equipment. The playroom was temporarily rearranged into an improvised concert venue and equipped with a portable electric piano. The CNR members organized a concert dedicated to colleagues from Palermo. The concert was performed by Andrea Bonetti (on a piano), a graduate of the Napolitan Conservatory "San Pietro a Majella", Christopher Stanly (on a guitar) and Veronika Kralj-Iglič (on a flute). Dr. Christopher Stanly, originally from India but presently working in the group of prof. Gabriella Pocsfalvi at CNR, and prof. Veronika Kralj-Iglič from University of Ljubljana, are members of the Ves4us project. Stanly's invitation to play in this event prompted him to learn the pieces entitled "Hallelujah" by L. Cohen and "Hotel California" by The Eagles group, especially for the event. The concert was highlighted by a new authoring song entitled "The sound of vesicles" by D. Zupanič, performed by the author and Andrea Bonetti and accompanied by an original movie assembled by the member of the Ves4us project dr. Annamaria Kisslinger. The movie featured the images of membrane shapes, extracellular vesicles and microalgae, which are the subject of the project. The concert contributed to a positive mood and inspiration of the participants of the minisymposium. Furthermore, it has confirmed that the scientific work can be a source of art and vice versa.

In March 4, a social event was organized by prof. Aleš Iglič, prof. Veronika Kralj-Iglič from University of Ljubljana and by Anna Romolo from Napoli. This event was dedicated to the friendship, music and collaboration between scientists and artists from both cities. The event took place in the historical Center of Napoli, in Palazzo Venezia , a historic building from the 14th century (2). In 1412, the Napoletan King donated the building to the Venetian Republic to serve as the seat of the Venetian Embassy. Within the Kingdom of Napoli it served the purpose for almost four centuries. Since then, it has been renovated several times. There is a beautiful garden with fruit trees and other plants within the facility, which was decorated for the purpose of the event. On the day of the concert, employees of the CNR Institute, their family members and friends have completely filled the palace hall. The performers were

musicians from Napoli and from Ljubljana and scientists from both cities, including the pianist, organist and composer Canio Fidanza, the violinist Vittorio Sbordone and the flutist Anita Prelovšek from Ljubljana. Anita Prelovšek chose a variation on the Rossini theme of the Opera entitled “Cinderella” for her solo performance. The program has joined the works of Italian, Slovenian and other classical music composers with an intermediate short part of the songs from 70. and 80. years of the last century (Fig.1). The transcriptions of the famous songs, the A. Piazzolla’s “Oblivion”and the Šoštakovič's “Second waltz” for the ensemble of two flutes, violin and piano, were made by D. Zupanič. The famous Neapolitan songs "O sole Mio" and "Funiculì Funiculà" were supported by a great enthusiasm of the audience.

Just before the concert, a researcher from CNR sang a soprano aria “Laudate Dominum” by WA Mozart, accompanied by Canio Fidanza on the piano. This excellent spontaneous performance was, according to the author of this contribution, an important event since it encouraged her to continue practicing singing that she abandoned for some time, and inspired her to start learning to play the piano. Thus she became actively involved in music again. A photographer Maria Manfredi from Napoli took camera records of the event and created an artistic movie featuring the highlights of the evening. The concert also included a social part where the audience and performers were able to learn and share opinions and experiences. We found that many of the institute’s employees frequently attend performances and concerts at the Napoletan Opera Theatre “San Carlo”. For example, we have learned from one of the participants, a PhD of Biomedicine, that he is very enthusiastic about music and that he has a subscription at the opera house. The event at the Palazzo Venezia was an inspiring feature for the scientists and a pleasant conclusion of the working day.

3. Concert in Ljubljana in the context of Socratic Lectures 2019

As a prelude to the Socratic Lectures minisymposium, an evening before the symposium, a social event with a concert dedicated to friendship and music took place at the “Fužine” castle in the suburbs of Ljubljana. The influence of the Venetian renaissance style can be noted in particular in a fresca entitled “The March of the Holy Three Kings” at the castle chapel (3). The chapel was built in the middle of the 16.th century (3). The program included many compositions of diverse styles (Fig. 2). There were instrumentalists, singers and dancers performing, including both professional musicians and scientists. A chorus “Evergreen” opened the evening with an old polyphone song “Cum decore”. A Russian pianist Elena Starceva-Somun, living in Slovenia, performed in duo with flutists Anita Prelovšek and Ilaria Griessler. A duo Al-Chemistry performed several author pieces. The duo is composed of scientists/musicians who presently live in Slovenia. Aleona Sultanova is originally from Kazakhstan. She is active in science also as an evaluator of the EU

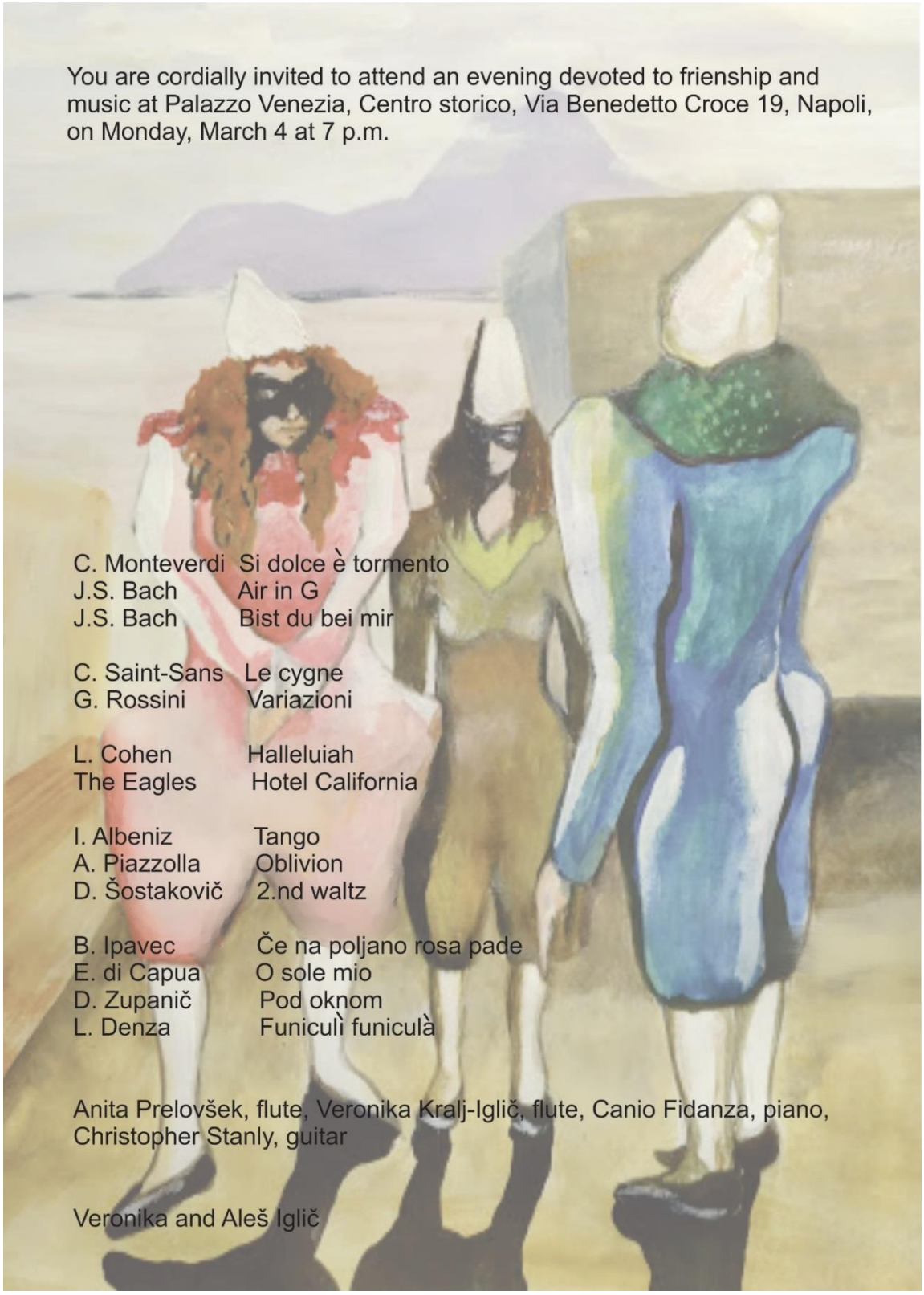

Figure 1. Invitation to the event at the Palazzo Venezia in Napoli, March 2019.

Commission projects, while in the field of art she performs as a composer, vocalist and pianist. Zoran Mosić is a food science engineer while in the field of art he plays the saxophone and sings. The genre of their songs is called “romance” in Russia. Among the songs, there was also one devoted to Nikola Tesla. Dance performances were delivered by Darja Eržen, Metka Penko-Natlačen and Stojan Natlačen-Penko. Metka Penko-Natlačen also sang soprano. She was accompanied on a piano by Jana Jamšek, while Jana Jamšek also performed as a soloist. As a surprise, members of the Laboratory of Clinical Biophysics dr. Manca Pajnič, Darja Božič, Ljubiša Pađen and Marko Jeran presented a composition of Slovenian popular songs, accompanied by Mitja Drab on a guitar. There were also some songs that Veronika Kralj-Iglič, Anita Prelovšek and Canio Fidanza (who was invited to Ljubljana for the occasion) had already performed together in Napoli, however, in Ljubljana, the violin part was played by Matej Venier. The event was a great oportunity for the symposium participants to meet old acquaintances and make new ones, which is a true value of international professional congresses. Luc Menaše wrote: "These meetings are only in a minor way what we learn from official proceedings and discussion records. The more important contents are the advantages that the individual participants experience “at the edge” and “between the lines” of the official program..." (4).

4. Scientists and music – a general impression

As we can see from the above described practices, the music is often an important part of the life of researchers and scientists (5). Some people actively engage in it, either by playing on an instrument, or singing, composing, preparing transcripts and organizing concerts. Some participate in listening to the music, have a profound knowledge of it or are at least its active fans. Many are faithful visitors to concerts and operas and are subscribed to symphonic and opera events.

A German sociologist, philosopher and musicologist, Theodor Adorno, distinguished in his famous typology of the listening relationship to music eight types of listeners, from the listener – an expert, that is the one that does not lose anything and is in every moment aware of what he is listening, to a non-listener who is indifferent or “anti-musical” listener. Between these two extremes, he identified six intermediate levels or types. Many of the above mentioned scientists pertain as a music audience to an expert type of a good listener type who listens well but is partly or completely unaware of the technical and structural elements, and an educated listener type who listens well, is informed and has a fetishist view upon music. The most abundant type listens to the music only for entertainment (6). According to Merriam's definition of the function of music (7), the role of music on the

events described in this contribution could be described as an aesthetic pleasure and striving for discovery. We can outline a thought written by A. Einstein: "If I were not a physicist, I would probably be a musician. I often think in music. I live my daydreams in music. I see my life in terms of music." (8).

Figure 2. Images of the event taking place at Palazzo Venezia, Napoli, on March 2019.

Figure 2. Invitation to the event at the Fužine castle, April 2019.

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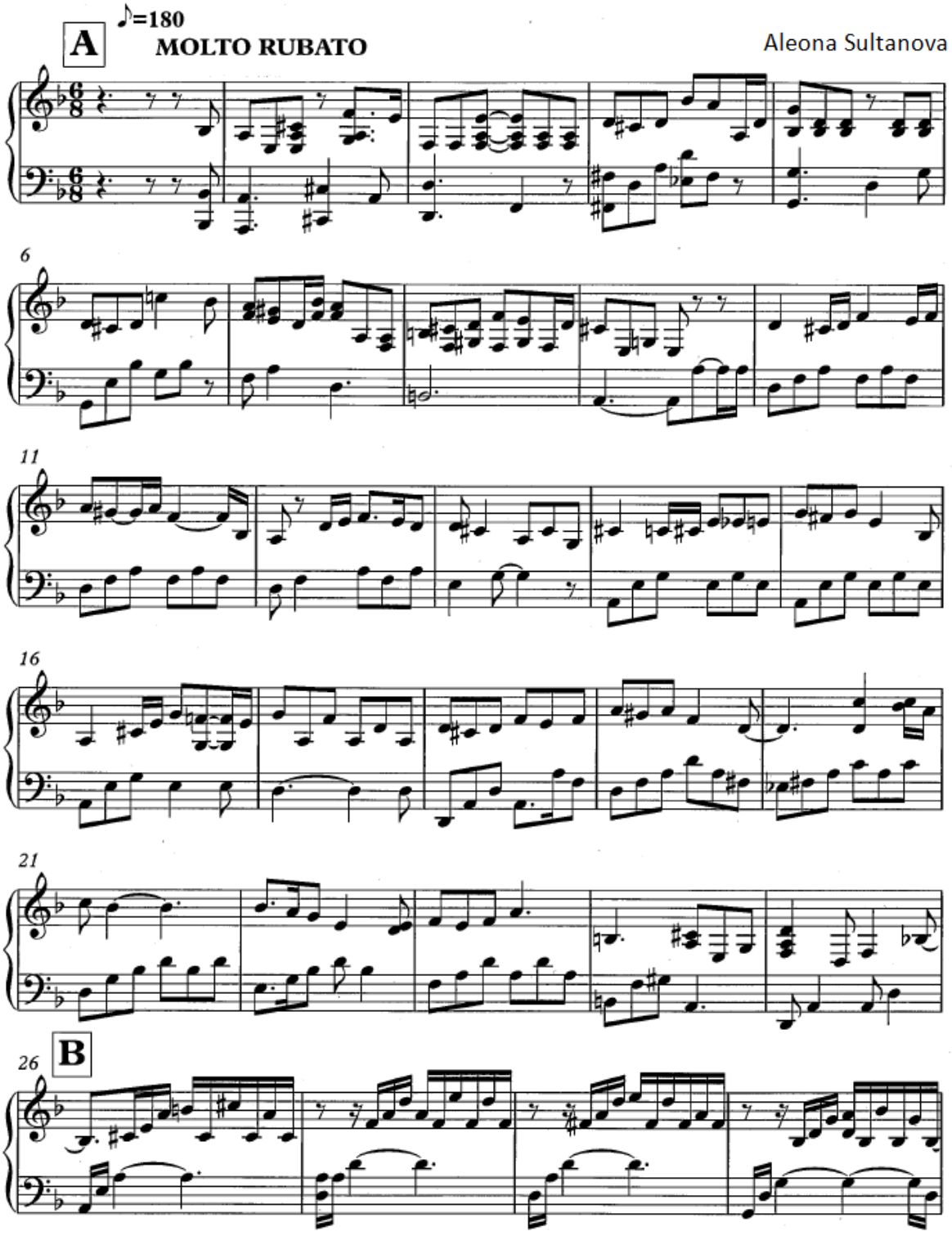
BARON VALVAZOR



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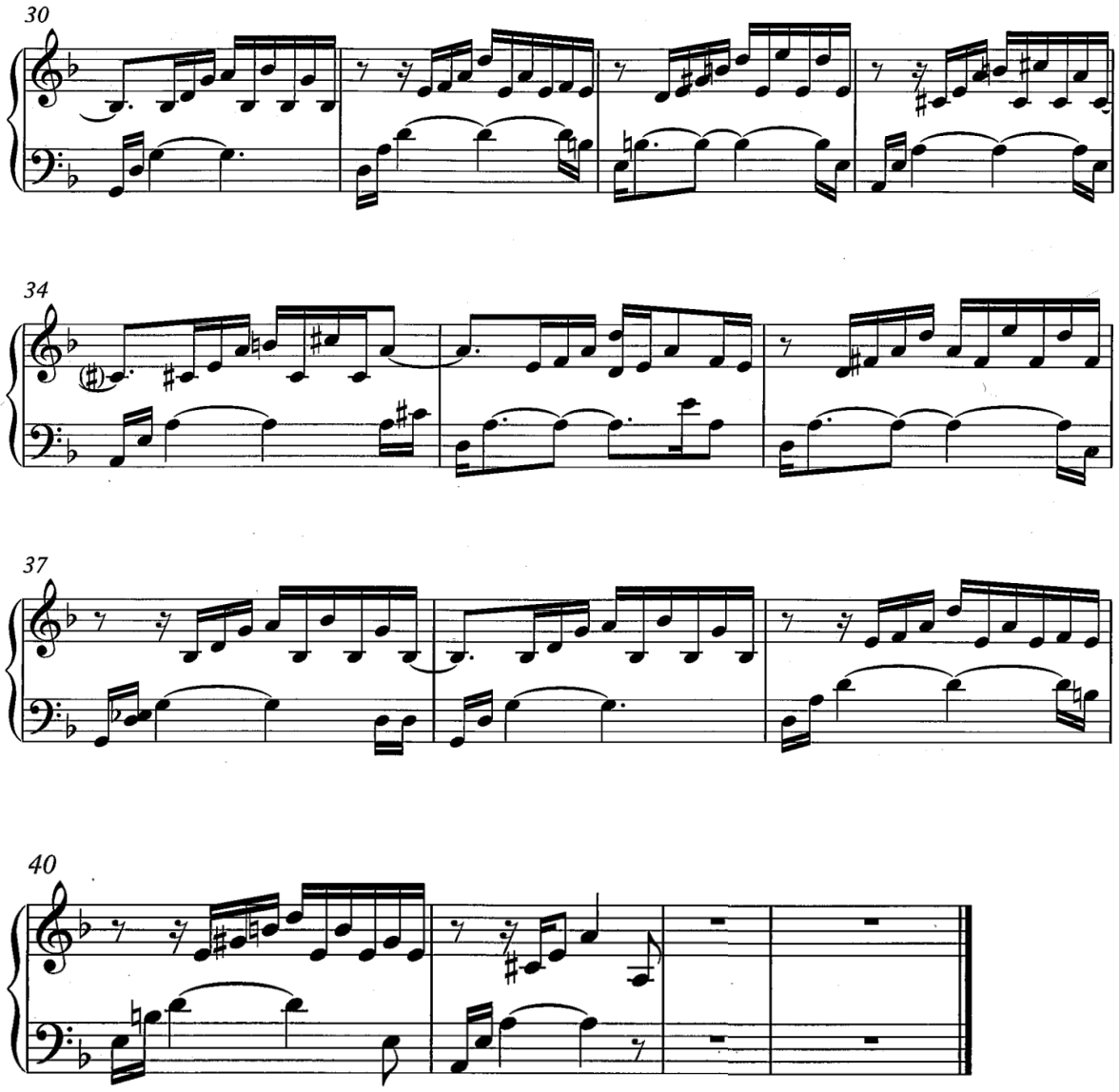
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БАРОН ВАЛЬВАЗОР

Lyrics, Music: Алена Султанова (Словения, Казахстан)

Жил барон Вальвазор три столетья назад, - дворянин, но ученый от бога,
И когда путешествовал в разных краях, - описал феномены природы,
И учился у всех просвещённых людей, расширяя свои горизонты,
что зачислен он был по заслугам своим в Королевское членство Европы

Refrain (in Russian)

Барон Вальвазор,
мрачен, угрюм этот взор
По Старому trg-у призрак пройдет
Он где-то в Любляне живет

Refrain (in Slovene)

Baron Valvazor
Mračna podoba oči
Na Starem so trgu videli vsi
Duha ki v Ljubljani živi

Сначала ты первый замок продал, потом - Богеншперк и свой дом, и книги свои на все деньги издал, наукой тебе был твой дом,
Четырнадцать лет скитаний с войной ты путником с верой прожил И книги, гравюры, монеты с собой как клад драгоценный носил

Refrain (in Russian):

Барон Вальвазор,
мрачен, угрюм этот взор
По Старому trg-у призрак пройдет
Он где-то в Любляне живет

Refrain (in Slovene):

Baron Valvazor
Mračna podoba oči
Na Starem so trgu videli vsi
Duha ki v Ljubljani živi

Об озере, что исчезает в мираж, о камнях, что в форме сердец
Вампирах, дурманах, металлов литье, - все это – семнадцатый век
И все состояние, что нажил тогда - в пятнадцать ты книг поместил
И умер ты в бедности, слава твоя - осталась в книгах твоих