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Aims & Scope

Compared to other fields, developments and innovations in the fields of medical, health and life sciences are very fast. In this century, where the human population is rapidly increasing and technology is developing rapidly, health problems are constantly changing and new solutions are constantly being brought to these problems. With the Covid 19 epidemic, it has emerged that a health problem affects all humanity and all areas of life. For this reason, this conference focused on the changes and innovations in the field of Medical, Health and Life Sciences.

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ICMeHeLS 2023: International Conference on Medical, Health and Life Sciences

Epigenetic Markers for Animal Health and Productivity in Livestock

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Abstract: Epigenetic markers are molecular modifications that affect gene expression without altering the DNA sequence. They are influenced by genetic and environmental factors and play crucial roles in animal health and productivity. Epigenetic processes include DNA methylation, histone modification, and chromatin remodeling. This review supports the recent discoveries on the impacts of epigenetic processes on livestock production and health traits, such as growth, development, disease resistance, and stress response. It also discusses the potential applications of epigenetic biomarkers in livestock management and improvement, as well as the challenges and gaps in livestock epigenetics research.

Keywords: DNA methylation; Histone modification; Chromatin remodeling; Gene expression; Phenotypic variation; Livestock productivity.

Introduction

Epigenetics is a fascinating and rapidly evolving field of research that can help us understand how our genes interact with our environment. It is the study of how the environment and other factors can change the way that genes are expressed. While epigenetic changes do not alter the sequence of a species's genetic code, they play an important role in development, health, and disease. Scientists who work in epigenetics explore the mechanisms that affect the activity of genes, such as DNA methylation, histone modification, and non-coding RNA. Epigenetic changes are influenced by various factors, such as nutrition, stress, age, and exposure to chemicals. Epigenetic changes are also inherited from one generation to the next (Ibeagha Awemu et al.,2015; Ieagha Awemu et al.,2021).

Epigenetic markers are measurable changes in the epigenome that reflect the status of epigenetic processes. They are used to assess the effects of epigenetic processes on livestock traits, such as productivity, disease resistance, stress response, and reproduction. They are also used to identify the genes and pathways involved in epigenetic regulation of livestock phenotypes (Ibeagha Awemu et al.,2015; Ieagha Awemu et al.,2021).

Epigenetic profiling is the analysis of epigenetic markers across the genome or at specific genomic regions. It is performed using various techniques, such as bisulfite sequencing, chromatin immunoprecipitation, or methylated DNA immunoprecipitation. They reveal the patterns of epigenetic variation among individuals, tissues, or conditions (Ibeagha Awemu et al.,2015; Ieagha Awemu et al.,2021).

Epigenetic applications are the potential uses of epigenetic information in livestock production and health management. Therefore, these epigenetic markers are involved improving animal breeding by incorporating epigenetic data into genomic selection, enhancing animal welfare by modulating epigenetic processes through

nutrition or environmental interventions, and developing epigenetic biomarkers for disease diagnosis or prognosis.

The Importance of Epigenetic Markers in Animal Productivity

Epigenetic markers are chemical modifications or molecules that can affect gene expression without altering the DNA sequence. They include DNA methylation, histone modifications, chromatin remodeling, and non-coding RNAs. Epigenetic markers can respond to environmental factors such as nutrition, pathogens, climate, and management practices, and influence animal phenotypes such as health, reproduction, growth, and production (Ibeagha Awemu & Zhao, 2015;Ieagha Awemu & Yu,2021).

Epigenetic markers are important for animal productivity because they can modulate the expression of genes involved in various biological processes that affect animal performance. For example, DNA methylation and histone modifications can regulate genes related to milk production, meat quality, feed efficiency, and disease resistance in livestock species[12]. Epigenetic markers can also mediate the effects of maternal nutrition, fetal programming, and early-life experiences on animal development and health (Ibeagha Awemu & Zhao, 2015; Ieagha Awemu &Yu, 2021).

Epigenetic markers can be used as tools to improve animal productivity through various strategies. For instance, epigenetic markers can be used to identify animals with desirable traits or to predict their breeding values based on their epigenetic profiles (Ibeagha Awemu & Zhao, 2015; Ieagha Awemu &Yu, 2021). Epigenetic markers can also be used to manipulate gene expression by using epigenetic drugs, dietary supplements, or gene editing techniques (Ibeagha Awemu & Zhao, 2015; Ieagha Awemu &Yu, 2021). Epigenetic markers can also be used to monitor animal health and welfare by detecting epigenetic changes associated with stress, inflammation, or infection (Ibeagha Awemu & Zhao, 2015; Ieagha Awemu &Yu, 2021).

Mechanisms of Epigenetic Markers in Animals

The mechanism of epigenetic markers in animals is the regulation of gene expression by modifying the accessibility and activity of the DNA without changing its sequence. Epigenetic markers are elicited by environmental cues or inherited from parents, and affect the metabolic function, behavior, and performance of animals and their offspring. Some of the main types of epigenetic mechanisms are:

- DNA methylation: This is the addition of a methyl group to a cytosine base, usually in a CpG dinucleotide context, which silence gene expression by preventing transcription factor binding or recruiting methyl-binding proteins that alter chromatin structure (Jin et al., 2011).
- Histone modifications: These are the addition or removal of various chemical groups (such as acetyl, methyl, or phosphate) to the amino-terminal tails of histone proteins, which activate or repress gene expression by changing the affinity between histones and DNA or by recruiting chromatin-modifying enzymes or transcriptional regulators. (Li et al., 2020).
- Non-coding RNAs: These are RNA molecules that do not encode proteins, but regulate gene expression at various levels, such as transcription, splicing, translation, or degradation. Examples of non-coding RNAs include microRNAs, long non-coding RNAs, circular RNAs, and small interfering RNAs (Li et al.,2020).
- Chromatin remodeling: This is the alteration of the nucleosome position or composition by ATP-dependent complexes, which enhance or inhibit gene expression by exposing or occluding regulatory elements or by exchanging histone variants with different properties (Li et al.,2020).

Measurement of Epigenetic Profile of Farm Animals

The epigenetic profile of animals can be measured by various methods that detect and quantify different types of epigenetic marks across the genome. Some of the common methods are:

- Bisulfite sequencing: This method converts unmethylated cytosines to uracils, while leaving methylated cytosines unchanged, and then sequences the DNA to identify the methylation status of each cytosine (Li et al.,2011).

- Chromatin immunoprecipitation (ChIP): This method uses antibodies to pull down DNA fragments that are bound by specific histone modifications or other chromatin-associated proteins, and then sequences or hybridizes the DNA to identify the genomic regions enriched for those modifications or proteins (Gade & Kalvakolanu, 2012).
- Non-coding RNA sequencing: This method isolates and sequences different classes of non-coding RNAs, such as microRNAs, long non-coding RNAs, or circular RNAs, and then maps them to the genome or transcriptome to identify their expression levels and targets (Cable et al., 2021).
- Single-cell epigenetic profiling: This method combines single-cell isolation techniques with epigenetic assays to measure the epigenetic marks of individual cells, and then uses computational methods to infer the epigenetic age or cell type identity of each cell (Clark et al., 2016).

Current Studies on Epigenetic Markers in Livestock

Current research on epigenetic markers in livestock has also demonstrated that epigenetic markers is used as tools to improve animal productivity and health through various strategies. For instance, epigenetic markers can be used to identify animals with desirable traits or to predict their breeding values based on their epigenetic profiles (Ibeagha Awem & Wang, 2020; Whelan et al., 2023). Epigenetic markers are also used to manipulate gene expression by using epigenetic drugs, dietary supplements, or gene editing techniques(Ibeagha Awem & Wang, 2020; Whelan et al., 2023). Epigenetic markers are also be used to monitor animal health and welfare by detecting epigenetic changes associated with stress, inflammation, or infection (Ibeagha Awem & Wang, 2020; Whelan et al., 2023).

However, current research on epigenetic markers in livestock faces several challenges and limitations that need to be addressed. Some of the challenges include the complexity and diversity of epigenetic mechanisms, the lack of standardized methods and protocols for epigenetic analysis, the ethical and social implications of epigenetic interventions, and the need for more funding and collaboration among researchers (Ibeagha Awem & Wang 2020; Whelan et al.,2023).Some of the limitations include the scarcity of epigenetic data and resources for livestock species, the difficulty of integrating epigenetic information with genomic and phenotypic data, the uncertainty of the stability and heritability of epigenetic marks, and the lack of causal evidence for the effects of epigenetic marks on animal traits (Ibeagha Awem & Wang, 2020; Whelan et al., 2023).

The Potential of Epigenetic Markers for Improving Animal Breeding Programs

The potential of epigenetic markers for improving animal breeding programs lies in their ability to provide additional information and control over gene expression and animal performance. Epigenetic markers can modulate the expression of genes involved in various biological processes that affect animal traits of economic importance. Epigenetic markers can also mediate the effects of maternal nutrition, fetal programming, and early-life experiences on animal development and health (Ibeagha Awemu & Zhao,2015; Wang & Ibeagha Awemu, 2020).

Epigenetic markers can be used as tools to improve animal breeding programs through various strategies. For instance, epigenetic markers can be used to identify animals with desirable traits or to predict their breeding values based on their epigenetic profiles (Ibeagha Awemu & Zhao, 2015; Wang & Ibeagha Awemu, 2020).Epigenetic markers can also be used to manipulate gene expression by using epigenetic drugs, dietary supplements, or gene editing techniques(Ibeagha Awemu & Zhao,2015; Wang & Ibeagha Awemu,2020).Epigenetic markers can also be used to monitor animal health and welfare by detecting epigenetic changes associated with stress, inflammation, or infection (Ibeagha Awemu & Zhao,2015; Wang & Ibeagha Awemu, 2020).

Challenges and Limitations in the Use of Epigenetic Markers

Epigenetic biomarkers are molecular features that can reflect the influence of environmental factors on gene expression, without altering the DNA sequence. They can provide valuable information about the function and regulation of genes, as well as the risk and progression of diseases. Epigenetic biomarkers can be measured using various technologies, such as PCR, sequencing, and pyrosequencing, on different types of biological samples, such as blood, tissue, urine, and saliva. However, there are several challenges and limitations in the use

of epigenetic biomarkers in the clinical laboratory, such as the lack of standardization, validation, and interpretation of epigenetic data, as well as the ethical and social implications of epigenetic testing (Rozek et al ., 2014).

Epigenetics is the study of how environmental factors can affect gene expression without changing the DNA sequence. Epigenetics encompasses four main research subfields: gene expression, molecular epigenetics, clinical epigenetics, and epigenetic epidemiology. Each subfield has a different focus and perspective on what constitutes the environment and how it interacts with genes and epigenetics. Epigenetics researchers use various discursive strategies to describe the relationships between genes, epigenetics, and environment, such as metaphors, analogies, and models (Pinel et al., 2019).

Epigenetic markers are molecular elements that mediate the communication between genes and environment. They can be understood as signals that enable a cell to remember past events and respond to current stimuli. Epigenetic markers can modulate gene expression by adding or removing chemical groups to DNA or histones, which are proteins that wrap around DNA. The most common epigenetic markers are DNA methylation and histone modifications. Epigenetic markers can be influenced by various environmental factors, such as diet, stress, infection, pollution, and drugs (Zovkic et al., 2013).

Epigenetic biomarkers have potential applications in various clinical settings, such as diagnosis, prognosis, classification, and treatment of diseases. For example, epigenetic biomarkers can be used to detect cancer at an early stage, predict the response and resistance to therapy, identify subtypes of cancer with different outcomes and therapeutic options, and monitor the recurrence and progression of cancer. Epigenetic biomarkers can also be used to assess the risk and susceptibility to complex diseases, such as cardiovascular disease, diabetes, obesity, and neurodegenerative disorders (Kamińska et al., 2019).

Machine learning is a branch of artificial intelligence that can analyze large and complex data sets using algorithms that learn from data. Machine learning can be applied to epigenetic data to identify patterns, associations, and predictions that are not easily detectable by conventional statistical methods. Machine learning can also help to integrate epigenetic data with other types of data, such as genomic, transcriptomic, proteomic, metabolomic, and phenotypic data. Machine learning can facilitate the discovery and validation of novel epigenetic biomarkers for clinical purposes (Rauschert et al., 2020).

Machine learning also faces some challenges and limitations in the analysis of epigenetic data. One challenge is the high dimensionality and sparsity of epigenetic data, which means that there are many more features (such as CpG sites) than samples (such as patients), and that many features have missing or zero values. Another challenge is the heterogeneity and variability of epigenetic data across different tissues, cells, individuals, populations, and environments. A third challenge is the causality and interpretability of machine learning models, which means that it is not always clear how machine learning models make predictions or what they imply for biological mechanisms or clinical implications (Brasil et al ., 2021).

Future Directions for Research on Epigenetic Markers in Animal Health and Productivity

a. Epigenetic variation is the diversity of epigenetic states among individuals or populations, which can result from genetic variation, environmental variation, or stochastic variation. Epigenetic variation can contribute to phenotypic variation and plasticity, as well as evolutionary potential and adaptation. Epigenetic variation can be measured using various techniques, such as bisulfite sequencing, chromatin immunoprecipitation, and RNA sequencing, which can reveal the patterns and levels of epigenetic modifications across the genome.

b. Epigenetic biomarkers are epigenetic features that can be used to diagnose, predict, or monitor diseases or traits in animals. Epigenetic biomarkers can reflect the effects of environmental factors on gene expression and disease susceptibility or resistance. Epigenetic biomarkers can also provide information about the function and regulation of genes, as well as the interactions between genes and environment. Epigenetic biomarkers can be detected in various biological samples, such as blood, tissue, milk, hair, and saliva.

c. Epigenetic processes can respond to environmental factors and underlying genotypes to influence animal health and productivity. For example, epigenetic processes can affect animal growth and development, milk yield and quality, wool quality and quantity, meat quality and quantity, reproduction and fertility, disease resistance and immunity, behavior and welfare, and adaptation and resilience. Epigenetic processes can also be manipulated by various interventions, such as nutrition, stress management, breeding strategies, and gene editing.

d. Epigenetic processes can also have transgenerational effects on animal health and productivity. For example, epigenetic processes can mediate the effects of maternal nutrition, stress, infection, or exposure to toxins on offspring development and performance. Epigenetic processes can also transmit the effects of paternal nutrition, stress, infection, or exposure to toxins on offspring health and fertility. Epigenetic processes can also influence the inheritance of acquired traits or adaptations across generations.

e. Epigenetic research faces some challenges and limitations in animal health and productivity. One challenge is the complexity and variability of epigenetic processes across different tissues, cells, individuals, species, and environments. Another challenge is the lack of standardization and validation of epigenetic methods and data analysis. A third challenge is the ethical and social implications of epigenetic research and applications, such as animal welfare, consumer acceptance, regulatory issues, and intellectual property rights.

Conclusion

Epigenetic markers are molecular features that reflect and modulate the effects of environmental factors on gene expression and phenotypic traits in animals. They have various implications for animal health and productivity, such as influencing stress response, immune function, metabolism, behavior, and adaptation. They can also be used as biomarkers for disease diagnosis, prognosis, classification, and treatment. In addition, they can be inherited across generations, mediating the effects of parental experiences on offspring performance. Moreover, they provide novel insights and opportunities for improving animal health and productivity. However, they face some challenges and limitations, such as the complexity and variability of epigenetic processes, the lack of standardization and validation of epigenetic methods and data analysis, and the ethical and social implications of epigenetic research and applications. Therefore, epigenetic research requires interdisciplinary collaboration, rigorous methodology, and careful consideration of its potential benefits and risks for animals and humans.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

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An Implantable Bio-Electro Scaffold for Brain Tissue Engineering

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Abstract One of the main challenges for neural tissue engineering is to design scaffolds suitable for implantation that allow cell proliferation to provide an environment similar to the extracellular matrix. Here, films of poly(vinyl alcohol-co-vinyl) acetate (PAcVA) were printed with gold-functionalized nanotubes of rotavirus VP6 protein (nVP6Au) in ordered patterns in serie and in parallel to form the µChipVP6Au. The obtained bio-electrical material resulted in a biocompatible scaffold suitable for the culture of the embryonic mouse hypothalamus cell line mHypoE-N1. Then, the bio-electrical scaffold was implanted in the mice's parietal cortex with no toxic effects observed. Our results showed that our bio-electrical implantable scaffold has tremendous potential as a platform for exogen cell implantation, a system for drug delivery, and an electrical scaffold for electrostimulation.

Keywords: Polyvinyl alcohol co-vinyl acetate, Neural tissue engineering, Brain implant, Nanotubes, Parkinson's disease.

Introduction

Conventional approaches to treating neurological disorders primarily rely on pharmacological and surgical interventions. Unfortunately, these methods often focus on providing symptomatic relief rather than addressing the root causes of the conditions. The current therapeutic landscape for neurological disorders presents significant challenges due to two key factors.

Firstly, a lack of structural support hinders the regeneration of damaged tissue and the filling of lesion cavities. Secondly, the unique characteristics of each medical case, with their inherent heterogeneity, further complicate the development of effective treatments. As a result, there is a pressing need for innovative and transformative approaches to tackle neurological disorders and provide meaningful, long-lasting solutions (Pina et al., 2019; Gilmour et al., 2020).

Tissue engineering has emerged as a promising and innovative approach that combines biological components with materials to restore damaged tissues. In the realm of neural tissue engineering, the quest for the ideal scaffold is underway. This scaffold should possess several key attributes to effectively support tissue regeneration. First and foremost, it must be non-toxic, ensuring neuronal cell viability, growth, and migration. Additionally, the scaffold should promote electrochemical communication between cells, mirroring the intricate signaling pathways of the brain. Furthermore, it should exhibit mechanical properties akin to the brain, providing the necessary support and flexibility. Lastly, an ideal scaffold would offer a controlled release of substances, facilitating the targeted delivery of therapeutic agents. By harnessing the potential of tissue engineering, we aim to unlock innovative solutions for restoring damaged neural tissue and advancing the frontiers of medical science (Zhang et al., 2014: Shafiee and Atala, 2017).

Hydrogels are promising materials for tissue engineering, offering many advantageous properties. Notably, their high-water content closely resembles that of soft tissues, making them an excellent mimic for biological environments. Hydrogels are polyelectrolytes that exhibit remarkable capabilities, such as the controlled release of small molecules with opposite charges. This ability to slow down cargo release significantly reduces the burst release issue. Furthermore, these hydrogels possess a unique attribute known as "smartness," which enables them to adapt their internal network in response to various external stimuli, including temperature, pH, ultrasound, glucose or urea concentrations, and potential differences. Termed "smart hydrogels," this class of materials has revolutionized the field of tissue engineering by facilitating the design of tailored implants that can be customized according to the specific needs of individual patients. With their impressive versatility and responsive nature, smart hydrogels are promising to advance regenerative medicine and develop personalized therapeutic solutions (Echeverria Molina et al., 2021; Peressotti et al., 2021).

Our previous research introduced an innovative synthetic scaffold, PAcVA, demonstrating the exceptional potential for mammalian cell culture and controlled drug release. This scaffold comprises poly(vinyl alcohol) crosslinked with glutaraldehyde and further functionalized with poly(L-lysine) and laminin. By manipulating the cross-linking degree, we could precisely regulate both the model molecule's release rate and the scaffold's mechanical properties. Our aim was to develop a material that closely resembles the stiffness of brain tissue. Building upon this foundation, our latest study introduces an exciting advancement: utilizing nanotubes derived from the VP6 protein of rotavirus (nVP6). To enhance its properties, we have further functionalized the nVP6 with gold nanoparticles (nVP6-Au) and coated the PAcVA scaffold with this composite. This coating provides several benefits, including creating a conductive surface, increased surface roughness, and a structure that mimics the architecture of the extracellular matrix. By incorporating these enhancements, we aim to establish a scaffold that supports cell growth and controlled drug release and replicates the intricate characteristics of the natural tissue environment (Plascencia-Villa et al., 2009; García-García et al., 2019).

In this study, we set out to create a remarkable nano-biorganometallic composite known as μChipVP6-Au. The composite is constructed through a three-layered approach, each layer serving a unique purpose. The superficial layer comprises a monolayer of neuronal cell culture, providing an ideal cell growth and interaction environment. This layer sets the foundation for the integration of our innovative composite. The intermediate layer is where the true innovation lies. We have implemented printed circuits of nVP6-Au onto the surface of the PAcVA scaffold. This strategic addition enhances the conductivity and surface roughness of the scaffold, unlocking a range of exciting possibilities. By incorporating nVP6-Au, we aim to optimize the performance of the composite, allowing for enhanced electrical properties and improved interaction with surrounding tissues. The lower layer consists of a PAcVA film with the unique ability to release substances in a controlled manner. This controlled release mechanism opens up possibilities for precise drug delivery and targeted therapy, ensuring optimal therapeutic outcomes. We conducted a proof-of-concept experiment to validate the feasibility and functionality of our μChipVP6-Au composite. The device was loaded with L-DOPA and intracranially implanted in mice exhibiting Parkinsonian-like symptoms. We aimed to restore motor coordination in these mice by administering L-DOPA in situ through our composite. Through rigorous evaluation, we determined the efficacy of our μChipVP6-Au in facilitating the restoration of motor function. By combining cutting-edge materials, innovative circuitry, and controlled release capabilities, our study showcases the immense potential of the μChipVP6-Au composite. Our findings open up new avenues for developing advanced therapeutics and personalized treatment strategies for neurological disorders such as Parkinson's disease.

Materials and Methods

Synthesis of PAcVA Films

PAcVA films were obtained according to the methodology reported by Villanueva-Flores et al. (2019).

Production and Characterization of nVP6

The nVP6-Au were obtained and characterized according to Plascencia-Villa et al. (2009).

µChipVP6-Au Construction

Electronic circuits were designed with the Autodesk Inventor® mechanical design software. Circuits were engraved in 1 mm of thickness in poly(methyl methacrylate) (PMMA) molds with a press machine (Minitech®, FP0R4) with a Nakanishi E3000 controller. PMMA molds were used to make stamp replicas in Poly (dimethyl siloxane) (PDMS) (Sigma, Sylgard®184) of 1 mm of thickness. PDMS polymerization was performed using a 10:1 ratio of base (Sylgard®, 3097366-1004) and curing agent (Sylgard®, 3097358-1004). The mixture was homogenized for 5 min with a Dremel® hand drill at 150 rpm and degassed under vacuum for 12 min. PDMS polymerization reaction was performed for 1 h at 60° C in a Luzeren® PCDE-3000 oven. Stamps were removed from the mold and incubated for 3 h at 150° C. Stamps were cut with a razor to adjust them to the size of the PAcVA films.

nVP6-Au printing patterns on PAcVA were performed by applying 80 µL of a solution of nVP6-Au at 2.1 mg mL^{-1} on the stamp's surface at room temperature protected from light for 15 min. The sample excess was removed with a micropipette and compressed air Silimex Aerojet 360® for 5 s. Subsequently, the seal was placed on PAcVA films of 0.5 mm of thickness, and a constant force of 1.0 N was applied for 5 min in a microcontact machine constructed by Cerón-Vera, F., and Hautefeuille M. Micrographs of the µChipVP6-Au were taken with a Nikon DS-Qi1Mc camera coupled to a Nikon Eclipse TE300 microscope.

Scanning Electron Microscopy (SEM) and X-Ray Absorption Spectroscopy (XAS)

Three mL of mHypoE-N1 ($50x10^3$ cells mL^{-1}) were cultured on the µChipVP6-Au placed on a *Chamber Slides™* (Thermo Fisher Scientific, 154534) for 8 days with the protocol described above and washed three times with Hank's solution (Thermo Fisher Scientific, 14025076) at room temperature. The cells were fixed with a 2.5% glutaraldehyde solution (Sigma, G7651) previously filtered through 0.22 µm membranes (Merck, SLGS033SB) at 4° C for 2 h. Samples were washed 3 times with deionized water and dehydrated with ethanol (Merck, 107017) in serial dilutions at room temperature: 10% 2 h, 20% 2 h, 30% 1 h, 40% 1 h, 50% 1 h, 60% 1 h, 70 % 1 h, 80% 1 h, 90% 1 h, 95% 1 h, 97.5% 1 h, 99% 1 h and 100% 1 h (3 times) avoiding cells exposure to air. Dehydrated samples were coated with carbon. SEM images and X-ray elemental analysis were performed with a JSM 5600-LV microscope.

Intracranial Implantation of µChipVP6-Au in a Murine Model of Parkinson Disease

Prior experiments, all protocols were evaluated, authorized, and performed according to the bioethical standards of the Bioethics Committee of the Institute of Biotechnology. For each experiment, 8 mice per group were analyzed. Experiments were performed using male adults C57/6BL mice, 8 weeks old, and 25-30 g of weight. Parkinsonian-like phenotype was induced based on the protocol described by Jackson-Lewis and Przedborski (2007). Four doses of 30 mg kg^{-1} of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-hydrochloride tetrahydropyridine (MPTP) (Sigma Aldrich, M0896) were administered with intervals of 2 h between each to groups of at least 8 mice each. With this MPTP dose, more than 90% of dopaminergic neurons are expected to die.

Behavioral Tests

Parkinsonian-like phenotype and the effect of the implantation of µChipVP6-Au were verified by measuring a) gross motor coordination, and c) fine motor coordination according to (Fleming et al. 2013; Jackson-Lewis and Przedborski 2007).

Surgical Procedure

Fourteen days after administration of MPTP, 20 mg kg^{-1} of pentobarbital (Pisa®) was administered intramuscularly as general anesthesia. Mice's heads were shaved and cleaned with a 10% iodine solution (GERMISIN®). Mice were placed in a mouse stereotaxic apparatus (Stoelting Co.), adjusting the ear canal fixators and jaw. The skin was incised 2 cm with a No. 10 scalpel blade (Carl Martin, 871B/10) longitudinally. It was dissected by planes, removing the periosteum until bregma was visible. Skull was climbed with a drill (Mototool Mini, D 80) up to the encephalic cortex. A sample of 2 mm^2 of µChipVP6-Au or PacVA films were implanted manually in the right parietal cortex. The control group was treated with an identical surgery procedure without introducing any implant. Skull osteosynthesis was performed with dental acrylic (QUARZ), covering the trepanation hole, and the integuments were sutured with Nylon 6-0 thread (Atramat®). After the surgical procedure, mice were kept individually in a box with a radiant lamp to heat.

Tissue Analysis by Hematoxylin-Eosin Staining

Five days post-operation, mice were sacrificed by cervical dislocation, and histological sections were cut in the implant zone. First, mice brains were extracted, placed in 12-well plates (Costar®, CLS3513-50EA), and covered with 4 mL of sterile PBS pH 7.2. PBS was removed to fix the tissue, and 4 mL of 10% formaldehyde (Sigma Aldrich, 252549) solution were added. Samples were incubated at room temperature for 24 h, protected from light. Successive dehydrations were performed by adding ethanol (Merck, 107017) at 70% for 1 h, 80% for 1 h, 95% for 1 h, and 100% for 1.5 h in sufficient quantity to cover the tissues. Ethanol was discarded, and xylene (Sigma Aldrich, 214736) was added three times, each incubating for 1.5 h. Mice brains were placed in cardboard molds of 3 cm^3, embedded in paraffin previously heated in a water bath at 60° C. Samples were dried for 2 h at room temperature. Then, blocks were cut into 5 µm thick sheets with a microtome (Abatec, KD-3368AM) and placed on glass slides (Fisherbrand™). Slides were dried in air for 30 min, protected from dust, and incubated at 45° C for 12 h in a Luzeren® PCDE-3000 oven. The slides were washed 3 times for 10 min to remove paraffin with xylene (Sigma Aldrich, 214736). To rehydrate the lamellae, 100%, 95%, and 80% ethanol (Merck, 107017) was added twice for 3 min. Each solution is in sufficient quantity to cover the lamellae completely. Ethanol was discarded, and samples were washed three times with distilled water. Tissue staining was performed by adding hematoxylin (Sigma, H3136) for 10 min in sufficient quantity to cover the slide completely. Then, lamellae were washed 3 times with tap water, each for 5 min, so that salts in the tap water favored hematoxylin color change from violet to purple. To remove excess staining in the samples, slides were rapidly dipped in a 0.5% hydrochloric acid (HCl) solution (Merck, 320331) diluted in absolute ethanol (Merck, 107017) 3 times and washed for 10 min each. Then, samples were washed 3 times with distilled water for 15 min each. Lamellae were immersed in 0.2% eosin (Merck, HT110116) for 1 min. Slides were washed with distilled water 3 times for 15 min each; slides were dehydrated, as mentioned before, with ethanol and treated with xylene. Samples were placed on glass slides (Corning, 12-553-464), and a 50% glycerol mounting medium (Sigma, G2025) was added. A coverslip was placed on the top (Corning, S17525B), taking care not to leave bubbles; the edges were sealed with transparent nail polish (Maybelline®, Super Stay 7d) until analysis. Micrographs were taken with a Nikon DS-Qi1Mc camera and a Nikon Eclipse TE300 microscope.

Intracranial Release of L-DOPA by µChipVP6-Au in Parkinsonian-like Mice

To validate the effectiveness of the µChipVP6-Au, we conducted an experiment where PAcVA films measuring 2 mm^2 were implanted into the brain cortexes of Parkinsonian-like mice. Before implantation, the materials were loaded with a dosage of 120 mg kg^{-1} of L-DOPA/Carbidopa (Cloisone® Psicofarma® 250 mg/25 mg), adjusted

according to the weight of the mice. To serve as controls, we included groups of Parkinsonian mice, healthy mice without surgical intervention, and Parkinsonian-like mice with either the µChipVP6-Au or PAcVA films without the loaded drug. The results from these groups were compared to a group that received the same drug dosage (30 mg kg^{-1} daily for 4 days, reaching a total of 120 mg kg^{-1}). We assessed spontaneous activity, as well as gross and fine movements, using previously established evaluation methods.

Results and Discussion

Construction of the µChipVP6-Au

As a scaffold for neuronal culture, PAcVA is limited by its poor roughness and electrical conductivity. Here, circuits in serie (Fig. 1a) and parallel (Fig. 1b) of nVP6-Au were printed on PAcVA films to create an electrical connection between distal points, which can serve as electrodes for neuronal electrical stimulation. As is shown in Figs. 1a and 1b, nVP6-Au pathways are well defined. Some gold aggregates are observed. On the one hand, the circuits in serie nVP6-Au are connected, creating a pathway of 8 cm in length and sharing the same input and exit of electrical current. This array allows us to evaluate the efficiency of the nVP6-Au printing. Any failure in the connectivity across the nVP6-Au pathway will interrupt the flux of electrical current. On the other hand, circuits in parallel have multiple nVP6-Au pathways of 1 cm in length and share the same input and exit of electrical current. Circuits in parallel show a higher resistance than circuits in serie; however, if a failure in the connectivity in the nVP6-Au pathways occurs, the current flux can continue through the others.

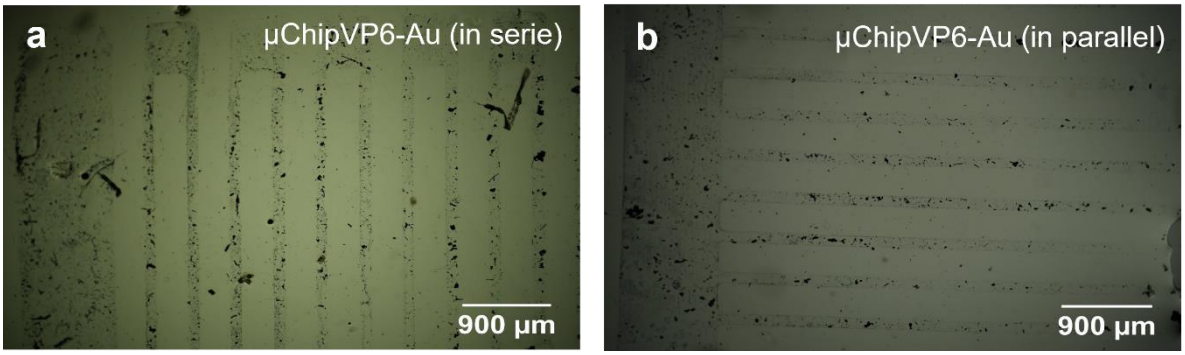

Figure 1. *nVP6-Au printed on PAcVA films to form µChipVP6-Au.* Microcrographs of electronic circuits of nVP6-Au in a) series and b) parallel.

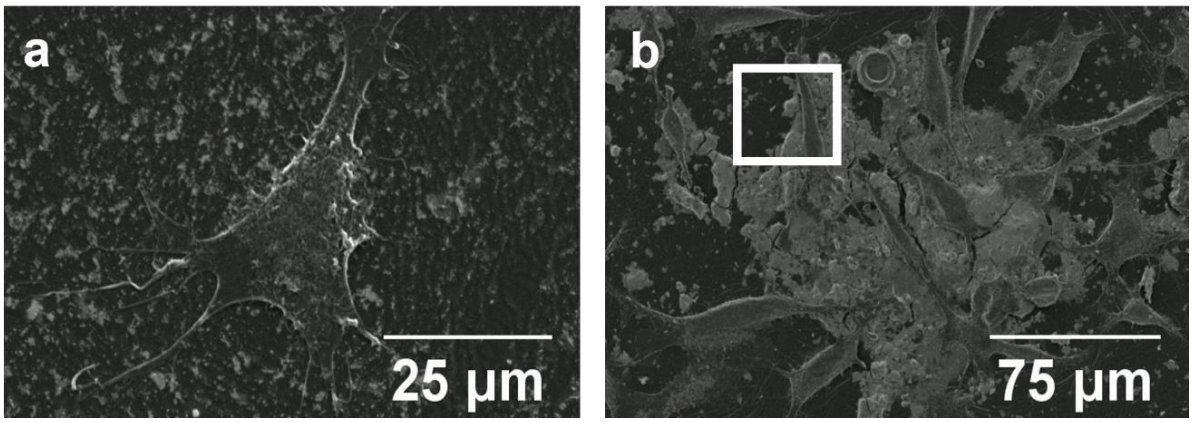

Figure 2. *Biocompatibility of µChipVP6-Au with mammalian cells.* Micrographs shown in (a) and (b) refer to SEM images of mHipoE-N1 cells grown on µChipVP6-Au at different magnifications.

Biocompatibility of µChipVP6-Au

Designing electric scaffolds for neural tissue engineering must consider structural and chemical differences between living and inert matter. µChipVP6-Au has soft elements, such as PAcVA and nVP6, and rigid elements, such as AuNPs. Interaction between substrate and neuron is a vital parameter to guarantee the cell viability and the effectiveness of the electrical contact. Figures. 2a and 2b, show mHypoE-N1 cells gown on the µChipVP6-Au. Neurite's extension to attach the cell to the substrate can be observed.

Brain Implantation of the µChipVP6-Au

Several devices can be successful *in vitro* but might fail under *in vivo* conditions, causing implant rejection, toxicity, inflammation, or undesired drug release profiles. The µChipVP6-Au was intracranially implanted in Parkinsonian-like mice to evaluate the *in situ* release of L-DOPA and to evaluate the restoration of motor coordination. Our results were compared to the same dose of drug administered via intraperitoneal. Three mice from each group were sacrificed on the fifth-day post-implantation. Histological sections were stained with hematoxylin-eosin to visualize inflammation, necrosis, or apoptosis processes. Hematoxylin forms hematein that stains the chromatin of the nucleus, ribosomal aggregations of the cytoplasm, and other acid structures in a blue-purple color. On the one hand, eosin stains basic cellular components such as cytoplasm in pink. Eosin staining is useful for identifying centrally nucleated fibers and the variation in cell diameter when tissue is inflamed (Prophet EB, 1992).

Fig. 3a shows a histological section of the cerebral cortex of mice that were not subjected to any surgical procedure where some reddish coloration corresponding to the basal number of eosinophils and no necrotic features are seen. Fig. 3b, shows a minimum and no pathological inflammation because of the surgical procedure. Fig. 3c and Fig. 3d show histological sections of the implantation site's periphery of PAcVA and µChipVP6-Au, respectively. No signs of apoptosis or necrosis were observed, or pathological inflammation associated with the presence of PAcVA or µChipVP6-Au in greater levels than that caused by the surgical procedure *per sé*.

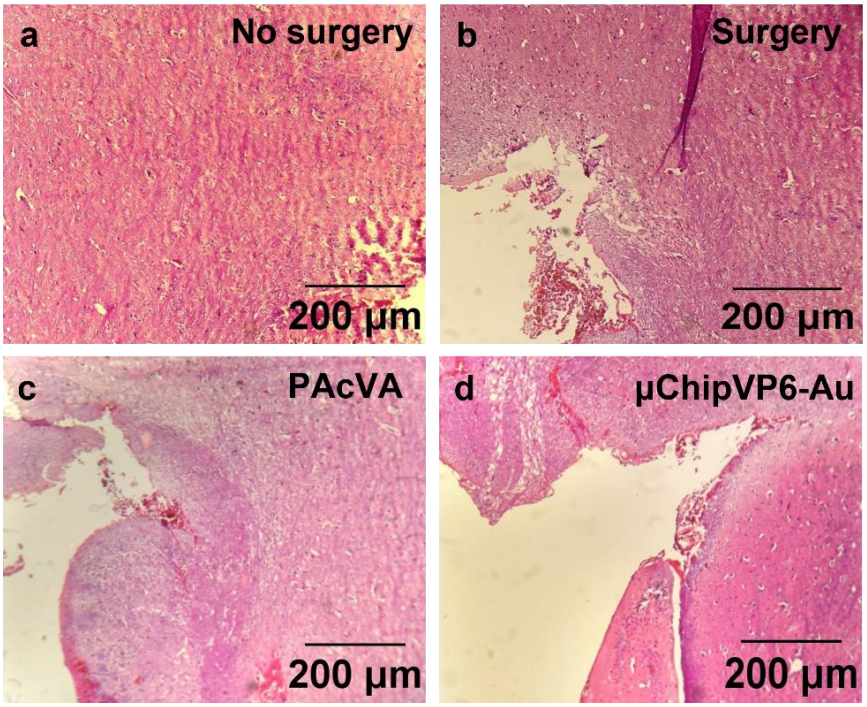

Figure 3. *Implantation of PAcVA and µChipVP6-Au in mouse cerebral cortex and hematoxylin-eosin histological staining.* a) No surgical procedure, b) Tissue after 5 days of surgery with no material implanted. Tissue after 5 days of surgery with 25 mm^2 of c) PAcVA and d) µChipVP6-Au.

L-DOPA *in situ* Release by the µChipVP6-Au in Parkinsonian-like Mice

Parkinson's disease (PD) is a neurodegenerative disorder of the nervous central system. PD is characterized by neuronal loss in the substantia nigra and striatal dopamine deficiency. PD symptoms are bradykinesia, tremor, dementia, depression, and anxiety. L-DOPA (L-3,4-dihydroxyphenylalanine) is the major drug administered for PD treatment. L-DOPA compensates dopamine depletion in PD (Poewe W., et al., 2017). Deep brain stimulation (DBS) can be prescribed for patients who do not respond to medication. DBS consists of electrical stimulation through an electrode that is implanted by stereotaxic surgery controlled by a subcutaneous pulse generator (Williams A., et al., 2010). Considering the electrical properties and capacity for controlled drug release of µChipVP6-Au, it can be applied for both PD treatments.

As a first approach, motor coordination in Parkinsonian-like mice was evaluated when treated with L-DOPA administered via intraperitoneal or through the brain implant. Groups of 8 Parkinsonian-like mice were implanted with PAcVA or µChipVP6-Au loaded with 120 mg kg^{-1} (mouse weight) of L-DOPA. As a positive control, Parkinsonian-like mice were intraperitonally administered with the same dose of L-DOPA. As negative controls, mice implanted with empty PAcVA or µChipVP6-Au were used. As it was expected, mice had a greater number of errors in the track as the width decreased.

The results of gross motor coordination are depicted in Fig. 4a, revealing a noteworthy trend. As anticipated, mice exhibited an increased number of errors on the track as the width decreased. However, our findings indicate that the administration of L-DOPA effectively restored gross motor coordination, mirroring the control group's performance. Importantly, this restorative effect was observed irrespective of whether L-DOPA was administered through the empty µChipVP6-Au or PAcVA methods, as both approaches demonstrated no discernible impact on the mice's behavior. Similarly, our investigation into fine motor coordination (Fig. 4b) yielded strikingly similar outcomes. Notably, the administration of L-DOPA via both intraperitoneal and intracranial routes successfully reinstated fine motor coordination, further highlighting the potential therapeutic efficacy of L-DOPA.

It is worth highlighting that Villanueva-Flores et al., (2019) reported that the drug release efficiency of PAcVA was less than 10% of the total amount of immobilized drug. Therefore, a 10-fold lower dose of L-DOPA was administered through the implant compared to intraperitoneal via to obtain the same results. A lower dose of administered L-DOPA could decrease the side effects of the drug (LeWitt PA., 2015). Additional studies are required to confirm it. Our results also demonstrate that ntVP6-Au do not play a significant role in the motor coordination of mice under our experimental conditions.

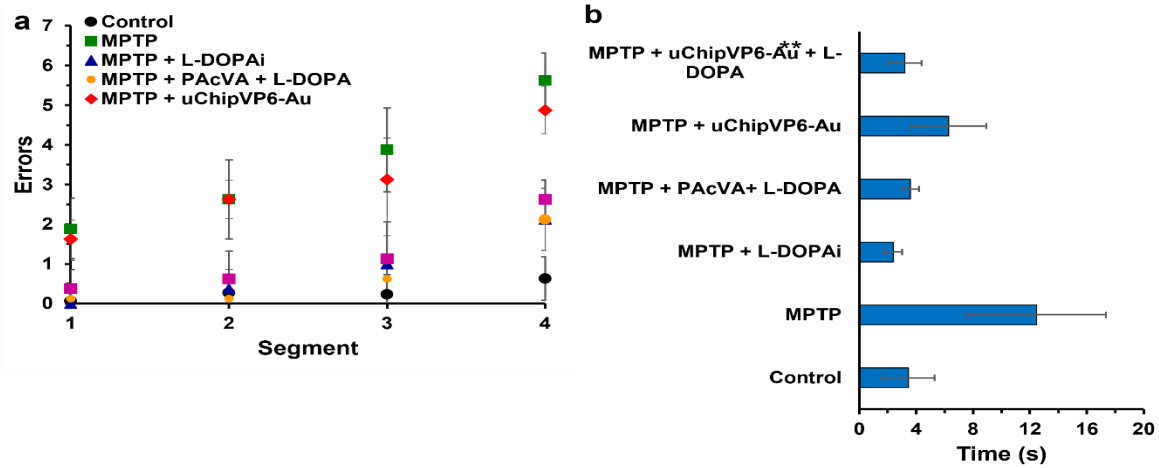

Figure 4. *Behavioral tests of Parkinsonian-like mice treated with 120 mg kg^{-1} of L-DOPA administered intraperitoneally (L-DOPAi) or intracranially (L-DOPA) through a PAcVA implant or the µChipVP6-Au.* The effect of PAcVA or µChipVP6-Au without L-DOPA was also analyzed as a negative control. a) Gross motor coordination, b) Fine motor coordination. (n = 8). Error bars represent the standard deviation.

Conclusions

This work contributes to the *state of the art* of scaffolds design for neural tissue engineering. To our knowledge, this is the first bio-organometallic implant based on viral proteins conjugated to precious metals printed on a new stimulus-responsive hydrogel. Unlike classical passive implants reported in the literature, μChipVP6-Au shows tremendous potential because it can be simultaneously applied as a scaffold for cell culture with the potential for cell replacement therapies, as well as *in situ* drug release and electrical stimulation. This expands the classical concept of passive scaffolds to active scaffolds that may play an interesting role in the future of regenerative medicine.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Author contributions

* FVF: Conceptualization, methodology, validation, formal analysis, investigation, writing original draft, and visualization.
* ACL: Conceptualization, methodology, validation, formal analysis, investigation,
* MH: Conceptualization, methodology, validation, formal analysis, investigation,
* LAP: Conceptualization, validation, formal analysis, resources, editing of original draft, supervision, and funding acquisition.

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Detecting Locus of Resistance to Scab in Local Apple Varieties in Uzbekistan Using Molecular Genetic Markers

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Abstract: The apple tree (Malus sp.) is the most cultivated plant in the world. Apple orchards cover a large area. scab (Venturia inaequalis) causes significant damage to the economies of many countries that are engaged in the production of apple trees. In Uzbekistan, most of the orchards are apple orchards. The present study includes a molecular genetic analysis of the resistance of local apple cultivars to pathogens Venturia inaequalis in local apple cultivars grown in Uzbekistan and the results of identification of resistance genes. The paper presents the results of molecular genetic analysis of the combination of scab resistance genes (Vf, Vfa1, Vfa2) found in 15% of samples. In 58.7% of the studied samples, a “high level of resistance” to scab was revealed.

Keywords: Polymorphism, Genetic markers, Phytopathogens, V. *inaequalis*

Introduction

The subfamily Maloideae to which the apple tree belongs includes 22-25 genera and about 600 species, which grow mainly in the temperate zone of the northern hemisphere. The genus Malus, belonging to this subfamily, is the most important in terms of the national economy. This genus has a wide distribution in various eco-geographical conditions. The range of the genus is within Europe, Asia and North America. The center of origin of the domestic apple tree is the Central Asian Genetic Center (Pereira-Lorenzo et al.,2009).

Eleven years ago, the nucleotide sequence of the apple tree was published (Tartarini et al., 1999), which opened up prospects for further study of the apple genome. The use of molecular genetic technologies, in particular, sequencing of the apple tree genome, revealed that the ancestor of Malus domestica is the wild species Malus sieversii (Velasco Riccardo et al., 2010).

SSR analysis has already been successfully applied to the apple tree to identify intraspecific polymorphism, determine varietal affiliation, and build a genetic map of the species Malus domestica (Gianfranceschi et al., 1996; Guilford P et al., 1997; Hokanson et al., 1998).

At present, a large number of microsatellite (SSR) apple markers are known and even their database, GDR (Genome Database for Rosaceae), has been created, which provides information on markers of many Rosaceae, including apple. Information about the apple markers themselves is available at https://www.rosaceae.org/search/markers. The first large group of SSR markers, in the amount of 140 pieces, which served as the basis for the database and further research, was developed by a group of European researchers (Liebhard et al., 2002). Scab is a widespread disease of the apple tree, causes a decrease in the quality of fruits, and in the years of epiphytosis is the cause of mass abscission of ovaries and premature leaf fall. Affecting seedlings, causes them to stun growth and reduce the quality of planting material. Different varieties of apple trees are affected by scab to varying degrees

Currently, at least 20 scab resistance genes are known. The first 8 of them: Va, Vb, Vbj, Vg, Vf, Vh, Vm and Vr are well studied and identified over 15 years ago. At present, the apple genome has markers linked to scab resistance genes Vf, Vh2, Vh4, and Vbj (Gessler et al., 2006). Later, several more genes were identified, and a new nomenclature was proposed.

Method

Sample Collection and DNA Isolation

109 samples were selected from about 70 varieties from the Surkhandarya, Khorezm regions of Uzbekistan and the Republic of Karakalpakstan.

Selection of Molecular Markers

Based on the analysis of the literature, markers identifying genes for resistance to apple scab, bacterial blight, and powdery rose were studied. STS marker VfC . Afunyan M.R. regularly belongs to the Vfa1, Vfa2, Vfa4 genes in the scab resistance locus Vf, the codominant Al-07, the dominant marker AM-19, disseminated by Tartarini et al., are specific to the Vf gene (Rvi6). There are also multiplex scab resistance kits using fluorescent markers CH-Vf (Vf), CH01d03 (Vg), CH02c02a (Vr2), Hi07h02 (Vm).

Table1. Molecular markers used to identify resistance genes

Disease	Marker	Gene	Primers	Product Size
Scab	VfC	Vfa1, Vfa2, Vfa4	F: 5`-ggtttccaaagtccaattcc-3` R: 5`-cgttagcattttgagttgac-3`	286, 484, 646 bp
	AL07	Vf, vf	F: 5'-tggaagagagatccagaaagtg-3' R: 5'-catccctccacaaatgcc-3';	466, 724 bp
	AM19	Vf	F: 5'-cgtagaacggaatttgacagtg-3' R: 5'-gacaaagggcttaagtgctcc-3'	526 bp

PCR Process

The PCR process for detection of resistance genes was carried out using a lyophilized ready-made PCR core. The total volume of the mixture of reaction products was 20 µl, including 1 µl of forward and reverse primers (0.1–0.5 µM), 1 µl of gDNA (≥20 ng) was added. The PCR amplification process was carried out in a Thermocycler VERITI amplifier according to the following program: initial denaturation at 94 °C for 5 minutes, then 35 cycles: 94 °C – 25 seconds, 56 °C – 30 seconds, 72 °C – 35 seconds , the final elongation was carried out at 72°C for 5 minutes. The amplification products were separated by gel electrophoresis on a 2.5% agarose gel. 100 bp DNA marker was used to determine the length of the amplicons.

Results and Discussion

The study included 109 local varieties of apple trees from different regions of Uzbekistan – Surkhandarya, Khorezm regions and Karakalpakstan, which were assessed for the presence of Vf genes associated with resistance to scab (Venturia inaequalis) using DNA markers.

The VfC 1/2 marker was used to identify the Rvi6 scab resistant gene in local apple varieties. PCR products should generate 286, 484, 646 b.p. fragments on the electrophorogram. Fragments 484, 646 bp produced by the VfC 1/2 marker belong to both resistant and susceptible genotypes of apple varieties to powdery mildew, and apple genotypes with a fragment of 286 bp indicate the presence of the powdery mildew resistance gene Rvi6 (Fig. 1).

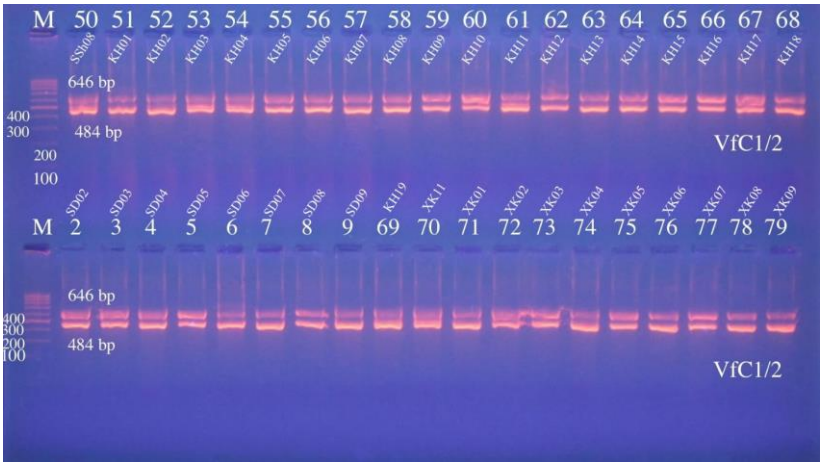

Figure 1. Amplicon fragments of the VfC1/2 marker. PCR product. M-marker.

The following distribution of allelic variants of genetic loci was revealed: Vfa1 (646 bp) was detected in 39 samples, Vfa2 (484 bp) in 106 samples, Vfa4 (286 bp) was not detected in any sample, AM19 Vf (526 bp) in 39, AL07 Vf (466 bp) was not detected in any sample, AL07 vf (724 bp) was present in all 109 samples (Fig. 2).

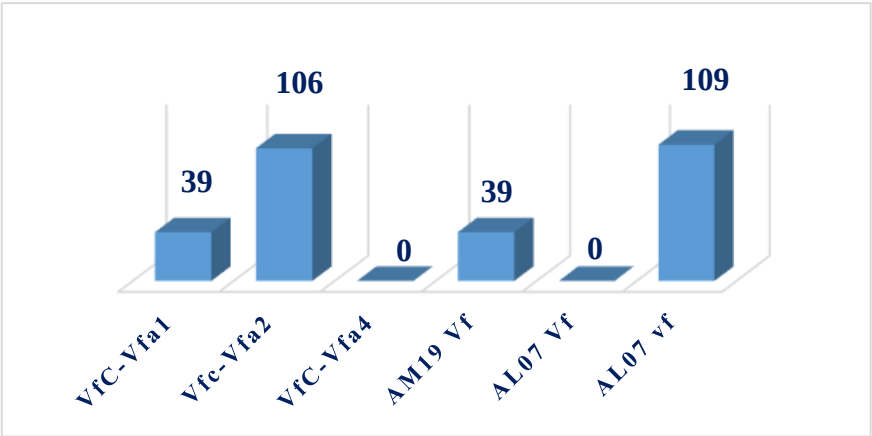

Figure 2. Distribution of occurrence of allelic variants of scab resistance genes for the three markers used

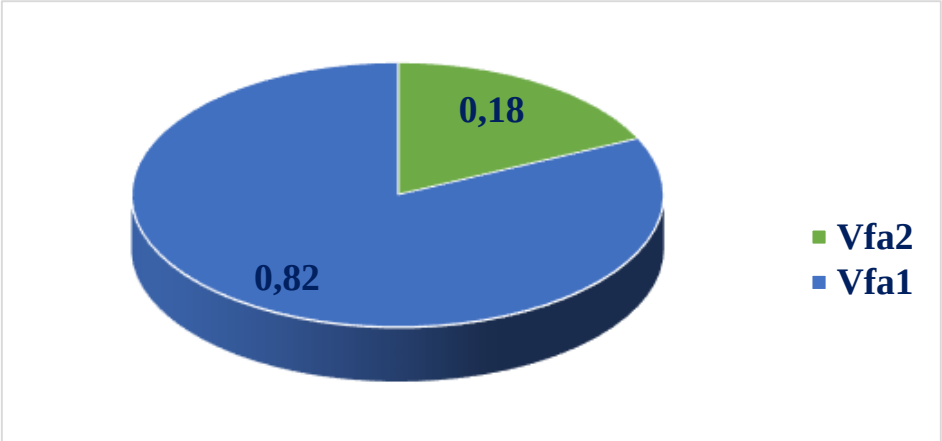

Figure 3. Distribution of Vfa1 and Vfa2 genotypes by VfC marker

Analysis of the distribution of genotypes revealed the presence of 2 homozygous genotypes for Vfa1, 72 homozygotes for the Vfa2 gene, and heterozygous Vfa1/Vfa2 genotypes were detected in 35 (32.1%) samples. The frequency distribution of Vf variants of the scab resistance gene in the apple population was presented as follows: Vfa1 - 0.18, Vfa2 - 0.82 (chi2=0.94; p=0.33). Thus, the frequency distribution of the three genotypes is in accordance with the equilibrium distribution of genotypes according to the Hardy-Weinberg law. A graphical representation of the frequency distribution of genotypes and alleles is shown in Fig. 3 and Fig. 4, respectively.

At least 10 scab resistance genes Vg, Vh2, Vh4, Vm, Vf, Va, Vbj, Vb, Vd and Vr2 are known (Ozgonen et al., 2007; Maric et al., 2010). For simplicity, Bus et al. (2009) proposed a new nomenclature for the above genes Rvi1, Rvi2, Rvi4, Rvi5, Rvi6, Rvi10, Rvi11, Rvi12, Rvi13 and Rvi15, respectively. In this work, we used the old nomenclature.

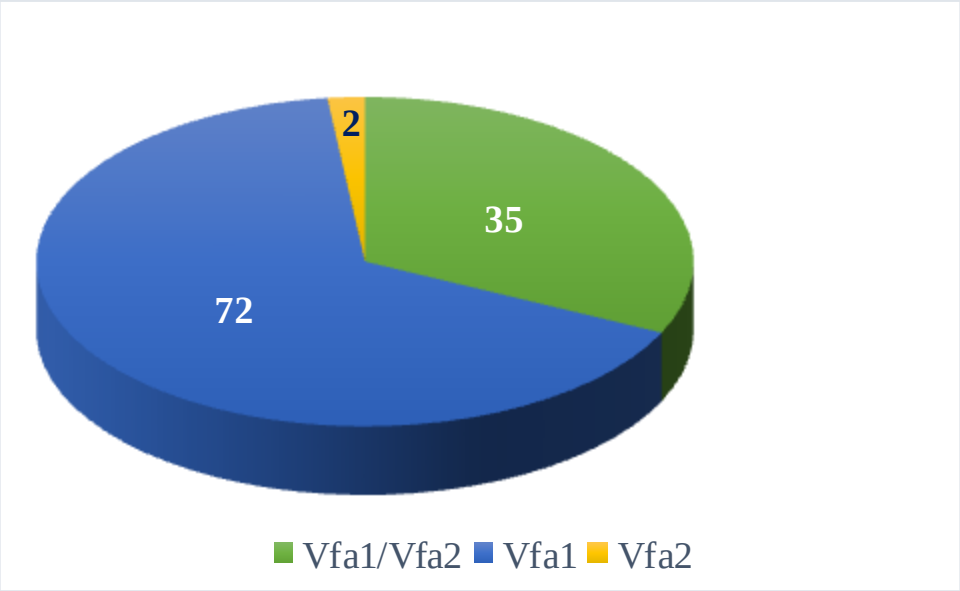

Figure 4. Frequency distribution of Vfa1 and Vfa2 variants by VfC marker

Of the 10 genes listed above, we decided to start with the genotyping of apple cultivars for the Vf genes, since this locus is the best studied and was one of the first identified in Malus floribunda, and this apple species has become the most widely used in breeding for the transfer of the resistance gene to commercial apple cultivars. scab.

In our study using the VfC marker, the Vfa1 gene was detected in 39 samples, Vfa2 in 106, and in 35 samples the presence of both (heterozygous) variants (Vfa1/Vfa2) was detected, while Vfa4 was not detected in any sample. It should be noted that in only two varieties of M.domestica, namely, "Besh barmok" (XS01) and "Kandolma" (XS13), the Vfa1 gene was detected in the homozygous state. Thus, according to the VfC marker, all the studied samples are to some extent resistant to scab (races 1 to 5).

According to previous studies, the use of the AL07 marker makes it possible to identify two variants of the Vf and vf gene (Tartarini et al., 1999; Patrascu et al., 2006), and the Vf variant found in Malus floribunda is dominant (manifested even in the heterozygous - Vf vf state) and is responsible for resistance to scab, while the vf variant is not associated with resistance.

In all analyzed samples for the AL07 marker, the Vf variant associated with resistance was not detected. Thus, according to the AL07 marker, all the studied samples are to some extent resistant to scab. Both markers - VfC and AL07 are codominant, i.e. they allow to detect the presence of at least two variants, while the AM19 Vf marker is a dominant marker, i.e. only one variant of the target Vf locus (yes/no) is detected during the analysis (Patrascu et al., 2006).

According to previous studies on a large sample of plants, the AM19 marker is much closer to the Vf genes than AL07; therefore, its linkage to the target resistance locus is less susceptible to recombination. According to the results of our study, the dominant marker AM19 made it possible to identify the Vf locus in 39 apple varieties.

Thus, the scab resistance gene for the AM19 marker was detected in 39 accessions, while the absence of this locus was found in 70 apple varieties.

Based on the obtained data of molecular genetic identification of Vf genes using three DNA markers, a table of the degree of resistance of each variety was compiled. The degree of resistance to each variety was assigned conditionally depending on the presence or absence of a locus of resistance or instability. Since the resistance locus Vf (AL07) was not detected in any sample in our study, its total contribution, as well as its recessive variant (vf), was not taken into account.

A value of 0 corresponds to the complete absence of marker loci associated with scab resistance genes (not detected in our study). A value of 1 was assigned if only the Vfa1 locus was present. A value of 2 was assigned if Vfa1 and Vfa2, or Vfa1 and Vf (AM19) loci were present. A value of 3 was assigned if only the Vfa2 locus or Vfa1, Vfa2 and Vf (AM19) was present. A value of 4 was assigned if both Vfa2 and Vf (AM19) loci were present in the variety. Analysis of the data showed that the main number (58.7%) of the samples has a 3 degree of stability, followed by samples with 2 and 4 degrees of stability 19.3% and 20.2%, respectively (Table 2).

Table 2. Distribution of varieties according to the degree of resistance to scab based on the genotype.

Degree of stability	Number of samples	Genotype (combinations of loci)
0	0	-
1	2	*Vfa1*
2	21	*Vfa1* + *Vfa2*, либо *Vfa1* + *Vf* (AM19)
3	64	*Vfa2*, либо *Vfa1*+*Vfa2* + *Vf* (AM19)
4	22	*Vfa2* + *Vf* (AM19)

The absence of the M.*floribunda* 821 locus in some resistant apple varieties presented in Uzbekistan indicates that these varieties may have been obtained without the participation of the genetic material of M. floribunda 821, or another locus, which provides resistance, was transferred to some races of the pathogen.

Conclusion

Among the studied samples of the apple population, the Vf and Vfa1 genes were found in 35.8% of the samples, and the Vfa2 gene in 95.4%. The distribution combination of resistance genes showed that the Vfa2 and Vfa1 gene was found in 18 samples (16%), the Vfa2 and Vf gene in 22 samples (20%), Vfa1 and Vf only in 1 sample, the Vfa2, Vfa1 and Vf genes were found in 16 samples (15%), Vfa2 and vf genes in 48 samples (44%).

Thus, it was revealed that among the local varieties of apple trees, Maisky and Yanar showed high resistance to scab in terms of molecular genetic indicators, and the varieties Atlas olma, Turkish, Khuboni, Renat Semerenko, Shoyi olma, Besh bermok showed resistance to scab. Of the 109 samples of local varieties of apple trees studied, molecular genetic analyzes revealed high resistance to scab in 19.6% of the samples.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

This article was presented as an poster presentation at the International Conference on Medical, Health and Life Sciences (www.icmehels.net) held in Budapest/Hungary on July 06-09, 2023.

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Bioactive Component Analysis of Dices of Pomegranate Fruits in Different Phenotype

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Abstract: Pomegranate fruit is a type of fruit belonging to the family of Lythraceae, consisting of shell, kernel, grains and dice. In this study, pomegranates of 3 different genotypes (local name: deve dişi, hicaz and nuz ekşisi) harvested (October-December), which are widely cultivated in the province of Gaziantep, Oğuzeli, were collected, and after identification, the dice sections were removed and dried in the shade. Total antioxidant and total oxidant levels, animicrobial activities, DNA protective activity and total flavanoid and total phenolic content were determined in 18 extracts in 3 different solvents (methanol, ethanol and DMSO) with solid-liquid extraction. Kirby-Bauer disc diffusion method was used to determine antimicrobial properties. According to the results obtained, 100% of Escherichia coli, Stenotrophomonas maltophilia, Staphylococcus aureus strains were resistant to all membrane extracts. While antioxidant levels of all membrane extracts were found highly and oxidant levels were found to be low. It was determined that the extracts have the potential to protect DNA against oxidative damage caused by UV and H_2O_2. Total phenolic determination by Folin-Ciocalteu method; Total flavonoid amount was measured by $AlCl_3$ using colorimetric method. Compared to the solvent and extraction methods used, high results were obtained in both phenolic and flavonoid determinations in all extracts, although they differed slightly among themselves. While the pomegranate fruit is consumed as food, the dice and shell parts are discarded. According to our study results, it is thought that alternative new products that can be used in complementary medicine can be obtained from pomegranate membranes that have a rich bioactive composition.

Keywords: DNA, UV, Pomegranate, Phenolic, Flavonoid

Introduction

Pomegranate is a type of fruit from the cinnagi family, consisting of hundreds of particles that form the fruit body with small seeds, with a slightly sour and sweet taste depending on the type, grown in temperate climates. Pomegranate basically consists of 4 main parts. These parts are; It consists of shell, kernel, grains, and white membrane. Pomegranate consists of 60-67% grains and 33-40% peel. The juice of pomegranate fruit is also made from 76-85% fruit grains and 45-61% whole fruit. Pomegranate fruit consists of 75% moisture, 1.6% protein, ascorbic acid 16 mg/1000 g, ash 0.7%, 0.58% acidity and high amount of minerals. The chemical composition of the pomegranate fruit varies according to the variety, growing region, climate, ripening, planting application and storage conditions (Jurenka, 2008) (Pooja et al., 2017). The pomegranate tree is a deciduous tree

and has spread to regions similar to the Mediterranean climate, which is the most suitable climate for the cultivation of superior pomegranates. Pomegranate is a temperate climate plant and requires high temperature levels to reach proper maturity. The maturity index is the ratio of soluble dry matter to total acidity, and this index is used to classify pomegranates as sweet, tart and negative. A maturity index of 31-98 is grouped as sweet, 17-24 as mayhos and 5-7 as sour species (Martinez et al., 2006).

The worldwide increase in interest in pomegranate and pomegranate-based products is not only related to the taste of the fruit, but there are many studies in scientific studies that prevent low-intensity and high-intensity cholesterol oxidation, lower blood pressure and reduce the development of vascular occlusion. In the emergence of these beneficial effects of pomegranate-based products, reference is made to phenolic compounds with antioxidant properties (Çam et al., 2009). Data from epidemiological studies show that consumption of fruits and vegetables high in phenolic compounds reduces mortality from cardiovascular, cerebrovascular diseases and cancer. Phenolic compounds show their beneficial effects by destroying free radicals. In recent years, the trend towards fruit juices such as grape juice and blueberry juice, which are high in phenolic antioxidants, has been increasing. Pomegranate juice is a popular fruit juice with its phenolic compounds and important biological activities (Husari et al., 2014; DiMarco-Crook et al., 2015).

The effects of pomegranate peel and seeds on health have been evaluated in different studies in the literature, but no study on pomegranate membranes has been found. Therefore, in this study, some bioactive component analyzes were studied in different extractions of pomegranate membranes.

Method

Antimicrobial Analysis

Kirby-Bauer disk diffusion method was used to determine the antimicrobial properties of 18 samples. The isolates were inoculated on MH agar medium using the spread method. This process was carried out in two stages. The density of the strains was adjusted according to the 0.5 McFarland standard and sowing was done. Paper discs containing 20 µl of plant extracts were placed on the surface of the petri dishes, which were inoculated immediately afterward, with the help of sterile forceps, and left for 24 hours of aerobic incubation at 35 °C. During this process, 22 mm between the discs and 14 mm from the edge of the petri dish so that the zones to be formed do not overlap each other. The distance was taken into account. Zone diameters were measured at the end of incubation. The results showed bacterial strains according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) document Version 10 criteria; assessed as resistant and susceptible. According to the results obtained, 100% of Escherichia coli, Stenotrophomonas maltophilia, Staphylococcus aureus strains showed resistance to all plant extract samples.

DNA Protective Activity

PBR322 plasmid DNA isolated from Escherichiacoli was used in the study to determine the DNA protective activity with pomegranate extracts created with ethanol, methanol and DMSO. The density of the gel used in the study was prepared according to the number of base pairs in the relevant DNA. With this method, it is aimed to determine whether the extracts we have created in the presence of UV rays and H_2O_2, which cause damage to DNA, have the potential to prevent DNA damage.

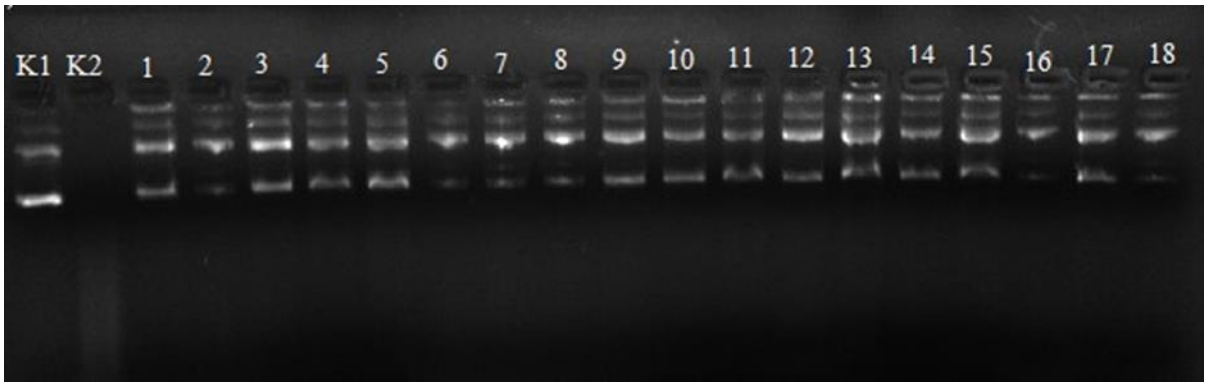

Figure 1. DNA protective activities band

It has been determined that the pomegranate extracts, especially the samples numbered 1,3,4,5,9,10,11,12,13,14,15 and 17, have a protective effect potential against oxidative damage caused by UV and H_2O_2. Therefore, it is thought that alternative new products can be produced by determining these compounds in pomegranate, which has a very rich bioactive composition. It is thought that the data obtained from this study have the potential to provide both ideas and data to new studies by creating data for researchers working in this field.

K1: Plazmit DNA (3 µl) + dH_2O (6 µl)
K2: Control: Plazmit DNA (3 µl) + dH_2O (6 µl)+ UV+ H_2O_2 (1 µl)

1. Plazmit DNA (3 µl) + NE Methanol Ultrasonic (4 hours) (5µl)+ UV+ H_2O_2 (1 µl)
2. Plazmit DNA (3 µl) + NE Ethanol (16 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
3. Plazmit DNA (3 µl) + NE Methanol (16 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
4. Plazmit DNA (3 µl) + NE DMSO Ultrasonic (4 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
5. Plazmit DNA (3 µl) + NE DMSO (16 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
6. Plazmit DNA (3 µl) + NE Ethanol Ultrasonic (4 hours) (5 µl) + UV+ H_2O_2 (1 µl)
7. Plazmit DNA (3 µl) + HN Ethanol Ultrasonic (4 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
8. Plazmit DNA (3 µl) + HN Ethanol (16 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
9. Plazmit DNA (3 µl) + HN Methanol (16 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
10. Plazmit DNA (3 µl) + HN Methanol Ultrasonic (4 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
11. Plazmit DNA (3 µl) + HN DMSO Ultrasonic (4 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
12. Plazmit DNA (3 µl) + HN DMSO (16 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
13. Plazmit DNA (3 µl) + DD DMSO Ultrasonic (4 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
14. Plazmit DNA (3 µl) + DD Methanol Ultrasonic (4 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
15. Plazmit DNA (3 µl) + DD DMSO (16 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
16. Plazmit DNA (3 µl) + DD Ethanol (16 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
17. Plazmit DNA (3 µl) + DD Methanol (16 hours) (5 µl)+ UV+ H_2O_2 (1 µl)
18. Plazmit DNA (3 µl) + DD Ethanol Ultrasonic (4 hours) (5 µl)+ UV+ H_2O_2 (1 µl)

Total Antioxidant and Total Oxidant Levels

Commercially available kits were used to determine the antioxidant capacity of pomegranate membrane extracts. Antioxidant substances in the sample convert the radical to the dark blue-green colored ABTS (2,2 AzinoBis (3-Ethyl Benzo Thiazoline-6- Sulfonic Acid) form of reduced ABTS, and the change in absorbance at 660 nm indicates the total antioxidant level of the sample. The synthetic antioxidant and Vitamin E analogue Trolox was used as a positive control (Halliwell et al., 1994). Rel Assay Diagnostics-TAS Assay Kit was used in this study. Total oxidant level determination test is calibrated with hydrogen peroxide. Ferrous ion in the presence of oxidant Oxidizes the chelator complex to ferric ion. The oxidation reaction intensity increases due to the presence of oxidants in the reaction medium. Ferric ion forms a colored complex in acidic medium. Color density is determined spectrophotometrically.

Table 1. Result of TAS

TAS Results mmol/L						
Sample No	1	2	3	4	5	6
	4,2322	4,2022	4,2285	4,2322	4,2472	4,191
Sample No	7	8	9	10	11	12
	4,1948	4,1873	4,2097	4,1873	4,1798	4,2509
Sample No	13	14	15	16	17	18
	4,2022	4,206	4,2285	4,1835	4,1723	4,1798

Table 2. Result of TOS

TOS Results mmol/L						
Sample No	1	2	3	4	5	6
	0,0273	0,0112	0,0169	0,0162	0,0069	0,0214
Sample No	7	8	9	10	11	12
	0,0064	0,0108	0,0233	0,0323	0,0564	0,1054
Sample No	13	14	15	16	17	18
	0,0114	0,0343	0,0179	0,0155	0,0137	0,0156

Mesurement of Total Phenolic Substance and Total Flavonoid Substance Amount

In the liquid extracts obtained by trying different extraction methods, the total amount of phenolic substance as gallic acid equivalent. The determination of the total flavonoid substance amount was measured as the equivalent of quercetin by UV-VIS spectrophotometer and photometric method. Total phenolic content was determined by the Folin-Ciocalteu method. Gallic acid was used as a standard and readings were taken at 760 nm. 0.5 N Folin reagent was prepared to be used in the experiment and 10% Na_2CO_3 was used as a color indicator. The stock standard was prepared at 1000 ppm with methanol and other standards were prepared by serial dilution. A calibration chart specific to the gallic acid standard was drawn and the total phenolic acid concentrations were determined with the absorbances obtained from the UV readings of the liquid extracts. Total flavonoid substance determination was made with $AlCl_3$, which is a colorimetric method. Quercetin was used as a standard and readings were made at 415 nm. A 2% $AlCl_3$ solution was prepared to be used in the experiment. The stock standard was prepared at 1000 ppm with methanol and other standards were prepared by serial dilution. A calibration chart specific to the quercetin standard was drawn and total flavonoid acid concentrations were determined by absorbances obtained from UV readings of liquid extracts.

Table 3. Total Phenolic Substance Results (mg\L)

Phenolic Substance Results (mg\L)						
Sample No	1	2	3	4	5	6
	6485,95	9137,97	6351,42	7679,43	5144,58	5276,38
Sample No	7	8	9	10	11	12
	3706,66	3507,8	4102,06	7057,79	4423,99	7127,8
Sample No	13	14	15	16	17	18
	4591,69	7043,05	11724,3	8581,92	7912,29	4336,3

Table 4. Total Flavonoid Substance Results (mg\L)

Flavonoid Substance Results (mg\L)						
Sample No	1	2	3	4	5	6
	495,98	2319,8	1148,55	1203,83	1647,7	1387,0
Sample No	7	8	9	10	11	12
	660,66	636,36	1462,01	1226,13	726,42	936,62
Sample No	13	14	15	16	17	18
	1248,83	1136,76	2080,32	2425,99	1297,01	1103,28

When the amounts of phenolic and flavonoid substances in the extracts prepared by co-solvent and co-extraction of three different pomegranate membranes were compared. In the extracts prepared with dimethylsulfoxide solvent and ultrasonic assisted extraction, the best phenolic substance results were obtained while the extracts prepared from the pulps of the muzzle were obtained in the classical extraction. When the amounts of flavonoid substances were examined, it was observed that the hijaz pomegranate membrane was high in ultrasonic assisted extraction. In the classical extraction, however, the nuz ekşi was found to be high.

Conclusion

In the pomegranate plant in Oguzeli, which is a priority in terms of rural development, only 45-50% of the fruit can be utilized in total by making only the juice of the pomegranate fruits or boiling the water and making the pomegranate syrup. The peel and seeds remaining during processing into pomegranate juice are industrially valuable waste products. Despite the active ingredient content, the peel, core and membrane parts (dice) are unfortunately in the category of waste materials due to the limited conditions and knowledge of the farmers. In our current study, it was determined that pomegranate membranes which are waste materials, showed good antioxidant properties and were UV protective against DNA. As a continuation of this rural development, we believe that the determination of the pharmacological bioactive components of the waste materials in the existing pomegranate processing plants and the evaluation of the materials released after the processing of the water will have an effective effect on the development in this area.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

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Analysis of Physiological and Biochemical Characteristics of Local Soybean Varieties which were Artificially Damaged with Phytopathogenic Fungi

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Abstract: According to the analysis of the results of the amount of chlorophyll "a" and "b" in the leaves of local soybean varieties under the influence of phytopathogenic micromycetes during the budding period was found that the chlorophyll "a" and chlorophyll "b" index of all soybean varieties decreased compared to the control. This situation indicates that the amount of chlorophyll "a" and chlorophyll "b" in the leaves of soybean plants is related to the presence of phytopathogenic micromycetes. When studying the effect of micromycetes *F. oxysporum* on the content of chlorophyll "b" in soybean leaves, the least effect was found in varieties Tomaris and Genetik-1 (respectively, the difference from the control = -9.3 and -9.6), and the local variety Sochilmas had the strongest influence (respectively, it showed that the difference from the control = -56.6 and -61.1). When studying the effect of micromycetes *A. alternata* on the amount of chlorophyll "b" in soybean plant leaves, the variety Genetik-1 had the least effect (respectively, the difference from Control = -15.4 and -14.3), and the strongest effect was on varieties Nafis and Sochilmas (respectively differences from control = -41.6 and -38.2).

Keywords: Soybean, Budding period, Flexibility, Chlorophyll, Phytopathogenic, Disease, Control, Experimental, Physiological.

Introduction

Soybean is one of the most important agricultural crops to meet the food demand of the world population. Soybean grains contain 18-24% fat, 36-40% protein, 26-34% carbohydrates, and 5-8% minerals (Arioglu, 2014). Legumes are highly valued worldwide as a low-cost alternative to meat and are the second most important food source after cereals. Legumes have a high nutritional value, providing people with proteins, essential amino acids, complex carbohydrates, minerals and vitamins (Maphosa, 2017). Soybean (*Glycine hispida Max (L.)*, or "golden bean"), which is considered a representative of this family, is an important plant on earth for its use in food and animal husbandry (Alexander, 1974).

Today, one of the main problems in all soybean-growing countries of the world is a contamination of soybeans with phytopathogenic micromycetes, which significantly damages the yield of this crop. 26-30% of the crop is

lost due to various diseases and external stress factors in soybean, like other crops worldwide (Oerke, 2004). So far, more than 30 diseases caused by fungi, bacteria, and viruses have been identified in soybean (Ranjan, 2019).

One of the most economically damaging diseases of leguminous plants is wilt disease caused by *fusarium* species. Many legume-growing countries suffer from this disease, which also affects the roots of the plant and causes the loss of a large part of the crop (Sampaio, 2020). More than 135 microorganisms have been studied in the soybean plant, but about 30 of them, mainly fungi, bacteria, and viruses, cause great economic damage (Roy, 2000).

Diseases caused by fungi are biotic stress factors that cause yield loss in legumes. Fungal diseases cause a reduction in the yield of leguminous crops from 15% to 80% (Horoszkiewicz-Janka, 2013). The spread of some fungal pathogens becomes an epidemic process, which causes the closure of plantations (Deneke, 2018). Alternaria disease in leguminous crops is caused by the fungus *alternaria alternata*, and this disease is one of the most common diseases in leguminous crops (Mamta Sharma, 2013). Some species of the genus *Alternaria* are saprophytes, while other species are phytopathogens that cause several diseases in agricultural crops (Thomma, 2003).

Pathogenicity tests showed that *Cephalosporium sp.*, *F. solani*, *F. oxysporum*, *F. verticillioides*, *R. solani* and *N. dahliae* were the most pathogenic fungi isolated from beans. The amount of chlorophyll "a", chlorophyll "b", and carotenoids significantly decreased in plants infected with these pathogens, while total phenols increased in diseased plants (Elwakil et al., 2009). These results showed that there is a correlation between the amount of total phenols, as well as chlorophyll "a", chlorophyll "b" and carotenoids in the plant tissues of the above fungal diseases.

Alternaria alternata causes leaf spot and leaf blight diseases in several agricultural crops, including soybeans, causing significant losses (Nelson, 2001). *Alternaria leaf spot*, caused by the necrotrophic fungus *A. alternata*, is one of the most severe foliar diseases in soybean-growing regions of the world, and its pathogenicity is increasing even in the current changing climate.

Method

As a research object, the local soybean varieties Genetic-1, To'maris, Baraka, Nafis, Sochilmas, and micromycetes of the *Fusarium* and *Alternaria* species, which is kept in the the unique scientific object collection of phytopathogens and other microorganisms of the Institute of genetics and plant experimental biology of the Academy of Sciences of the Republic of Uzbekistan were used. Scientific research was carried out in 2021-2022 in lysimeters and laboratory conditions of the regional experimental station of the Institute of Genetics and Experimental Biology of Plants of the Academy of Sciences of the Republic of Uzbekistan, located in the Kibray district of the Tashkent region.

Field experiments were conducted in specially protected lysimeters. In the 1st background (control) of the experiment, clean (phytopathogen-free) seeds of soybean varieties were sown in the soil. In background 2 (experiment), soybean seeds were sown together with sterile oat grain infected with phytopathogenic micromycetes. The infection levels of soybean varieties with phytopathogenic micromycetes were determined by visual assessment of plants at growth and development stages.

Chlorophyll "a", chlorophyll "b", total chlorophyll, carotenoid content in plant leaves from physiological indicators during the budding period of plants under lysimeter conditions in the influence of phytopathogenic micromycetes on local and foreign varieties of soybean Nayek Sumanta, et. all.. (2014.) was determined by the following formula given in works.

Ch-a=13.36A664 – 5.19 A649
Ch-b=27.43A649 – 8.12 A664
C x+c=(1000A470 –2.13Ca- – 97.63Cb)/209

Results and Discussion

The amount of chloroplast pigments in the leaves of soybean cultivars artificially infested with phytopathogenic fungi during the budding phase was studied. When the amount of chlorophyll "a" was studied during the

budding period of soybean, it was found that the amount of chlorophyll "a" in plants artificially infested with *F. oxysporum* and *A. alternata* was reduced in different degrees compared to the control plants. Among the soybean varieties infected with *F. oxysporum*, Baraka, Tomaris and Genetik-1 varieties had a high amount of chlorophyll "a" (3.19±0.11 mg/g, 3.28±0.41 mg/g and 3,01±0.24 mg/g), while Nafis and Sochilmas varieties had low values (1.66±0.34 mg/g and 1.61±0.27 mg/g, respectively) (Table 1) .

Table 1. Amount of pigments during budding period of local soybean cultivars.

Varieties	chlorophyll "a"			chlorophyll "b"		
	Control	*F.oxysporum*	*A.alternata*	Control	*F.oxysporum*	*A.alternata*
Genetic-1	3,83±0,26	3,01±0,24	3,08±0,11	1,74±0,06	1,32±0,32	1,09±0,61
Baraka	3,28±0,23	3,19±0,11	2,95±1,00	1,96±0,40	1,00±0,57	1,14±0,16
Tumaris	4,01±0,18	3,28±0,41	3,04±0,41	1,77±0,27	1,18±0,25	1,29±0,27
Nafis	3,15±0,09	1,66±0,34	1,88±0,18	1,93±0,09	1,10±0,14	0,83±0,23
Sochilmas	2,75±0,28	1,61±0,27	1,90±0,39	1,17±0,18	0,69±0,23	0,75±0,28

Among the soybean varieties infected with *A. alternata*, Genetik-1 and To'maris varieties had high chlorophyll "a" quantity (3.08±0.11mg/g and 3.04±0.41mg/g, respectively), while Sochilmas variety had a low quantity (1.90±0.39 mg/g). When the amount of chlorophyll "b" in the leaves of soybean cultivars was studied during the budding period, it was found that the amount of chlorophyll "b" in the plants artificially infested with *F. oxysporum* and *A. alternata* was reduced in different degrees compared to the control. Among the soybean varieties infested with *F. oxysporum*, the amount of chlorophyll "b" was high in To'maris and Genetik-1 varieties (1.18±0.25 mg/g and 1.32±0.32 mg/g, respectively), in Sochilmas variety had a low index (0.69±0.23mg/g).

Among the soybean varieties infested with *A. alternata*, To'maris variety had a high chlorophyll "b" quantity (1.29±0.27mg/g), while Sochilmas variety had a low quantity (0.75±0.28mg/g). The amount of carotenoids in the leaves of soybean cultivars was also studied. As a result of the action of phytopathogenic fungi, it was found that the amount of carotenoids in the leaves increased in different degrees compared to the control. Among soybean varieties which were artificially infected with *F. oxysporum*, the highest carotenoid content index was found in To'maris variety (1.50±0.18 mg/g), and the lowest indicator was found in Sochilmas variety (0.99±0.09 mg/g) (2 -table).

Table 2. Carotenoid content index results

№	Varieties	Total chlorophyll			Carotenoid		
		Control	*F.oxysporum*	*A.alternata*	Control	*F.oxysporum*	*A.alternata*
1	Genetic-1	5,57±0,10	4,34±0,40	4,17±0,66	1,32±0,10	1,30±0,25	1,62±0,34
2	Baraka	5,24±1,28	4,19±0,74	4,09±0,12	0,91±0,70	1,39±0,16	1,04±0,14
3	Tumaris	5,78±0,33	4,46±0,48	4,33±0,49	0,64±0,25	1,50±0,18	1,48±0,25
4	Nafis	5,08±0,15	2,76±0,41	2,71±0,33	0,90±0,23	1,11±0,18	1,19±0,16
5	Sochilmas	3,92±0,45	2,30±0,30	2,65±0,33	0,71±0,27	0,99±0,09	1,09±0,04

Amount of pigments during budding period of local soybean cultivars.Among soybean varieties which were artificially infested with A. alternata, the highest index of carotenoid content was determined in Genetic-1 variety (1.62±0.34mg/g), and the lowest index in Baraka variety (1.04±0.14mg/g). In conclusion, when the amount of chloroplast pigments of local soybean varieties artificially infested with phytopathogenic fungi was studied, it was found that To'maris, Genetik-1 and Nafis varieties were more resistant to F. oxysporum and A. alternata than other varieties.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors

Acknowledgements or Notes

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Two Cases of Left Hip Heterotopic Ossification Treated with Surgery and their Outcomes Postoperatively

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Abstract: 52-year-old woman was admitted to Trauma and Orthopedics department (TOD) due to septic arthritis (SA) in left hip joint (LHJ). Patient underwent three consecutive open debridement procedures. During the treatment, an intracerebral hematoma was discovered. Upon rehabilitation patient indicated severe pain in LHJ that impaired movement. Three months after admission CT showed heterotopic ossification (HO) in LHJ. Patient was recommended to complete treating postinfectious complications and refer to TOD for further evaluation and discussing treatment options. Five months after the first presentation, complete mechanical block could be seen in LHJ due to HO. Open left hip ossificate resection and LHJ redressation were performed. During procedure significant increase in range of motion (ROM) was obtained. Iatrogenic transection of the femoral nerve occurred and was treated surgically. After surgery, patient was subjected to early rehabilitation and later forwarded to rehabilitation department. Increase of active and passive ROM remained. 34-year-old man was admitted to TOD due to polytrauma, including multiple hip and acetabular fractures. Patient underwent three surgical interventions due to fractures. Month after admission, postoperative deep tissue infection in the fracture and Covid-19 were discovered and treated. Two years later, patient returned to TOD complaining about significant impairment of ROM in LHJ due to HO, which was surgically removed. Surgical treatment is rarely chosen for treatment of HO since alleviation of pain is not worth the risks, and HO can reoccur, preferring treatment with exercise and indomethacin, but when a significant increase of ROM is expected, surgical treatment is justified. These case reports show how surgical treatment of HO with mixed origin in patients with good expected outcomes and high involvement in their own rehabilitation should be at least considered. It is important to emphasize both pre and postoperative rehabilitation and prophylaxis.

Keywords: Heterotopic ossification, Myositis ossificans, Postinfectious osteoarthritis, Mechanical block, Range of motion, Septic arthritis

Background

Heterotopic ossification (HO) is a frequent complication in rehabilitation settings that consists of the formation of mature, lamellar bone in the extraskeletal soft tissue. The suggested classification proposes division in three types – traumatic, *myositis ossificans progressiva* and neurogenic HO. It most often develops after joint

arthroplasty, burns, stroke, or spinal cord injury. The most common locations for HO development are areas highly susceptible to trauma, such as knee, elbow, hip and shoulder joints. (Mavrogenis et al., 2011). A rare pediatric form of HO is *myositis ossificans progressiva*, a rare metabolic disease where the muscle ossifies. About 10-20% of patients with traumatic brain injury develop HO; however, the prevalence has been reported from 0,2% to 4% after thermal injury and up to 30% after certain types of hip arthroplasty or acetabular fractures. *(*Zhu et al., 2015) Factors impacting the development of HO are the mechanism of injury, duration of immobilization and degree of spasticity. Moreso pressure ulcers, long bone fractures and edema have also been noted as factors increasing the risk of development of HO. (Ranganathan et al., 2015; Weppner et al., 2012) Newer research has also found correlation in HO in patients after hip arthroplasty with African American race, osteoporosis and low estrogen states (Singh et al., 2022).

There are no guidelines for definitive treatment of HO; however prophylaxis and perioperative use of non-steroidal anti inflammatory drugs (NSAIDs) have shown beneficial outcomes. The mechanism proposed is that by inhibiting the osteogenic progenitor cells from differentiating into bone progenitor cells, it can help reduce the risk of development of HO. *(Ranganathan et Al. 2015)* Since risk factors include immobilization and spasticity, passive and active development of movement in the affected joint is suggested. In most cases prophylaxis can prevent or alleviate the symptoms associated with HO, the most severe of them being pronounced reduction of range of motion (ROM), pain, nerve compression and ankylosis of the affected joint (Sun & Hanyu-Deutmeyer, 2022).

Surgical treatment is indicated in cases when the patient has decreased functionality due to the ossificates, the pain cannot be alleviated or there are signs of vascular or neural compression (Denomandie et al., 2018). The available data suggest different approaches for the surgery. While some research suggests waiting 12-18 months before the ossificate has completely matured depending on the etiology, other researchers encourage to perform early surgical intervention to minimize the risk of developing an intra-articular pathology, osteoporosis and ankylosis. Different surgical approaches have been described in various sources, depending on the localization of the ossificates. (Genet et al., 2009; Ranganathan et al., 2015; Yoon et al., 2018). Radiation therapy is also suggested for prophylaxis postoperatively to decrease the risk of re-development of HO (Lee et al., 2022).

Case Reports

Mixed Origin Heterotopic Ossification of the Left Hip

A 52-year-old woman was admitted to the Emergency Department at Riga East Clinical University Hospital "Gaiļezers" after generalized seizure with disturbed consciousness. According to anamnesis, the patient was abusing alcohol for a long period of time. Upon further evaluation the patient described pain in left hip and knee joints with decrease in ROM. Based on the patient's allegations, there was no previous pain or decreased ROM in upper mentioned joints.

Evidence of inflammation was found within the blood draw and LHJ aspirate. Patient was hospitalized in Riga East Clinical University Hospital Toxicology and Sepsis clinic. Upon arrival, the patient's condition was severe, requiring mechanical ventilation and treatment in the intensive care unit. A *Staphylococcus Aureus* sepsis was discovered the same day and due to multi organ dysfunction syndrome with renal failure the patient underwent hemodialysis.

Upon admission patient had Blood alcohol level 0,2 g/L, Leukocytes 7,42 10^9/L, Neutrophils 6,85 10^9/L, Erythrocytes 3,24 10^12/L, Hemoglobin 10,50 g/dL, Hematocrite 30,9%, Thrombocytes 81 10^9/L INR 0,88 Fibrinogen 0,52 g/L, CRP 231,7, ALAT 81, ASAT 377, Potassium 3,14 mmol/l, Procalcitonin 8,12 ng/mL, and Creatinine kinase 3408 U/L.

The urine sample showed positive Nitrite test, Protein 3 g/L, Erythrocytes 200 Ery/mkL, cloudy appearance, Albumin 150 mg/L, Albumin/Creatinine ratio 33,9 mg/mmol and Protein/Creatinine ratio 170 mg/mmol. Urine was positive for *Candida sp.* 100 000 CFU/mL.

The X-ray showed signs of an osteoarthritis (Figures 1&2) while the CT showed fluid collection in the LHJ between *m. obturatorius externus* and *m. pectineus* (3,5 x 5,6 x 3,1 cm) (Figures 3&4), LHJ III-degree degenerative osteoarthritis with synovitis, subcortical cysts and marginal osteophytes and liver steatosis. The CT of thoracic cavity and head was without any major changes.

Bronchioalveolar fluid was positive for *Staphylococcus aureus* < 1000 CFU/mL.

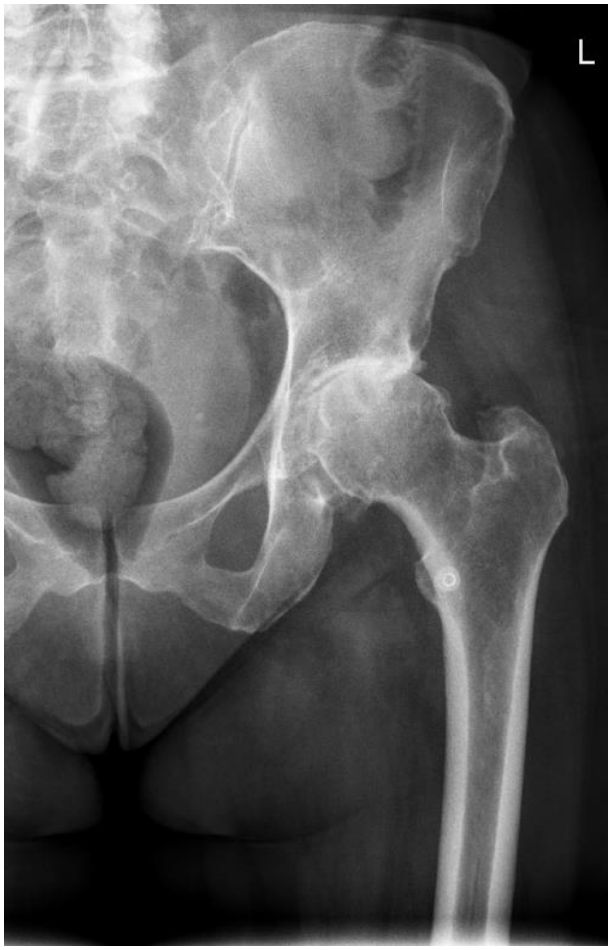

Figure 1. LHJ degenerative osteoarthritis, X-ray AP projection.

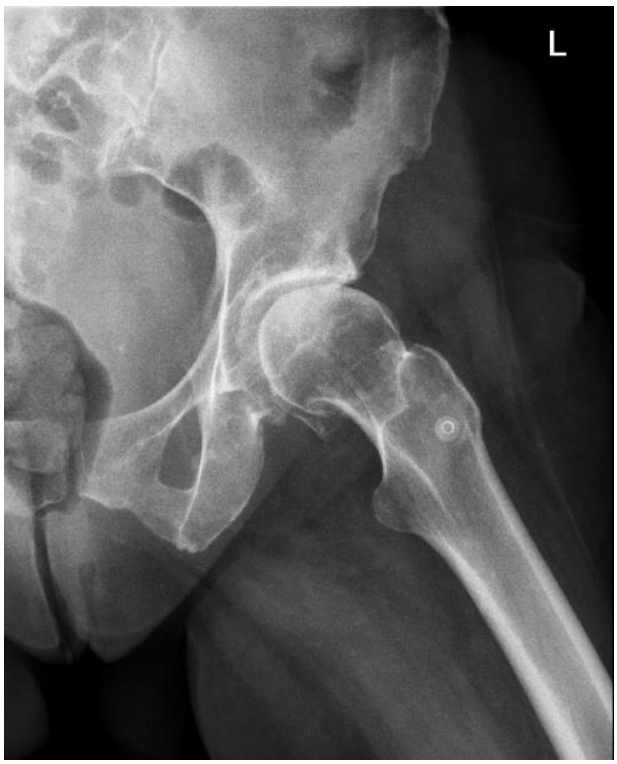

Figure 2. LHJ degenerative osteoarthritis, X-ray Lauenstein projection.

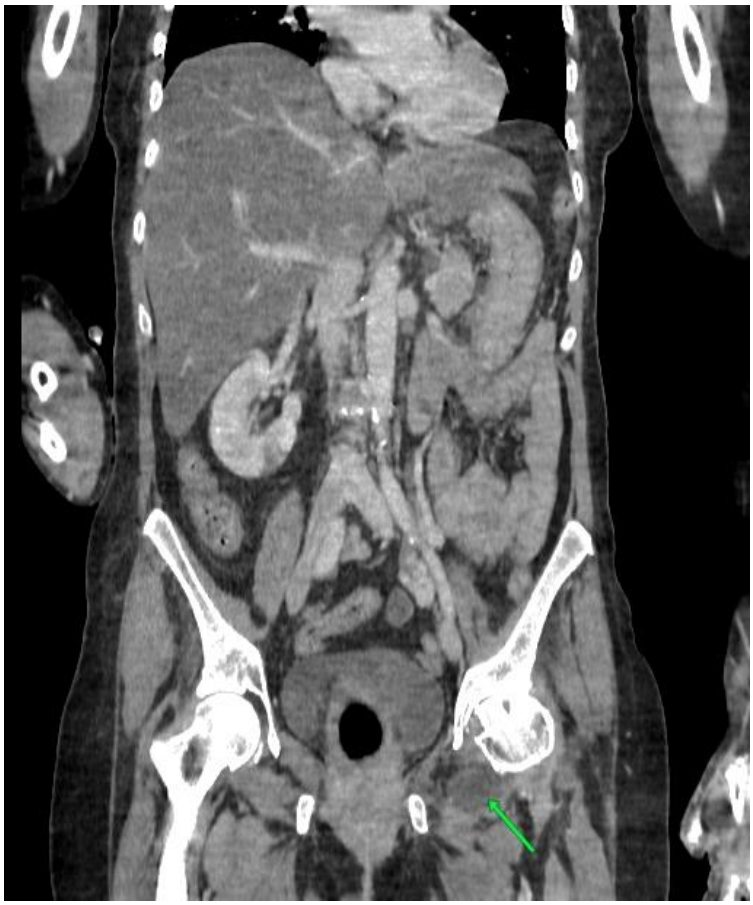

Figure 3. Fluid collection in LHJ area, CT with intravenous contrast coronal plane.

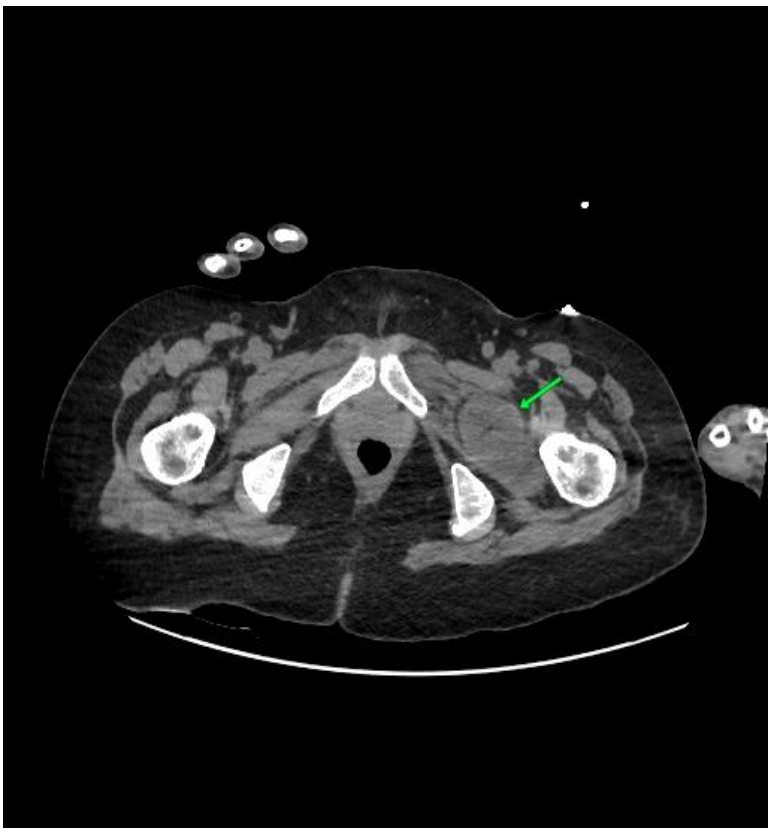

Figure 4. Fluid collection in LHJ area. CT axial plane.

The patient started antibacterial treatment with Ciprofloxacin 400 mg intravenously twice a day. On the second day, Ampicillin 2 g six times a day intravenously was added. Due to hypotension, the patient received continuous perfusion of inoconstrictors. On the third day, ciprofloxacin was switched to ceftriaxone 2 g two times a day based on hospital internal policy for treatment of infection without confirmed causative microorganism. On day six, the patient started treatment with oxacillin 2 g 6 times a day and continued it for the duration of the stay in the hospital. Three days after the initial admission an MRI was performed on the patient to evaluate possible infectious locus in the spine. In these scans an abscess could be seen clearly (Figure 5).

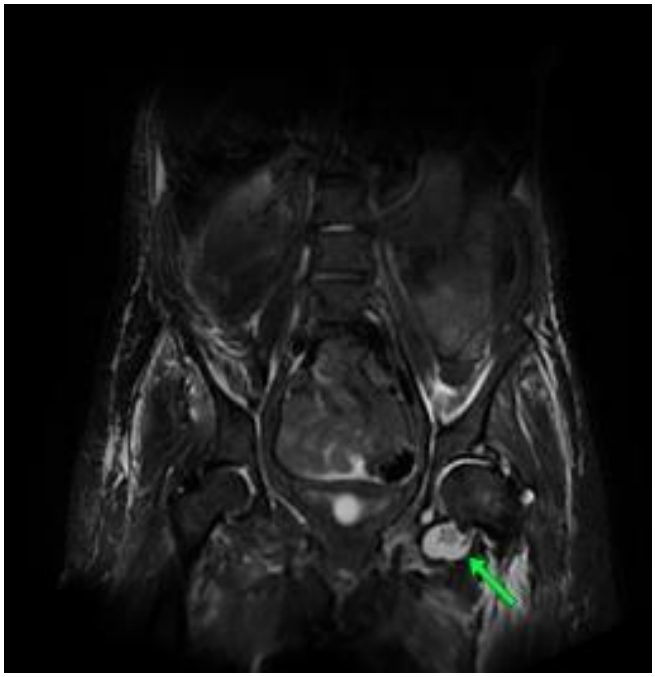

Figure 5. Intraarticular abscess, MRI coronal plane..

Four days after admission, a left hip arthrocentesis was performed, and opaque purulent fluid was obtained with a little amount of cells and no other significant changes. It was sent for microbial evaluation and was positive for *Staphylococcus Aureus* <1000 CFU/mL in two out of three samples. Since the patient was unfit for surgery, in the ICU in a mini open way a drain was inserted through hip adductors to ensure the drainage of pus from the affected joint until the patient was stable enough for surgical treatment.

A week after initial admission, multi resistant *Enterococcus sp.* 10 000 CFU/mL, Alpha-hemolytic *Streptococcus viridans* 10 000 CFU/mL and multi-resistant *Staphylococcus lugdunensis* 100 000 CFU/mL were found in the bronchoalveolar fluid. The lung X-ray showed worsening of the condition in comparison to the first one with left lung atelectasis. Since the patient underwent endotracheal intubation with subsequent mechanical lung ventilation, it is most likely that the patient acquired ventilator associated pneumonia.

Leukocytosis had escalated to 11,14 10^9/L with predominant neutrophilia (9,56 10^12/L). Hemoglobin 8,50 g/dL, Thrombocytes had elevated to 107 10^9/L after the patient received thrombocyte mass infusions. CRP had lessened to 155,9 mg/L, Albumin had lessened to 21,5 g/L, Potassium 3,17 mmol/L, Procalcitonin 2,35 ng/mL, Interleukin-6 had grown from 119,3 to 693,4 pg/mL in two days. Based on laboratory and clinical findings, patient had an active infectious process.

A week after the day of admission, the patient underwent open left hip debridement procedure with irrigation and drainage through an iliofemoral approach. During the debridement, purulent content was found in the joint. The sample taken came back negative, possibly due to antibacterial treatment already started. Eight days after admission and the first day after the debridement the patient underwent tracheostomy and continued to receive mechanical ventilation. On the CT, a pneumonia of both lungs could be seen with pulmonary hypertension and congestion in the small circulation circle. The abdominal CT scan showed multiple spleen ischemia areas, ascites, cyst in the left kidney. Fluid collections around left *m. gluteus medius* and *m. iliopsoas* could be found however the initial abscess between *m. obturatorius externus* and *m. pectineus* had lessened in size.

The histological evaluation of tissue samples taken from the arthritis site showed villous chronic synovitis, however, no microorganisms were found. The patient had multiple possible origins of the arthritis, however, none of the microbial samples matched.

Due to left hip pyogenic arthritis, the patient underwent three surgical interventions in two weeks after being stationed. The first surgical intervention was an arthrotomy and drainage, and an irrigation and draining a week after the first with the resection of *m. iliopsoas* due to necrosis of the muscle. The third intervention was another irrigation and draining in combination with antibacterial treatment after two more weeks. The last procedure included femoral head denervation due to hip arthritis with arthrosis. After the second procedure no microorganisms were found in any of the seven tissue samples sent for microbiological evaluation. An arthrocentesis of the left knee also showed no signs of microbial colonization. The patient continued treatment with Vancomycin and Oxacillin due to many other infectious complications such as central venous catheter infection, ventilation associated pneumonia and urinary tract infection.

Five weeks after the patient was admitted, an intracerebral hematoma in the right parietal lobe with signs of lysis was found un an MRI scan (Figure 6). A neurosurgeon consultant was invited and saw no indications for operative treatment.

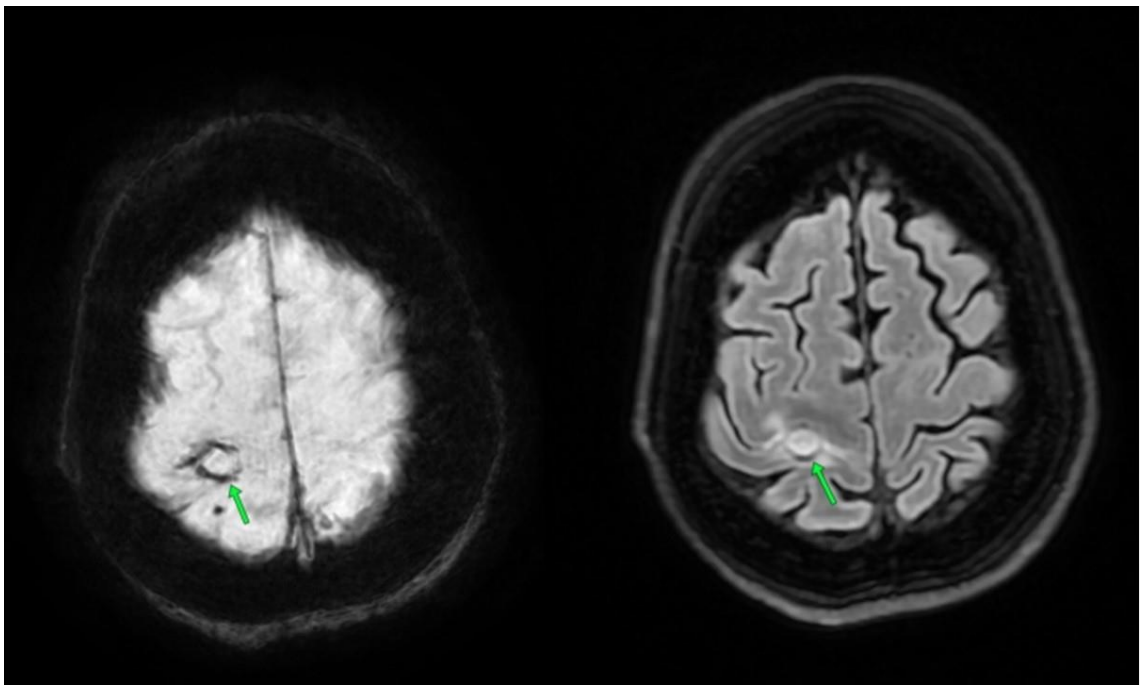

Figure 6. Right parietal lobe ICH, MRI SWI, T2 Flair, axial plane.

An echocardiogram was performed where a bacterial endocarditis of mitral valve and stage IV mitral regurgitation was found with the primary source being the left hip arthritis. The previous echocardiogram performed upon admission a month earlier showed no signs of bacterial endocarditis. A blood sample at the time showed Leukocytosis 11,17 10^9/L, CRP 59,70 mg/L, LDH 379,00 U/L, Procalcitonin 0,10 ng/mL, however, the patient had been receiving intravenous antibacterial treatment for a month. Due to intracerebral hematoma, surgical intervention was postponed after repeated consultations of neurologist and cardiac surgeon in three more weeks.

Seven weeks after the initial admission patient is stabilized, extubated and no longer undergoes hemodialysis. Both laboratory and imaging findings show leftover signs of an infection, however, the main site of the infection, the LHJ arthritis is treated with no signs of arthritis remaining. The patient was prepared for discharge to continue rehabilitation and undergo mitral valve replacement surgery at Pauls Stradins Clinical University Hospital. Afterwards the patient was transferred to Riga East clinical university hospital branch "Biķernieki" Rehabilitation clinic, where the patient undergoes rehabilitation. Two and a half months after initial arrival in ED, a CT for hip joint was performed. Osteodestructive lesions due to progression of the previously confirmed osteoarthritis in the LHJ are found (Figure 7). Around the left hip joint and in the upper part of soft tissue in the left thigh multiple ossificates and small surrounded fluid collections are seen (Figures 8 & 9).

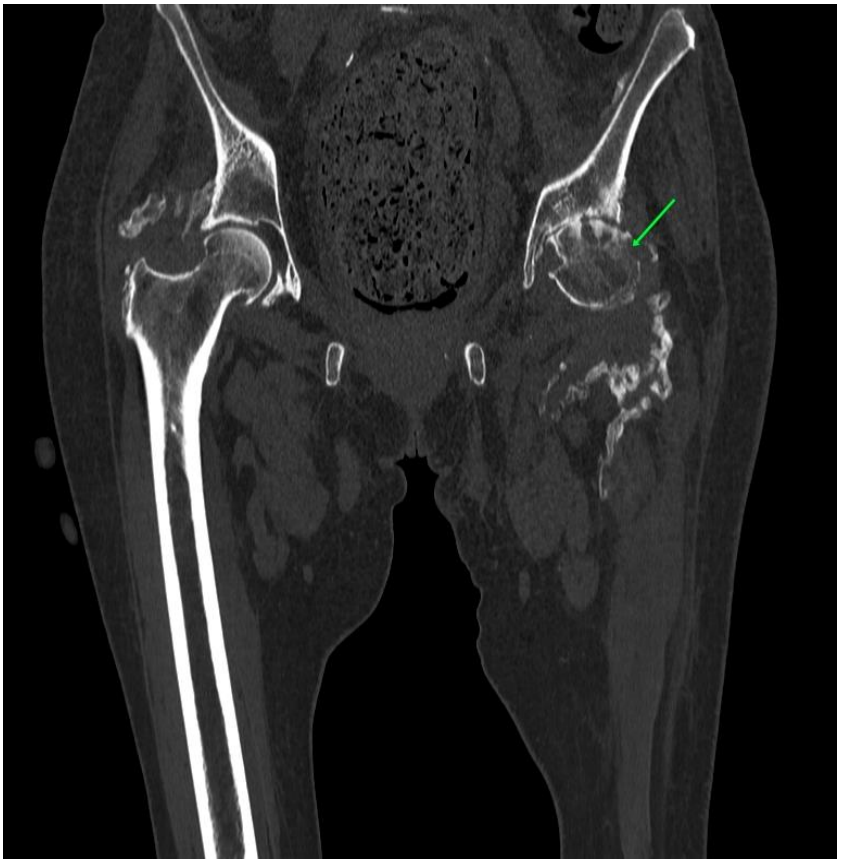

Figure 7. Osteodestructive lesions of hip joint, CT coronal plane.

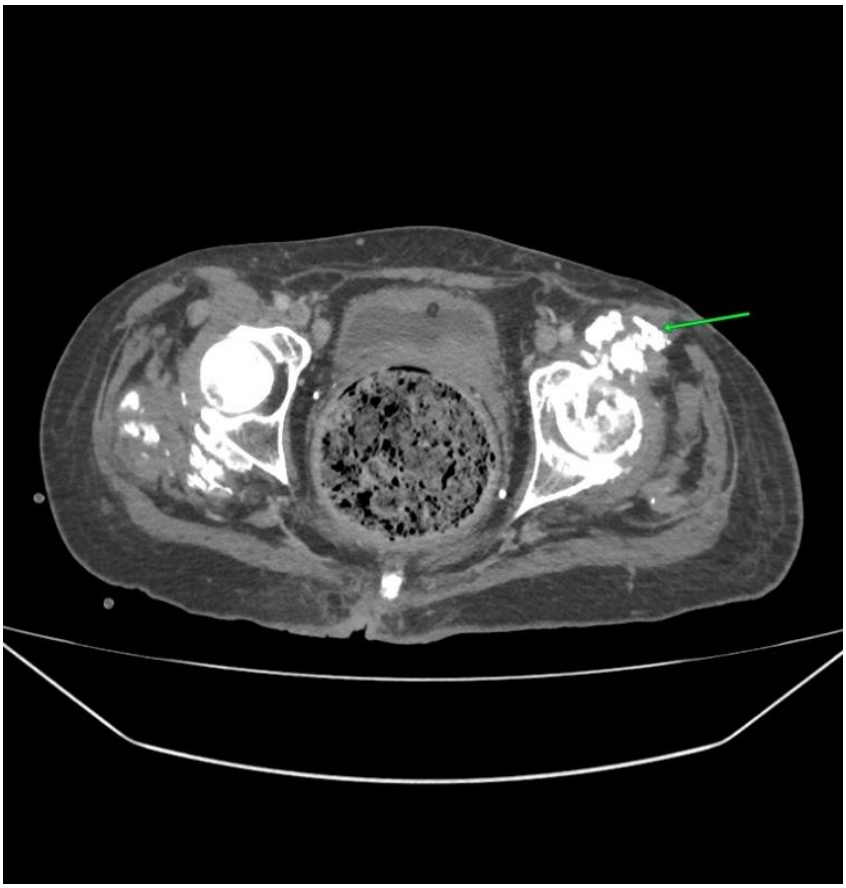

Figure 8. Ossificates around LHJ, CT axial plane.

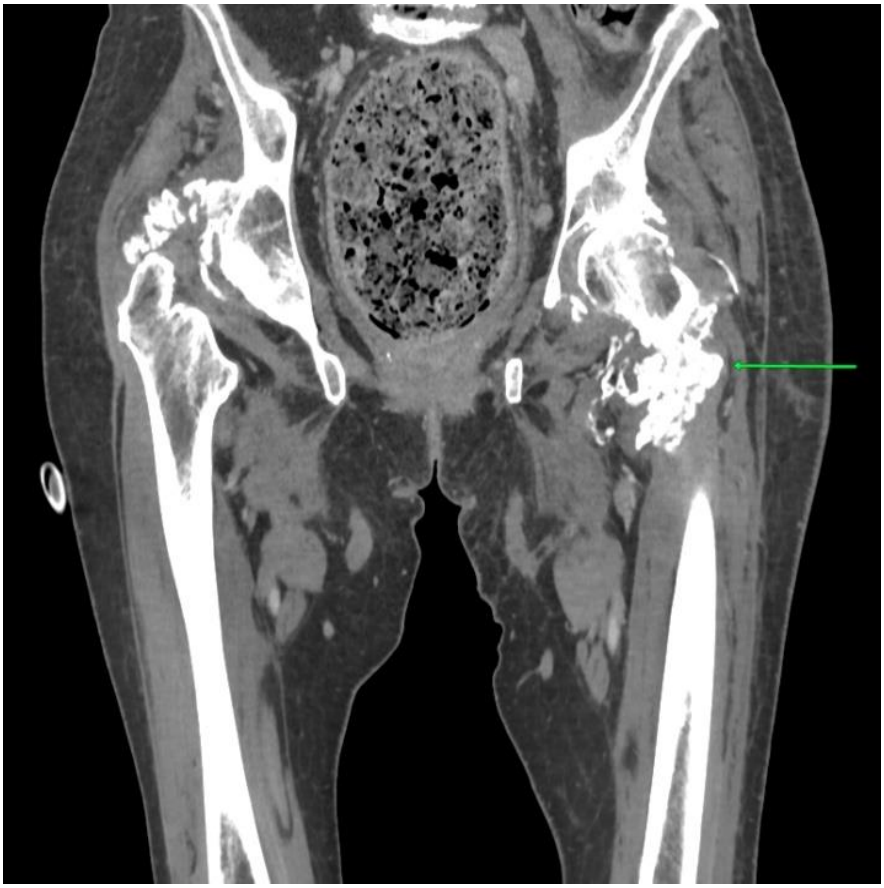

Figure 9. Ossificates around LHJ, CT coronal plane.

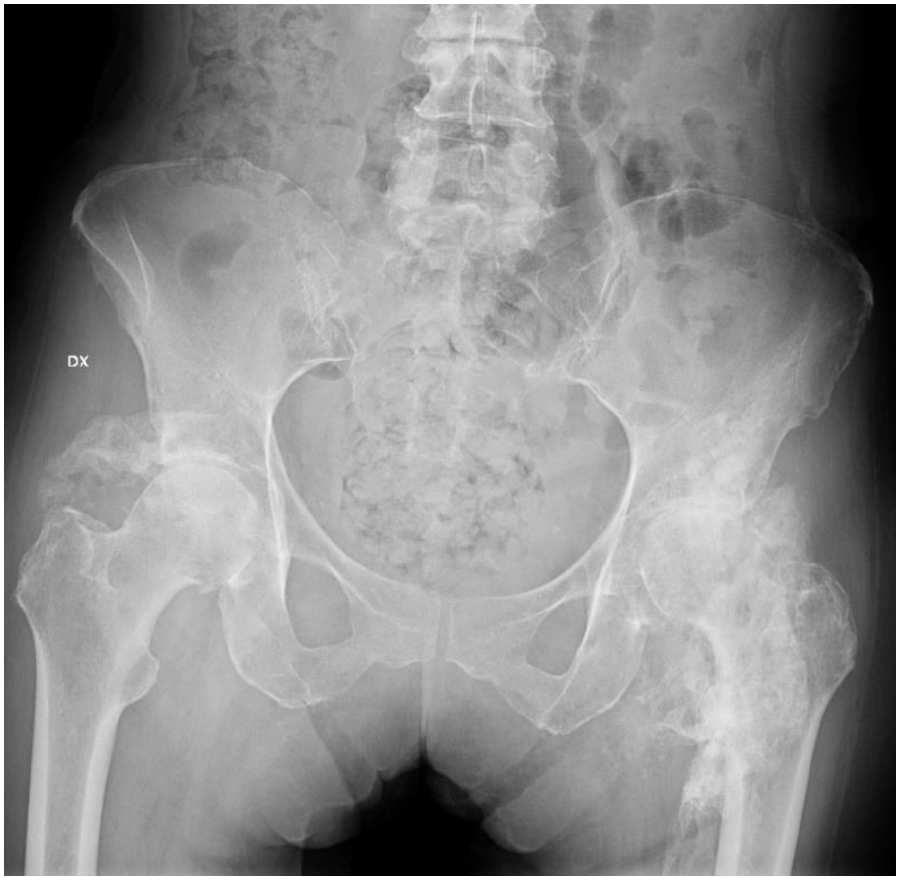

Figure 10. Left hip joint heterotopic ossification, X-ray AP projection.

Three months after the initial presentation, patient was transferred to Pauls Stradins Clinical University Hospital for treatment of mitral valve bacterial endocarditis with grade IV insufficiency and multiple vegetations with a diameter up to 1 cm. During and after the admission for the valve replacement surgery that was performed successfully, the patient continues to take peroral antibacterial treatment with Cefazolin for at least two more weeks after discharge. Two weeks after being transferred, the patient returned to the Rehabilitation clinic and continued to undergo rehabilitation in two stages. An increase in functioning was seen, however, LHJ was with severely decreased ROM, with a limited movement - flexion of 15 degrees. In the left knee ROM had improved with flexion possible up to 90 degrees. In five and a half months after the initial admission, an X-ray is done and massive HO can be seen (Figure 10).

Nine months after the initial hospitalization, patient returns to TOD to undergo removal of left hip HO. The stage of the ossification is Brooker III. (Hu et al.,2015). Upon arrival patient complains about significant restriction of movement in LHJ – extension zero degrees, flexion 20 degrees, passive and active abduction, adduction, inner and outer rotation are not possible at all. The lack of ROM makes difficult daily activities, such as hygiene, sitting and transportation. The Harris Hip score before the treatment was 27,08% (Kauffman et al., 2007). Based on previous CT scans, patient now has massive HO in the frontal part of left hip area in the soft tissues, that blocks the movement. The same can be seen twenty days before the surgical treatment of the ossificates on a CT scan. (Figures 11, 12 & 13) Due to complete mechanical blockage patient undergoes surgical excision of the ossificates (Figure 14), tenolysis, myolysis, *musculus rectus femoris* separation and reattachment to *spina iliaca anterior inferior.*

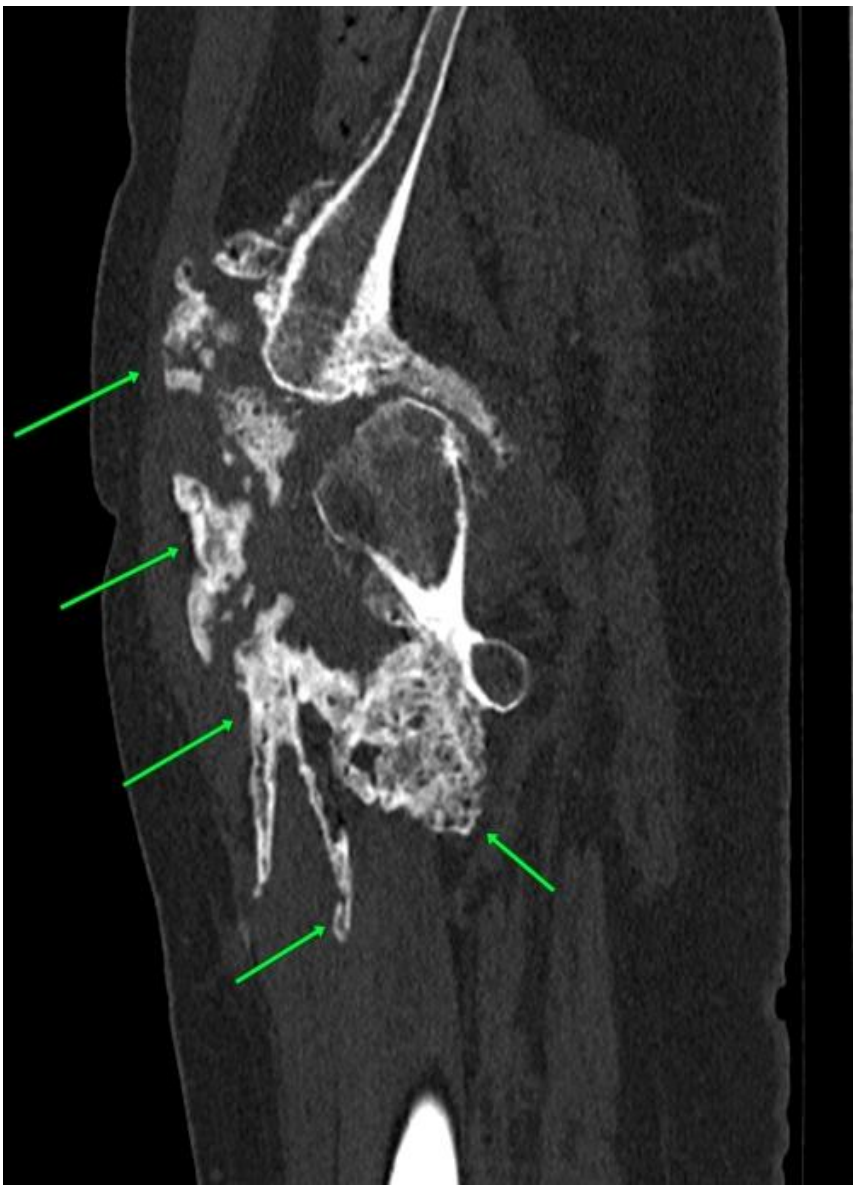

Figure 11. Left hip heterotopic ossification, CT sagittal plane.

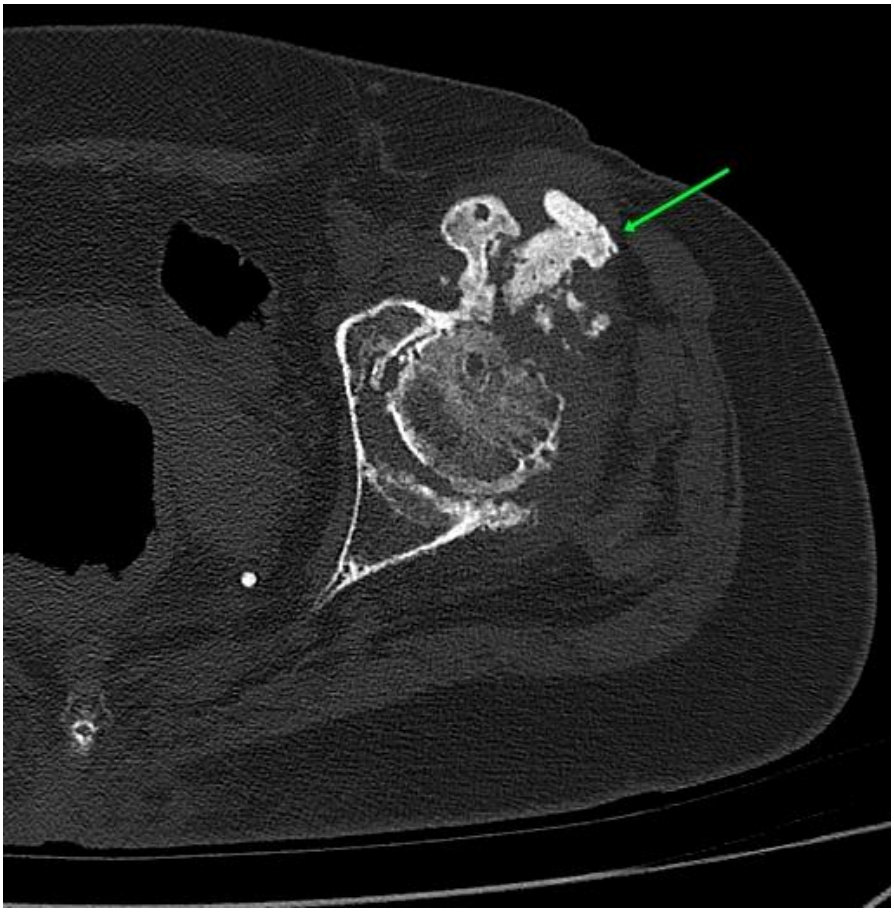

Figure 12. Left hip heterotopic ossification, CT axial plane.

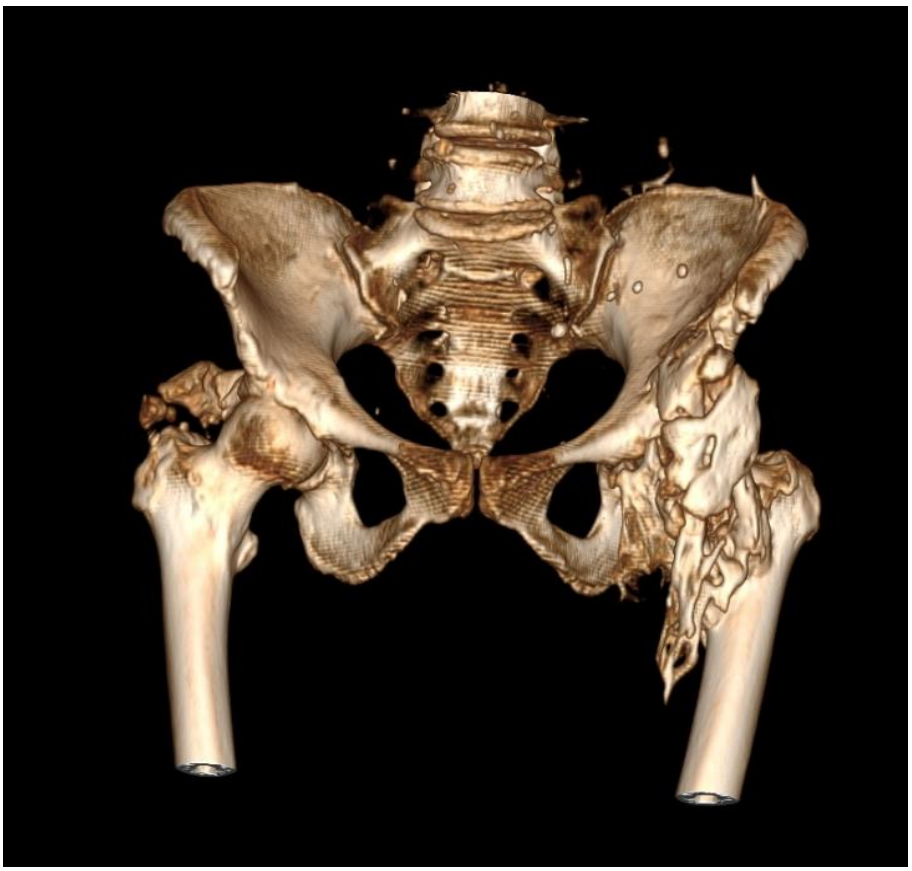

Figure 13. 3D reconstruction of left hip heterotopic ossification.

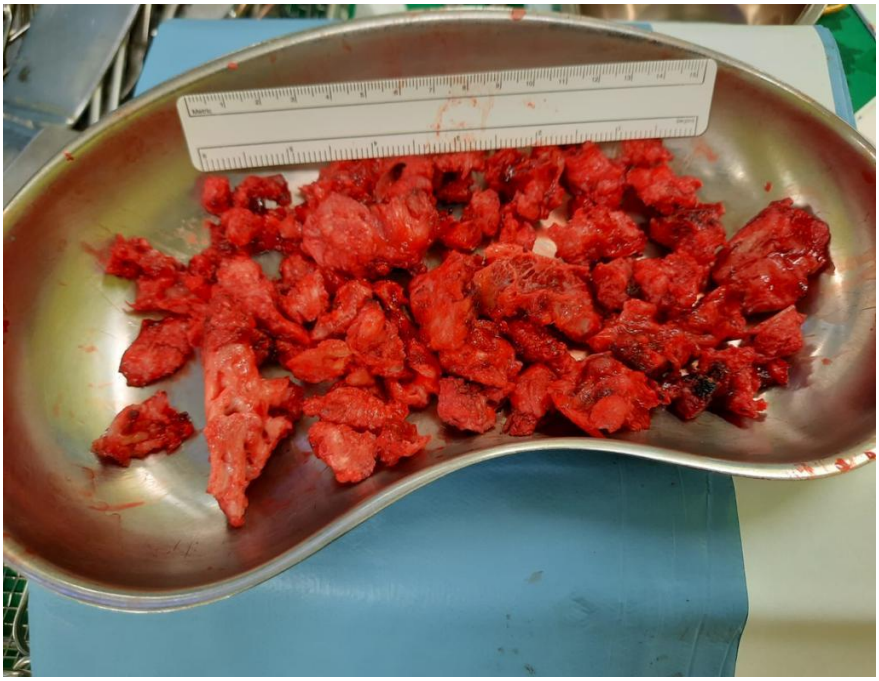

Figure 14. Excised ossificates.

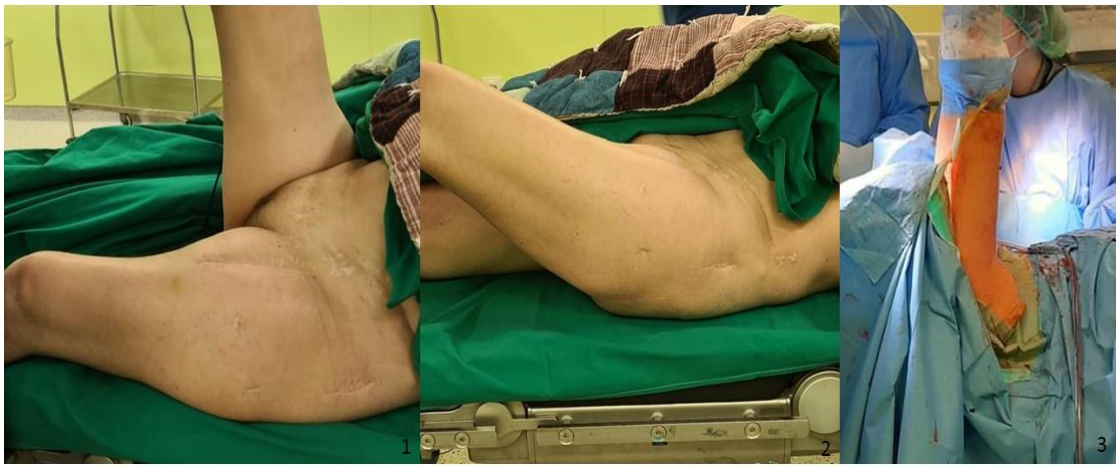

Figure 15. Passive flexion of RHJ (1) and LHJ before (2) and after (3) surgery.

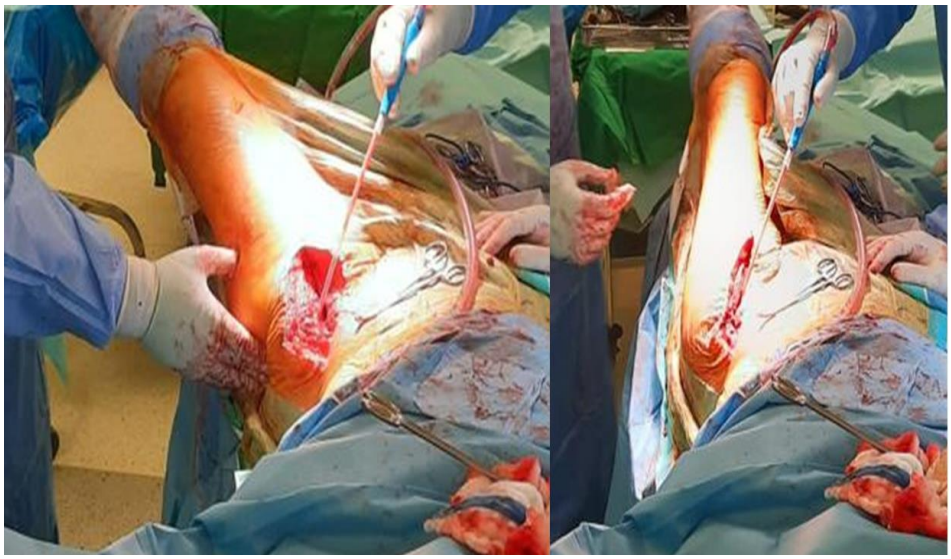

Figure 16. Passive abduction and adduction in LHJ during surgery.

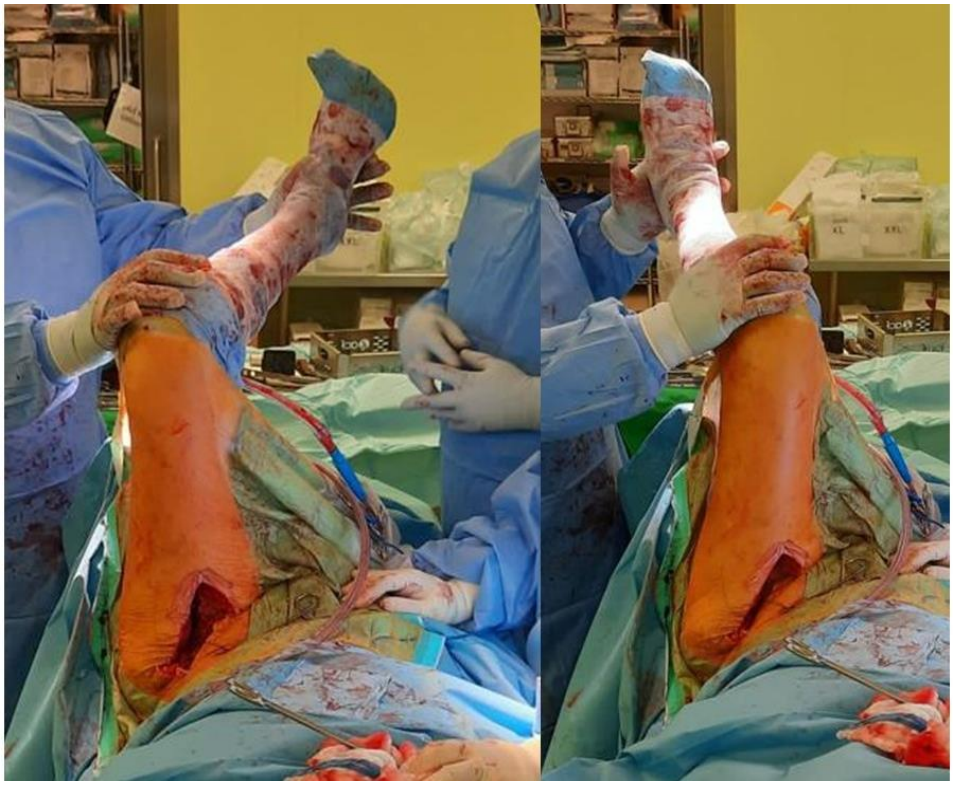

Figure 17. Passive internal and external rotation in LHJ during surgery.

During the procedure, the *nervus femoralis sinistra* was iatrogenically transected and was surgically repaired end-to-end by a microsurgeon. After the HO resection, left hip joint manipulation under anesthesia was performed to increase ROM with achieving – flexion of 90 degrees (Figure 15), abduction and adduction of 25 degrees (Figure 16), internal rotation of 20 degrees, and external rotation of 10 degrees (Figure 17). After the procedure, the patient continued rehabilitation in the Trauma and Orthopedics department for a week and later was transferred to the Rehabilitation clinic for further treatment. The patient had to use foot orthosis for the treatment of left foot contracture obtained after hemiparesis of the left side due to intracerebral hematoma, plantar and dorsiflexion weakness, as well as a knee brace due to *nervus femoralis sinistra* lesion and *musculus quadriceps femoris sinistra* weakness.

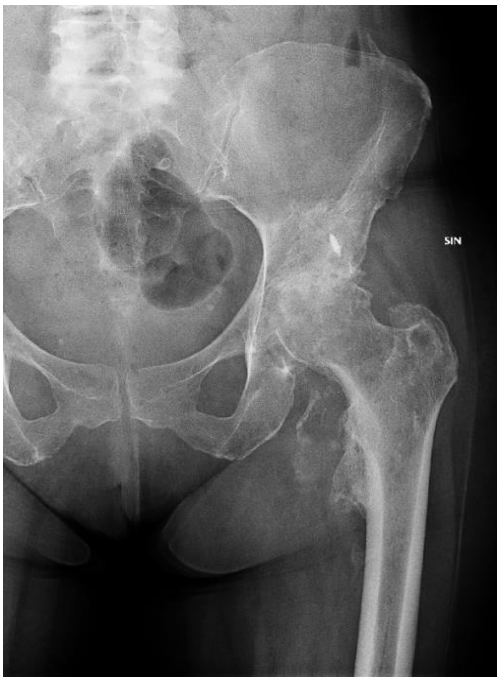

Figure 18. LHJ six weeks after surgery, X-ray AP projection.

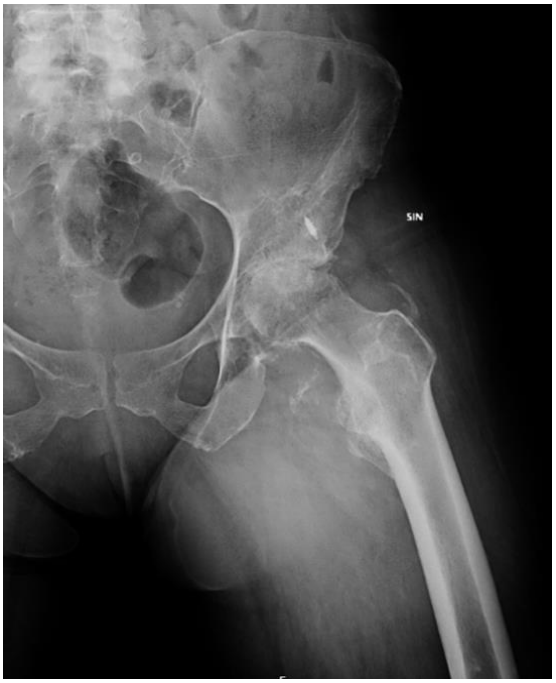

Figure 19. LHJ six weeks after surgery, X-ray Lauenstein projection.

Twelve weeks after the HO resection, the patient still had not regained full motor and sensory function in the left thigh due to nerve lesion. The flexion in LHJ was retained, but still there was some impairment in abduction and adduction. The foot and knee ROM had improved, and the patient was satisfied with the results and continues rehabilitation to improve independence and ability. The Harris Hip Score after the surgery was 47,80%. Small ossificates could be seen once more in the control X-ray, but did not impair the movement in the joint. (Figure 18 & 19)

Post-Traumatic Left Hip Heterotopic Ossification

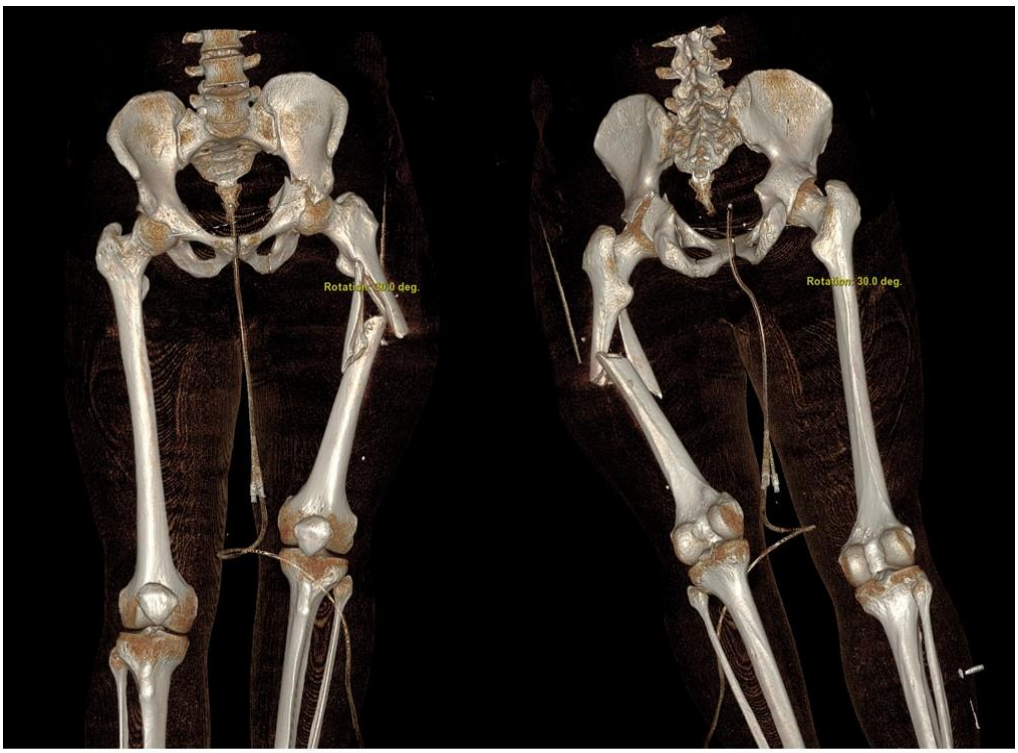

Figure 20. 3D reconstruction of lower limb fractures after polytrauma.

A 34-year-old man was admitted to ED due to polytrauma after a car accident. Upon arrival a full check-up bin accordance to polytrauma protocol standards was performed with a CT scan, confirming subarachnoid hemorrhage, III-degree liver rupture, bilateral multiple rib fractures, left acetabulum T type fracture with dislocation, pelvic ring left side sacroiliac joint dislocation, left femur subtrochanteric multifragmented fracture with dislocation, and left tibia *eminentia intercondylaris* avulsion fracture. (Figure 20.)

A blood sample was drawn and showed blood alcohol level of 2,04 g/L, INR 0,92, Leukocytes 20,78 10^9/L with Neutrophilia (14,60 10^/L) and Lymphocytosis (5,32 10^9/L), mild anemia Hemoglobin 12,10 g/dL, Creatinine 144,33 mkmol/L and Troponin T-HS 45,62 ng/L. No other significant abnormalities were found. The patient continued treatment in the ICU and underwent skeletal traction for temporary fixation of the left femur and acetabular fractures. On the second day Leukocytosis had shrunk twice (11,93 10^9/L) with Neutrophilia 8,96 10^9/L, Hemoglobin had lessened to 10,40 g/dL, ALAT was 713,54 U/L, ASAT 546,14 U/L, CRP had grown to 175,12 mg/L, Creatinine kinase 3045,47 U/L, Procalcitonin 0,519 ng/mL.

On the fifth day after the admission, the patient underwent a mini-open femoral fracture reduction with cerclage wiring and intramedullary osteosynthesis with a proximal femoral nail. The next day the patient underwent open reduction and internal fixation (ORIF) of left sacroiliac joint and anterior part of T type acetabular fracture osteosynthesis with plate and screws through modified iliofemoral approach with *spina iliaca anterior superior* osteotomy. Two weeks later the patient underwent ORIF of the posterior column through the Kocher-Langenbeck approach with plate and screws.

Two weeks after the third surgical intervention, the site of incision showed signs of inflammation. An aspirate from the surgical site was obtained and sent for microbial evaluation. It showed positive for Enterococcus faecalis <10 CFU/mL and *Staphylococcus epidermidis* <1000 CFU/mL. Both strains were multi resistant with confirmed sensitivity to Vancomycin. The blood sample showed Leukocytosis 9,16 10^9/L, Erythrocyte sedimentation rate 43 mm/h, CRP 28,6 mg/L and Procalcitonin 0,062 ng/mL, suggesting an early recognition of the infection. Due to a left acetabulum deep tissue infection a decision on surgical treatment was made and four days later the patient underwent open debridement, irrigation, and drainage. Additional tissue samples for microbial evaluation were taken with four of the six samples coming back positive for *Staphylococcus epidermidis* and one for *Enterococcus faecalis*. Incisional negative pressure wound therapy dressing (NPWT) was implemented. Patient started antibacterial treatment with Vancomycin 1g twice a day and continued it for six weeks.

Three days after the discovery of the deep tissue infection, the patient tested positive for SARS CoV-2 infection with corresponding clinical symptoms, such as fever, cough, sore throat, myalgia and fatigue. Patient was isolated in Covid-19 department and stayed there for ten days. After SARS CoV-2 infection, patients continued treatment in TOD where wounds were healing primarily and NPWT was removed five weeks after the debridement procedure. Postoperative period had no complications, however the patient mentioned a burning sensation in the left foot, suggesting *n. ischiadicus sinistra* lesion. Five days later the patient was discharged to continue treatment at home.

Two years after the trauma and the following treatment, the patient returns to TOD complaining of impaired movement in LHJ, the only possible movement being active flexion of the hip for no more than 70 degrees with neutral abduction and adduction. An X-ray discovered HO in the left hip of the patient. (Figure 21 & 22) The Harris Hip Score before surgical treatment was 76,50, which is high; however, the limitation of movement impaired patients' ability for daily functioning as well as job duties. The patient was informed about possible complications and risks of a surgical intervention and agreed to surgical treatment.

The patient underwent an open left hip joint frontal and lateral HO resection with partial *m. rectus femoris* separation and refixation to *spina iliaca anterior inferior* and placement of deep surgical drains. After HO resection, the patient was prescribed Aspirin 100 mg two times a day for deep venous thrombosis and HO prophylaxis and indomethacin 75 mg once a day for two weeks.The drain was removed upon lessening of the drained fluid and the patient was discharged three days after the surgery with provided recommendations.

Twelve weeks after the treatment, the patient was satisfied with the results and the control X-ray showed no HO recurrence. (Figure 23) ROM had increased to 100 degree flexion in neutral abduction and adduction position. *N. ischiadicus* neuropathy had also lessened. The Harris hip score had grown to 95%.

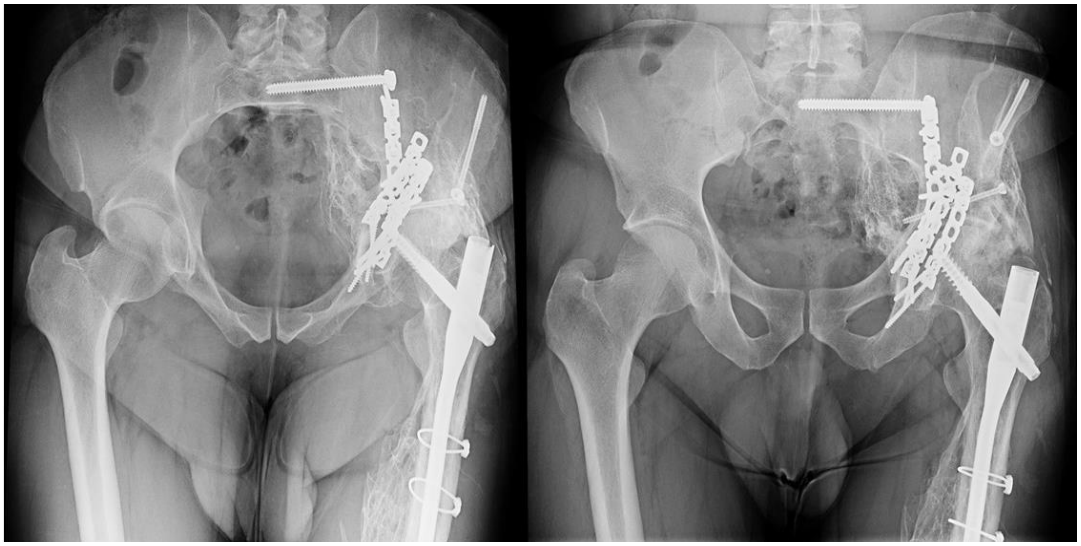

Figure 21. Heterotopic ossification of LHJ, inlet and AP projection, X-ray

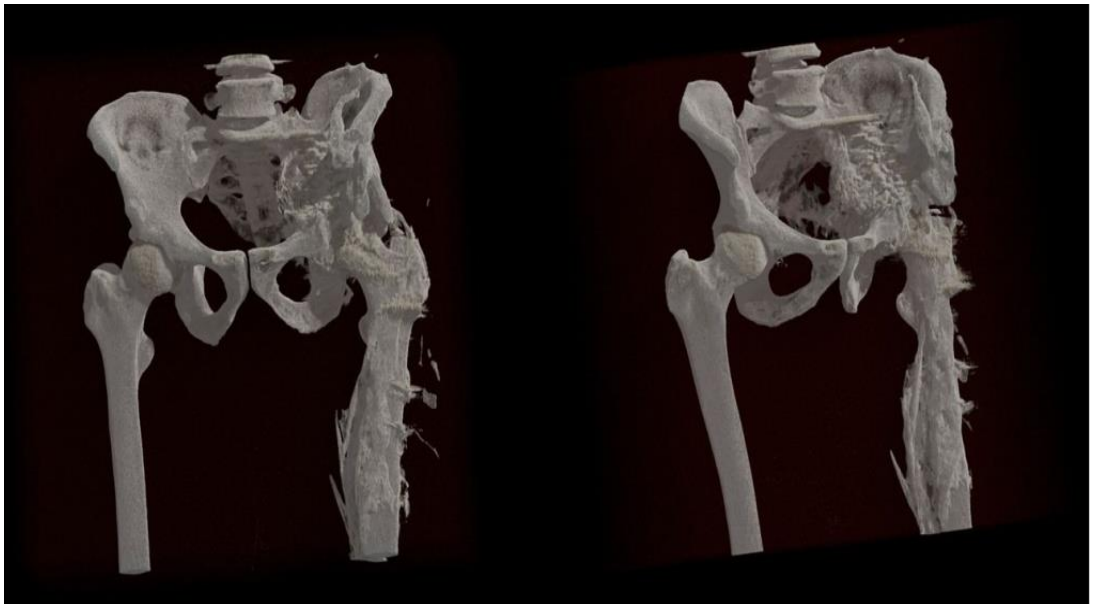

Figure 22. LHJ HO 3D reconstruction.

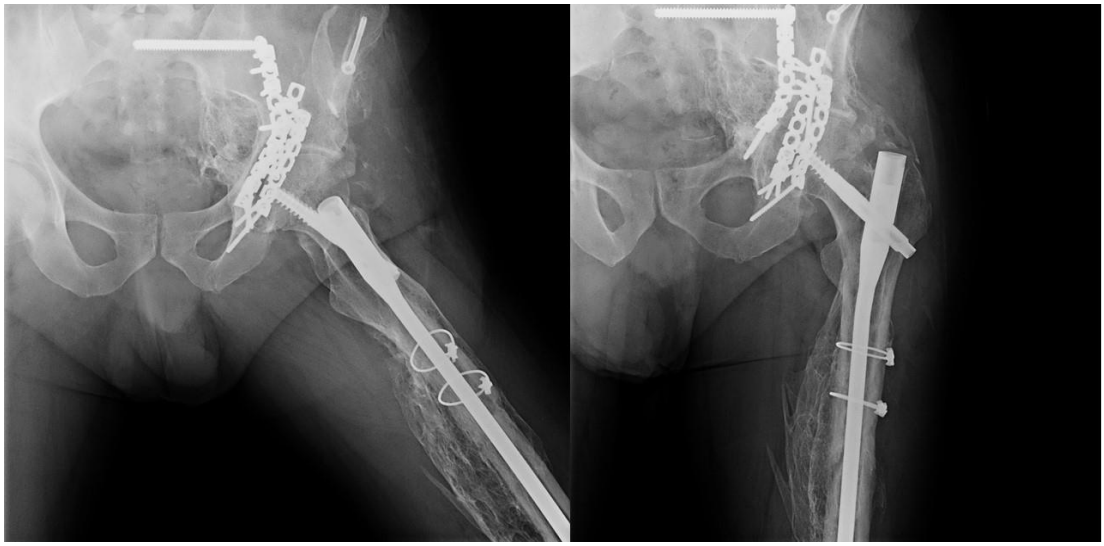

Figure 23. LHJ HO, X-ray Lauenstein, AP projection.

Discussion

In this report a case of mixed origin and posttraumatic heterotopic ossification were reported. Although the surgical treatment usually is not the first line treatment, in these cases it showed a beneficial outcome for the patients with increased quality of life, functioning and regained ROM in the left hip joint afterwards. The resection was done nine months after the initial arrival in hospital and eight months after first sighting of the HO in patient imaging results and two years after the initial trauma for the second patient.

Surgical treatment is not the first line of treatment in HO cases due to risks associated with postoperative complications as well as the recurrence of the ossification. It is suggested to perform it in cases where there is lack of clinical improvement with nonoperative treatment and appearance of progressive disability. (Ranganathan et al., 2015)

An early functional surgery without waiting for "cold" appearance on bone scintigraphy does not affect the recurrence of the ossification. (Genêt et al., 2012; Genêt et al., 2015; Denomandie et al., 2018; Vasileiadis et al., 2015). Furthermore, surgery should not be delayed in case of complete joint ankylosis or joint fusion that brings more destructive outcomes upon the patient. The loss of ROM in the patient should be a more important factor than waiting for maturation of the ossificates. It was found that early surgical intervention minimizes the development of intra-articular pathology, osteoporosis and the resultant complications without increasing the risk of recurrence of HO. (Genet et al., 2009) The patients should however be consulted on the risks due to potential injury of the surrounding soft tissue, such as delayed wound healing, infection, nerve injury and recurrent contracture. The opinions in literature on the timing of the ossificate resection varies depending on the origin of the pathology, in case of traumatic HO suggesting resection at six to nine months and in case of neurogenic HO at 18 months. (Mavrogenis et al., 2011; Ranganathan et al., 2015)

Some of the risk factors mentioned in the literature for the recurrence of HO are incomplete resection and not waiting for complete maturation, since some isles of developing ossificates can be unseen both radiographically and during surgical resection. It was found that resection done before 180 days post injury had a higher risk of recurrence in patients with traumatic amputation induced HO (Pavey et al., 2015). Another issue is the increased risk of infection due to the newly-formed bone being a highly vascularized structure. As surgical intervention itself can induce an inflammatory state and increase the risk of recurrence, the timing of the surgery is still debatable. Moreso the results of recurrence of HO following traumatic brain injury is inconsistent both in time and magnitude. (Davis et al., 2013; Juarez et al., 2018).

Nevertheless the pre and post operative prophylaxis with indomethacin and radiation therapy seems to produce beneficial results for the patient. There are several studies that have focused on different NSAIDs both selective and non selective and thus far indomethacin has shown the highest benefit. However, aspirin or acetylsalicylic acid has shown beneficial effects on occurrence of HO, especially after hip arthroplasty. Based on its wide spectrum of pharmacological effects, it can alleviate pain, reduce risk of venous thrombosis and occurrence after surgical removal of HO (Nunley et al., 2011; Wang et al., 2023). The studies mentioned did not focus on HO after traumatic or neurogenic incident and several other studies also showed beneficial effects of other NSAIDs such as ibuprofen, celecoxib, dexketoprofen with highest benefit from indomethacin and celecoxib (Migliorini et al., 2020; Legosz et al., 2019).

Conclusion

In conclusion surgical treatment of HO in patients with good expected outcomes and high involvement in their own rehabilitation should be at least considered. It is important to emphasize both pre and postoperative rehabilitation and prophylaxis. Several factors should be considered before the surgery, including the origin and risk factors of HO, available data on recurrence and bone maturation in these patients as well as risks following the surgery.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

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* The authors express gratitude to the patients for consenting to the publication of this case report.

Footnotes

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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Redox State and Biogenic Elements in Osteoporosis with Different Localization

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Abstract: The redox state of the human body is the result of a complex influence of enzymes, metabolites, biogenic elements, and endogenous lifestyle factors. With age, unfavorable changes occur and degenerative and pathogenic processes are unlocked, various diseases appear, such as osteoporosis. In the present work, the radical scavenging activity (RSA%) and the blood serum conclusions of copper and magnesium were investigated in patients with osteoporosis of the hip, as well as in patients with osteoporosis of the lumbar spine. 66 menopausal and postmenopausal women aged 63.41±7.81 were investigated - newly diagnosed with osteoporosis and controls. Exclusion criteria were as follows: diabetes, endocrine and liver disease; intake of estrogenic and biogenic elements. Bone mineral density (BMD) was measured in all patients using dual-energy X-ray absorptiometry (DEXA). Patients with T-Score ≤ -2.5 were divided into two groups – with osteoporosis of the hip and with osteoporosis of the lumbar spine. A control group with T-Score > -1.0 was formed. Serum RSA% was established using the experimental method for determining antioxidants ABTS decolonization assay. Serum copper and magnesium levels were determined using atomic absorption analysis. According to the RSA% indicator, we obtained the following values: 73.30 ± 9.84 in the group with lumbar vertebrae osteoporosis; 68.87 ± 13.26 in the hip osteoporosis group; 55.67 ± 1.37 in the control group. When monitoring the radical scavenging activity, depending on the localization of the disease, we detected higher values in patients with osteoporosis of the lumbar spine. Serum copper and magnesium concentrations of all patients

were elevated relative to the controls, but there was no statistical difference between groups with different disease localization. Patients with osteoporosis were found to have a higher protective antioxidant capacity in response to increased free-radical processes in the body and cellular metabolic disorders.

Keywords: Osteoporosis, Hip, Lumbar spine, RSA, Bioelements.

Introduction

The redox state in living organisms is the balance between the opposing functions of oxidizing agents and reducing agents in them. It is determined by the type and quantitative ratio of active oxidants, such as reactive oxygen species (ROS) and nitrogen species (RNS), and active enzymatic and non-enzymatic antioxidants. (Lazarova et al., 2019) ROS are free radicals, products of cellular metabolism that are primarily generated in the mitochondria. They are necessary for the human body as signaling substances for cells. Simultaneously with this process, there are also biochemical processes for trapping free radicals by antioxidants. With a reduced concentration of antioxidants in the body and increased production of ROS, secondary chain-radical processes begin with lipids, proteins, and DNA molecules, resulting in more aggressive oxygen species. The systemic imbalance between oxidizers and reducers, the so-called oxidative stress leads to irreversible changes and loss of functions of molecules, cells, and organs. (Schieber et al., 2014). It is the cause of the appearance of various diseases, one of which is osteoporosis.

Oxidative stress affects a significant proportion of menopausal women (Sakac et al., 2000). Oxidative stress impairs bone remodeling and lowers bone density (Asenova et al., 2020). The antioxidant activity of women with osteoporosis is increased. This is believed to be a response of the bone marrow stem cells, which respond to the higher concentration of oxidants by increasing their radical scavenging activity (RSA%). This explains the higher antioxidant activity of the patients compared to that of the control group of women without osteoporosis (Romάn et al., 2017). The reason for this is the fact that ROS in high concentration, which exists in a deteriorated redox state, also attacks enzymes-metalloproteins. In the oxidative destruction of Cu, Zn-SOD, the protein is degraded and copper ions are released (Kwon et al., 2000; Valko et al., 2016). Our previous study confirmed an increased serum concentration of the trace elements copper and zinc, with the Cu/Zn molar ratio corresponding to the degree of the disease. (Tomova et al., 2020). The released copper ions are redox active and participate in secondary radical reactions and increase antioxidant activity in patients with osteoporosis (Tomova et al., 2022). The increase of radicals in the body impairs bone homeostasis and decreases bone mineral density (BMD) (de Romaña et al., 2016; Valko et al., 2005). Conversely, BMD is positively affected by dietary antioxidant supplementation in postmenopausal women (X. Wu et al., 2017; Rondanelli et al., 2021; Mazzanti et al., 2015).

Results published on micronutrient concentrations in menopausal and postmenopausal women to this date are conflicting and do not explain their role in bone homeostasis. Published studies to determine the level of radical scavenging capacity in patients with osteoporosis and osteopenia are few and do not demonstrate synergistic relationships with the level of essential elements, BMD, and anthropometric indicators.

Osteoporosis depends on many factors and requires multivariate statistical analysis to study the relationships between the different clinical indicators characteristic of this disease. Therefore, in our study, we applied multivariate statistical analysis to the obtained data from 59 patients. The aim was to clarify the relationship between bone density, the level of biogenic elements copper, zinc, magnesium, iron, and calcium, and the level of antioxidant activity (AOA) in newly diagnosed patients with osteopenia and osteoporosis. The results were surprising. After the clustering of the data, not two, but three clusters were formed describing three different stages of bone metabolism disturbance with a different interval in the variations of the studied indicators.

The cluster of patients with the lowest level of BMD had the highest levels of Mg and Cu, and the lowest levels of Zn, Fe, AOA, and BMI. The factor analysis and principal component analysis performed grouped Mg and Cu as well as Ca as discriminating parameters related to the state of reduced bone density. They determine over 70% of the total variation of the system (Tomova et al., 2022). 3D-plot of factor loadings reveals that AOA is a hidden factor related to the degree of bone density reduction. But bone density and degree of disease (osteopenia or osteoporosis) do not fall into the same cluster, which can be explained by the large difference in bone density in different places of localization of the disease (Tomova et al., 2022). This determined the division of patients according to disease location in our subsequent studies.In the present work, the radical scavenging activity (RSA%), the concentrations of copper and magnesium in blood serum of patients with osteoporosis of the hip, as well as of patients with osteoporosis of the lumbar spine, were investigated.

Materials and Methods

66 menopausal and postmenopausal women aged 63.41 ± 7.81 years - newly diagnosed with osteoporosis and controls - were studied. The exclusion criteria were as follows: diabetes, endocrine and liver diseases; intake of estrogens, and biogenic elements. The bone mineral density (BMD) of all participants was measured using dual-energy X-ray absorptiometry (DEXA).

Patients with t-Score ≤-2.5 are divided into two groups – with osteoporosis of the hip and with osteoporosis of the lumbar vertebrae. A control group with t-score >-1.0 was also formed. The bone mineral density (BMD) of all participants was measured using dual-energy X-ray absorptiometry (DEXA). Patients with t-Score ≤-2.5 are divided into two groups – with osteoporosis of the hip and with osteoporosis of the lumbar vertebrae. A control group with t-Score >-1.0 was also formed.

The body mass index (BMI) of all participants was measured. Venous blood was drawn from all controls and patients by a standard procedure following quality assurance requirements in the pre-analytical phase. After centrifugation of the blood at room temperature for 10 min, serum was separated for analysis of RSA%, copper, and magnesium. The serum was frozen if not analyzed immediately. Determination of RSA% is performed within 14 days of sample collection.

Methods of Analysis

Spectrophotometric ABTS-test for Determination of RSA% in Serum

To determine the redox status of the patients, blood serum was examined using the experimental spectrophotometric method for the determination of antioxidants, ABTS-test (Re et al., 1999). The stable green radical cation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) was added to the samples. The staining intensity of the samples decreases after interaction with the antioxidants from the blood serum. We measured the absorbance of the serum samples at 734 nm. The change in absorbance is an indicator of RSA, i.e. the intensity of the ongoing processes and their impact on the patient's condition. A calibration curve RSA%/Trolox, μmol/L was constructed, according to which RSA% was recalculated into Trolox equivalent (TE) - antioxidant capacity, per μL of serum. The calibration curve has a very good degree of linearity.

Flame Atomic Absorption Analysis of Copper and Magnesium in Serum

Blood serum copper and magnesium levels were determined by flame atomic absorption analysis (AAnalyst, Perkin Elmer). For magnesium analysis, serum was diluted 1:50 with 0.25% LaCl3 solution before quantitative analysis. For copper analysis, serum was diluted 1:3 with bidistilled water before quantitative analysis.

Statistical Analysis:

The obtained values for the studied indicators and anthropometric data are presented as mean values ± SD for the respective groups. The statistical significance of all data was assessed by analysis of variance with an unpaired t-test. The statistically significant difference between groups: patients with osteoporosis of the hip; patients with osteoporosis of the lumbar vertebrae and a control group we determined at $p < 0.05$. From the obtained degree of linearity of the calibration curve RSA%/Trolox, μmol/L the coefficient of determination was calculated to be 99.83. The univariate regression analysis performed showed that 99.83% of the changes in RSA% value were due to the changes in Trolox concentration.

Results and Discussion

Results

The evaluation was done by the analysis of variance with unpaired Student's t-test between different groups with $P < 0.05$ for statistical significance (Table 1). The results of Table 1 are graphically presented in Figure 1. According to the RSA% indicator, presented as TE, we obtained values of 7.24 ± 0.82 in the lumbar spine osteoporosis group; 6.79 ± 1.16 in the hip osteoporosis group; 5.46 ± 0.04 in the control group (Table2).

Table 1. Mean serum levels of RSA % , Mg, Cu, X mean ± SD

	Controls	Patients with osteoporosis with reduced hip bone density	Patients with osteoporosis with reduced bone density of the lumbar spine
Number of subjects examined	n = 14	n = 14	n = 37
BMD g/cm^2	1.13 ± 0.13	0.602 ± 0.49	0.729 ± 0.043
RSA%	55.67 ± 1.38	68.87 ± 13.26	73.30 ± 9.84
Age	62.29 ± 8.34	65.71 ± 6.43	62.78 ± 7.91
BMI, kg/m^2	28.86 ± 4.74	21.75 ± 3.33	24.44 ± 4.07
Mg, mmol/l	0.82 ± 0.07	0.96 ± 0.22	0.92 ± 0.19
Cu, µmol/l	19.74 ± 3.42	21.69 ± 5.21	21.11 ± 5.7

Table 2. t-test, P < 0.05 for statistical significance

Parameter	Patients with hip osteopenia vs controls	Patients with lumbar spine osteopenia versus controls	Patients with hip osteoporosis versus lumbar spine osteoporosis
BMD g/cm^2	P < 0.05	P < 0.05	P < 0.05
RSA %	P > 0.05	P < 0.05	P > 0.05
Възраст	P > 0.05	P > 0.05	P > 0.05
BMI, kg/m^2	P < 0.05	P < 0.05	P < 0.05
Mg, mmol/l	P < 0.05	P < 0.05	P > 0.05
Cu, µmol/l	P > 0.05	P > 0.05	P > 0.05

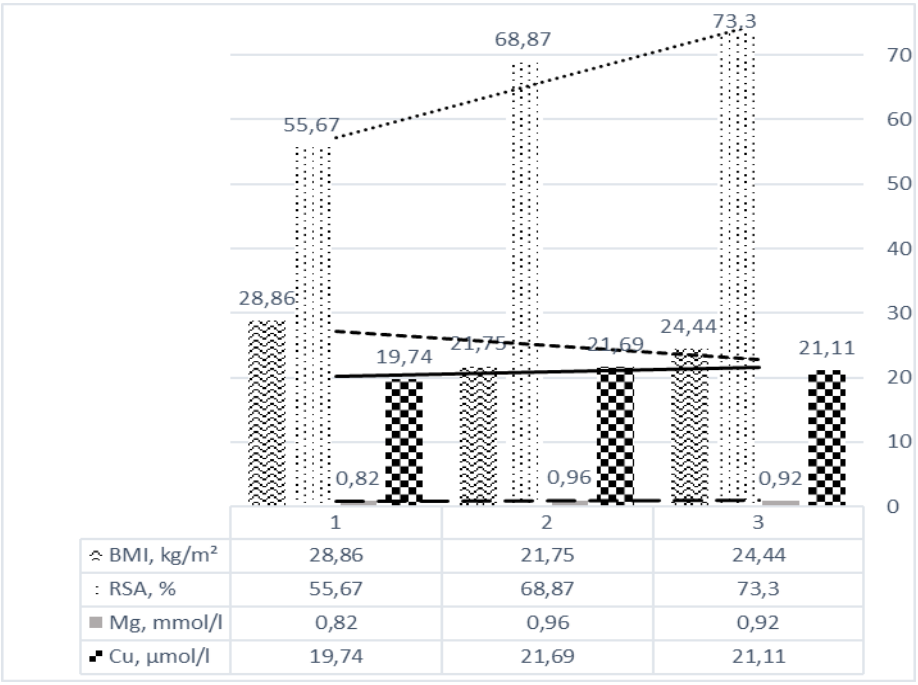

Figure 1. Mean values of BMI, RSA%, Mg, Cu, for the 3 groups (1 – control group, 2 – patients with osteoporosis of the hip, 3 – patients with osteoporosis of the lumbar vertebrae)

Discussion

The obtained results of the t-test according to age and BMD indicators confirm the correct selection of the studied participats. According to the BMI indicator, the groups have statistically significant differences and confirm what has been proven by previous researchers, that with a BMI > 27, the probability of having osteoporosis significantly decreases (Boyanov, 2006).

Serum copper and magnesium concentrations of all patients were elevated relative to the controls, but there was no statistical difference between groups with different disease localization. Patients with osteoporosis were found to have a higher antioxidant activity в сравнение с контролите. When monitoring the radical scavenging

activity, depending on the localization of the disease, we detected higher values in patients with osteoporosis of the lumbar spine.

Conclusions

Patients with osteoporosis have higher antioxidant activity in response to increased free-radical processes in the body

As bone density decreases, the level of serum RSA increases, as a result of increased ROS production. The higher level of radicals probably enhances the production of liver enzymes such as Cu, and Zn-SOD. On the other hand, the kinetics of radical reactions give us reason to consider that the overproduction of radicals damages the protein structure of oxidoreductases, especially in patients with slower biochemical mechanisms.

The serum concentration of copper is elevated in osteoporosis patients and leads to increased RSA

During the destruction of metalloproteins, redox-active metal ions are released. The higher level of radicals also damages cell membranes, making it easier for free copper to pass into the blood. The increased concentration of copper ions in the serum initiates secondary radical processes and further increases the patients' RSA.

The localization of osteoporosis determines the degree of imbalance of the redox state

The difference in BMD of the lumbar spine and the hip determines a different degree of imbalance of the redox state, RSA.

RSA% as a marker for localization of osteoporosis

To establish RSA% as a marker for localization of decreased bone density, it is necessary to: increase the number of participants; select participants with close values of BMI.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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New Findings in the Ethnobotany of Uzbekistan

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Abstract: This research provides information about the significance and use of some wild flora plants such as *Atraphaxis pyrifolia* Bunge and *Megacarpaea gigantea* Regel in folk medicine by the local population of some districts of Samarkand and Navoi regions of the Republic of Uzbekistan. The above-listed species are little known to official medicine, but local traditional healers have been using them for many years and have a proven record of applying these plants in the treatment of various human diseases. For example, infusion of leaves of *Atraphaxis pyrifolia* is used by local people for cardiovascular diseases, insomnia, and as a sedative, seeds of *Megacarpaea gigantea* for treatment of kidney and cholelithiasis, as well as inflammation of the kidneys. However, the plant raw materials are collected from natural places of distribution which may cause a process of the natural deterioration of their supply in future and the sale of dried parts is carried out by traders in the markets or local traditional healers. Thus, this study implies how crucial it is to develop a prospect for the use of medicinal plants in Uzbekistan, and its significance in terms of improving the economy of the region, improving the quality of life of the population and improving the health of the nation.

Keywords: Medicinal plant, Human diseases, Flora, Uzbekistan, Ethnobotany.

Introduction

For centuries, medical plants herbs have been widely used in traditional medicine and are still actively applied in a variety of cases. Initially, by trial and error, and then with some skill and experience, a person began to use natural remedies of plant origin to treat diseases or ailments, and thus knowledge of useful plants with their medicinal effect appeared (Fitzgerald et al., 2020). The use of medicinal plants/herbs has been gradually improving over many generations, and eventually, this was a crucial step which lead to a rise in the development of traditional medicine. The official definition of traditional medicine can be seen as “a body of knowledge, skills and practices based on theories, beliefs and experiences, inherent in different cultures, explainable or inexplicable, used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses” (World Health Organization, 2000).

Nowadays, the actual database of flora in Uzbekistan has more than 4385 wild species. Since the publication of the first edition of the “Flora of Uzbekistan”, at least 712 additional wild species have been added to the national control list, while the six-volume edition of the Flora of Uzbekistan (1941–1962) contains 4148 species (138 families, 1023 genera), including 3663 native and 485 aboriginal species (Li et al., 2020). According to Belolipov et al. (2015), the largest number of plant species of the natural flora of Uzbekistan with medicinal qualities is found in such families as Apiaceae Lindl., Lamiaceae Martinov, Asteraceae Bercht. & J. Presl, Brassicaceae Burnett., Rosaceae Juss., Liliaceae Juss. and Fabaceae Lindl. In addition, over 200 species are broadly used in traditional medicine.

In recent years, the Republic of Uzbekistan has been implementing consistent reforms and adopting a number of governmental decisions on the protection of medicinal plants, the rational use of natural resources, and the construction of plantations for the cultivation of medicinal plants and their (processing/recycling, refining). Of

the wild representatives of the flora of Uzbekistan, 112 species of medicinal plants are registered for use in scientific medicine, of which 70 species are actively used in the pharmaceutical industry (Eshpulatov et al., 2021).

It must be noted that with the latest technological evolution of medicine production and the process of producing synthetic chemicals, many different people and ethnic groups still often use medicinal herbs to treat various diseases and ailments. However, for several reasons, some of them are not registered in the state registries of pharmacopoeial species, are poorly studied, or are under research.

Materials and Methods

We reviewed the markets of Samarkand and Navoi regions to obtain general information about the healing properties of medicinal plants mainly used by the locals and their commercial form. The survey of the local population was conducted in the form of a questionnaire with the acknowledgement of the respondents. The interviews were conducted strictly following the rules of the Code of Ethics established by the International Society for Ethnobiology (The ISE Code of Ethics, 2006). All data received from the respondents were recorded in a special form.

Results and Discussion

As a result of these scientific trips to the Samarkand and Navoi regions, we conducted an ethnobotanical analysis/survey of people involved in the process of preparation and sale of medicinal herbs and traditional healers. During the expeditions in the Kattakurgan and Narpay districts of the Samarkand region, as well as the city of Navoi of the Navoi region, medicinal, spicy-aromatic, food plant species from the local flora and those imported from foreign countries were identified. As a result, we have identified about 50 plant species belonging to various genera and families sold in the markets of the studied regions (refer to the table below).

Table 1.Taxonomic composition of the examined plants

№	Plant family	Number of genus	Number of species
1	Apiaceae Lindl.	3	4
2	Araceae Juss.	1	1
3	Asteraceae Bercht. & J.Presl	9	9
4	Berberidaceae Juss.	1	1
5	Brassicaceae Burnett.	1	1
6	Crassulaceae J.St.-Hil.	1	1
7	Cucurbitaceae Juss.	2	2
8	Equisetaceae Michx. ex DC.	1	1
9	Fabaceae Lindl.	4	4
10	Gentianaceae Juss.	1	1
11	Hypericaceae Juss.	1	2
12	Iridaceae Juss.	1	1
13	Lamiaceae Martinov	5	5
14	Lauraceae Juss.	2	2
15	Papaveraceae Juss.	1	1
16	Poaceae Barnhart	1	1
17	Polygonaceae Juss.	3	3
18	Ranunculaceae Juss.	1	1
19	Rosaceae Juss.	3	3
20	Rubiaceae Juss.	1	1
21	Solanaceae Juss.	1	1
22	Urticaceae Juss.	1	1
23	Zingiberaceae Martynov	2	2
TOTAL:		47	49

The given table above demonstrates that the leaders in the number of species among medicinal, spicy-aromatic, food plant species belong to the family Asteraceae (9/18.3%), then following with by several families - Lamiaceae (5/10.2%), Apiaceae (4/8.2%), Fabaceae (4/8.2%) and Polygonaceae (3/6.1%). Finally the

remaining families are represented by either two or one species. In this analysis, we took into account all plant species, regardless of their practical application and place of origin, that is, whether the studied species were representatives of the flora of Uzbekistan or introduced plants or imported plants from near and far abroad.

Over the course of these studies, several types and medicinal collections were identified that are used by the locals as a method of treatment for many well-known diseases. For example, *Atraphaxis pyrifolia*, *Astragalus sieversianus* Pall., *Leonurus turkestanicus* V.Krecz.&Kuprian., *Hypericum perforatum* L., *Ziziphora pedicellata* Pazij et Vved., *Mentha piperita* L., *Melissa officinalis* L., *Cichorium intybus* L., *Berberis integerrima* Bunge, *Megacarpaea gigantea*, *Crataegus turkestanica* Pojark., *Arum korolkowii* Regel, *Equisetum arvense* L., *Achillea millefolium* L., *Helichrysum maracandicum* Popov ex Kirp., *Hypericum scabrum* L., *Tussilago farfara* L., *Salvia sclarea* L., *Rhodiola hetrodontha* (Hook. f. et Thomson) Boriss., *Rheum maximowiczii* Losinsk., *Inula grandis* Schrenk, *Urtica dioica* L., *Tanacetum pseudachillea* C. Winkl., *Cichorium intybus* L., *Rosa webbiana* Wall. ex Royle, *Berberis integerrima* Bunge, etc.

However, almost all species of identified plants have long been used both in traditional and modern medicine, except for the little-known local species of *Atraphaxis pyrifolia* and *Megacarpaea gigantea*. Both species grow on the territory of the Republic of Uzbekistan, and the local population collects the raw plant materials. The habitat of *Megacarpaea gigantea* includes the Kok-Suv ridge of the Western Tien Shan and the Zaravshan, Aktau, Nuratau, and Gissar ridges of the Pamir-Alai mountain system. The species occurs most frequently in Samarkand and Kashkadarya oblasts, where it is mainly collected by the local population for ethnobotanical purposes.

Atraphaxis pyrifolia can be found on the slopes of the lower and middle mountain zones in the Tashkent, Ferghana, Namangan, Samarkand, Jizzak, Kashkadarya, Surkhandarya and Navoi regions of Uzbekistan. When conducting ethnobotanical research, an important aspect is a search for new recipes for the preparation of medicinal decoctions, infusions, teas, ointments, collections, and other remedies and medicines used by the locals only to introduce them to a larger audience.

According to local traditional healers and people who are trading various herbal products, *Megacarpaea gigantea* seeds have been commonly used for many decades for medicinal purposes. As a remedy for kidney stones, cholelithiasis and inflammation of the kidneys, the people of these areas use the seeds of this plant. It is recommended to chew one seed half an hour before a meal for a period of 1-3 months. An infusion of the leaves of *Atraphaxis pyrifolia* is recommended to drink to improve heart activity and blood circulation, also for headaches, insomnia, and tinnitus, and to increase the general tone of the body. For this purpose, take 1 tsp dried crushed leaves and pour 200 ml into a glass of boiling water, then cool at room temperature, strain, and squeeze the remaining raw material. Drink 1/4 cup 3-4 times a day.

Conclusion

In summary, the analysis of the state of use of medicinal plants of domestic flora showed the need to increase ethnobotanical research in all regions of the country. As part of this study, we have identified promising but understudied medicinal plants from the local flora - *Atraphaxis pyrifolia* and *Megacarpaea gigantea*, with their original recipes created and tested over generations by traditional healers and residents. Today, due to the low toxicity and milder effect on the body of herbal medicines, many residents of our country use wild plants of local flora for the treatment and prevention of diseases. In this regard, it is necessary to conduct targeted research to develop the production of local herbal remedies, improve technologies for isolating biologically active substances, and establish their chemical structure, modification and purification.

Scientific Ethics Declaration

The author declares that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the author.

Acknowledgements or Notes

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Rapid Multiplex PCR for Species Authentication: Current Prospects and Challenges

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Abstract: Multiplex PCR is a powerful technique that allows simultaneous amplification of multiple DNA targets in a single reaction. It has been widely applied for species authentication in various food matrices, such as meat, fish, dairy, and honey products. Species authentication is important for ensuring food safety, quality, traceability, and compliance with religious, ethical, and environmental standards. However, multiplex PCR also faces some challenges and limitations, such as primer design, optimization, validation, interference, and interpretation of results. This review summarizes the current state of the art of multiplex PCR for species authentication, highlighting its advantages, applications, challenges, and future directions. It also provides some practical guidelines and recommendations for designing and performing multiplex PCR assays for reliable and accurate species identification.

Keywords: Multiplex PCR, Species authentication, Meat products; Species-specific DNA; Food fraud, Food quality.

Introduction

Food authentication is the process of verifying the identity, origin, and quality of food products and ingredients. It is essential for ensuring food safety, traceability, and compliance with religious, ethical, and dietary preferences of consumers (Safdar & Juenjo, 2015; Fanelli et al., 2021). However, food authentication is also challenging due to the complexity, diversity, and adulteration of food matrices, as well as the lack of standardized and reliable methods for detection and differentiation of food species (Safdar et al.,2013; Cichna Markl et al.,2023).

Among the various analytical techniques available for food authentication, multiplex PCR has emerged as a powerful and versatile tool that allows simultaneous amplification of multiple DNA targets in a single reaction. Multiplex PCR can provide rapid, sensitive, specific, and cost-effective identification of food species from raw and processed samples. It can also detect adulteration, substitution, or mislabeling of food products with undesired or prohibited animal species (Safdar et al.,2013; Fanelli et al., 2021).

However, multiplex PCR also faces several technical and practical challenges that limit its widespread application and acceptance in food authentication. These challenges include designing optimal primers, selecting appropriate annealing temperatures, balancing primer concentrations, avoiding primer-dimer formation, and ensuring specificity and sensitivity of the reaction. Moreover, multiplex PCR requires validation,

standardization, and harmonization of methods and protocols across different laboratories and regions (Ali et al., 2014;Ruslan et al., 2022).

In this review, we aim to provide a comprehensive overview of the current status and future prospects of multiplex PCR for food authentication. We will discuss the methods, and applications of multiplex PCR for species identification in various food matrices. We will also highlight the challenges and solutions of multiplex PCR for food authentication. Finally, we will suggest some directions for future research and development in this field.

Principles and Methods of Multiplex PCR

The principle of multiplex PCR is similar to that of conventional PCR, except that more than one pair of primers is added to the reaction mixture. Each primer pair anneals to a specific region of the template DNA and directs the synthesis of a complementary strand by a thermostable DNA polymerase. The resulting amplicons can be detected by various methods, such as gel electrophoresis, capillary electrophoresis, hybridization, or fluorescence (Markalatos et al., 2022; Perkel, 2012).

The methods of multiplex PCR vary depending on the type and number of targets, the choice of probes, and the detection system. Some of the common methods are:

- **TaqMan® probes:** These are hydrolysis probes that consist of an oligonucleotide with a fluorophore at the 5' end and a quencher at the 3' end. During PCR, the probe anneals to a specific target sequence between the primers. When the polymerase extends the primer and reaches the probe, it cleaves the probe and releases the fluorophore, which emits fluorescence. The fluorescence signal is proportional to the amount of target DNA. Different probes can be labeled with different fluorophores for multiplexing (Markalatos et al.,2022; Perkel, 2012).
- **Molecular beacons:** These are hairpin-shaped probes that have a fluorophore at one end and a quencher at the other end. In the absence of target DNA, the probe forms a stem-loop structure that brings the fluorophore and the quencher close together, preventing fluorescence. When the probe binds to a complementary target sequence during PCR, it opens up and separates the fluorophore from the quencher, allowing fluorescence. Different probes can be labeled with different fluorophores for multiplexing (Markalatos et al.,2022; Perkel, 2012).
- **FRET (fluorescence resonance energy transfer) probes:** These are pairs of probes that hybridize to adjacent regions of the target DNA during PCR. One probe is labeled with a donor fluorophore and the other with an acceptor fluorophore. When the probes are in close proximity, the donor fluorophore transfers energy to the acceptor fluorophore, which emits fluorescence at a different wavelength. The fluorescence signal indicates the presence of target DNA. Different pairs of probes can be labeled with different combinations of fluorophores for multiplexing (Markalatos et al., 2022; Perkel, 2012).
- **SYBR® Green:** This is a dye that binds to double-stranded DNA and fluoresces when excited by light. During PCR, as more amplicons are generated, more dye binds and more fluorescence is produced. The fluorescence signal is proportional to the amount of total DNA in the reaction. However, this method cannot distinguish between specific and nonspecific products, so it requires additional analysis such as melting curve analysis or gel electrophoresis to confirm the identity and size of the amplicons (Safdar et al., 2015).

Applications of Multiplex PCR for Species Authentication

Species authentication is the process of verifying the identity and origin of animal species in food products and ingredients. It is important for ensuring food safety, quality, traceability, and compliance with religious, ethical, and dietary preferences of consumers. It is also essential for preventing food fraud, adulteration, substitution, or mislabeling of food products with undesired or prohibited animal species (Safdar et al., 2013; Safdar & Juenjo, 2015). It is a valuable technique for species authentication, as it can provide rapid, sensitive, specific, and cost-effective identification of multiple animal species in a single reaction. Multiplex PCR can detect DNA from different animal species in various food matrices, such as raw and processed meat, fish, seafood, dairy, and honey products. Multiplex PCR can also discriminate between closely related species, such as sheep and goat, or chicken and duck.

Multiplex PCR has been applied successfully for species identification in foods of various origins, such as meat (Lopez Andreo et al.,2006; Izadpanah et al., 2018), fish , and seafood products (Santaclara et al.,2006; Safdar&

Juenjo, 2015; Vakshiteh et al.,2016). Some practical examples of multiplex PCR methods for species authentication are:

- A multiplex PCR method using TaqMan probes for the detection of sheep/goat, bovine, chicken, duck and pig DNA in meat products (Izadpanah et al.,2018). The method was able to detect 0.1% of adulteration in binary mixtures and 0.01% in ternary mixtures.
- A multiplex PCR method using molecular beacons for the detection of horse, donkey, beef, pork, chicken and turkey DNA in meat products (Lopez Andreo et al.,2006). The method was able to detect 0.01% of adulteration in binary mixtures and 0.001% in ternary mixtures.
- A multiplex PCR method using FRET probes for the detection of Atlantic salmon (Salmo salar), rainbow trout (Oncorhynchus mykiss), European sea bass (Dicentrarchus labrax) and gilthead sea bream (Sparus aurata) DNA in fish products. The method was able to detect 0.1% of adulteration in binary mixtures (Santaclara et al.,2006; Vakshiteh et al., 2016; Verez Bagnis et al.,2017).
- A multiplex PCR method using SYBR Green for the detection of shrimp (Penaeus monodon), crab (Scylla serrata), squid (Loligo duvauceli) and cuttlefish (Sepia pharaonis) DNA in seafood products. The method was able to detect 0.01% of adulteration in binary mixtures (Santaclara et al.,2006;Safdar& Juenjo, 2015; Vakshiteh et al., 2016).
- **Foodborne pathogen detection:** Multiplex PCR can be used to detect and differentiate various foodborne pathogens, such as bacteria, viruses, fungi, and parasites, in food samples. Multiplex PCR can provide rapid and accurate identification of the causative agents of foodborne diseases, such as Salmonella, Escherichia coli, Listeria, Campylobacter, Vibrio, Norovirus, Hepatitis A virus, Aspergillus, Fusarium, Cryptosporidium, and Giardia (Malorny et al., 2003; Wang et al.,2014;Wang et al., 2015). Multiplex PCR can also be used to detect antimicrobial resistance genes or virulence factors in foodborne pathogens (Wang et al.,2015).
- **Food allergen detection:** Multiplex PCR can be used to detect and quantify food allergens, such as peanuts, soybeans, milk, eggs, wheat, fish, shellfish, and nuts, in food products (Vakhshiteh et al.,2016). Multiplex PCR can provide sensitive and specific detection of food allergens to prevent allergic reactions in consumers. Multiplex PCR can also be used to detect cross-contamination or mislabeling of food products with allergenic ingredients (Holzhauser et al.,2009; Roder et al.,2011).
- **Food authenticity detection:** Multiplex PCR can be used to detect and verify the origin and quality of food products and ingredients. Multiplex PCR can provide reliable identification of animal species, such as meat, fish, seafood, dairy, and honey products (Vakhshiteh et al.,2016). Multiplex PCR can also be used to detect adulteration or substitution of food products with cheaper or inferior materials (Safdar& Juenjo,2015). Multiplex PCR can also be used to detect genetically modified organisms (GMOs) or organic products in food products (Holst Jensen et al.,2003; QIAGE,n.d.).
- **Food mycotoxin detection:** Multiplex PCR can be used to detect and quantify mycotoxins, such as aflatoxins, ochratoxins, fumonisins, trichothecenes, and zearalenone, in food products. Multiplex PCR can provide rapid and accurate detection of mycotoxins to prevent toxic effects in consumers. Multiplex PCR can also be used to detect the presence of toxigenic fungi or molds in food products (Li et al.,2011; Li et al.,2012).

Challenges and Limitations of Multiplex PCR

Optimization of reaction conditions: Multiplex PCR requires careful optimization of various parameters, such as primer design, primer concentration, annealing temperature, magnesium concentration, and cycle number, to achieve efficient and specific amplification of multiple targets in a single reaction. Optimization of multiplex PCR is often laborious, time-consuming, and empirical, and may require trial-and-error experiments or software programs to find the optimal conditions for each target. In addition, optimization of multiplex PCR may vary depending on the type and quality of the food sample, the presence of inhibitors or contaminants, and the detection system used (Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al.,2018).

Separation and detection of products: Multiplex PCR requires a reliable and sensitive method for separating and detecting the amplified products. Traditional methods, such as agarose gel electrophoresis or capillary electrophoresis, may not be suitable for separating multiplex PCR products that differ by only a few base pairs or have overlapping sizes. Moreover, these methods may not be able to quantify the products accurately or distinguish between specific and nonspecific products. Therefore, multiplex PCR often requires more advanced methods, such as real-time PCR with fluorescent probes or melting curve analysis, or high-resolution

electrophoresis with automated systems (Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al., 2018).

Quality control and validation: Multiplex PCR requires rigorous quality control and validation procedures to ensure the accuracy and reliability of the results. Quality control measures include using appropriate controls, such as positive controls, negative controls, internal controls, and external controls, to monitor the performance of the reaction and detect any errors or contamination. Validation procedures include verifying the specificity, sensitivity, reproducibility, robustness, and limit of detection of the multiplex PCR method for each target and each food matrix. Validation procedures may also require comparison with other methods or reference standards (Perkel,2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al.,2018).

Strategies to Overcome the Challenges of Multiplex PCR

Strategies to overcome the challenges of multiplex PCR in food science are:

Primer design: Primer design is crucial for successful multiplex PCR, as it affects the specificity, sensitivity, and efficiency of the reaction. Primers should be designed to have similar melting temperatures, lengths, and GC contents, and to avoid complementarity or cross-hybridization with each other or with nonspecific regions of the template DNA. Primers should also be designed to amplify products of different sizes or with different fluorescent labels for easy separation and detection. Primer design can be facilitated by using software programs or databases that can check for primer properties and potential interactions (Perkel,2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al.,2018).

Primer concentration: Primer concentration is another important factor for optimal multiplex PCR, as it affects the balance and yield of the products. Primer concentration should be adjusted empirically or using a universal primer concentration to ensure equal amplification of all targets. Primer concentration should also be optimized to avoid primer-dimer formation or nonspecific amplification. Primer concentration can be optimized by using gradient PCR or serial dilutions ((Perkel,2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al.,2018).

Annealing temperature: Annealing temperature is a critical parameter for specific and efficient multiplex PCR, as it affects the hybridization of primers to their target sequences. Annealing temperature should be optimized to ensure optimal binding of all primers without compromising specificity or sensitivity. Annealing temperature can be optimized by using gradient PCR or empirical testing (Perkel,2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al., 2018).

Magnesium concentration: Magnesium concentration is a key component for multiplex PCR, as it affects the activity and fidelity of the DNA polymerase. Magnesium concentration should be optimized to ensure optimal enzyme performance without causing nonspecific amplification or primer-dimer formation. Magnesium concentration can be optimized by using gradient PCR or serial dilutions (Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al., 2018).

Cycle number: Cycle number is a variable parameter for multiplex PCR, as it affects the quantity and quality of the products. Cycle number should be optimized to ensure sufficient amplification of all targets without causing saturation or degradation of the products. Cycle number can be optimized by using standard curves or empirical testing (Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al.,2018).

Additives or enhancers: Additives or enhancers are optional components for multiplex PCR, as they can improve the reaction efficiency and specificity. Additives or enhancers can include synthetic additives, such as Factor MP (QIAGEN), that promote stable and efficient annealing of multiple primers; buffer components, such as betaine, DMSO, or formamide, that reduce secondary structure formation or increase primer melting; or enzyme stabilizers, such as BSA, that prevent enzyme inhibition or degradation(Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al., 2018).

Quality control measures: Quality control measures are essential for reliable and accurate multiplex PCR results, as they can monitor the performance and validity of the reaction. Quality control measures can include using appropriate controls, such as positive controls, negative controls, internal controls, and external controls, to detect any errors or contamination; using validation procedures, such as verifying the specificity, sensitivity, reproducibility, robustness, and limit of detection of the multiplex PCR method for each target and each food

matrix; and using comparison methods or reference standards to confirm the results (Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al., 2018).

Validation the Specificity and Sensitivity of a Multiplex PCR Assay

Some strategies to validate the specificity and sensitivity of a multiplex PCR assay for food allergen detection are:

- **Specificity:** Specificity is the ability of the multiplex PCR assay to detect only the intended food allergens and not other food components or contaminants. Specificity can be validated by testing the multiplex PCR assay with various food matrices, both positive and negative for the target allergens, as well as with potential cross-reactive or interfering substances, such as other allergens, plant or animal species, or DNA inhibitors. Specificity can also be validated by comparing the multiplex PCR assay with other methods, such as ELISA or mass spectrometry, or with reference standards, such as certified reference materials or proficiency testing samples (Garber et al.,2020; Waiblinger et al., 2022).
- **Sensitivity:** Sensitivity is the ability of the multiplex PCR assay to detect low levels of the target food allergens in food samples. Sensitivity can be validated by testing the multiplex PCR assay with serial dilutions of known concentrations of the target allergens in various food matrices, both raw and processed. Sensitivity can also be validated by determining the limit of detection (LOD) and the limit of quantification (LOQ) of the multiplex PCR assay for each target allergen in each food matrix. LOD and LOQ can be calculated by using statistical methods, such as probit analysis or linear regression, based on the signal-to-noise ratio or the standard deviation of the blank (Garber et al., 2020; Waiblinger et al., 2022).

Multiplex PCR for Simultaneous Detection of Multiple Foodborne Pathogens

We can use multiplex PCR for simultaneous detection of multiple foodborne pathogens in complex matrices by following these steps:

- **Selecting the target genes:** The target genes should be specific and conserved for each foodborne pathogen, and preferably located on the chromosome rather than on plasmids or phages. The target genes should also be related to the virulence or pathogenicity of the foodborne pathogens, such as toxin genes, invasion genes, or resistance genes (Garrido Maestu et al.,2019; Chen et al.,2021).
- **Designing the primers and probes:** The primers and probes should be designed to have similar melting temperatures, lengths, and GC contents, and to avoid cross-hybridization or primer-dimer formation. The primers and probes should also be designed to amplify products of different sizes or with different fluorescent labels for easy separation and detection. The primers and probes should be tested individually and in combination before multiplexing (Garrido Maestu et al.,2019; Chen et al.,2021).
- **Optimizing the reaction conditions:** The reaction conditions, such as primer concentration, annealing temperature, magnesium concentration, cycle number, and additives or enhancers, should be optimized to ensure efficient and specific amplification of all targets in a single reaction. The reaction conditions should also be optimized to overcome the inhibitory effects of complex matrices, such as food components or contaminants. The reaction conditions should be optimized by using gradient PCR, serial dilutions, standard curves, or empirical testing (Garrido Maestu et al.,2019; Chen et al.,2021).
- **Separating and detecting the products:** The products should be separated and detected by using a reliable and sensitive method, such as real-time PCR with fluorescent probes or melting curve analysis, or high-resolution electrophoresis with automated systems. The products should be quantified by using standard curves or internal controls. The products should also be verified by using quality control measures, such as positive controls, negative controls, external controls, or validation procedures (Garrido Maestu et al.,2019; Chen et al.,2021).

Future Perspectives and Recommendations

Increasing the multiplexing capacity: Multiplex PCR can be improved by increasing the number of targets that can be detected simultaneously in a single reaction. This can be achieved by using novel probe technologies, such as padlock probes, molecular inversion probes, or universal tag arrays, that can generate thousands of unique signals for different targets. Alternatively, multiplex PCR can be combined with other

techniques, such as microarrays, next-generation sequencing, or digital PCR, that can increase the throughput and resolution of the analysis (Mafra et al., 2008; Sint et al., 2012).

Enhancing the sensitivity and specificity: Multiplex PCR can be enhanced by increasing the sensitivity and specificity of the detection of low-abundance or closely related targets in complex matrices. This can be achieved by using novel amplification strategies, such as loop-mediated isothermal amplification, rolling circle amplification, or helicase-dependent amplification, that can amplify the target sequences without thermal cycling or primers. Alternatively, multiplex PCR can be combined with other techniques, such as immunomagnetic separation, aptamer-based capture, or CRISPR-Cas systems, that can enrich or edit the target sequences before or after amplification (Mafra et al.,2008; Sint et al., 2012).

Developing point-of-care devices: Multiplex PCR can be developed into point-of-care devices that can provide rapid and accurate detection of foodborne pathogens or allergens in situ. This can be achieved by using microfluidic platforms, such as lab-on-a-chip or paper-based devices, that can integrate sample preparation, amplification, and detection steps in a miniaturized and automated format. Alternatively, multiplex PCR can be combined with other techniques, such as lateral flow assays, electrochemical biosensors, or smartphone-based readers, that can provide simple and portable detection systems (Mafra et al.,2008; Sint et al., 2012).

Some ethical and social implications of using multiplex PCR for food safety are:

- **Consumer protection:** Multiplex PCR can provide consumer protection by ensuring the authenticity, quality, and safety of food products and ingredients. Multiplex PCR can prevent food fraud, adulteration, substitution, or mislabeling of food products with undesired or prohibited animal species, allergens, GMOs, or organic products. Multiplex PCR can also prevent foodborne diseases, allergic reactions, or ethical/religious violations by detecting foodborne pathogens or allergens in food products (Settani & Corsetti,2007; Ali et al.,2014).
- **Regulatory compliance:** Multiplex PCR can provide regulatory compliance by meeting the standards and requirements of food labeling and traceability. Multiplex PCR can provide accurate and reliable identification and quantification of food components or contaminants in various food matrices, both raw and processed. Multiplex PCR can also provide rapid and cost-effective detection of food components or contaminants in situ or in the field. Multiplex PCR can facilitate the enforcement of food regulations and the prevention of food fraud (Settani & Corsetti,2007).
- **Ethical responsibility:** Multiplex PCR can provide ethical responsibility by respecting the rights and preferences of consumers, producers, and animals. Multiplex PCR can inform consumers about the origin, quality, and safety of food products and ingredients, and allow them to make informed choices based on their dietary, ethical, or religious preferences. Multiplex PCR can also protect producers from unfair competition or false accusations by verifying the authenticity and quality of their products. Multiplex PCR can also reduce animal suffering by minimizing the use of animals for testing or experimentation (Settani & Corsetti,2007;Safdar& Juenjo,2015).

Conclusion

It is concluded that multiplex PCR is a powerful technique that can simultaneously amplify multiple target sequences in a single reaction, providing rapid, sensitive, and specific detection of various food components or contaminants. It has been widely applied in food analysis for species authentication, foodborne pathogen detection, food allergen detection, food authenticity detection, and food mycotoxin detection. However, it also faces some challenges, such as optimization of reaction conditions, separation and detection of products, quality control and validation of methods, and ethical and social implications of results. In addition, it can be improved by using novel probe technologies, amplification strategies, detection systems, microfluidic platforms, and point-of-care devices. Furthermore, it can also be combined with other techniques, such as microarrays, next-generation sequencing, digital PCR, immunomagnetic separation, aptamer-based capture, CRISPR-Cas systems, lateral flow assays, electrochemical biosensors, or smartphone-based readers. Finally, multiplex PCR has great potential for future applications in food safety and quality control.

Scientific Ethics Declaration

The author declares that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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