Prof. dr hab. n. med.
Artur Stolarczyk
specjalista ortopedii
i traumatologii

Warszawski Uniwersytet Medyczny

Wydział Lekarski

Olga Adamska

Badania nad wpływem farmakologicznie wywołanej cukrzycy na morfologiczne zmiany oraz wpływem melatoniny na stężenie markerów biochemicznych stresu oksydacyjnego w więzadłach stawu kolanowego

Evaluation of the influence of pharmacologically- induced diabetes mellitus on alterations in morphological appearance and influence of melatonin supplementation on the concentration of biochemical markers of oxidative stress in the ligaments in knee joint

Rozprawa na stopień doktora w dziedzinie nauk medycznych i nauk o zdrowiu

w dyscyplinie nauki medyczne

Promotor:

Prof. dr hab. n. med. Artur Stolarczyk

Klinika Ortopedii I Rehabilitacji

Warszawskiego Uniwersytetu Medycznego

Warszawa 2023r.

Słowa kluczowe: cukrzyca, stres oksydacyjny, melatonina, morfologia więzadeł, staw kolanowy

Keywords: diabetes mellitus, oxidative stress, melatonin, morphology of ligaments, knee joint

Kieruję serdeczne podziękowania do Promotora niniejszej rozprawy doktorskiej, Pana prof. dr hab. n. med. Artura Stolarczyka, Kierownika Kliniki Ortopedii i Rehabilitacji WUM za inspirację do podjęcia badań, wsparcie merytoryczne w przygotowaniu rozprawy oraz nieustającą mobilizację do pracy.

Chciałabym ponadto bardzo podziękować Sz. P. prof. dr hab. n med. Agnieszce Cudnoch-Jędrzejewskiej oraz całemu Zespołowi Katedry i Zakładu Fizjologii Doświadczalnej i Klinicznej WUM za przychylność i inspirację, czego efektem było umożliwienie mi pracy w laboratorium oraz życzliwa gotowość do wsparcia.

Moim Najbliższym, w szczególności Mamie i Tacie, dziękuję za cierpliwość, wyrozumiałość, troskę i wsparcie w trakcie okresie powstawania pracy.

The animal study protocol was approved by the Institutional Ethics Committee of Warsaw School of Applied Sciences.

Protocol code: WAW2/027/2019

Date of approval: 25/02/2019

Table of contents

1. List of abbreviations

SPRD- Sprague– Dawley rats

AGEs- advanced glycation end-products

DM- diabetes mellitus

ECM- extracellular matrix

RAGE- receptor for advanced glycation end-products

Col1A1- collagen type 1

Col3A1- collagen type 3

MMP/s- metalloproteinase/s

VEGF- vascular endothelial growth factor

LSS- lumbar spinal stenosis

LF- ligamentum flavum

ROS- reactive oxygen species

PPARs- peroxisome proliferator- activated receptors

ER- endoplasmic reticulum

PDL- periodontal ligament

TMMPs- tissue inhibitors of MMPs

ATF6- activating transcription factor 6

PKR- double-stranded RNA-activated protein kinase

PERK- endoplasmic reticulum kinase

IRE1- inositol requiring enzyme 1

STZ- streptozotocin

STZ DM- streptozotocin- induced diabetes mellitus

SOD- superoxide dismutase

GST- glutathione S-transferase

TAS- tissues total antioxidant status

TOS- total oxidant status

ROS- reactive oxygen species

OSI- oxidative stress index

MDA- malondialdehyde

GSH- glutathione

NO- nitric oxide

2. Context

Connective tissue aging is accelerated by a progressive accumulation of advanced glycation end-products (AGEs). Formation of AGEs is characteristic for diabetes mellitus (DM) progression, and affects only specific proteins with relatively long half-lives. This is the case of fibrillar collagens that are highly susceptible to glycation. While collagen provides a framework for plenty of organs, the local homeostasis of specific tissues has been seriously afflicted. Among the many age- and diabetes- related morphological changes affecting human connective tissues there is concurrently reduced healing capacity, flexibility and quality among ligaments, tendons, bones and skin. Although DM provokes a wide range of known clinical disorders, the exact mechanisms of connective tissue alteration is still being investigated. Most of them rely on animal models in order to conclude patterns of the damage.

Major interest of this study was to perform the surgical dissection of the left medial collateral ligament (MCL) on each rat model and to perform a sham procedure (being a preparation of the skin and fascia of the right MCL) to visualize the collagenous structure of interest, but without proceeding to dissection of MCL Furthermore the aim was to detect the influence of melatonin on biochemically expressed oxidative stress. The sacrifice resulted in enhancing the current state of art, by collecting tissues for histopathological, biomechanical and research studies for possible enhancing the knowledge concerning therapeutic approach on humans suffering from DM and its complications. It is planned to identify alterations in microarchitecture of MCL and remodeling of its morphology as well as to analyse differences of damage to ligaments in hyperglycemic rats compared to rats with normoglycemia, using histological, and biochemical evaluation.

The purpose of the present study was to ensure whether DM in fact, intensifies molecular principles of alteration, regeneration and healing process of the ruptured MCL in rat model of streptozotocin-induced DM. Another aim was to discover a melatonin supplementation effect on the morphological, histological and biochemical results of the tissue.

(1) Does MCL derived from diabetic rats show higher fibrosis, elastin degradation or degree of calcification than non-diabetic rats? (2) Does the microarchitecture differ between the groups and further diminishes biomechanical activity of the tissue?

3. Introduction

A lack of physiological action of insulin in DM induces an impaired metabolism of carbohydrates, lipids, and proteins, which are the necessary components for cellular homeostasis and tissue activities [1]. Altered glucose metabolism impacts on all the basic processes taking place in the organism and remains the reason for impaired body functioning and regenerative abilities [2]. DM is a complex metabolic disorder, which has several direct and indirect effects on multiple processes, starting from simple to more complex: chemotaxis, phagocytosis, bacterial killing, heat shock, protein expression, antioxidant synthesis, oxygen-free-radical generation, growth factor depletion, glucocorticoid concentration, cell proliferation, up-regulation of apoptosis, and extracellular matrix (ECM) synthesis [3,4]. The cellular and molecular background for those changes and irreversible degradation are further evaluated and, so far, the pathophysiology of tendinopathies has been clarified comprehensively. Despite the ligaments remaining frequently injured in patients in whom insulin resistance or DM developed and the fact that they are the key structures in joint balance, the influence of body hyperglycaemia is poorly understood [5].

Ligaments are compositions of collagenous tissue that create the joints and link bones together. Two-thirds of the inner biochemical content of ligaments is water, and one-third consists of solid components. Those in the majority consist of collagen (type I collagen accounting for 85% of the collagen), proteoglycans, elastin and other proteins, and glycoproteins such as actin, laminin, and integrins [6]. Ligaments play a crucial role in the motoric system by responding to loads and micro-injuries that affect them during the whole lifetime with an increased mass and stiffness. All the life activities together with ageing, maturation, tension, and exercise given to the joint affect the biomechanical properties of ligaments. While they play a key role in functioning and weight bearing, there is clinical evidence of alteration of their properties caused by DM, but the current knowledge is not comprehensive. Joint degenerative and inflammatory diseases such as commonly diagnosed osteoarthritis (OA), pending pain, and reduced activities of daily living are the top complaints of patient who attend orthopaedic outpatient clinics. The pain that accompanies the acceleration of pro-inflammatory molecules is the number-one cause of disability worldwide [7,8,9,10]. DM, on the other hand, will likely affect 500 million people worldwide by 2030 [8]. Diabetes is the seventh leading cause of death [9], while DM and OA affect about 1 billion

people worldwide [10]. Both remain a serious public health issue; therefore, they require extensive investigation into their mutual dependencies.

3.1. The Formation of Advanced Glycation End Products (AGEs) Is Considered to Be the Biggest Risk Factor for the Development of Diabetic Complications

Persistent hyperglycaemia provokes glucose interference with plasma molecules, covalent formations with lipids, and proteins via non-enzymatic glycation, which determines the alteration of molecular mechanisms and the overall tissue condition [10].

Protein glycation and the formation of AGEs play a crucial role in the pathogenesis of diabetic complications. AGEs create intra- and extracellular cross-links with proteins, sometimes lipids and nucleic acids, which participate in the progression of diabetic complications. They cause disruption in the molecular conformation, rearrange enzymatic activity, and disorganise molecule-receptor functioning [4,11,12]. The investigation of cellular receptors for AGEs (RAGE) revealed the pathophysiological bases for the alteration of intracellular signalling as well as expression of important genes, pro-inflammatory molecules, and free-radical release [6]. The activated RAGE promotes the chain reaction of reactive oxygen species and triggers the transcription of factor NF-κB [4,13]. Cross-link formation in collagen fibres dictates the development of pro-inflammatory molecules, which then accelerate the creation of a disorganised cell phenotype [14,15,16].

3.2. Negative Impact of Diabetes on the Musculoskeletal System

Beyond the most commonly occurring complications of DM, musculoskeletal disorders are commonly found, receiving unproportionally little attention in general. The evidence shows that musculoskeletal injuries in individuals with DM may occur due to the AGEs, increasing collagen crosslinking and generating abnormal collagen deposits in tendons and ligaments as well as abnormal thickening and joint rigidity [15,16]. Research concerning orthopaedic problems of patients with DM remains scarce, but musculoskeletal disabilities provoke substantial burdens on quality of life. When vascular and neurological deficits appear, they underline the prudence of the control of a glycaemic state in orthopaedics in terms of recovery [17]. Figure 1 presents possible complications of DM on orthopaedic patients.

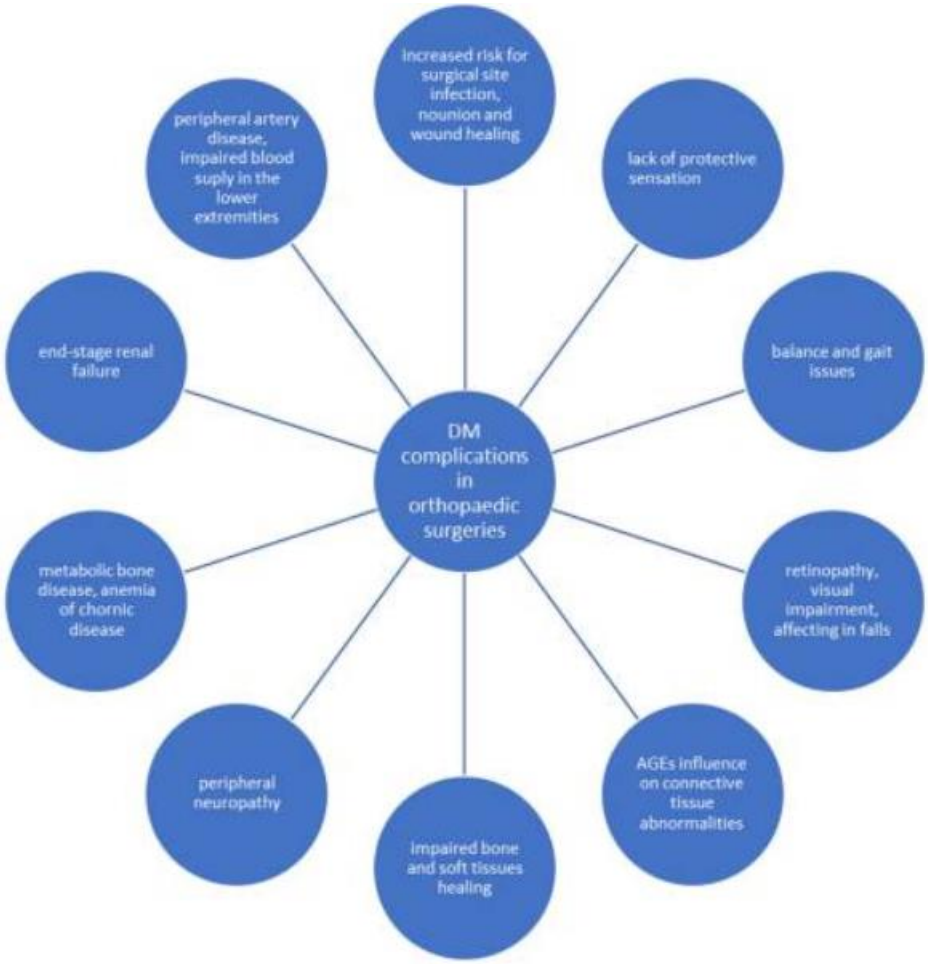

Figure 1. Potential impact of DM complications on patients undergoing orthopaedic surgeries [8].

Since 12% of all patients visiting orthopaedists suffer from DM, it is important to order the current knowledge about its effects on the musculoskeletal system [18].

A study conducted by Frisch et al. reported the connection between DM in patients undergoing surgery and an elevated complication ratio due to the poor quality of soft tissues, followed by prolonged hospital stays and higher perioperative mortality [19].

In general, surgery induces metabolic stress, catabolic hormone secretion, and breaking of an anabolic hormone action such as insulin. Patients with DM, who do not have an impaired insulin secretion and are treated with a major surgery, have an even more exacerbated metabolic stress-related response for increased insulin demand. Major surgery provokes functional insulin insufficiency [20]; therefore, diabetic patients are at higher risk for a procedure-induced infection and poor glycaemic control [19,21,22].

Studies consistent support that well-controlled DM is key for a successful surgery. Management by a multidisciplinary team and attention to discharge planning are the key aspects of care during and after orthopaedic surgery performed on patients with metabolic disorders [20,21,22]. However, there are currently no guidelines for orthopaedic departments to provide patients with a holistic approach to their metabolic disorders.

3.3. Heterogeneity in Connective Tissue Treatment Modalities—Tendons and Ligaments

It is generally recognized among the population worldwide that ageing societies carry out less physical activity. Additionally, joint stiffness is provoked by increased quantities of collagen cross-linking, an effect of AGEs. Due to differential histological formation between tendons and ligaments, the pathophysiologies of both tissues are heterogeneous. Furthermore, the prognosis for ligament healing is much worse [23,24].

Despite ligaments and tendons being functionally and grossly similar, mainly composed of collagen type I (Col1A1) and collagen type 3 (Col3A1), they still present as heterogeneous according to histological and biochemical characteristics. Plenty of studies concern tendon alteration facilitated by DM, but the literature is lacking regarding ligament redaction. The differences in tissue-specific collagen-maturation processes in fibroblasts creating ligaments and tendons are still unknown, and they provide the reason for the inferiority of ligaments in terms of collagen synthesis, proliferation, and migration. Studies found it to be a crucial aspect of the relatively poor healing potential of ligaments in comparison to tendons [21,22].

Experimental studies relying on animal models compared the morphological and biochemical features of ligaments and tendons and revealed a higher ratio of enzymatic content of the lysine hydroxylase 2/lysine hydroxylase 1 in ligaments. The expression of lysyl oxidase has a regulatory effect on the amount of enzymatic cross-linking. The levels of Col1A1 and Col3A1 were additionally greater in the case of the ligament matrices than in the tendon matrices. Ligament- and tendon-derived cells have distinct collagen-maturation processes at the cellular level, and collagen maturation of ligament cells is not necessarily inferior to that of tendons with regard to collagen synthesis and maturation [23].

The literature shows the decreased proliferation rate, higher turnover, and breakdown of fibroblasts derived from soft tissues induced by DM. These suggest that excessive proliferation together with an altered structure of the fibroblasts may contribute to a poor

production of collagen [24,25]. Further investigations evidence that DM fibroblasts have impaired migration and phenotypic change, increase in matrix metalloproteinase 9 (MMP-9), and diminished production of vascular endothelial growth factor (VEGF) [26,27,28].

3.4. Rheumatoid arthritis (RA) and T1DM

It has long been analysed that people with RA have a higher risk of T1D, and vice versa, but recent studies have concluded that the association of RA and T1D appears to be limited and specific to those RA patients with positive anti-citrullinated peptide antibodies. The risk of developing RA in later life was attributed partly to the presence of a specific allele 620 W PTPN22, possibly representing a common pathway for both autoimmune diseases. To date, both animal and human studies have yielded conflicting and inconsistent results linking DM with OA initiation and progression, and more rigorous data are needed [11,12]. Hyperglycaemia induces pro-inflammatory mediators, local toxicity, hypertrophy, and apoptosis in soft tissues of the joints [29]. DM induces chondrocytes to become hypertrophic and produce catabolic factors such as Interleukin 1ß and Fibroblast Growth Factor 2, which increases ECM degradation [29,30]. They also facilitate the further production of inflammatory mediators such as nitric oxide, prostaglandin E2, and ROS.

The study conducted by Njoto I. et al. confirms that DM with characteristic perlecan expression is a trigger for OA [30,31]. Radojčić MR et al. revealed that there are associations between the novel ECM biomarker C1M and local and systemic interleukin 6 with synovitis and pain, finding a net loss of collagen elevation and increase in levels of MMP-2, -9, and -13. Furthermore, cathepsin K is responsible for the degradation of C1M, and thus, the osteoclast's main protease, which enables its release from bone. It is a soft connective-tissue-degradation biomarker found to be released in an ex vivo model of synovitis as well as in RA, ankylosing spondylitis, and osteoarthritis. Inflammation then involves the majority of cytokines, which are essential in cellular communication and important mediators in the aberrant metabolism of articular structures [32].

3.5. Manifestation of ligaments pathologies in diabetic patients- specific findings

It has long been analysed that people with RA have a higher risk of T1D, and vice versa, but recent studies have concluded that the association of RA and T1D appears to be limited and specific to those RA patients with positive anti-citrullinated peptide antibodies. The risk of

developing RA in later life was attributed partly to the presence of a specific allele 620 W PTPN22, possibly representing a common pathway for both autoimmune diseases. To date, both animal and human studies have yielded conflicting and inconsistent results linking DM with OA initiation and progression, and more rigorous data are needed [11,12]. Hyperglycaemia induces pro-inflammatory mediators, local toxicity, hypertrophy, and apoptosis in soft tissues of the joints [29]. DM induces chondrocytes to become hypertrophic and produce catabolic factors such as Interleukin 1ß and Fibroblast Growth Factor 2, which increases ECM degradation [29,30]. They also facilitate the further production of inflammatory mediators such as nitric oxide, prostaglandin E2, and ROS.

The study conducted by Njoto I. et al. confirms that DM with characteristic perlecan expression is a trigger for OA [30,31]. Radojčić MR et al. revealed that there are associations between the novel ECM biomarker C1M and local and systemic interleukin 6 with synovitis and pain, finding a net loss of collagen elevation and increase in levels of MMP-2, -9, and -13. Furthermore, cathepsin K is responsible for the degradation of C1M, and thus, the osteoclast's main protease, which enables its release from bone. It is a soft connective-tissue-degradation biomarker found to be released in an ex vivo model of synovitis as well as in RA, ankylosing spondylitis, and osteoarthritis. Inflammation then involves the majority of cytokines, which are essential in cellular communication and important mediators in the aberrant metabolism of articular structures [32].

3.6. Histological and biochemical changes in ligaments specifically in diabetic patients

The process of ageing facilitates morphological and histological changes in the appearance of connective tissue. The glistening white appearance of connective tissue under the microscope is not recognizable anymore at a certain extent of ageing. DM was found to be a cofactor for exacerbating this change even more abruptly. Severely affected fragments show up greyish and amorphous, disproportionally distinguished into fusiform or nodular thickening portions [45].

Histopathological screening reveals a decreased number of fibroblasts and tenocytes together with a significantly decreased number of collagen fibres. Furthermore, they seem disintegrated and frayed, and their elasticity is consistent. The prognosis is rather poor due to the fact that detection of the condition usually takes place when the ligaments and tendons

are already significantly thickened. In such an advanced stage, the normal daily-life functioning remains impaired and both the quantitative and qualitative features of the tissue are irreversibly decreased. Moreover, disturbed blood flow as a result of the progression of DM disorganizes the fundamental components of the tissue, which hinders the recovery [46]. Investigation of the molecular and cellular pathophysiology of ligaments must be prioritised in order to achieve satisfactory remission. The current findings of mechanisms altered specifically in ligaments are summarised in Table 1.

Table 1. Characteristics of studies on properties of ligament alterations among diabetic populations.

Literature	Species model	Groups	Duration of DM	Analysed tissue	Found correlations
Li K. et al., 1995[27]	Sprague- Dawley rats	SCG (n=22) DM (n=28) DM-IT (n=7)	1 week	Ligament (MCL)	The length, thickness, and cross-sectional area of the DM MCL were significantly smaller than the control values - consistent with the reduced quantities of collagen in ligaments. MCL cell density was smaller in DM group and compared to DM-IT, but DM-IT showed improvement in properties compared to untreated DM.
Vincente A. et al., 2020[28]	Sprague- Dawley rats	CG (n= 20) DM (n=20) DM-IT (n=20)	11 days	Ligament (PDL)	Force applied to PDL of DM rats caused more inflammatory response, more oxidative stress, and a greater extent of orthodontic tooth movement than in normoglycemic rats. Stress produces a greater disorganization of PDL in diabetic rats together with higher MMP-8 and MMP-9 expressions. Greater expression of it is observed in diabetic patients, which leads to increased collagen and gelatins degradation. This provokes poor regenerative features and worse prognosis of mechanical recovery after trauma and mechanical stress
Njoto I. et al., 2018[30]	*Rattus norvegicus* strain Wistar	CG (n= 3) DM (n= 15)	61 days	Cartilage (chondrocytes and pericellular matrix)	Increase in glycemia of animal model interfere with chondrocytes shape and formation. Hyperglycemia provokes production of pro-inflammatory mediators, such as: AGEs, local toxicity to joint tissues and apoptosis.
Njoto I. et al., 2019[31]	*Rattus norvegicus* strain Wistar	CG (n= 5) DM (n= 15) DM-IT (n=)	21 days; 28 days; 42 days	Ligament (ACL)	Protein expression of perlecan in ligaments gradually decreased over time within DM groups. Hyperglycemia predisposes articular cartilage damage, higher severity of the osteoarthritis disease and reaches into the intracellular compartment.

Xin L. et al., 2010[57]	Sprague-Dawley rats	CG (n=24) DM (n=24)	8 weeks	Ligament (PDL)	DM group showed increased expression of MMP-1 and Col-III and decreased expression of Col-I in PDL. The DM group appeared to have worse recovery from damage caused by orthodontic movement. DM showed alterations in immune response, inflammation, extracellular matrix synthesis and collagen destruction.
Tan J. et al., 2022[47]	Genetically diabetic C57BLKS/J-*Lepr^{db}* (*db*/*db*) mice and their C57BLKS/J wild-type littermates	DM (n= 10) IG (n= 10) CG (n= 8)		Ligament (PDL)	The mRNA expression levels of GRP78, ATF6, PERK, and XBP1 were highest in DM, followed by IG and the lowest in CG. Hyperglycemia activates ER stress. DM and IG microscopic observations showed disorganised cell arrangement in PDL, necrotic tissue, inflammatory cells, inflammation, granulation tissue hyperplasia, disordered fibroblasts.
Tang L. et al., 2022[58]	Genetically diabetic C57BLKS/J-*Lepr^{db}* (*db*/*db*) mice and their C57BLKS/J wild-type littermates	DM (n= ?) CG (n= ?)	8 weeks	Ligament (PDL)	DM produced ROS with an increased MDA levels indicating lipid peroxidation. SOD and GSH-Px level refer to essential antioxidative scavengers of ROS, were significantly decreased in the serum of DM. The intracellular DNA damage measurement occurred based on markedly increased 8-OHdG expression in DM. The telomere oxidative damage (accelerated telomere shortening) was detected through an expression of 53BP1 and the colocalization of 53BP1 and TRF2 increase in the PDL of DM group.
Li H. et al., 2008[59]	Sprague–Dawley rats	DM (n= ?) CG (n= ?)	12 weeks	Ligament (Posterior longitudinal ligament tissues of cervical spine)	Hyperglycemia increases gene expression and protein synthesis of collagen types I and III, particularly, in cells of the posterior longitudinal ligament.

Tan J et al. found that, in an in vivo-induced hyperglycaemic microenvironment, high glucose inhibits the osteogenic differentiation of the periodontal ligament (PDL), thus inhibiting the synthesis and secretion of the collagen matrix and calcium salts to an adequate degree [47]. Endoplasmic reticulum (ER) stress activated by overstimulation mainly involves three unfolded protein-response signalling pathways, including activating transcription factor 6 (ATF6) pathway, double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) pathway, and inositol-requiring enzyme 1 (IRE1) pathway. While moderate ER stress can effectively protect the body, excessive ER stress causes degeneration [48,49]. Song X et al. evidenced that ER causes apoptosis in PDL and vascular calcification in a

rat model [50]. In the previous study of Tan J et al., ER stress was also found to reduce the osteogenic differentiation ability of PDL when influenced by tumour necrosis factor-α [46]. Both indicate that an inflammatory microenvironment causes drastic interruption throughout the physiological functioning of cell mechanisms in ligaments [49,51].

The balance of MMPs and tissue inhibitors of MMPs (TIMPs) is crucial for the stabilisation of the ECM, with an MMP/TIMP imbalance associated with the pathologic breakdown of the ECM [28,51,52,53]. In diabetes, collagen is rapidly degraded by elevated MMP levels and the fibroblast function is disturbed [54,55,56].

Summary

A wide range of studies investigating the influence of diabetes on tendons in animal models have been conducted. Although tendons and ligaments differ significantly from their hierarchical structure, which lets them adapt to their function, there are also many similarities between the two tissues. There are no preclinical or clinical data focusing on biomechanical and histological changes in ligament properties in the individuals with DM, as it is evidenced in the case of tendons. While the cellular and molecular mechanisms of these alterations are still ambiguous, further well-designed clinical studies are needed to establish which diabetes-induced molecules influence the ligamentous fibres, causing their degeneration.

4. Hypotheses and Objectives

4.1. Hypotheses

1. According to the conducted studies, the correlation of AGEs, excessive induction of MMPs release, formation of reactive oxygen species, and accumulation of ECM inflammatory cytokines seem to play a crucial role in complications of diabetic ligaments.

2. Diabetes-induced ligament alteration differs from normal ageing and occurs in the ECM within two patterns:
(1) an increased nonenzymatic cross-link of proteins by glycation at lysine residues and
(2) a decreased rate of proteoglycan synthesis. Diabetic alterations of ligaments may especially originate from excessive stimulation and interaction with PPARs, JAK/STAT pathways, ER stress, and ROS and AGEs accumulation.

3. The present literature points to the contribution of DM to the loss of elastin fibres that occurs in ligament complications following DM.

4.2. Objectives

1. Identification of alterations present among MCL microarchitecture and tracking the mechanisms, that affect ligamentous functioning via assessment of diabetic and normoglycemic groups. Classification of the changes occurring in ligaments using histopathological screening and the Sairyo scale of fibrosis, loss of elastic fibers and calcification.
2. Analysis of differences in ligament remodeling and damage after surgical interventions and sham procedures in rats with pharmacologically induced diabetes (hyperglycemia) compared to normoglycemic control group.
3. Analysis of the concentrations of inhibitors of the autooxidative activity of lipid peroxidase, nitric oxide, glutathione S-transferase, ceruroplasmin, albumin, uric acid in the tissue homogenate and blood plasma in rats with pharmacologically induced diabetes (hyperglycemia) compared to normoglycemic control group.
4. Analysis of the influence of melatonin supplementation on the concentration of oxidative stress biochemical markers in tissue homogenates and blood plasma coming from pharmacologically induced diabetes groups and normoglycemic control group.
5. Analysis of differences in concentration of the biochemical markers of oxidative stress between the groups with normoglycemia and hyperglycemia undergoing ligament surgery.

5. Materials and methods

5.1. Animal material and organisation

Forty (40) Male Sprague–Dawley (SPRD) rats with a weight ranging from 280 to 300 g, 12 weeks of age were purchased from Central Laboratory of Experimental Animal Models, Medical University of Warsaw, Warsaw, Poland. The study protocol was approved by the ethics committee. The animals used in the study were equally randomly separated into four group.

Group 1 (n = 10) general control group: the group which was not induced with DM and fed on a normal diet.

Group 2 (n = 10) melatonin-supplemented control group: the group which was fed on a normal diet and supplemented with 3 mg/kg/day melatonin *per os* (p.o.) for 4 weeks.

The group 3 and 4 were injected with a streptozotocin (STZ) at a dose of 60 mg/kg of body weight, dissolved in a freshly pre-pared buffer (0.1 mol/L citrate, pH 4.5). The rats were fasted for 8h before STZ injection. Solution was injected into rats by intraperitoneal (i.p.) meant to induce diabetes [60]. The anesthetics and painkillers were avoid, because the procedure itself requires only the temporary immobilization for an injection. Application of the analgesic or anesthetic drugs would require additional immobilization of the animal and additional injection, and thus subjecting the animal to unnecessary stress. Fasting blood glucose level were assessed on the day of the intraperitoneal injection of STZ and 72 hours after the injection, a venous blood glucose test was performed by glucometer strips. The animal models were immobilized in a plastic tube and placed on the heating platform to dilate the vessels in their tails. The drop of blood was obtained by puncturing the vein with a needle. Glucose measurement was performed using the device to detect the blood glycemia levels. For the purpose of blood glucose measurement, the anesthetics and/ or painkillers were not used either. This is because animals that are under anesthesia, experience disturbed thermoregulation processes and it could provoke overheating. Only rats with hyperglycemia (glucose level over 250 mg/dL) were considered diabetic and used in the present study.

72h after the STZ injection, rats with consistent blood glucose levels ≥200mg/dl for three consecutive days were regarded as successful diabetic models. All 8 models underwent successful DM induction.

Group 3 (n = 10) diabetic group: the group in which DM was induced by a i.p. injection of “60 mg/kg” STZ and which was not supplemented with melatonin.

Group 4 (n = 10) melatonin-supplemented diabetic group: the group in which DM was induced by a i.p. injection of “60 mg/kg” STZ and which was supplemented with 3 mg/kg/day melatonin p.o. for 4 weeks.

5.2. Experimental animals

Rats were fed a commercial diet. Plasma glucose concentration was recorded weekly to monitor the hyperglycemia in group 2 and normoglycemia in group 1. Venous blood was sampled and tested on glucometer strips. The animal models were immobilized in a plastic tube and placed on the heating platform to dilate the vessels in their tails. The drop of blood was obtained by puncturing the vein with a needle. The animals were weighted weekly, based on which the condition of the animals was observed.

The rats were conditioned in plastic cages with a metal lid in the abundance of 2 rat models per cage form 1 week before the study. The cages were translucent, guarantying the eye-contact in between the animals. The animals received a commercial diet, and had free access to water. The rats were placed in a conventional lab housing with a 12h light/12h dark cycle at temperature of 21 ± 2°C and 55% ± 10% humidity. Additionally, the playground was launched.

All the animals received the housing condition according to the criteria outlined in the Guide for the Care and the Use of Laboratory Animals prepared by EU Directive 2010/63/EU for animal experiments. The ethic regulations were followed in accordance with national and institutional guidelines for the protection of animals’ welfare during experiments. The study was designed according to the ARRIVE guidelines. The study was conducted in the university departments, preorganized to conduct animal-based research. The staff that worked with the animals was experienced in inducing the DM in SPRD rats. The rats represent the most common species used in experiments concerning the musculoskeletal system and post-traumatic regeneration.

5.3. Tissue preparation

Animals from all of the groups received a surgical dissection of MCL that was performed on the left lower extremity of an animal, a sham procedure was performed on the right lower extremity of the same animal to simulate the surgical conditions in surrounding tissues and to compare the process of regeneration. The procedure was performed under general anesthesia (ketamine 75 mg/kg of body weight + xylazine 7 mg/kg of body weight). After disinfecting of the left knee, on the medial side a longitudinal cut from 3 mm below the knee joint to 3 mm above the joint was made along its long axis, then the skin and fascia was cut to reveal MCL (Figure 2). The ligament was dissected and cut in the middle. Fascia, ligaments and skin were closed with a surgical suture (Vicryl 6.0, Ethicon) (Figure 2). A sham operation was performed on the right limb. All steps were performed exactly the same as the operation of dissecting the left MCL, except that the ligament after preparation remained intact. During the operation, the prophylaxis was maintained and animal comfort was prioritized. Table 2 summarizes the division of the animals into the groups and the figure 3 represents the schematic overview of the study design. Two experienced scientists and I performed the surgical interventions.

5.4. Controlled treatment with melatonin supplementation

Melatonin (Sigma M-5250) supplementations achieved animals belonging to the group II and IV at a dose of 3 mg/kg/day for 4 weeks after undergoing the surgical procedures.

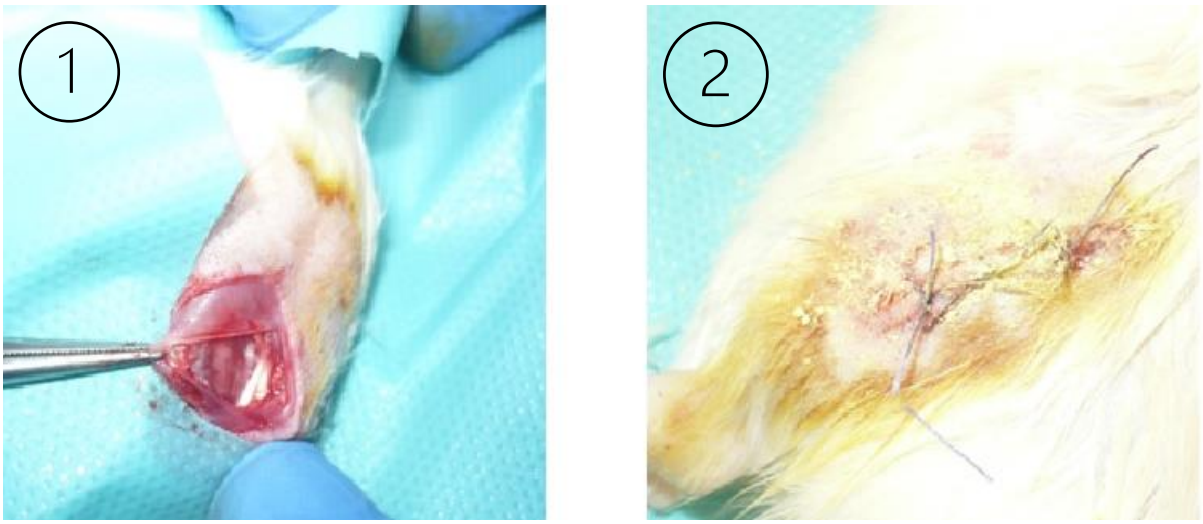

Figure 2. 1) Animal model surgical site during the procedure of ligament preparation in the knee joint. 2) Sutured surgical wound of the experimental animal model after the intervention in the ligament of the knee joint.

Table 2. The experimental groups.

Group	Surgical intervention	Additional interventions	Number of rats
Main Group I	Normoglycemia/ one-sided surgical dissection of the left MCL/ simulated surgical dissection of the right MCL (sham procedure) Total resection of the MCL, that was surgically dissected (28 days post-operatively)		10
Main Group II	Normoglycemia/ one-sided surgical dissection of the left MCL/ simulated surgical dissection of the right MCL (sham procedure) Total resection of the MCL, that was surgically dissected (28 days post-operatively)	Oral melatonin supplementation for 4 weeks; starting after the surgical procedures	10
Main Group III	DM/ one-sided surgical dissection of the left MCL/ simulated surgical dissection of the right MCL (sham procedure) Total resection of the MCL, that was surgically dissected (28 days post-operatively)		10
Main Group IV	DM/ one-sided surgical dissection of the left MCL/ simulated surgical dissection of the right MCL (sham procedure) Total resection of the MCL, that was surgically dissected (28 days post-operatively)	Oral melatonin supplementation for 4 weeks; starting after the surgical procedures	10
	Summary		40

5.5. Samples collection

5.5.1. Histological assessment

The operating surgeon obtained tissue from the central portion of the MCL (entire layer) to minimize tissue damage and optimize the harvest of the ligament only. After all fat tissue was removed, the tissue specimens were washed with saline to remove blood and bodily fluid contaminants. All specimens were transported under sterile conditions to the laboratory within 20 min of excision. The harvesting and processing procedures were identical in all rats. Two experienced scientists and I performed the surgical interventions.

The specimens were fixed in 4% formaldehyde in phosphate-buffered saline and prepared for paraffin embed- ding. Twenty consecutive sections, each 4-µm thick, were cut on a

microtome and treated with the appropriate stains. A light microscope (Nikon, Japan) was used to view and photograph the specimens.

5.5.2. Fibrosis and elastic fiber loss grading

Four cross-sections of each specimen were randomly selected for histologic analysis and graded independently by four of the authors (A.S., A.W., M.P. and O.A.), all of whom were blinded to the group. The severity of fibrosis (based on Masson trichrome staining) was graded on four-point scale, according to the guidelines set by Sairyo et al. [61]: 0—normal tissue showing no fibrotic region; grade 1—fibrosis of less than 25% of the entire area; 2—fibrosis of 25–50% of the entire area; grade 3—fibrosis of 50–75% of the entire area; 4—more than 75% fibrosis. The same method was used to evaluate elastin fiber loss (based on elastica van Gieson and Orcein staining). Average grades of fibrosis and elastin fiber loss were calculated for every sample. Results are presented in Figure 3.

5.5.3 Tissue homogenates

Rats from all groups were terminally anesthetized after 6 weeks by an intraperitoneal injection of 1.5-2 ml/kg (199.95-267 mg/kg of body weight of sodium pentobarbital + 40.05-53.4 mg/kg of body weight of pentobarbital). Blood samples were drown by cardiac puncture into tubes containing EDTA. This method is recommended to collect a high-quality single large blood amount from the animal. Blood was allowed to clot at room temperature followed by centrifugation. Serum was divided into aliquots and stored at −20 °C. Ligament samples were also collected from different experimental groups for histopathological and biochemical examination.

In the first part, ligaments harvesting was undergone immediately and homogenized in volume/tissue ratio of 100 mM phosphate buffer (pH 7.4), containing 22 mg% EDTA. Organs from different experimental groups were fixed in 10% buffered formalin for 24 h. After fixation, tissues were washed in tab water; then dehydrated in ascending series of ethanol 70, 90, 95 and 100%, respectively, followed by clearing in xylene and embedding in paraffin wax at 55C°. Five sections (4 µm thickness) were cut from each tissue followed by staining with hematoxylin and eosin. Briefly, slides were put in xylene to dissolve the paraffin wax, then in absolute alcohol for two min, 95% alcohol for one min, 70% alcohol for one min. Slides were stained with hematoxylin for five min then washed in tab water. Excess stain was

removed in acid alcohol for few seconds. Sections were stained with eosin for four to five min and washed in water then absolute alcohol (95%) and xylene.

The tissue homogenate was then stored at -20 ^{o}C for determination of lipid peroxides, total thiols, activity of catalase, superoxide dismutase (SOD) and glutathione S-transferase (GST). The ligament homogenates were furthermore retrieved to analyse the concentrations of oxidative biomarkers statuses: tissues total antioxidant status (TAS), total oxidant status (TOS), ROS activities, and oxidative stress index (OSI). Furthermore the lipid peroxidation was determined using malondialdehyde (MDA) and glutathione (GSH). Blood sample was centrifuged to separate plasma and undergone the measures of fasting blood glucose, total lipid, triglyceride levels, cholesterol levels, lipid peroxides, nitric oxide (NO), total thiols, total albumine and activities of catalase, uric acid, superoxide dismutase (SOD) and glutathione S-transferase (GST).

5.6. Biochemical analyses

5.6.1. Lipid peroxidase and nitric oxide

Lipid peroxide concentration was detected in obtained plasma and tissue homogenates. The product of the reaction between malondialdehyde and barbituric acid was measured. NO was determined in plasma as nitrite concentration after reduction of nitrate to nitrite. The reaction took place at 22 ^{o}C for 20 min and the absorbance at 546nm was measured using $NaNO_3$ solution as standard [62].

5.6.2. SOD, catalase, GST, MDA and ceruloplasmin activities

SOD activity was measured in three tubes: plasma, hemolysates and tissue homogenates and was calculated using its inhibitory activity of the autooxidation of epinephrine at alkaline medium [63]. GST activity in hemolysates and tissue homogenates was measured through the chemical determination using 1-chloro2,4-dinitrobenzene as substrate [64]. Catalase activity in hemolysate and tissue homogenates was determined based on its ability to decompose H_2O_2 and measured at 240 nm [65]. Serum MDA was determined by rat MDA ELISA Kit (MBS268427, MyBioSource, USA). SOD activity was determined by rat SOD ELISA Kit (MBS266897, MyBioSource, USA). Bio-Rad protein assay was used to measure total protein

concentration in tissue homogenates. Ceruloplasmin activity was determined using a para-phenylenediamine dihydrochloride method [66].

5.6.3. Total plasma thiols, uric acid and albumin

Total thiols was detected using the chemical method described by Ellman [67]. Albumin detection was performed colorimetrically with a commercial kit (Sclavo Diagnostics, Italy). Uric acid was determined by enzymatic colorimetric method using commercial kit (Biocon, BurbachyGermany).

5.6.4. Plasma cholesterol, triglycerides and total lipids

Cholesterol concentration was determined using an enzymatic colorimetric kit (Biocon). Triglycerides concentration was measured using an enzymatic hydrolysis of triglycerides with subsequent determination of liberated glycerol by colorimetry (Boehringer). Total lipids were chemically determined by the phosphovanillin method [68].

5.6.5. Total protein and blood glucose

Total proteins in plasma and tissue homogenates of ligaments and muscles were determined by a Biurertartrate method using commercial kit (Sclavo Diagnostics). Blood glucose level was determined by commercial kit (Biocon).

5.6.6. Oxidative status

In the second part, ligaments harvesting was undergone and bone samples were excised and homogenized in cold 0.9% NaCl using glass equipment to obtain a 10% (w/v) homogenate. The homogenates were centrifuged at 10,000x*g* for 15 min at 4°C so as to obtain the necessary clear supernatants required for the experiments. TAS, TOS, ROS activities, and OSI were assessed by the method described by Erel [69] and Erel [70].

5.6.7. Lipid peroxidation parameters

MDA analyses were performed in the bone tissue as mentioned earlier and the levels were given as nmol/g/protein [71].

Determination of glutathione GSH in the bone tissue: GSH levels were determined as mentioned earlier. The GSH level was determined as mg/dL/g protein [72].

5.7. Schematic summary of study design

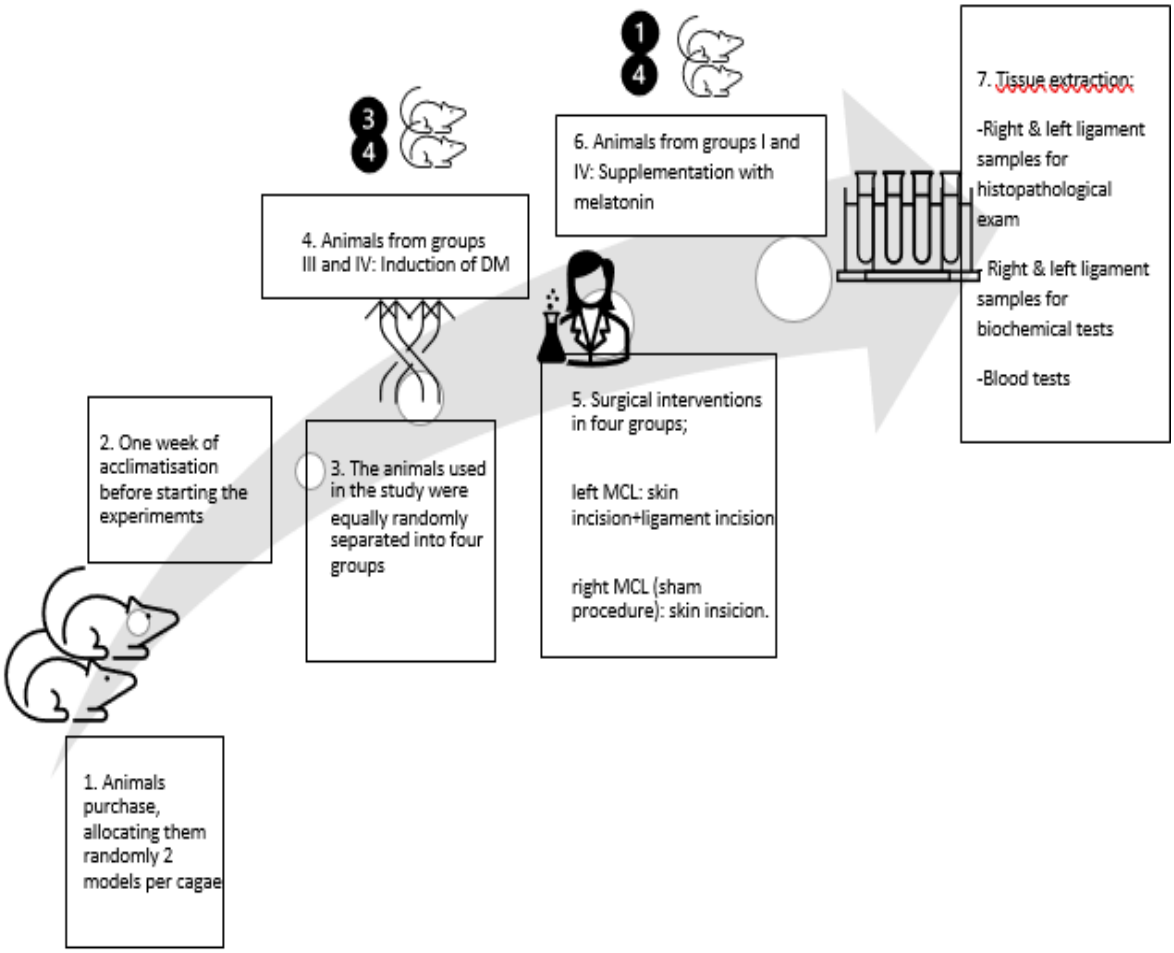

Figure 3. Schematic chronologic representation of the study design.

6. Statistical analysis

The collected data were analysed using the STATISTICA 13.3 TIBCO Software Inc. statistical package. Data are expressed as mean ± SD for all parameters in plasma, erythrocyte lysate and tissue homogenates. The evaluation of the distribution of the analyzed variables was verified with the Shapiro-Wilk test.

When comparing two dependent variables and the distribution is consistent with a normal distribution, the student's t-test was used. When comparing the two dependent variables and non-compliance of the distribution with the normal distribution were used Wilcoxon pair order test.

In the case of multiple independent samples (such as comparison of animal models from different groups) and the distribution does not match to the normal distribution, the Kruskal-Wallis ANOVA test was used. Post-hoc group comparisons were carried out using the Newman-Keuls test. To determine the occurrence statistically significant differences between specific variables were used multiple comparisons of average ranks for all trials (such as comparison of parameters from single animal model).

Trait correlations were assessed using the Spearman test:

- Rxy > 0 positive correlation - when the value of X increases, so does Y,
- Rxy = 0 no correlation - when X increases, Y sometimes increases and sometimes decreases,
- Rxy < 0 negative correlation – when X increases, Y decreases.

The correlation strength was determined according to the following guidelines:

- Rxy = 0 variables are not correlated
- 0 < Rxy < slight correlation
- 0.1 < Rxy < 0.3 weak correlation
- 0.3 < Rxy < 0.5 average correlation
- 0.5 < Rxy < 0.7 high correlation
- 0.7 < Rxy < 0.9 very high correlation
- 0.9 < Rxy < 1 almost complete correlation

The statistical significance level of $p<0.05$ was adopted.

7. Results

7.1. Histological screening

Parameters described as “after STZ injection” were measured 24 hours after the ip. injection of STZ. Values presented in the tables are compared in a following manner: group I is a control group for group II and III, and group II is a control group for group IV. All of the animals from group 3 and 4 successfully underwent the procedure of DM induction.

Table 3. indicates statistical significance of worsened status of the MCL of the left extremity than the right extremity at the endpoint of surgical dissection of the MCL and the sham procedure in the same individual according to the Sairyo score. The histological evaluation was undergone based on the imaging, that examples are presented in the Figure 4. Specifically noticeable was a significant fibrosis in the left MCL compared to right MCL in group III with a score ranging 3.43 ± 0.88; $P<0.05$ and in group I ranging 1.44 ± 0.7; $P<0.05$. Third part (1.44 of Sairyo score) of dissected ligament tissue from normoglycemic group appeared fibrotic and three fourth of diabetic dissected MCL were fibrotic. Increased loss of elastin fiber loss was observed in the dissected MCL among all groups $P<0.05$. Melatonin supplemented group was preserved from elastin fiber loss in comparison to slightly intact control group achieving scores 0 and 0.5 ± 0.2, $P<0.05$ for right MCL in the groups respectively, and 0 and 1.05 ± 0.55,$P<0.05$ for left MCL, respectively. The samples of right MCL in all groups showed statistically significant more calcifications than tissue retrieved from left MCL from the same groups. The non-diabetic group presented with calcification in dissected MCL (1.05, ± 1.7) and no sign of calcifications among right MCL with statistical significance ($P<0.05$).

Figure 4 visualizes the specifical differences in the architecture of the tissues in different groups. Specifically, the specimens belonging to group III present with hypercellularity and fibrosis in comparison to the integrated, linear fibroblasts in control group. What is interesting, the group IV presents with improved overall quality of dissected ligaments.

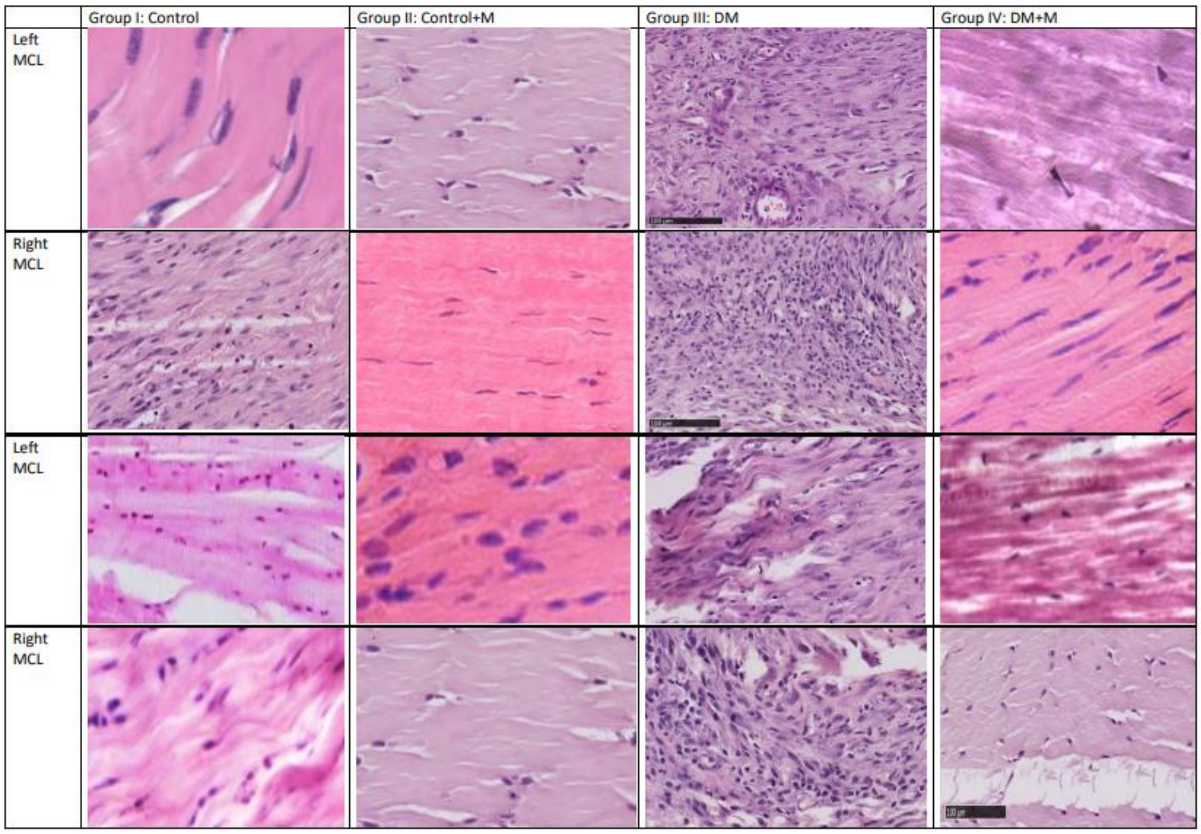

Figure 4. Histology stained sections (longitudinal dissection) are corresponding to the two groups: normoglycaemia and diabetes mellitus. Each column contains light microscopy images obtained from a single representative model and from the same section, scale bar shown on the left bottom corner (100µm). Each line is indicated for one model, from which the tissue was retrieved. The samples imaging was used to assess the status of the tissue. Results are presented in tables 3 and 4.

Table 3. Analysis of animals by group. Meaning of the abbreviations: Control, Control group; Control + M, control group + melatonin supplementation; DM, diabetic group; DM + M, diabetic group + melatonin supplementation. Data are expressed as "mean ± SD." [a]$p < 0.05$ versus control group; [b]$p < 0.05$ versus diabetic group.

	Group I: Control/ Right MCL	Group I: Control/ Left MCL	Group II: Control+M Right MCL	Group II: Control+M Left MCL	Group III: DM/ Right MCL	Group III: DM/ Left MCL	Group IV: DM+M/ Right MCL	Group IV: DM+M/ Left MCL
Sairyo score	0	1.44 (± 0.7)	0.25 (± 0.3) [a]	0.5 (± 0.11) [a]	2.25 (± 0.5) [a]	3.43 (± 0.88) [a]	0.5 (± 0.15) [b]	0.75 (± 0.05) [b]
Elastin fiber loss	0.5 (± 0.2)	1.05 (± 0.55)	0 [a]	0 [a]	0.7 (± 0.2)[a]	2.3 (± 0.9) [a]	1.25 (± 0.2) [b]	1.0 (± 0.1) [b]
Calcifi-cations	0	1.05 (± 1.7)	0 [a]	0.25 (± 0.05) [a]	0.67 (± 0.2) [a]	1.97 (± 0.28)[a]	1.0 (± 0.35) [b]	0.96 (± 0.14) [b]

Optic quantitative analysis of ligaments is presented in table 4. connective tissue of right and left MCL from group I appeared with typical ligament components with statistical significance (10, 10; P<0.05; P<0.05). In the case of group II, the right MCL appeared with characteristic pattern (10; P<0.05) and in the case of the left MCL, the minority of samples (8; P<0.05) appeared typically, but 2 of the samples presented with single lymphocytic infiltration, loosened structure, mast cells infiltration and increased fibroblasts as in comparison to control (P<0.05).

Histopathological screening of the left MCL from group III revealed a loosened structure of the connective tissue (8; P<0.05) together with lymphocytic infiltration (5; P<0.05), increased fibroblasts quantity (5;P<0.05)and mast cells infiltration. (3; P<0.05) and features of increased fibroblasts activity influencing on the overall optically assessed quality of the tissue. The overall hypertrophy of the tissue, hypercellularity and invalid fibroblasts function was described in half of the samples (10; P<0.05) when compared to group I.

The right MCL from group III in minority presented the characteristic pattern of the tissue (4;P<0.05), with increased fibroblasts (4;P<0.05), mast cells infiltration (4;P<0.05), loosened structure (4;P<0.05) and lymphocytic infiltration (2;P<0.05). The overall hypertrophy of the tissue, hypercellularity and invalid fibroblasts function was described in half of the samples (5; P<0.05) when compared to group I.

Group IV presented with characteristic pattern of the right MCL in 9 samples witch is a statistically significant value in comparison to group II ($P<0.05$). The lymphocytic infiltration appeared in 2 samples ($P<0.05$), with increased fibroblasts (5;$P<0.05$), mast cells infiltration (3;$P<0.05$) and loosened structure (4;$P<0.05$). Only 1 sample was assessed as being a bad quality ($P<0.05$) similarly to corresponding group II. Left MCL of the group IV presented with characteristic pattern of the right MCL in 8 samples witch is a statistically significant value in comparison to group II ($P<0.05$). The lymphocytic infiltration appeared in 3 samples ($P<0.05$), with increased fibroblasts in 2 samples ($P<0.05$), mast cells infiltration (1;$P<0.05$) and loosened structure (2;$P<0.05$). Quality of 2 samples was assessed as bad, comparable to group II ($P<0.05$).

Furthermore all the samples of left MCL from group III seemed disintegrated and frayed in their elasticity is consistent (90%; $P=0.001$). Some of the samples were found mildly hypertrophic (2 ± 0.92; $P=0.001$) and some intensively (6 ± 0.22; $P=0.001$) hypertrophic. Right MCL from group III seemed hypercellular (8; $P>0.05$), with lymphocytic infiltration (2; $P=0.00865$). However, the structure differed from the physiological appearance of the ligament tissue. Numerous collagen fibers seemed loosened with mast cells infiltrations in some cases (4; $P=0.8567$). The overall screening revealed significantly better qualitative measures of the melatonin supplemented diabetic group from corresponding diabetic value ($P=0.01$). The process of dissection facilitates morphological and histological changes in appearance of MCL. The glistening white appearance under the microscope was not recognizable anymore. DM was found to be a cofactor for exacerbating it even more abruptly. Severely affected fragments show up grayish and amorphous, disproportionally distinguished into fusiform or nodular thickening portions. Melatonin supplementation in group III brought statistically significant improvement in all the morphological and histological measures, $P<0.05$. Melatonin supplementation in group II diminished elastin fiber loss in comparison to control group from 0.5 ± 0.2, $P<0.05$ and 0, $P<0.05$ for right MCL group I and right MCL group II, respectively. Furthermore the same observation concerned the left MCL from group I with a score 1.05 ± 0.55, $P<0.05$ and 0 for group II. In other cases, melatonin supplementation in group II did not cause any significant changes

Table 4. Optic quantitative analysis of the ligaments from group I to IV using the light microscope. Meaning of the abbreviations: Control, Control group; Control + M, control group + melatonin supplementation; DM, diabetic group; DM + M, diabetic group + melatonin supplementation. Data are expressed as " [a]$p < 0.05$ versus control group; [b]$p < 0.05$ versus diabetic group.

	Group I: Control/ Right MCL	Group I: Control/ Left MCL	Group II: Control+M Right MCL	Group II: Control+M Left MCL	Group III: DM/ Right MCL	Group III: DM/ Left MCL	Group IV: DM+M/ Right MCL	Group IV: DM+M/ Left MCL
Characteristic pattern for the tissue	10	10	10 [a]	8 [a]	4 [a]	5 [a]	9 [b]	8 [b]
Lymphocytic infiltration	1	1	0 [a]	1 [a]	2 [a]	5 [a]	2 [b]	3 [b]
Loosened structure	0	2	1 [a]	1 [a]	4 [a]	8 [a]	4 [b]	2 [b]
Mast cells infiltration	0	0	0 [a]	2 [a]	4 [a]	3 [a]	3 [b]	1 [b]
Increased fibroblasts	0	0	1 [a]	2 [a]	4 [a]	5 [a]	5 [b]	2 [b]
Bad quality assessed optically: hypertrophy, fibroblasts hyperactivity, hypercellularity	0	2	1 [a]	0 [a]	5 [a]	10 [a]	1 [b]	2 [b]

7.2. Laboratory blood plasma tests

Diabetic rats presented with characteristic abnormalities, that are shown in Table 5. In diabetic rats from group III blood glucose, total lipids, cholesterol, and triglyceride presented significantly increased values ($P<0.05$) when compared to control animals. However the diabetic subgroup who was frequently administered melatonin presented with reduced concentration of blood glucose, triglyceride, total lipids, and cholesterol ($P<0.05$).

In all measured parameters, melatonin administration in control group II did not show any significant changes in comparison to healthy control group I. Fasting blood glucose levels from untreated DM group increased significantly in comparison to healthy control and presented increased values in DM+M group, but significantly lower than dose in DM group, 258 ± 38, 154 ± 19; $P<0.05$, respectively.

All of total lipid, triglyceride levels, cholesterol levels increased significantly in non-treated diabetic group 3, 650 ± 50, 2.75 ± 0.25 and 6.2 ± 0.6, respectively, in comparison to healthy controls (470 ± 25, 1.5 ± 0.12 and 2.65 ± 0.15 for total lipid, triglyceride and cholesterol, respectively (Table 5). A significant decline in total lipid, triglyceride levels, cholesterol levels

was observed in melatonin treated diabetic group IV (375 ± 35, 1.5 ± 0,2, 3.18 ± 0.2) in comparison to non-treated diabetic group.

Table 5. Fasting blood glucose levels before (1) and after (2) STZ injection, total lipid (3), triglyceride levels (4) and cholesterol levels (5) represented thought statistical analysis concerning different experimental groups. Meaning of the abbreviations: Control, Control group; Control + M, control group + melatonin supplementation; DM, diabetic group; DM + M, diabetic group + melatonin supplementation. Data are expressed as "mean ± SD." [a]$p < 0.05$ versus control group; [b]$p < 0.05$ versus diabetic group.

		Group I: Control	Group II: Control+M	Group III: DM	Group IV: DM+M	P value
1	Fasting blood glucose before STZ injection (mg/dL)	93 (± 7)	91 (± 3) [a]	90 (± 13) [a]	95 (± 6) [b]	0.0022
2	Fasting blood glucose after STZ injection (mg/dL)	99 (± 5)	102 (± 11)	258 (± 38) [a]	154 (± 19) [b]	0.0017
3	Total lipid (mg/dL)	470 (± 25)	420 (± 55) [a]	650 (± 50) [a]	375 (± 35) [b]	<0.001
4	Triglycerid levels (mmol/L)	1.5 (± 0.12)	1.65 (± 0.35) [a]	2.75 (± 0.25) [a]	1.5 (± 0.2) [b]	0.0047
5	Cholesterol levels (mmol/L)	2.65 (± 0.15)	3.22 (± 0.18) [a]	6.2 (± 0.6) [a]	3.18 0.2) [b]	<0.001

7.3. Oxidative status of plasma

Table 6 presents the values with increased plasma levels of lipid peroxides and uric acid (P<0.05) in diabetic rats and reduced albumin concentration in diabetic rats (P<0.05). Nitric oxide was increased (P>0.05) in diabetic rats, while total thiol and ceruloplasmin, were reduced (P>0.05) when compared to control. Treatment with melatonin significantly increased total thiol and ceruloplasmin activity. Also treatment with melatonin significantly decreased (P<0.001, P<0.05) lipid peroxides and uric acid, respectively. Rats treated with melatonin only showed a decrease (P>0.05) in nitric oxide.

Table 6. Levels of lipid peroxides, nitric oxide and antioxidants in plasma of rats. Meaning of the abbreviations: Control, Control group; Control + M, control group + melatonin supplementation; DM, diabetic group; DM + M, diabetic group + melatonin supplementation. Data are expressed as "mean ± SD." [a]$p < 0.05$ versus control group; [b]$p < 0.05$ versus diabetic group.

	Control	Control+M	DM	DM+M	P value
LPO (nmol/ml)	0.28 (± 0.01)	0.33 (± 0.1) [a]	0.65 (± 0.15) [a]	0.16 (± 0.01) [b]	0.0011
Nitric oxide (ng/ml)	137.28 (± 12.25)	132.55 (± 7.5) [b]	271.63 (± 16.5) [a]	142.65 (± 30.45) [b]	0.0567
Total thiols (nmol/ml)	0.98 (± 0.11)	1.13 (± 0.46) [a]	0.32 (± 0.08) [a]	1.24 (± 0.19) [b]	0.9827
SOD (ng/ml)	128.72 (± 15.45)	114.85 (± 14.13) [a]	102.36 (± 10.05) [a]	140.65 (± 8.26) [b]	<0.001
Ceruloplasmin (mg/l)	161.65 (± 10.58)	176.37 (± 11.5) [a]	102.84 (± 10.91) [a]	216.22 (± 9.96) [b]	0.1765
Albumin (g/l)	43.18 (± 2.17)	39.96 (± 3.15) [a]	29.37 (± 4.56) [a]	39.28 (± 1.99) [b]	<0.001
Uric acid (mmol/l)	0.56 (± 0.45)	0.49 (± 0.23) [a]	0.71 (± 0.4) [a]	0.51 (± 0.03) [b]	0.0321

7.4. Oxidative status of ligament homogenates

Ligament homogenates showed a significant increase in lipid peroxides in diabetic rats compared to the controls (P<0.05) (Table 7). GST activities increased significantly (P<0.001) in diabetic rats treated with melatonin. Total thiols presented with similar values (P<0.05) in rats treated with melatonin as in control group. Treatment with melatonin significantly increased (P<0.05, P<0.001) activities of superoxide dismutase and catalase, respectively, compared to the DM group and showed similar range of values to control.

Table 7. Levels of lipid peroxides (LPO), total thiols, and the activities of superoxide dismutase (SOD), catalase and glutathione S-transferase (GST) in the ligament homogenates of the different experimental groups. Meaning of the abbreviations: Control, Control group; Control + M, control group + melatonin supplementation; DM, diabetic group; DM + M, diabetic group + melatonin supplementation. Data are expressed as "mean ± SD." [a]$p < 0.05$ versus control group; [b]$p < 0.05$ versus diabetic group.

		Control	Control+M	DM	DM+M	P value
1	LPO (pmol/mg Hb)	11.28 (± 2.27)	12.01 (± 1.5) [a]	22.65 (± 4.65) [a]	10.16 (± 1.12) [b]	0.0032
2	Total thiols (nmol/mg Hb)	22.68 (± 1.81)	24.16 (± 2.47) [a]	14.82 (± 2.38) [a]	25.64 (± 3.19) [b]	0.0727
3	GST (nmol/min/mg Hb)	224.7 (± 35.5)	222.5 (± 27.9) [a]	162.28 (± 20.46) [a]	298.25 (± 35.56) [b]	<0.001
4	SOD (ng/mg Hb)	5.65 (± 0.5)	4.25 (± 0.73) [a]	2.99 (± 0.83) [a]	5.58 (± 0.84) [b]	0.0032
5	Catalase (U/mg Hb)	0.65 (± 0.12)	0.72 (± 0.18) [a]	0.29 (± 0.16) [a]	0.61 (± 0.09) [b]	<0.001

TAS, TOS, ROS levels, and OSI rates of ligament homogenates are presented in Figure 5. In the diabetic groups, it was found that TAS levels decreased, while TOS, ROS levels, and OSI rates increased. These changes were found to be meaningful when compared to the control group ($p < 0.0001$; $p < 0.0001$; $p < 0.0001$; $p < 0.0001$, respectively). Treatment with melatonin to diabetic rats mitigated these alterations ($p < 0.0001$; $p < 0.0001$; $p < 0.0001$; $p < 0.0001$, respectively).

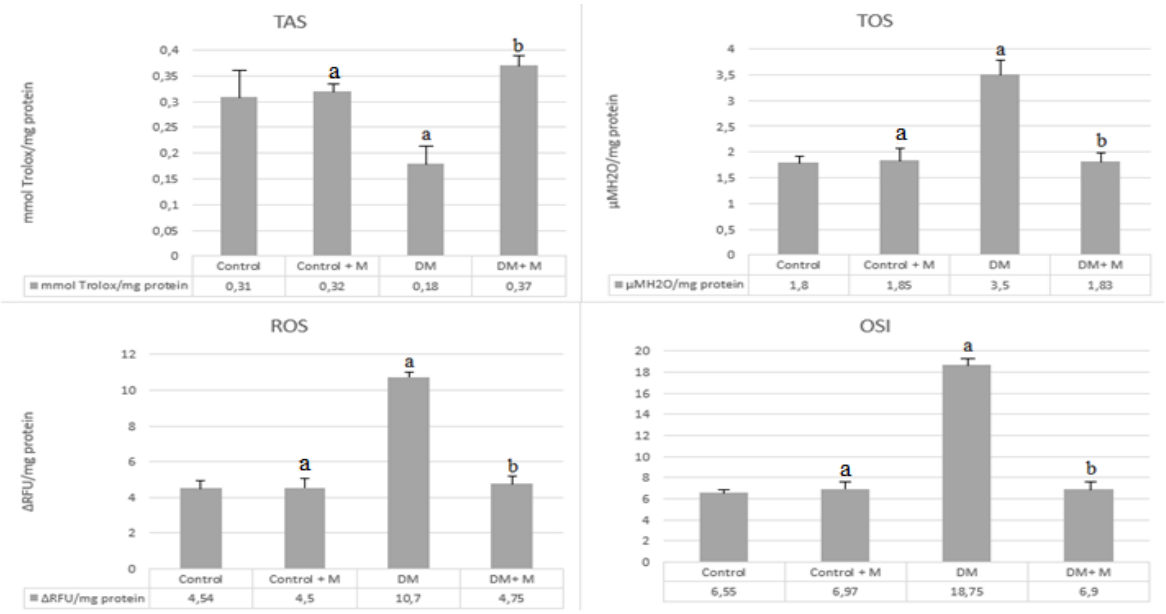

Figure 5. Ligament homogenates TAS, TOS, ROS levels, and OSI rates of all groups. Meaning of the abbreviations: Control, Control group; Control + M, control group + melatonin supplementation; DM, diabetic group; DM + M, diabetic group + melatonin supplementation; OSI, oxidative stress index; ROS, reactive oxygen species; TAS, total antioxidant status; TOS, total oxidant status. Data are expressed as "mean ± SD." $^{a}p < 0.05$ versus control group; $^{b}p < 0.05$ versus diabetic group.

Figure 6 presents the values of MDA and GSH in the groups. The highest MDA value in the ligament homogenates was obtained in DM group. MDA levels in the bone tissue in groups with DM and melatonin supplementation was lower than in the DM group, but higher than those in all other groups. The GSH values appeared higher in the melatonin supplemented groups than in those without supplementations. The highest GSH levels were measured upon diabetic group supplemented with melatonin.

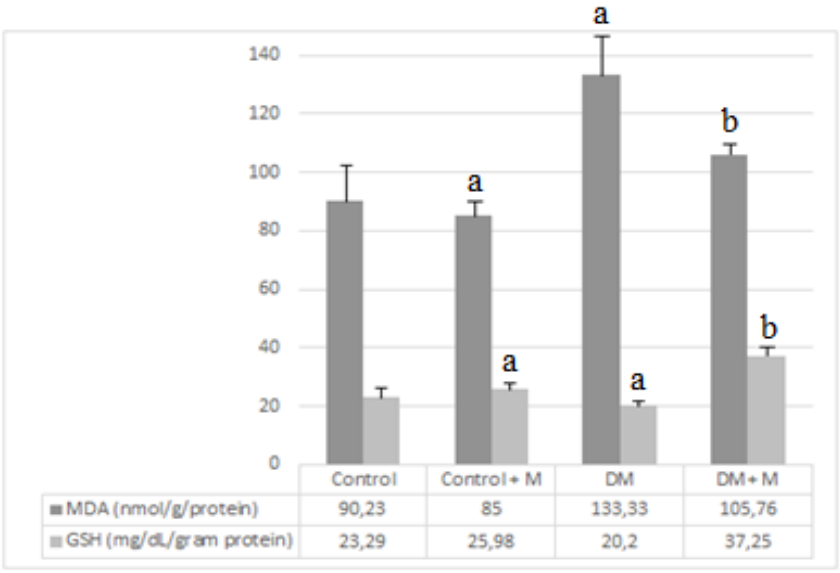

Figure 6. Levels of MDA and GSH in the ligament homogenate of the groups. Meaning of the abbreviations: Control, Control group; Control + M, control group + melatonin supplementation; DM, diabetic group; DM + M, diabetic group + melatonin supplementation; MDA, Malondialdehyde; GSH, glutathione. Data are expressed as "mean ± SD." $^{a}p < 0.05$ versus control group; $^{b}p < 0.05$ versus diabetic group.

8. Discussion

Ligaments play a unique and important role in the musculoskeletal function and are underestimated causes of diseases. The knowledge about the molecular mechanism ongoing in the connective tissue is not as advanced as in other skeletal tissues [73,74]. This is partly because, until recently, the transcription factors crucial for the formation and maintenance of ligaments, remained unknown and there is still a lot to discover. The recent findings of Scleraxis (Scx), Mohawk (Mkx) and early growth response factor (Egr1) and their specific roles in ligament development, maturation, homeostasis and regeneration of mature tissues has substantially advanced the possibilities to conduct a research on ligaments [75]. The future experiments may focus on concepts regarding the developmental, functional and disease-associated changes in ligament cells and extracellular matrix (ECM) with a focus on Mkx, Scx and Egr1, as it gives hope for investigating the inhibitory pathways possible to downstream in order to regress pathological changes among ligaments [76]. Tendon and ligament tissues, despite incoming from the different developmental processes, are closely related. The basic structures of these tissues and the gene expression profiles of their major cell type (tenocytes) are rather similar. Due to other functions, the regulatory pathways differ and morphologically they are predisposed to heterogenous roles [77]. Therefore, the available findings in tendinopathies are not well adopted in the concept of ligament regeneration and it requires further results. Depending upon the types of tissue, wound healing may occur by predominantly re-eptihelialization, by re-epithelialization with substantial new connective tissue formation, or by a combination of both plus new bone formation. The insult addressed to different cell types provokes different imbalances in their homeostasis.

Systemic diseases with long-lasting inflammation impacts the organism in various ways [77,78]. DM is a prevalent metabolic disorder that impairs barrier function and healing responses throughout the human body. The mechanisms are different, including cell proliferation and migration, altered level of inflammation and reduced formation of new connective tissue and bone. In particular, DM inhibits the expression of mitogenic growth factors and simultaneously stimulates that of pro-inflammatory cytokines through epigenetic mechanisms. Moreover, hyperglycemia and oxidative stress induced by DM, prevent the

expansion of mesengenic cells that are involved in both soft and hard tissue wounds [79-81]. Those mechanisms are greatly investigated on animal models and it further requires comprehensive study to address all the arising issues.

Tendon and ligament damage caused by injury or overuse, or associated with aging and arthritis is a common clinical problem, because damaged tissues heal very slowly and rarely recover completely. Similar concern is related to chronic inflammation in case of obese patients, that alike DM inhibits appropriate mechanism to fight the destructive mechanisms. Not many researches correlate the damage to ligaments with possible systemic diseases and rather with trauma. But on the other hand, the diabetic debilitating effect on tissue homeostasis is known. Some studies focus on changes in ligament histological appearance in arthritic knees that are not related to major trauma but are presumably caused by mediators, such as cytokines or matrix degrading enzymes, in the arthritic joints that affect tendon and ligament cells and the ECM [77]. The clinical representations are nonspecific and include pain, functional limitations that are highly prevalent, with >50% of individuals older than 60 years of age being affected [78]. Injury and aging changes to the ECM of tendon and ligament in the knee joints, particularly the ACL, represent major risk factors for the development of osteoarthritis (OA) [80,81]. The ligaments are essential for knee kinematics and in the setting of ACL deficiency, the articular cartilage as well as the menisci are more susceptible to arthritic changes. A large number of OA patients without a history of ligament injury have ACL deficiency at the time of total knee arthroplasty [82], and a correlation between the radiologic OA grade and the histological grade of ACL degeneration has been reported in end-stage OA [83]. It has been reported that fewer than half of subjects with ACL rupture recall a knee injury, suggesting that this is a risk factor for knee OA, and it remains under-recognized [84]. Starting from an undifferentiated knee pain, progressing to end-stage OA is quite pessimistic, but unfortunately quite realistic scenario of patient suffering from DM with no new insight, leading to novel therapeutic approaches [83,84]. There is a significant interest in understanding the cellular mechanisms responsible for expedited healing response in ligament tissue and how they are impacted by systemic disease.

Ligaments represent a fibrous connective tissue that connect muscle and bone and stabilize the body through carrying the mechanical forces [85,86]. The ECM utilizes the majority of

the overall volume of ligament and it is hierarchically organized in several distinct layers. It consists predominantly of type I collagen [87,88] and small amounts of other collagens, including types III, IV, V and VI [89,90]. The small but significant part take proteoglycans, such as decorin, fibromodulin, biglycan and lumican. Their important role is to organize and lubricate collagen fiber bundles [88]. Several animal studies conducted on tendons visualized that disruption of these proteoglycans in mice leads to abnormal collagen fibrils [87,88]. The ECM, organized in triple-helix polypeptide chains, determines the density and lateral and longitudinal stacking of microfibrils and leads to the assembly of fibrils with increasing diameter and mechanical strength. Fascicles are vascularized and innervated, which is essential for tendon homeostasis and response to injury [86,89]. The elasticity of tendons and ligaments is due to the large amount of type I collagen [91].

Talking about cell types in ligament, the principal are fibroblast-like cells, termed tenocytes, or ligament fibroblasts that are located between parallel chains of collagen fibrils [89-96]. Under normal conditions, they tend to be quiescent and have very low proliferation rates. Then ECM production is responsive to changes in the mechanical load [97]. Tenomodulin is specifically expressed in mature tendon and ligament cells [98]. The deletion of biglycan and fibromodulin in mice causes the depletion of tendon progenitor cells, suggesting that these ECM components provide a substantial environment for stem cells [97]. The identification of specific molecular markers for tendon progenitor cells in adult tissues will help elucidate the contribution of this subset to tendon homeostasis and regeneration.

Asahara et al hypothesized that Egr-1 expression may be compromised in DM which may lead to impaired collateral vessel growth, which elucidates impaired healing and homeostasis [90]. Heterogeneity in connective tissue treatment modalities- skin, tendons and ligaments Even though it is generally recognized among the population worldwide that aging societies decline physical activity, the joint stiffness is provoked by increased quantities of collagen cross-linkings, that are not only the effects of AGEs and differ among tendons and ligaments [99,100]. Despite that ligaments and tendons are functionally and grossly similar, they present with heterogeneous histological and biochemical characteristics. Plenty of studies concern skin, oral cavity and finally tendon alteration facilitated by DM, but the literature lacks on ligament redaction. The differences in tissue-

specific collagen maturation processes in ligament-derived fibroblasts are still unknown and they cover the reason for ligaments inferiority in terms of collagen synthesis, proliferation and migration in comparison to tendons. Therefore studies found it to be a crucial aspect of relatively poor healing potential of ligaments in comparison to tendons [101,102]. Experimental studies relying on animal models compared the morphological and biochemical features of ligament and tendons and revealed a higher ratio of enzymatic content of the lysine hydroxylase 2/lysine hydroxylase 1 in ligaments. The expression of lysyl oxidase brings a regulatory effect on the amount of enzymatic cross-linking. The levels of Col1A1 and Col3A1 were additionally greater in case of ligaments matrix than in the tendon matrices. Ligaments and tendons- derived cells have distinct collagen maturation processes at the cellular level and collagen maturation of ligaments' cells is not necessarily inferior to that of tendon with regard to collagen synthesis and maturation [99]. However, findings show the decreased proliferation rate, higher turnover and breakdown of fibroblasts derived from soft tissues induced by DM. These suggest that excessive proliferation together with an altered structure of the fibroblasts may contribute to poor production of collagen [100,102]. Further investigations evidence that DM fibroblast have impaired migration and phenotypic change, increase in matrix metalloproteinase 9 (MMP-9) and diminished production of vascular endothelial growth factor (VEGF) [103-106]. Molecular underground of ligament healing in DM- possible approaches. Unlike skin wounds, ligaments and joints injuries lack dermal appendages such as hair follicles, sweat glands and sebaceous glands. This is significant since the appendages contain stem cells that promote the healing process, which may be particularly important in healing of connective tissue. DM delays both the early and late stages of oral wound healing [93]. Diabetic wounds have increased inflammation as noted by greater numbers of neutrophils [93,94]. Re-epithelialization is enhanced by TNF inhibitors in diabetic animals but not in normal animals, indicating that the increased levels of TNF in the diabetic wounds is problematic whereas the normal level of TNF does not inhibit the healing process [95]. In skin wounds, DM reduces the conversion from a M1 macrophage phenotype to a M2 macrophage phenotype, which may contribute to prolonged inflammation [96].

Diabetic skin wounds also have a greater senescence-associated secretory phenotype (SASP) with increased inflammatory cytokine expression (eg IL-1a, IL-6, IL-8, and CXCL2) and MMP

expression [97]. Moreover, aged mice have a similar increase in the SASP phenotype as diabetic mice, suggesting that there may be similar mechanisms that delay healing with both aging and DM [97]. Of translational relevance, targeted expression of CXCR2 in primary human dermal fibroblasts led to paracrine induction of nuclear p21. Furthermore, a selective agonist to CXCR2 was able to reverse delayed healing in diabetic mice and accelerate ex vivo human skin wound healing. Collectively, these data suggest an unappreciated role for CXCR2 in mediating cellular senescence in pathological wound repair [97]. Ligament wounds have reduced angiogenic response when compared to skin wounds in general. The excessive angiogenesis of wounds on the skin may be due to the inflammation [107]. Inflammatory signals such as IL-1β and TNF promote neovascularization [108,109] and are linked to aberrant angiogenesis in rheumatoid arthritis [110]. Moreover, the experiment focused on oral wound healing found that VEGF expression by the oral keratinocytes is FOXO1-dependent, what differentiate it from the skin, and its deletion impairs angiogenesis and wound healing parameters in periodontal ligaments (PDL) [108,111]. It is important to investigate whether ligament healing represents FOXO1-dependent healing pathways, because inhibition of this molecule has been investigated to increase the potential to recovery. Jeon HH et al in their study promote the primary benefit of FOXO1 inhibition in early phases of wound healing, by an enhanced production of connective tissue matrix, increased myofibroblast formation, and greater angiogenesis in diabetic wounds. A distinctive feature of diabetic wound healing is increased and prolonged inflammation. Furthermore, FOXO1 inhibition reduces the production of pro-inflammatory mediators such as TNF-α and IL-1β and enzymes that facilitate reactive oxygen species formation. This is important since reducing prolonged inflammation improves the healing of wounds in type 2 DM animals [111]. Thulasingam et al promote the importance of proper glycemic control due to possible complication prevention. They found that Egr-1 signalling was impaired in DM mice; however, it can be rescued by insulin treatment. The mechanism by which impaired arteriogenesis occurs in DM is not fully understood, however there was observed the correlation of the decreased expression of Egr-1 and decreased collateral artery diameter in the diabetic mice. Important downstream targets of Egr-1, namely, ICAM-1 and uPA, were found to be decreased, too [112]. ICAM-1 plays a critical role in endothelial monocyte adhesion, which is essential for arteriogenesis. An earlier study identified an

upregulation of ICAM-1 mRNA in growing collateral arteries after induction of arteriogenesis by femoral artery ligation [113].

Studies link a higher prevalence of lumbar spinal stenosis (LSS) to the comorbidities, including DM. Ligamentum flavum (LF) hypertrophy includes fibrocartilaginous pathological degeneration [114-117]. Specimens of the LSS patients presented with increased infiltration of inflammatory cells and were stained positively for MMP-3, MMP-9, vimentin, fibronectin, and increased ROS [116-117]. Shemesh S. et al revealed enhanced elastin fibres loss in DM patients and the positive correlation of this loss to fasting plasma glucose [118]. Lin et al found that culturing rat connective tissue in hyperglycemia for up to 48 hours, not only causes decreases in the expression of collagen type 1, but also scleraxis and tenomodulin, apoptosis and decreased proliferation [119]. Wu et al conducted a study, concluding on the increased expression of genes including mohawk, biglycan, and transforming growth factor β-1 [120].

Diabetes- induced cytokine release activates the JAK/STAT pathway that is responsible for the majority of cell types development, including chondrocytes, osteocytes and fibrocytes. An error occurring in order to the local or systemic inflammation causes an immediate response in disturbed signalling in this tightly regulated process. Damerau A. et al observed that activated by inflammatory molecules JAK/STAT signalling stimulates osteoclasts, which results in an enhanced bone resorption. Furthermore it contributes to the production of matrix damaging enzymes within the synovial fluid that lead to cartilage and ligaments destruction and bone erosions in late stages [121]. Endoplasmic reticulum (ER) stress activated by overstimulation addressed to cells, mainly involves three unfolded protein response signalling pathways, including activating transcription factor 6 (ATF6) pathway, double-stranded RNA-activated protein kinase (PKR) like endoplasmic reticulum kinase (PERK) pathway, and inositol requiring enzyme 1 (IRE1) pathway. While moderate ER stress can effectively protect the body, excessive ER stress causes degeneration as it happens during DM involvement [122,123]. Song X et al evidenced ER effects in apoptosis of periodontal ligament (PDL) and vascular calcification in a rat model [124]. In the previous study of Tan J et al, ER stress was also found to reduce the osteogenic differentiation ability of PDL when influenced by tumour necrosis factor-α (55). Both indicate that inflammatory

microenvironment causes drastic interruption throughout the physiological functioning of cell mechanisms in ligaments [123,125]. The balance of MMPs and tissue inhibitors of MMPs (TIMPs) is crucial for the stabilization of the ECM, with an MMP/TIMP imbalance associated with pathologic breakdown of the ECM [102,123-125]. DM provokes rapid collagen degradation, by elevated MMP levels and fibroblast function is disturbed [124,125]. The undergone review shows the possible directions for future investigations of rapidly arising DM complications. Clear molecular explanation may allow for preventive measures in human, in order to eliminate complications of OA in patients.

The surgical stress and trauma to the tissue increases fibrosis, calcifications and decreases elasticity in the connective tissue of the ligaments in rats. Despite the same observations concern both groups, the inflammatory environment and probable devastating role of molecules involved in the permanent inflammatory processes, decrease the ligament biomechanical properties abruptly. It evidences that DM impacts the musculoskeletal system and the quality of connective tissue seriously. Implication of those findings must be used as a treatment strategy of common musculoskeletal complaints.

Long-term hyperglycemia can damage many tissues, cells, and cell membranes. Oxidative stress increases in diabetic patients, due to increased levels of reactive oxygen species (ROS). Organisms do have defense systems such as antioxidant enzymes and molecule that reduce and mitigate the harmful effects of ROS. Melatonin, a potential antioxidant molecules, is an effective scavenger of different ROS such as hydroxyl and peroxyl radicals.

Melatonin is a hormone synthesized from tryptophan mainly in the pineal gland. It is involved in the regulation of circadian and seasonal rhythms, sleep, retinal functions, glucose metabolism in adipocytes, and regulates food intake and the secretion of various cytokines such as leptin. It also plays a role in the oxidant systems as an antioxidant. Thus, it can be a potential antioxidant scavenger in DM and may help prevent of harmful effects of free radicals.

Diabetes mellitus DM, which involves several systems, is a disease characterized by acute and chronic complications [126]. There have been several reports to the effect that diabetes has a negative impact on bone metabolism and bone mineral density [127,128]. Albright and

Reifenstein [127] were the first to point out the co-presence of diabetes and osteoporosis in 1948, while Meema and Meema [128] showed in 1967 that diabetes was an osteoporotic condition. The decrease in bone mineral density particularly in type I diabetes was noted [129], and the pathogenesis of increased bone loss was reported to be attributable to insulin deficiency [130]. It was demonstrated in a study which included rats with STZ-induced diabetes that diabetes caused bone destruction by significantly increasing urinary excretion of calcium-phosphorus [131]. The results of the concerned study also proved the fact that diabetes had a negative effect on bone metabolism. Melatonin hormone, which is secreted by the pineal gland in darkness and according to the circadian rhythm, plays a part in the regulation of many physiological functions in the body [132-134]. As it is both fat and water soluble, melatonin can reach all cell organelles, including the nucleus [135]. This property gives melatonin an advantageous position in the protection of DNA against oxidative stress [136]. Consequently, melatonin is regarded as being a strong antioxidant which prevents the oxidative damage resulting from lipid peroxidation [136-138]. It has been argued that melatonin which is known to contribute to the carbohydrate.

Many approaches have been used in the effective management of type 2 diabetes mellitus. A recent paradigm shift has focused on the role of adipose tissues in the development and treatment of the disease [139]. Brown adipose tissues and white adipose tissues are the two main types of adipose tissues with beige subsets more recently identified [140]. They play key roles in communication and insulin sensitivity. However, WAT has been shown to contribute significantly to endocrine function. WAT produces hormones and cytokines, collectively called adipocytokines, such as leptin and adiponectin. These adipocytokines have been proven to vary in conditions, such as metabolic dysfunction, type 2 diabetes, or inflammation. The regulation of fat storage, energy metabolism, satiety, and insulin release are all features of adipose tissues. As such, they are indicators that may provide insights on the development of metabolic dysfunction or type 2 diabetes and can be considered routes for therapeutic considerations. The essential roles of adipocytokines vis-a-vis satiety, appetite, regulation of fat storage and energy, glucose tolerance, and insulin release, solidifies adipose tissue role in the development and pathogenesis of diabetes mellitus and the complications associated with the disease [141]. The b-cell toxicity caused by streptozotocin is apparently due to injury in b-cell and elevation of local free radicals in b-cell

after increasing free radicals in other body organs [142]. In the present study, diabetic rats induced by streptozotocin showed the expected elevation in plasma glucose, total lipids, triglycerides and cholesterol, confirming abnormalities of glucose and lipids in diabetes [143]. Treatment of diabetic rats with melatonin decreased blood glucose, triglyceride, total lipids and cholesterol levels. Also, melatonin reduced blood glucose levels, although some hyperglycemia remained. These results are in agreement with Farid et al. [144] and Ahmad Hajam et al. [145], they found that melatonin treatment reduced blood glucose levels to control levels and reduced hyperlipidemia in diabetic rats. Some authors have tried to explain the possible mechanism of hypoglycemic effects of melatonin. Several lines of evidence support a beneficial role of melatonin in glucose tolerance, including human epidemiologic studies, clinical trials and genetic studies. Furthermore, prolonged melatonin treatment (>12h) enhances post-exposure glucose-stimulated insulin secretion from cultured non-diabetic human islets [146]. It has been also suggested melatonin stimulates both glucagon and insulin release from cultured human islets [147]. Low urinary levels of the primary metabolite of melatonin, 6-sulfatoxy melatonin, have been prospectively associated with increased insulin resistance and risk of T2D [148, 149]. Also, in patients with T2D and insomnia, a significant decrease in glycated hemoglobin (HbA1c) levels was found after a 5-month open label trial of repeated nighttime melatonin administration, although HbA1c levels were not affected in the preceding 3-week randomized, double blind, crossover trial [150]. Moreover, a genetic study together with a recent functional genomics study has reported that rare coding variants in the melatonin receptor gene MTNR1B, that inhibit melatonin binding or signaling, are collectively associated with increased risk of T2D [151, 152]; although recent well-powered analysis of coding variants from exome and genome sequencing studies do not support these initial findings [153]. For clinical practice, since most glycemic assessments are performed in the morning, clinicians may find that people have better glucose tolerance in the absence of melatonin. However, if the test is done very early in the morning or if patients display late chronotypes – i.e., in both cases testing would occur especially early relative to their circadian phase – then melatonin may be still elevated and thereby impair glucose control. Therefore, this aspect may be considered when interpreting the glycemic test result [154].

There is an evidence that streptozotocin-induced diabetes releases free radicals [155]. As glutathione is closely linked to glucose metabolism via NADPH of hexose monophosphate shunt, it is logical that free radical metabolism is altered in diabetes. Changes in SOD and catalase activity, glutathione and vitamin E levels have been reported [156]. In the present experiment, streptozotocin treatment caused a significant increase in the lipid peroxidation in plasma, erythrocytes lysate, and liver and kidney. Basically, in diabetic rats increased lipid peroxidation was associated with hypertriglyceridemia [157]. Moreover, the lipid content of cell membranes seems to be disrupted by diabetes as proved by increased nonenzymatic glycation, lipid peroxidation and cholesterolyphospholipid ratio. Increased glycation of collagen and plasma proteins in diabetes may stimulate the oxidation of lipids, which in turn may stimulate auto-oxidative reactions of sugars enhancing damage to both lipids and proteins in the circulation and continuing the cycle of oxidative stress [158]. NO synthase is present in pancreatic b-cells and may be involved in the release of insulin under normal physiological conditions. NO is a gas that serves as a ubiquitous signaling molecule participating in physiological activities of various organ systems. Nitric oxide is produced in the endocrine pancreas and contributes to synthesis and secretion of insulin. The potential role of NO in insulin secretion is disputable - both stimulatory and inhibitory effects have been reported. Available data indicate that effects of NO critically depend on its concentration. Different isoforms of NO synthase control this and have the potential to decrease or increase insulin secretion [159]. In the present study, plasma nitrite as end product of nitric oxide activity was elevated in the untreated diabetic rats. Similar results were obtained already on other studies [160-162]. NO reacts with oxygen yielding nitrogen dioxide or peroxnitrities; both are strongly oxidative and more cytotoxic than nitric oxide itself [163]. Uric acid is considered as one of non-enzymatic antioxidant, but increased production of uric acid means increased free radical production due to activation of the xanthine oxidase enzyme system [164]. In our experiment, uric acid levels were increased in diabetic rats. This may be due to metabolic disturbance in diabetes reflected in high activities of xanthine oxidase, lipid peroxidation and increased triglyceride and cholesterol. Moreover, protein glycation in diabetes may lead to muscle wasting and increased release of purine, the main source of uric acid as well as in activity of xanthine oxidase. Reduced antioxidant levels as a result of increased free radical production in diabetes have been reported [165]. In the present study, streptozotocin treatment caused a significant depletion

of both enzymatic and non-enzymatic antioxidants in plasma or erythrocyte lysates or the homogenates of both ligaments and muscles. SOD and catalase activities in streptozotocin induced diabetic rats were very reasonable. Accordingly, SOD treatment can protect in vivo or in vitro against the high toxic potential of the superoxide radicals in alloxan-induced diabetic rats [166]. It was also found a significant decrease in plasma ceruloplasmin and albumin, however, uric acid was increased. In our experiment melatonin treatment generally normalizes oxidative stress in streptozotocin diabetic rats. Melatonin treatment reduced oxidative stress (lipid peroxidation, nitric oxide and uric acid) while elevating enzymatic and non-enzymatic antioxidant systems in blood, ligament and muscle homogenates. Anwar et al. (1998) reported that melatonin had a potent reducing effect on the production of lipid peroxides in rats exposed to cytotoxic drugs [167,168]. The reduction of nitric oxide levels may be due to inhibition of nitric oxide synthase enzyme activity by melatonin [169]. Moreover, melatonin significantly inhibited the accumulation of cGMP levels induced by L-arginine or sodium nitroprusside and finally reduced nitric oxide production [170]. Nitric oxide, involved in the neuropathy which is one of the complication of diabetes as a result of oxidative stress, is reduced after melatonin treatment [171]. Storr et al. reported that melatonin inhibited nitric oxide synthase enzyme and reduced nitric oxide production. The reduction in uric acid levels after melatonin treatment may be due to reduction of lipid peroxidation, triglyceride and cholesterol, while elevation of these substances may increase uric acid synthesis. Melatonin is one of the most important pineal indols and it has a potent free radical scavenger [172]. Melatonin treatment increased SOD and GST activities in plasma, ligament and muscle homogenates. Moreover, Ferlazzo et al. [173] reported that melatonin demonstrated powerful lipophilic antioxidant and free radical scavenging action. Melatonin has been proven to be twice as active as vitamin E, believed to be the most effective lipophilic antioxidant. Melatonin-induced signal transduction through melatonin receptors promotes the expression of antioxidant enzymes as well as inflammation-related genes. Melatonin also exerts an immunomodulatory action through the stimulation of high-affinity receptors expressed in immunocompetent cells [174]. It is evident from the present study that melatonin supplementation may help in the prevention and protection against the free radicals production in diabetes. The antioxidant effects of melatonin caused an excellent elevation in the antioxidant systems activity and reduced oxidative stress.

Diabetes poses a serious risk for decreased bone tissue quality, leading to osteoporosis. Furthermore, increased oxidative stress and free radical products are also hazardous for joints [175]. Lipid peroxidation, which is among the most harmful effects of free radical products, and MDA, which is one of its end products, must have osteoclast activity [175]. Therefore, oxidant stress is a major mediator of bone loss and joint capsule, ligaments and tendon damage in diabetes [176-178]. DM is also accompanied by an increased risk of osteopenia and bone fracture. It is generally confirmed that osteopenia develops together with oxidative stress, which increases in diabetic conditions in mice with a STZ-induced diabetes [176].

In the present study, the highest MDA levels in ligament tissues were found in diabetic groups which were not supplemented with melatonin. This result is consistent with the reports of the above-cited researchers who stated that oxidant stress that increased in diabetes had a negative impact on bone metabolism. What is more, it was reported that endurance exercise was able to enhance the bone quality by restoring bone blood flow [177].

However this results are heterogenous from the study conducted by Bicer et al. [177]. This study interestingly, found out continuously high bone MDA levels in the diabetic group, despite subjected them to swimming exercise. Those elevated MDA values that were established in the bone tissue demonstrate that the lipid peroxidation which is enhanced in diabetic rats cannot be offset by exercise. Similarly, diabetic patients were reported to have elevated plasma thiobarbituric acid reactive substances values after rest and exercise [178].

The results of the concerned study show for the first time that exercise starts oxidative stress in diabetes [178]. Moreover, these studies supported the studies which had argued that patients with type I diabetes suffer from increased lipid peroxidation even in the absence of complications [179]. When the results of the above-cited researchers were evaluated completely, it was not surprising that high bone MDA values occurs in the diabetic group and diabetic group subjected to swimming exercise. The results agreed with what was found in the present study. MDA values in the bone tissue in control group was found to be lower than the values in the diabetic group. Decreased MDA values established in control group and melatonin supplemented group is an important discovery, as the results obtained

in them indicate that melatonin supplementation suppresses MDA values in diabetic groups, irrespective of exercise. It was reported that melatonin had a protective effect on bone tissue [180] and prevented lipid peroxidation in the bone tissue, which increases in diabetes, by activating antioxidant defense mechanisms, and consequently, that melatonin has a protective effect in diabetes [181,182]. That melatonin supplementation was reported to inhibit elevated MDA levels in various tissues of rats subjected to swimming exercise [183] also supports the results we found in control group and melatonin supplemented group. Melatonin-supplemented diabetic control group had the highest bone tissue GSH values.
This result demonstrates that melatonin supplementation significantly increases GSH levels in both exercised rats and diabetic rats, irrespective of whether or not they were subjected to swimming exercise. Melatonin supplementation was also reported to significantly increase antioxidant activity in rats with induced diabetes [183,184]. It is generally accepted that melatonin inhibits lipid peroxidation, which increases in diabetes, by activating antioxidant defense mechanisms, and consequently, that melatonin has a protective effect in diabetes [185-188]. Melatonin supplementation to rats subjected to 30-min swimming exercise was shown to elevate GSH levels in various tissues, which were in harmony with the elevated GSH levels that we found.

In our study MDA values in the bone tissue in group with diabetic rats supplemented with melatonin were found to be lower than the values in diabetic group without supplementation, but higher than those in all other groups. The results obtained in them indicate that melatonin supplementation suppresses MDA values in diabetic groups. It was reported that melatonin had a protective effect on tissues throughout the body [188-192] and prevented lipid peroxidation in the bone tissue. Within this result we confirm it presents the same protective effects in bones. Melatonin-supplemented diabetic group had the highest bone tissue GSH values. This result demonstrates that melatonin supplementation significantly increases GSH levels in diabetic rats. Melatonin supplementation was also reported to significantly increase antioxidant activity in rats with induced diabetes (65-68). It is generally accepted that melatonin inhibits lipid peroxidation[170-172]. Melatonin supplementation to rats subjected to 30-min swimming exercise was shown to elevate GSH levels in various tissues [177], which were in harmony with the elevated GSH levels that we found.

During metabolism of streptozotocin, a variety of toxic intermediates are produced. Besides alkylating agents like methyl cations and methyl radicals [189], it has been shown that ROS are produced by streptozotocin as well [193]. Moreover, streptozotocin liberates NO which has been proposed to be one of the key intermediates of its toxicity. Taken together, streptozotocin-induced diabetes increases oxidative stress through generation of free radicals [194], lipid peroxidation, superoxide dismutase, protein glycosylation [195], decreased levels of catalase and glutathione peroxidase [196], as well as DNA single-strand breaks [192]. In the serum of animals with streptozotocin-induced diabetes, melatonin remarkably reduces the degree of both lipid peroxidation and protein glycosylation [194,196], decreases the levels of cholesterol, triglyceride, low-density lipoprotein [195], sialic acid [196] and glucose [197], as well as possibly regulating the activities of antioxidant enzymes [197].

Nevertheless, the most pronounced effect of melatonin administration was the prevention of an increase in NO levels in blood plasma during streptozotocin-induced diabetes [172], which implies that melatonin may operate as an NO scavenger and carrier. Despite this fact, another investigation concluded that the protective effects of melatonin against streptozotocin-caused β-cell damage may be related to interference with DNA damage and poly (ADP-ribose) polymerase activation rather than through effects on NO pathways [144]. Also, streptozotocin-induced diabetes resulted in lower melatonin levels in the pancreas, kidney and duodenum compared to the control, thus suggesting that the lower amplitude of melatonin in target organs induced by streptozotocin might contribute to the desynchronization of daily rhythms and might also weaken the antioxidant capacity of tissues [145]. Since it readily passes all biological membranes to reach intracellular organelles, many cells can synthesize melatonin, presumably to scavenge the oxygen- and nitrogen-based reactants produced in these cells, and, moreover, its membrane receptors are widespread in mammals and mediate some of the melatonin's actions. It has been determined that the effects of melatonin on insulin secretion are mediated through the melatonin receptors. By inhibiting cAMP and/or cGMP pathways, melatonin reduces insulin secretion. However, it has been shown that melatonin activates the PLC/IP_3 pathway, which mobilises Ca^{2+} from intracellular stores and, subsequently, increases insulin secretion. Meanwhile, insulin secretion, both *in vivo* and *in vitro*, exhibits a circadian rhythm, apparently generated within the islets, which is influenced by melatonin by inducing a phase

shift in insulin secretion. The observation that clock genes exhibit circadian expression in pancreatic tissue could be an indicator of the generation of circadian rhythms in the pancreatic islets themselves. Also, plasma melatonin levels and AA-NAT are decreased in type 2 diabetes patients. Taken together, these results indicate a close interrelationship between insulin and melatonin, which may be significant for the genesis of diabetes [193-198].

Oxidative stress is of great importance in the formation of complications related to DM. It has been spread widely that oxidative stress develops in presence of an excessive production of superoxide. The proper on time diagnosis of oxidative stress state in diabetes is crucial, because changes occur neatly everywhere throughout the body and disable functioning. The biochemical analyses that was undergone in the present study proves the existence of oxidative stress in diabetes. These parameters are ROS, TOS, TAS, and OSI and they indicate the level of accumulated oxidants and possible tissue damage caused by DM [196]. The typical pathophysiology of oxidative stress provokes ROS and TOS increase and TAS level decrease. It is explained by TAS activity in effective reduction of oxidative stress complications and furthermore it shows an increased antioxidant level [197].

Melatonin has ability to protect cells against the harmful effects of free radicals, including reactive oxygen and nitrogen species. Besides, Melatonin diminishes the levels of oxidative stress in organisms directly by inducing the antioxidant defense systems [198-200]. Melatonin can decrease oxidative stress by stimulating gene expression of antioxidant enzymes such as SOD and GSH-Px [201]. Ahmad Haja et al. found that 1 mg/kg of melatonin given to the diabetic group for 4 increased the GSH level, CAT, and SOD activities, but decreased LPO level in pancreas [145]. Albazal et al. that GSH level, CAT, and SOD activities decreased in diabetic animals, while LPO and ROS levels increased [203]. Treatment with melatonin at 10 mg/kg/day for 7 weeks reversed negative impact of oxidative stress [202]. Another study reported that administration of 10 mg/kg melatonin to diabetic rats for 14 days improved oxidative stress parameters significantly [203]. In the diabetic group, GSH, TAS levels, and GPx activity diminished, while LPO, ROS, and TOS levels increased. It was observed that oxidative stress parameters were reversed upon administering melatonin to diabetic rats by reducing oxidative stress [204]. In the present study, oxidative stress parameters such as TAS, TOS, ROS, and OSI were studied. It was found that the oxidative

stress in the bone tissue caused by diabetes decreased upon the administration of melatonin for 4 weeks. When both the effect of melatonin on antioxidant enzymes and free radical scavenging effect are considered, the outcomes are compatible with previous reports.

The results of the present study indicated that melatonin supplementation prevents increased free radical production and inhibits antioxidant activity resulting from diabetes in the bone tissue. Melatonin has many positive effects and antioxidant properties. In the present study, the effect of melatonin on diabetes was examined using different doses and biochemical parameters. It was observed that melatonin had a healing and restored normal biochemical parameters. The data indicate that the use of melatonin with or without insulin may be effective in preventing or at least retarding the development of some diabetic complications.

9. Conclusions

In order to address the objectives of the study:

1. Histological evaluation of the MCL microarchitecture from the groups with induced diabetes were characterized by structural fibrosis, cellular hypertrophy, relaxed collagen fibers with lymphocytic infiltration, the presence of mast cells and hyperactivity of fibroblasts. These features indicate intensely impaired regenerative capacity, the presence of inflammation and remodeling.
2. Surgical intervention within the ligament contributed to the impaired healing potential and loss of the morphological structure in the group of animals with induced diabetes. The group with normoglyceamia represented much lower intensity of the worsened status of the tissue, suggesting diabetic influence on the process.
3. In the tissue homogenate formed from the ligaments of animals from the group with induced diabetes, there was an increase in the inhibitors of the autooxidative activity of lipid peroxidase, which is a determinant of inflammatory changes. This indicates a high level of oxidative stress within these tissues. Melatonin supplementation in a group of animals with induced diabetes contributed to an increase in the concentration of antioxidants, that exposes the protective effect on the ligaments in hyperglycemia. In the case of melatonin supplementation in the group of normoglycemic animals, melatonin supplementation did not cause significant changes.
4. The results suggest the decreased antioxidant activities among ligaments exposed to chronic hyperglycaemia. Treatment with melatonin significantly improved the potential of antioxidant mechanisms.
5. These finding evidence the degenerative influence of DM on the ligament tissue, inhibiting physiological protective mechanism for oxidative stress. These changes were found to be meaningful when compared to the control group, in which the proportions were opposite to enable an adequate response to oxidative stress. Treatment with melatonin to diabetic rats mitigated alterations and improved the antioxidant status of ligaments from diabetic group.
6. Lipid peroxidation, which is one of the effects of free radical products, and MDA, which is one of its end products are responsible for induction of apoptosis in

ligaments. This findings proves that diabetes and the accompanying oxidative stress are the main mediators of the loss of normal ligament morphology. Decreased MDA values found in the diabetic group with supplemented melatonin indicate that it attenuated the effects of hyperglycaemia on ligaments.

7. Melatonin supplementation significantly increases antioxidant activity in rats with induced diabetes. Inhibition of lipid peroxidation, intensified in diabetes under the influence of melatonin, was observed.

10. Abstract in English

Introduction

Lack of physiological action of insulin in DM induces an impaired metabolism of carbohydrates, fat and proteins. Those are the necessary components for cellular homeostasis and tissue activities. Altered glucose metabolism impacts on all the basic processes taking place in the organism and remains the reason for impaired body functioning and regenerative abilities. DM is a complex metabolic disorder, which has several direct and indirect effects on multiple processes, starting from simple to more complex: chemotaxis, phagocytosis, bacterial killing, protein expression, antioxidant synthesis, free radicals inhibition, glucocorticoid concentration, cells proliferation. The cellular and biochemical background for those changes and irreversible degradation need to be further evaluated in order to enhance the knowledge about the exact mechanism altering the skeletal system. Due to the significant epidemiology of diseases concerning the skeletal system, it is of great importance to further evaluate the mechanisms. Those hypotheses created a concept of undertaking the study, that aims to investigate the histopathological and biochemical alterations provoked by pharmacologically- induced DM in ligaments and to assess the regenerative potential of the tissue.

Ligaments are compositions of collagenous tissue that create the key elements of physiological locomotory function. In two-thirds of their inner biochemical content, ligaments consist of water and in one-thirds of solid components, which in majority consist of collagen (type I collagen accounting for 85% of the collagen) and proteoglycans, elastin and other proteins and glycoproteins such as actin, laminin and the integrins. Ligaments play a crucial role in the motoric system by keeping responding to loads and micro injuries that affect them during the whole lifetime with an increased mass and stiffness. All the life activities together with aging, maturation, tension and exercise given to the joint, affect the biomechanical properties and regenerative potential of ligaments. There is a clinical evidence of alteration of their properties caused by DM and aging, that facilitate the loss of function and ability of regeneration throughout the ligaments. DM will likely affect 500 million people worldwide by 2030 according to WHO reports. Therefore, the key aspect in the context of patient health care seems to be the introduction of efforts aiming at eliminating or delaying complications caused by DM. The discovery of the histopathological and biochemical basis of

ligament degeneration as a direct factor causing changes within the ligaments is the basis for further research aimed at introducing targeted treatment techniques.

Objectives:

6. Identification of alterations present among MCL microarchitecture and tracking the mechanisms, that affect ligamentous functioning via assessment of diabetic and normoglycemic groups. Classification of the changes occurring in ligaments using histopathological screening and the Sairyo scale of fibrosis, loss of elastic fibers and calcification.
7. Analysis of differences in ligament remodeling and damage after surgical interventions and sham procedures in rats with pharmacologically induced diabetes (hyperglycemia) compared to normoglycemic control group.
8. Analysis of the concentrations of inhibitors of the autooxidative activity of lipid peroxidase, nitric oxide, glutathione S-transferase, ceruroplasmin, albumin, uric acid in the tissue homogenate and blood plasma in rats with pharmacologically induced diabetes (hyperglycemia) compared to normoglycemic control group.
9. Analysis of the influence of melatonin supplementation on the concentration of oxidative stress biochemical markers in tissue homogenates and blood plasma coming from pharmacologically induced diabetes groups and normoglycemic control group.
10. Analysis of differences in concentration of the biochemical markers of oxidative stress between the groups with normoglycemia and hyperglycemia undergoing ligament surgery.

Materials and methods

The study was conducted on forty (40) male Sprague-Dawley rats weighing 280 to 300 g, 12 weeks of age. Rats were housed in plastic cages with a metal lid at 2 animal models per cage from 1 week prior to testing for acclimation. The cages were transparent, which guaranteed visual contact between the animals. The animals received commercial food and had free access to water. Cages with models were placed in laboratory rooms with a 12-hour light/12-hour dark cycle at a temperature of 21 ± 2°C and a humidity of 55% ± 10%.

The animals were randomized into four equal groups, the I and II groups (20 rats) received saline subcutaneously and continued to function as controls. The III and IV groups (20 rats) were injected with a single dose of streptozotocin (STZ) at a dose of 60 mg/kg body weight, dissolved in freshly prepared buffer (0.1 mol/L citrate, pH 4.5). Rats were fasted for 8 hours prior to STZ injection. 72 hours after STZ injection, rats with a constant blood glucose level of ≥200 mg/dL for three consecutive days were considered successful diabetic models. 20 models passed successful DM induction. Rats with DM were fed a diet rich in fat and sugar. Plasma glucose and cholesterol were recorded weekly to monitor hyperglycemia in groups three and four and normoglycemia in groups I and II. Venous blood was collected and tested on glucometer strips. All animals underwent surgery to cut the left tibial collateral ligament in the hind limb and suture it and suture the access site to create inflammation to study the regenerative capacity of animals with normal carbohydrate metabolism and with pharmacologically induced diabetes. Each animal then underwent sham surgery to access and suture the right tibial collateral ligament in the hindlimb without ligament intervention.

After the animals had undergone surgeries, groups II and IV were given melatonin supplementation for 4 weeks.

The animals were euthanized 6 weeks after the start of the experiment. From each model, connective tissue was collected from the ligament subjected to the intervention and the ligament from the other limb, which was subjected to sham surgery. Two samples of the material were collected from each of the model's ligaments into two test tubes for histological evaluation and the formation of a tissue homogenate for biochemical evaluation. In addition, blood was taken for laboratory tests.

All animals were housed in accordance with the criteria set out in the guide for the care and use of laboratory animals, prepared under the EU Directive 2010/63/EU for the purposes of animal experiments. Ethical principles were followed in accordance with national and institutional guidelines for the protection of animal welfare during experiments. The study was designed in accordance with the ARRIVE guidelines. The study was conducted in university departments, previously organized to conduct research on animals. The personnel who worked with the animals had experience in inducing DM in SPRD rats. Rats represent the most common species used in musculoskeletal and post-traumatic recovery experiments.

Results

There was a positive feedback between plasma glucose values of fasting animals and their body weight and degree of elastin degradation, fibrosis and calcification within the ligaments. All animals from the diabetic group successfully underwent the DM induction procedure and gained significantly more body weight compared to the control group. Histopathological examination of normoglycemic rats from groups I and II and diabetic rats from groups III and IV was assessed using the Sairyo scale. The results showed statistically significant worse parameters within the left MCL undergoing surgical intervention in the group of animals with induced diabetes compared to the ligament tissues of the left MCL of animals with normal glycemia.

Particularly notable was the significant fibrosis in the left MCL compared to the right MCL in group III with a score in the range of 3.43 ± 0.88; $P<0.05$ and in group I in the range of 1.44 ± 0.7; $P<0.05$. Group I showed a Sairyo coefficient of 1.44 ± 0.7 within the dissected ligamentous tissue from the left MCL. In the dissected MCL, an increased loss of elastin fibers was observed in all groups $P < 0.05$. The melatonin supplemented group showed little loss of elastin fibers compared to the control group, with scores of 0 and 0.5 ± 0.2, respectively, $P < 0.05$ for the right MCL and 0 and 1.05 ± 0.55, respectively, $P < 0.05$ for the left MCL. The right MCL samples in all groups showed statistically significantly more calcification than the left MCL tissues in the same groups. The non-diabetic group presented calcifications in the dissected MCL (1.05, ± 1.7) and no signs of calcifications in the right MCL with a statistically significant score ($P < 0.05$).

Compared to control group, plasma chemistry studies in diabetic rats showed elevated plasma lipid peroxidase and uric acid levels ($P=0.0011$) and decreased albumin levels ($P=0.0321$). Compared to corresponding control group, nitric oxide was increased ($P=0.0567$) in diabetic rats, while total thiols and ceruloplasmin were decreased ($P>0.05$) compared to corresponding control group. Melatonin treatment significantly increased total thiol and ceruloplasmin activity. Also, melatonin treatment significantly decreased ($P<0.001$, $P<0.05$) lipid peroxides and uric acid, respectively.

Compared to control group, ligament tissue homogenates from a group of diabetic rats showed a significant increase in lipid peroxidase (P=0.0032). GST activity was significantly increased (P<0.001) in melatonin-treated diabetic rats. Total thiols showed reduced values in the tissues of diabetic rats compared to the control group. Melatonin treatment significantly increased (P<0.05, P<0.001) superoxide dismutase and catalase activity, respectively, compared to the DM group and showed a similar range of values compared to the control group.

In the tissue homogenate of ligaments taken from rats with induced diabetes, TAS levels were found to decrease, while TOS, ROS and OSI levels increased. These changes turned out to be significant compared to the control group (p <0.0001; p <0.0001; p <0.0001; p <0.0001, respectively). Melatonin treatment of diabetic rats alleviated these changes, in the group of tissue homogenates from the ligaments taken from melatonin-induced diabetic rats, the results became statistically significant (p < 0.0001; p <0.0001; p < 0.0001, respectively). ; p 0.0001). The highest value of MDA in bone tissue was obtained in the DM group. Bone MDA levels in the DM and melatonin-supplemented groups were lower than in the DM group but higher than in all other groups. GSH values turned out to be higher in the groups with supplemented melatonin than in the groups without supplementation. The highest GSH levels were measured in the melatonin-supplemented DM group.

Tissues from the diabetic group with supplemented melatonin showed reduced concentrations of MDA in and increased GSH values in the ligaments of rats. The results of the study confirmed the protective effect of melatonin supplementation on the ligaments of the knee joint by preventing lipid peroxidation. This result demonstrates that melatonin supplementation significantly increases antioxidant activity in rats with induced diabetes. It is assumed that melatonin inhibits lipid peroxidation, which intensifies in diabetes, by activating antioxidant defense mechanisms. Therefore, melatonin has a protective effect on the ligaments in diabetes in terms of their functionality. The results of this study show that melatonin supplementation prevents the increased production of free radicals and inhibits the antioxidant activity resulting from diabetes in the ligaments.

Conclusions

1. Tissues from animals from the group with induced diabetes were characterized by structural fibrosis, cellular hyperplasia, loosened authorized collagen with lymphocytic infiltration, the presence of mast cells in the hyperreactivity of fibroblasts. These features indicate intense impaired regenerative capacity, coupled with inflammation and remodelling.

2. Surgical intervention signs of increased inflammatory changes in a group with diabetes, suggesting the implications of the disease on the regenerative capacity.

3. Diabetes via the streptozotocin treatment, revealed to enhance the lipid peroxidation, which in turn stimulated auto-oxidative reactions of lipids and proteins. Melatonin supplementation in a group of animals with induced diabetes, which causes an increase in antioxidant enhancement, thus a protective effect on the supplement in a hyperglycaemic environment. In the case of melatonin supplementation in the group of normoglycemic animals, melatonin supplementation is never found to change.

4. Examination of the tissue homogenate of diabetic rats in terms of determining the level of antioxidant activity, resulted in concluding the diabetes induces reduced antioxidant activity. Melatonin treatment increases the ability to respond the oxidative stress.

5. The study proves that diabetes inhibits the physiological protective mechanism against oxidative stress. Melatonin treatment of diabetic rats alleviated changes and improved the antioxidant status of diabetic ligaments.

6. Lipid peroxidation, which is one of the effects of free radical products, and MDA, which is one of its end products, protein apoptosis induction activity, which proves that diabetes and the accompanying oxidative stress are the main mediators of the loss of normal ligament morphology. Decreased MDA values found in the diabetic group with supplemented melatonin indicate that it attenuated the effects of hyperglycaemia on ligaments.

7. Melatonin supplementation significantly increases antioxidant activity in rats with induced diabetes. Inhibition of lipid peroxidation, intensified in diabetes under the influence of melatonin, was observed.

11. Abstract in Polish

Wstęp

Brak fizjologicznego działania insuliny w cukrzycy powoduje upośledzenie metabolizmu węglowodanów, tłuszczów i białek. Są to składniki niezbędne do homeostazy komórkowej i aktywności tkankowej. Zaburzony metabolizm glukozy implikuje negatywny wpływ na wszystkie podstawowe procesy zachodzące w organizmie i pozostaje przyczyną upośledzonego funkcjonowania i zdolności regeneracyjnych organizmu. Cukrzyca jest złożonym zaburzeniem metabolicznym, które ma kilka bezpośrednich i pośrednich skutków dla wielu procesów, począwszy od prostych do bardziej złożonych: chemotaksji, fagocytozy, infekcji, ekspresji białek, syntezy przeciwutleniaczy, degradacji wytwarzanymi wolnych rodników tlenowych, stężenia glikokortykoidów, proliferacji komórek i innych. Komórkowe i molekularne tło tych zmian a zarazem ich nieodwracalnej degradacji pozostaje wciąż nieoczywiste, natomiast kwestia wpływu zmian wywołanych cukrzycą na układu ruchu jest nadal niezbadany. Ze względu na dużą epidemiologię chorób układu kostno-stawowego, pozostaje naglącym problemem klinicznym. Na podstawie rozważań wpływu zaburzeń metabolizmu zrodziła się koncepcja badania, które ma na celu przybliżenie obrazu histopatologicznego oraz biochemicznego tkanek, poddanego niewyrównanej farmakologicznie indukowalnej cukrzycy oraz zbadania możliwości wpłynięcia, tudzież odwrócenia zaistniałych zmian.

Więzadła to kluczowe elementy prawidłowo funkcjonującego układu ruchu, biochemicznie stanową kompozycję tkanki kolagenowej, które tworzą stawy i łączą ze sobą kości. W 2/3 więzadła składają się z wody, natomiast pozostałą część tworzą: kolagen, proteoglikany, elastyna i inne białka i glikoproteiny takie jak aktyna, laminina i integryny. Więzadła odgrywają kluczową rolę w układzie ruchu, stabilizując stawy w reakcji na obciążenia i mikrourazy, które dotykają je przez całe życie. Wszystkie czynności życiowe wraz ze starzeniem się, dojrzewaniem i ćwiczeniami fizycznymi wpływają na właściwości biomechaniczne więzadeł oraz ich potencjał regeneracyjny. Cukrzyca obok procesu fizjologicznego starzenia organizmu stanowi czynnik utraty funkcjonalności w obrębie więzadeł, co następnie przekłada się na upośledzenie dotychczasowych funkcji spełnianych przez tkankę. Prognozy WHO szacują, że epidemiologia cukrzyca do 2030 r. sięgnie 500

milionów ludzi na świecie. Zatem kluczowym aspektem w kontekście opieki zdrowotnej nad pacjentami wydaje się wprowadzanie działań służących niwelowaniu bądź opóźnianiu wywołanych przez cukrzycę powikłań. Odkrycie histopatologicznego i biochemicznego podłoża degeneracji więzadeł jako bezpośredniego czynnika wywołującego zmiany w obrębie więzadeł stanowi podstawę do dalszych badań, służących wprowadzeniu celowanych technik leczniczych.

Cele:

1. Identyfikacja zmian w mikroarchitekturze więzadła, poznanie mechanizmów degeneracyjnych, skutkujących utratą funkcji na podstawie porównania tkanki szczura z cukrzycą z tkanką zdrowego szczura oraz klasyfikacja zmian z wykorzystaniem histologicznej oceny skali nasilenia włóknienia, utraty włókien elastycznych i zwapnienia w obrębie więzadeł wg Sairyo.
2. Analiza różnic w przebudowie i uszkodzeniu więzadeł po interwencjach operacyjnych i procedurach pozorowanynch u szczurów z farmakologicznie wywołaną cukrzycą (hiperglikemią) w porównaniu do grupy kontrolnej z normoglikemią.
3. Analiza stężeń inhibitorów aktywności autooksydacyjnej peroksydazy lipidowej, tlenku azotu, S- transferazy glutationu, ceruroplazminy, albuminy, kwasu moczowego w homogenacie tkankowym oraz osoczu krwi zwierząt z grupy z farmakologicznie wywołaną cukrzycą oraz grupy z normoglikemią.
4. Analiza wpływu suplementacji melatoniny na obniżenie stężenia markerów biochemicznych stresu oksydacyjnego w homogenacie tkankowym oraz osoczu krwi pochodzących od zwierząt z farmakologicznie wywołaną cukrzycą oraz normoglikemią.
5. Analiza różnic w stężeniach markerów biochemicznych stresu oksydacyjnego w grupie badanej i kontrolnej oraz pomiędzy grupami z farmakologicznie wywołaną cukrzycą oraz od zwierząt z normoglikemią, poddanych zabiegom operacyjnym w obrębie więzadeł.

Materiał i metody

Badanie zostało przeprowadzone z udziałem czterdziestu (40) szczurów, samców rasy Sprague-Dawley o wadze od 280 do 300 g, w wieku 12 tygodni. Szczury przebywały w

plastikowych klatkach z metalową pokrywą w liczbie 2 modeli zwierzęcych na klatkę od 1 tygodnia przed badaniem, celem aklimatyzacji. Klatki były przezroczyste, co gwarantowało kontakt wzrokowy między zwierzętami. Zwierzęta otrzymywały karmę komercyjną i miały swobodny dostęp do wody. Klatki z modelami umieszczono w pomieszczeniach laboratoryjnych z cyklem 12 godzin światła/12 godzin ciemności w temperaturze 21 ± 2°C i wilgotności 55% ± 10%.

Zwierzęta podzielono losowo na cztery równe grupy, pierwsza i druga grupa (20 szczurów) otrzymała roztwór soli fizjologicznej podskórnie i dalej funkcjonowały jako grupy kontrolne. Trzeciej i czwartej grupie (20 szczurów) wstrzyknięto dootrzewnowo pojedynczą dawkę streptozotocyny (STZ) w dawce 60 mg/kg masy ciała, rozpuszczonej w świeżo przygotowanym buforze (0,1 mol/L cytrynian, pH 4,5). Szczury głodzono przez 8 godzin przed wstrzyknięciem STZ. 72 godziny po wstrzyknięciu STZ szczury ze stałym poziomem glukozy we krwi ≥200 mg/dl uznano za udane modele cukrzycowe. 20 modeli przeszło udaną indukcję DM. Szczury z cukrzycą karmiono dietą komercjalną. Krew żylną pobierano i badano na paskach glukometru.

Wszystkie zwierzęta poddano operacji przecięcia lewego więzadła pobocznego piszczelowego w tylnej kończynie i zaszycia go oraz zaszycia miejsca dostępu, celem wytworzenia stanu zapalnego, by zbadać zdolności regeneracyjne zwierząt z prawidłową gospodarką węglowodanową oraz z farmakologicznie wywołaną cukrzycą. Każde ze zwierząt następnie poddano pozorowanej operacji uzyskania dostępu operacyjnego do prawego więzadła pobocznego piszczelowego w tylnej kończynie i zaszycia go bez interwencji w obrębie więzadła.

Po poddaniu zwierząt zabiegom chirurgicznym, grupy drugą oraz czwartą poddano suplementacji melatoniny, która trwała 4 tygodnie.

Zwierzęta poddano eutanazji po 6 tygodniach od rozpoczęcia eksperymentu. Od każdego modelu pobrano tkankę łączną z obrębu więzadła poddanego interwencji oraz więzadła z drugiej kończyny, która podległa operacji pozorowanej. Z każdego z więzadeł modelu pobrano po dwie próbki materiału do dwóch probówek w celu oceny histologicznej oraz utworzenia homogenatu tkankowego do oceny biochemicznej. Ponadto pobrano krew do badań laboratoryjnych.

Wszystkie zwierzęta miały zapewnione warunki utrzymania zgodnie z kryteriami określonymi w przewodniku dotyczącym opieki nad zwierzętami laboratoryjnymi i ich wykorzystywania, przygotowanym na mocy Dyrektywy UE 2010/63/UE do celów doświadczeń na zwierzętach. Zasad etycznych przestrzegano zgodnie z krajowymi i instytucjonalnymi wytycznymi dotyczącymi ochrony dobrostanu zwierząt podczas eksperymentów. Badanie zostało zaprojektowane zgodnie z wytycznymi ARRIVE. Badanie przeprowadzono na wydziałach uniwersyteckich, wcześniej zorganizowanych do prowadzenia badań na zwierzętach. Personel, który pracował ze zwierzętami, miał doświadczenie w indukowaniu DM u szczurów SPRD. Szczury reprezentują najczęściej spotykany gatunek wykorzystywany w eksperymentach dotyczących układu mięśniowo-szkieletowego i regeneracji pourazowej.

Wyniki

Wystąpiło sprzężenie zwrotne dodatnie między wartościami glukozy w osoczu krwi zwierząt na czczo a ich masą ciała i stopniem degradacji elastyny, zwłóknienia i zwapnienia w obrębie więzadeł. Wszystkie zwierzęta z grupy cukrzycowej pomyślnie przeszły procedurę indukcji DM i uzyskały istotnie większą masę ciała w porównaniu do grupy kontrolnej. Badanie histopatologiczne szczurów z normoglikemią z grup I i II oraz szczurów z cukrzycą z grup III i IV oceniano za pomocą skali Sairyo. Wyniki pokazały statystycznie istotne gorsze parametry w obrębie lewego MCL poddawanego interwencji chirurgicznej w grupie zwierząt z indukowaną cukrzycą w porównaniu do tkanek więzadeł lewego MCL zwierząt z prawidłową glikemią.

Szczególnie zauważalne było znaczące zwłóknienie w lewym MCL w porównaniu z prawym MCL w grupie III z wynikiem w zakresie 3,43 ± 0,88; $P<0.05$ oraz w grupie I w zakresie 1,44 ± 0,7; $P<0,05$. Grupa I wykazało współczynnik Sairyo w granicach 1,44 ± 0.7 w obrębie wypreparowanej tkanki więzadłowej z lewego MCL. W wypreparowanym MCL zaobserwowano zwiększoną utratę włókien elastyny we wszystkich grupach $P <0.05$. Grupa z suplementowaną melatoniną wykazało małą utratę włókien elastyny w porównaniu do grupy kontrolnej, uzyskując odpowiednio wyniki 0 i 0.5 ± 0.2, $P <0.05$ dla prawego MCL oraz odpowiednio 0 i 1.05 ± 0.55, $P <0.05$ dla lewego MCL. Próbki prawego MCL we wszystkich grupach wykazywały statystycznie istotnie więcej zwapnień niż tkanki pobrane z lewego MCL

z tych samych grup. Grupa bez cukrzycy prezentowała zwapnienia w wypreparowanym MCL (1.05, ± 1.7) i brak oznak zwapnień wśród prawego MCL ze statystycznie istotnym wynikiem(P <0.05).

W porównaniu do grupy kontrolnej, badania biochemiczne osocza szczurów z grupy poddanych cukrzycy wykazały podwyższone stężenie peroksydazy lipidowej i kwasu moczowego w osoczu (P=0.0011) i obniżone stężenie albumin (P=0.0321). W porównaniu do grupy kontrolnej tlenek azotu był zwiększony (P=0.0567) u szczurów z cukrzycą, podczas gdy całkowite tiole i ceruloplazmina były zmniejszone (P>0.05) w porównaniu z grupą kontrolną. Leczenie melatoniną znacząco zwiększyły całkowitą aktywność tioli i ceruloplazminy. Również leczenie melatoniną znacząco obniżyło (P<0.001, P<0.05) odpowiednio nadtlenki lipidów i kwas moczowy.

W porównaniu do grupy kontrolnej, homogenaty tkankowe więzadeł z grupy szczurów poddanych cukrzycy wykazały znaczny wzrost peroksydazy lipidowej (P=0.0032). Aktywność GST znacznie wzrosła (P <0.001) u szczurów z cukrzycą leczonych melatoniną. Całkowite tiole wykazywały obniżone wartości w tkankach szczurów z grupy cukrzycowej w porównaniu z grupą kontrolną. Leczenie melatoniną znacznie zwiększyło (P<0.05, P<0.001) aktywność odpowiednio dysmutazy ponadtlenkowej i katalazy w porównaniu z grupą DM i wykazało podobny zakres wartości w porównaniu do grupy kontrolnej.

W homogenacie tkankowym z więzadeł pobranych od szczurów z indukowaną cukrzycą stwierdzono obniżenie poziomu TAS, podczas gdy poziomy TOS, ROS i wskaźniki OSI wzrosły. Zmiany te okazały się znaczące w porównaniu z grupą kontrolną (odpowiednio $p < 0,0001$; $p < 0,0001$; $p < 0,0001$; $p < 0,0001$). Leczenie melatoniną szczurów z cukrzycą złagodziło te zmiany, w grupie homogenatów tkankowych z więzadeł pobranych od szczurów z grupy z indukowaną cukrzycą leczonych melatoniną, wyniki przybrały statystycznie istotne wartości (odpowiednio $p < 0,0001$; $p < 0,0001$; $p < 0,0001$; p 0,0001). Najwyższą wartość MDA w tkance kostnej uzyskano w grupie DM. Poziomy MDA w tkance kostnej w grupach z DM i w grupie z suplementowaną melatoniną były niższe niż w grupie DM, ale wyższe niż we wszystkich pozostałych grupach. Wartości GSH okazały się wyższe w grupach z suplementowaną melatoniną niż w grupach bez suplementacji. Najwyższe poziomy GSH zmierzono w grupie DM z suplementowaną melatoniną.

Tkanki z grupy z cukrzycą suplementowaną melatoniną wykazały obniżone stężenie MDA w oraz podwyższone wartości GSH w więzadłach szczurów. Wyniki badania potwierdziły ochronny wpływ suplementacji melatoniny na więzadła stawu kolanowego, poprzez zapobieganie peroksydacji lipidów. Wynik ten pokazuje, że suplementacja melatoniną znacznie zwiększa aktywność przeciwutleniającą u szczurów z indukowaną cukrzycą. Przyjmuje się, że melatonina hamuje peroksydację lipidów, która nasila się w cukrzycy, poprzez aktywację antyoksydacyjnych mechanizmów obronnych. W związku z tym melatonina wykazuje działanie ochronne na więzadła w cukrzycy w kontekście ich funkcjonalności. Wyniki niniejszego badania wykazują, że suplementacja melatoniną zapobiega zwiększonej produkcji wolnych rodników i hamuje aktywność antyoksydacyjną wynikającą z cukrzycy w więzadłach.

Wyniki badania osocza krwi zwierząt z grupy z indukowaną cukrzycą są analogiczne. Wykryto statystycznie istotny wzrost stężenia peroksydazy lipidowej w porównaniu z grupą kontrolną. Ponadto zauważono znamienny wzrost stężenia tlenku azotu, dysmutazy nadtlenkowej, S-transferazy glutationu, ceruroplazminy, albuminy, kwasu moczowego w porównaniu z grupą kontrolną. Stężenie całkowitych tioli, ceruroplazminy, albuminy jako markerów protekcyjnych było znamiennie niższe w porównaniu z grupą kontrolną. Suplementacja melatoniny w grupie zwierząt z indukowaną cukrzycą przyczyniła się do zwiększenia stężenia przeciwutleniaczy, takich jak dysmutaza nadtlenkowa oraz katalaza. Wyniki pobrane od grupy zwierząt z indukowaną cukrzycą z suplementacją melatoniny przedstawiały wartości istotnie statystycznie porównywalne z grupą kontrolną. W przypadku suplementacji melatoniny w grupie zwierząt z normoglikemią, suplementacja melatoniny nie spowodowała istotnych zmian. Zbadanie homogenatu tkankowego pod względem całkowitego statusu antyoksydacyjnego przyniosło wynik statystycznie niższy w porównaniu z grupą kontrolną. Całkowity status oksydacyjny, poziom reaktywności cząstek tlenu oraz współczynnik stresu oksydacyjnego wykazały zaś istotnie statystycznie wyższe w porównaniu z grupą kontrolną z normoglikemią.

Wnioski

1. Tkanki pochodzące od zwierząt z grupy z indukowaną cukrzycą charakteryzowały się włóknieniem strukturalnym, przerostem komórkowym, rozluźnionymi włóknami kolagenowymi z naciekiem limfocytarnym, obecnością komórek tucznych i nadreaktywnością fibroblastów. Cechy te wskazują na intensywnie upośledzone zdolności regeneracyjne, obecność stanu zapalnego i przebudowy.
2. Interwencja chirurgiczna w obrębie więzadła przyczyniła się do nieprawidłowej regeneracji i uszkodzenia struktury więzadła u zwierząt z indukowaną cukrzycą. W grupie zwierząt z normoglikemią zaobserwowano niższe nasilenie zmian zapalnych, co sugeruje implikacje choroby metabolicznej na zdolności regeneracyjne tkanki.
3. W homogenacie tkankowym utworzonym z więzadeł zwierząt z grupy z indukowaną cukrzycą wystąpił wzrost aktywności autooksydacyjnej peroksydazy lipidowej spowodowany streptozotocyną, który jest wyznacznikiem zmian zapalnych w obrębie więzadeł. Również stężenie innych molekuł pośrednio świadczących o obniżonej zdolności regeneracyjnej więzadeł w homogenacie tkankowym pobranym od zwierząt z grupy z indukowaną cukrzycą było podwyższone. Wskazuje to na wysoki poziom stresu oksydacyjnego w tych tkankach. Suplementacja melatoniną w grupie zwierząt z indukowaną cukrzycą przyczyniła się do wzrostu stężenia przeciwutleniaczy, wykazując tym samym ochronny wpływ na tkanki w środowisku hiperglikemii. W grupie zwierząt normoglikemicznych suplementacja melatoniną nie powodowała istotnych zmian.
4. Badanie homogenatu tkankowego pod kątem całkowitego statusu antyoksydacyjnego dało wynik świadczący o cukrzycy jako determinancie zmniejszonej aktywności antyoksydacyjnej więzadeł narażonych na przewlekłą hiperglikemię. Leczenie melatoniną znacząco zwiększyło całkowitą zdolność odpowiedzi na stres oksydacyjny.
5. Badanie dowodzi, że cukrzyca wywiera negatywny wpływ na więzadła, hamując fizjologiczny mechanizm ochronny przed stresem oksydacyjnym. Leczenie melatoniną szczurów z cukrzycą złagodziło zmiany i poprawiło status antyoksydacyjny więzadeł z grupy cukrzyków.
6. Peroksydacja lipidów, która jest jednym z najbardziej szkodliwych skutków produktów wolnorodnikowych, oraz MDA, który jest jednym z jej produktów końcowych, wykazują aktywność do indukcji apoptozy tkanek, co dowodzi, że cukrzyca i towarzyszący jej stres

oksydacyjny są głównymi mediatorami zanikania prawidłowej morfologii więzadeł. Obniżone wartości MDA ustalone w grupie z cukrzycą z suplementowaną melatoniną wskazują, że tłumi ona negatywny wpływ hiperglikemii na więzadła.

7. Suplementacja melatoniną znacznie zwiększa aktywność przeciwutleniającą u szczurów z indukowaną cukrzycą. Zaobserwowano zahamowanie peroksydacji lipidów, nasilonej w cukrzycy pod wpływem melatoniny.

12. Bibliography

1. Colagiuri S. Definition and Classification of Diabetes and Prediabetes and Emerging Data on Phenotypes. Endocrinol Metab Clin North Am. 2021 Sep;50(3):319-336. doi: 10.1016/j.ecl.2021.06.004.
2. Shemesh S, Sidon E, Kaisler E, Sheinis D, Velkes S, Ohana N, Benayahu D. Diabetes mellitus is associated with increased elastin fiber loss in ligamentum flavum of patients with lumbar spinal canal stenosis: results of a pilot histological study. Eur Spine J. 2018 Jul;27(7):1614-1622. doi: 10.1007/s00586-017-5315-0.
3. Ali R, Khamis T, Enan G, El-Didamony G, Sitohy B, Abdel-Fattah G. The Healing Capability of Clove Flower Extract (CFE) in Streptozotocin-Induced (STZ-Induced) Diabetic Rat Wounds Infected with Multidrug Resistant Bacteria. Molecules. 2022 Mar 31;27(7):2270. doi: 10.3390/molecules27072270.
4. Passarelli M, Machado UFF. AGEs-Induced and Endoplasmic Reticulum Stress/Inflammation-Mediated Regulation of GLUT4 Expression and Atherogenesis in Diabetes Mellitus. Cells. 2021 Dec 29;11(1):104. doi: 10.3390/cells11010104.
5. Shi L, Rui YF, Li G, Wang C. Alterations of tendons in diabetes mellitus: what are the current findings? Int Orthop. 2015 Aug;39(8):1465-73. doi: 10.1007/s00264-015-2775-x.
6. Leong NL, Kator JL, Clemens TL, James A, Enamoto-Iwamoto M, Jiang J. Tendon and Ligament Healing and Current Approaches to Tendon and Ligament Regeneration. J Orthop Res. 2020 Jan;38(1):7-12. doi: 10.1002/jor.24475. Epub 2019 Sep 30.

7. WHO. About Diabetes. 2014. Available online: https://www.who.int/diabetes/action_online/basics/en/index3.html (accessed on 21 September 2022).
8. Gustafson B, Hedjazifar S, Gogg S, Hammarstedt A, Smith U. Insulin resistance and impaired adipogenesis. Trends Endocrinol Metab. 2015 Apr;26(4):193-200. doi: 10.1016/j.tem.2015.01.006.
9. WHO. Global Report on Diabetes. 2016. Available online: https://www.who.int/publications/i/item/9789241565257 (accessed on 21 September 2022).
10. Cui A, Li H, Wang D, Zhong J, Chen Y, Lu H. Global, regional prevalence, incidence and risk factors of knee osteoarthritis in population-based studies. EClinicalMedicine. 2020 Nov 26;29-30:100587. doi: 10.1016/j.eclinm.2020.100587.
11. Liao KP, Gunnarsson M, Källberg H, Ding B, Plenge RM, Padyukov L, Karlson EW, Klareskog L, Askling J, Alfredsson L. Specific association of type 1 diabetes mellitus with anti-cyclic citrullinated peptide-positive rheumatoid arthritis. Arthritis Rheum. 2009 Mar;60(3):653-60. doi: 10.1002/art.24362.
12. Armitage LH, Wallet MA, Mathews CE. Influence of *PTPN22* Allotypes on Innate and Adaptive Immune Function in Health and Disease. Front Immunol. 2021 Feb 25;12:636618. doi: 10.3389/fimmu.2021.636618.
13. Arivazhagan L, López-Díez R, Shekhtman A, Ramasamy R, Schmidt AM. Glycation and a Spark of ALEs (Advanced Lipoxidation End Products) - Igniting RAGE/Diaphanous-1 and Cardiometabolic Disease. Front Cardiovasc Med. 2022 Jun 24;9:937071. doi: 10.3389/fcvm.2022.937071.

14. Hudson DM, Archer M, King KB, Eyre DR. Glycation of type I collagen selectively targets the same helical domain lysine sites as lysyl oxidase-mediated cross-linking. J Biol Chem. 2018 Oct 5;293(40):15620-15627. doi: 10.1074/jbc.RA118.004829.

15. Velayoudom-Cephise FL, Cano-Sanchez M, Bercion S, Tessier F, Yu Y, Boulanger E, Neviere R. Receptor for advanced glycation end products modulates oxidative stress and mitochondrial function in the soleus muscle of mice fed a high-fat diet. Appl Physiol Nutr Metab. 2020 Oct;45(10):1107-1117. doi: 10.1139/apnm-2019-0936.

16. Singh VP, Bali A, Singh N, Jaggi AS. Advanced glycation end products and diabetic complications. Korean J Physiol Pharmacol. 2014 Feb;18(1):1-14. doi: 10.4196/kjpp.2014.18.1.1.

17. Wukich DK. Diabetes and its negative impact on outcomes in orthopaedic surgery. World J Orthop. 2015 Apr 18;6(3):331-9. doi: 10.5312/wjo.v6.i3.331.

18. Stolarczyk A, Sarzyńska S, Gondek A, Cudnoch-Jędrzejewska A. Influence of diabetes on tissue healing in orthopaedic injuries. Clin Exp Pharmacol Physiol. 2018 Jul;45(7):619-627. doi: 10.1111/1440-1681.12939.

19. Galway U, Chahar P, Schmidt MT, Araujo-Duran JA, Shivakumar J, Turan A, Ruetzler K. Perioperative challenges in management of diabetic patients undergoing non-cardiac surgery. World J Diabetes. 2021 Aug 15;12(8):1255-1266. doi: 10.4239/wjd.v12.i8.1255.

20. Duggan E, Chen Y. Glycemic Management in the Operating Room: Screening, Monitoring, Oral Hypoglycemics, and Insulin Therapy. Curr Diab Rep. 2019 Nov 20;19(11):134. doi: 10.1007/s11892-019-1277-4.

21. Lai J, Li Q, He Y, Zou S, Bai X, Rastogi S. Glycemic Control Regimens in the Prevention of Surgical Site Infections: A Meta-Analysis of Randomized Clinical Trials. Front Surg. 2022 Mar 25;9:855409. doi: 10.3389/fsurg.2022.855409.

22. Lenguerrand E, Beswick AD, Whitehouse MR, Wylde V, Blom AW. Outcomes following hip and knee replacement in diabetic versus nondiabetic patients and well versus poorly controlled diabetic patients: a prospective cohort study. Acta Orthop. 2018 Aug;89(4):399-405. doi: 10.1080/17453674.2018.1473327.

23. Kato S, Saito M, Funasaki H, Marumo K. Distinctive collagen maturation process in fibroblasts derived from rabbit anterior cruciate ligament, medial collateral ligament, and patellar tendon in vitro. Knee Surg Sports Traumatol Arthrosc. 2015 May;23(5):1384-92. doi: 10.1007/s00167-013-2773-8.

24. Loots MA, Lamme EN, Mekkes JR, Bos JD, Middelkoop E. Cultured fibroblasts from chronic diabetic wounds on the lower extremity (non-insulin-dependent diabetes mellitus) show disturbed proliferation. Arch Dermatol Res. 1999 Feb-Mar;291(2-3):93-9. doi: 10.1007/s004030050389.

25. Black E, Vibe-Petersen J, Jorgensen LN, Madsen SM, Agren MS, Holstein PE, Perrild H, Gottrup F. Decrease of collagen deposition in wound repair in type 1 diabetes independent of glycemic control. Arch Surg. 2003 Jan;138(1):34-40. doi: 10.1001/archsurg.138.1.34.

26. Lerman OZ, Galiano RD, Armour M, Levine JP, Gurtner GC. Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia. Am J Pathol. 2003 Jan;162(1):303-12. doi: 10.1016/S0002-9440(10)63821-7.

27. K-C L, Zernicke RF, Barnard RJ, A F-Y L. Response of immature diabetic rat bone-ligament junctions to insulin and exercise. Clin Biomech (Bristol, Avon). 1995 Sep;10(6):331-336. doi: 10.1016/0268-0033(95)00034-i.

28. Vicente A, Bravo-González LA, Navarro JA, Buendía AJ, Camacho-Alonso F. Effects of diabetes on oxidative stress, periodontal ligament fiber orientation, and matrix metalloproteinase 8 and 9 expressions during orthodontic tooth movement. Clin Oral Investig. 2021 Mar;25(3):1383-1394. doi: 10.1007/s00784-020-03446-7.

29. Willett TL, Kandel R, De Croos JN, Avery NC, Grynpas MD. Enhanced levels of non-enzymatic glycation and pentosidine crosslinking in spontaneous osteoarthritis progression. Osteoarthritis Cartilage. 2012 Jul;20(7):736-44. doi: 10.1016/j.joca.2012.03.012.

30. Njoto I, Soekanto A, Ernawati E, Abdurrachman A, Kalim H, Handono K, Soeatmadji DW, Fatchiyah F. Chondrocyte Intracellular Matrix Strain Fields of Articular Cartilage Surface in Hyperglycemia Model of Rat: Cellular Morphological Study. Med Arch. 2018 Nov;72(5):348-351. doi: 10.5455/medarh.2018.72.348-351.

31. Njoto I, Kalim H, Soeatmadji DW, Handono K, Fatchiyah F. Effect of Hyperglycemia to The mRNA Level and Protein Expression of Perlecan at Rat Model of Osteoarthritis with Diabetes Mellitus Type 1. Med Arch. 2019 Jun;73(3):144-148. doi: 10.5455/medarh.2019.73.144-148.

32. Radojčić MR, Thudium CS, Henriksen K, Tan K, Karlsten R, Dudley A, Chessell I, Karsdal MA, Bay-Jensen AC, Crema MD, Guermazi A. Biomarker of extracellular matrix remodelling C1M and proinflammatory cytokine interleukin 6 are related to synovitis and pain in end-stage knee osteoarthritis patients. Pain. 2017 Jul;158(7):1254-1263. doi: 10.1097/j.pain.0000000000000908.

33. Asadian L, Haddadi K, Aarabi M, Zare A. Diabetes Mellitus, a New Risk Factor for Lumbar Spinal Stenosis: A Case-Control Study. Clin Med Insights Endocrinol Diabetes. 2016 May 5;9:1-5. doi: 10.4137/CMED.S39035.

34. Anekstein Y, Smorgick Y, Lotan R, Agar G, Shalmon E, Floman Y, Mirovsky Y. Diabetes mellitus as a risk factor for the development of lumbar spinal stenosis. Isr Med Assoc J. 2010 Jan;12(1):16-20. PMID: 20450123.

35. Lotan R, Oron A, Anekstein Y, Shalmon E, Mirovsky Y. Lumbar stenosis and systemic diseases: is there any relevance? J Spinal Disord Tech. 2008 Jun;21(4):247-51. doi: 10.1097/BSD.0b013e31813707af.

36. Abate M, Schiavone C, Pelotti P, Salini V. Limited joint mobility in diabetes and ageing: recent advances in pathogenesis and therapy. Int J Immunopathol Pharmacol. 2010 Oct-Dec;23(4):997-1003. doi: 10.1177/039463201002300404.

37. Maruf MH, Suzuki A, Hayashi K, Habibi H, Salimi H, Terai H, Tamai K, Hoshino M, Toyoda H, Yamada K, Takahashi S, Ohyama S, Hori Y, Nakamura H. Increased advanced glycation end products in hypertrophied ligamentum flavum of diabetes mellitus patients. Spine J. 2019 Oct;19(10):1739-1745. doi: 10.1016/j.spinee.2019.06.001.

38. Kim HJ, Lee KW, Cho HG, Kang KT, Chang BS, Lee CK, Yeom JS. Indirect effects of decompression surgery on glycemic homeostasis in patients with type 2 diabetes mellitus and lumbar spinal stenosis. Spine J. 2015 Jan 1;15(1):25-33. doi: 10.1016/j.spinee.2014.06.016.

39. Kim KT, Cho DC, Sung JK, Kim CH, Kang H, Kim DH. Changes in HbA_{1c} levels and body mass index after successful decompression surgery in patients with type 2 diabetes mellitus and lumbar spinal stenosis: results of a 2-year follow-up study. Spine J. 2017 Feb;17(2):203-210. doi: 10.1016/j.spinee.2016.08.029.

40. Lin YC, Li YJ, Rui YF, Dai GC, Shi L, Xu HL, Ni M, Zhao S, Chen H, Wang C, Li G, Teng GJ. The effects of high glucose on tendon-derived stem cells: implications of the

pathogenesis of diabetic tendon disorders. Oncotarget. 2017 Mar 14;8(11):17518-17528. doi: 10.18632/oncotarget.15418.

41. Wu YF, Wang HK, Chang HW, Sun J, Sun JS, Chao YH. High glucose alters tendon homeostasis through downregulation of the AMPK/Egr1 pathway. Sci Rep. 2017 Mar 7;7:44199. doi: 10.1038/srep44199.

42. Poulsen RC, Knowles HJ, Carr AJ, Hulley PA. Cell differentiation versus cell death: extracellular glucose is a key determinant of cell fate following oxidative stress exposure. Cell Death Dis. 2014 Feb 20;5(2):e1074. doi: 10.1038/cddis.2014.52.

43. Sabatini M, Bardiot A, Lesur C, Moulharat N, Thomas M, Richard I, Fradin A. Effects of agonists of peroxisome proliferator-activated receptor gamma on proteoglycan degradation and matrix metalloproteinase production in rat cartilage in vitro. Osteoarthritis Cartilage. 2002 Sep;10(9):673-9. doi: 10.1053/joca.2002.0827.

44. Damerau A, Gaber T, Ohrndorf S, Hoff P. JAK/STAT Activation: A General Mechanism for Bone Development, Homeostasis, and Regeneration. Int J Mol Sci. 2020 Nov 26;21(23):9004. doi: 10.3390/ijms21239004.

45. Mei YM, Li L, Wang XQ, Zhang M, Zhu LF, Fu YW, Xu Y. AGEs induces apoptosis and autophagy via reactive oxygen species in human periodontal ligament cells. J Cell Biochem. 2020 Aug;121(8-9):3764-3779. doi: 10.1002/jcb.29499.

46. Kjaer M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. Physiol Rev. 2004 Apr;84(2):649-98. doi: 10.1152/physrev.00031.2003.

47. Tan J, Zhou Y, Luo J, Wu X, Liu H, Wang W, Li Z, Zhong M, Wu L, Li X. High glucose inhibits the osteogenic differentiation of periodontal ligament stem cells in periodontitis

by activating endoplasmic reticulum stress. Ann Transl Med. 2022 Feb;10(4):204. doi: 10.21037/atm-22-6.

48. Oakes SA, Papa FR. The role of endoplasmic reticulum stress in human pathology. Annu Rev Pathol. 2015;10:173-94. doi: 10.1146/annurev-pathol-012513-104649.

49. Elvira B, Vandenbempt V, Bauzá-Martinez J, Crutzen R, Negueruela J, Ibrahim H, Winder ML, Brahma MK, Vekeriotaite B, Martens PJ, Singh SP, Rossello F, Lybaert P, Otonkoski T, Gysemans C, Wu W, Gurzov EN. PTPN2 Regulates the Interferon Signaling and Endoplasmic Reticulum Stress Response in Pancreatic β-Cells in Autoimmune Diabetes. Diabetes. 2022 Apr 1;71(4):653-668. doi: 10.2337/db21-0443.

50. Snyder JT, Darko C, Sharma RB, Alonso LC. Endoplasmic Reticulum Stress Induced Proliferation Remains Intact in Aging Mouse β-Cells. Front Endocrinol (Lausanne). 2021 Aug 31;12:734079. doi: 10.3389/fendo.2021.734079.

51. Tan J, Zhou L, Xue P, An Y, Luo L, Zhang R, Wu G, Wang Y, Zhu H, Wang Q. Tumor Necrosis Factor-α Attenuates the Osteogenic Differentiation Capacity of Periodontal Ligament Stem Cells by Activating PERK Signaling. J Periodontol. 2016 Aug;87(8):e159-71. doi: 10.1902/jop.2016.150718.

52. Van Doren SR. Matrix metalloproteinase interactions with collagen and elastin. Matrix Biol. 2015 May-Jul;44-46:224-31. doi: 10.1016/j.matbio.2015.01.005.

53. Xiao D, Yang R, Gong L, Zhang Y, Xie Y, Ni S. Plantamajoside inhibits high glucose-induced oxidative stress, inflammation, and extracellular matrix accumulation in rat glomerular mesangial cells through the inactivation of Akt/NF-κB pathway. J Recept Signal Transduct Res. 2021 Feb;41(1):45-52. doi: 10.1080/10799893.2020.1784939.

54. Wang YF, Zuo ZH, Luo P, Pang FS, Hu JT. The effect of cyclic tensile force on the actin cytoskeleton organization and morphology of human periodontal ligament cells. Biochem Biophys Res Commun. 2018 Dec 2;506(4):950-955. doi: 10.1016/j.bbrc.2018.10.163.

55. Vander Horst MA, Raeman CH, Dalecki D, Hocking DC. Time- and Dose-Dependent Effects of Pulsed Ultrasound on Dermal Repair in Diabetic Mice. Ultrasound Med Biol. 2021 Apr;47(4):1054-1066. doi: 10.1016/j.ultrasmedbio.2020.12.024.

56. Lobmann R, Ambrosch A, Schultz G, Waldmann K, Schiweck S, Lehnert H. Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. Diabetologia. 2002 Jul;45(7):1011-6. doi: 10.1007/s00125-002-0868-8.

57. Li X, Zhang L, Wang N, Feng X, Bi L. Periodontal ligament remodeling and alveolar bone resorption during orthodontic tooth movement in rats with diabetes. Diabetes Technol Ther. 2010 Jan;12(1):65-73. doi: 10.1089/dia.2009.0085.

58. Tang L, Li T, Chang Y, Wang Z, Li Y, Wang F, Sui L. Diabetic oxidative stress-induced telomere damage aggravates periodontal bone loss in periodontitis. Biochem Biophys Res Commun. 2022 Jul 23;614:22-28. doi: 10.1016/j.bbrc.2022.04.039.

59. Li H, Liu D, Zhao CQ, Jiang LS, Dai LY. High glucose promotes collagen synthesis by cultured cells from rat cervical posterior longitudinal ligament via transforming growth factor-beta1. Eur Spine J. 2008 Jun;17(6):873-81. doi: 10.1007/s00586-008-0662-5.

60. Watanabe N, Woo SL, Papageorgiou C, Celechovsky C, Takai S. Fate of donor bone marrow cells in medial collateral ligament after simulated autologous transplantation. Microsc Res Tech. 2002 Jul 1;58(1):39-44. doi: 10.1002/jemt.10115.

61. Sairyo K, Biyani A, Goel VK, Leaman DW, Booth R Jr, Thomas J, Ebraheim NA, Cowgill IA, Mohan SE. Lumbar ligamentum flavum hypertrophy is due to accumulation of

inflammation-related scar tissue. Spine (Phila Pa 1976). 2007 May 15;32(11):E340-7. doi: 10.1097/01.brs.0000263407.25009.6e.

62. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol. 1988 Oct 1;141(7):2407-12. PMID: 3139757.

63. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972 May 25;247(10):3170-5. PMID: 4623845.

64. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem. 1974 Nov 25;249(22):7130-9. PMID: 4436300.

65. Luck, H., 1963. Catalase. In: Bergmeyer, H.U. (Ed.), Methods Of Enzymatic Analysis. Academic Press, New York, pp.885–888.

66. Schosinsky KH, Lehmann HP, Beeler MF. Measurement of ceruloplasmin from its oxidase activity in serum by use of o-dianisidine dihydrochloride. Clin Chem. 1974 Dec;20(12):1556-63. doi: 10.1093/clinchem/20.12.1556.

67. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959 May;82(1):70-7. doi: 10.1016/0003-9861(59)90090-6.

68. Knight JA, Anderson S, Rawle JM. Chemical basis of the sulfo-phospho-vanillin reaction for estimating total serum lipids. Clin Chem. 1972 Mar;18(3):199-202. PMID: 5020813.

69. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem. 2004 Apr;37(4):277-85. doi: 10.1016/j.clinbiochem.2003.11.015.

70. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem. 2005 Dec;38(12):1103-11. doi: 10.1016/j.clinbiochem.2005.08.008.

71. Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol. 1990;186:421-31. doi: 10.1016/0076-6879(90)86135-i.

72. Atroshi F, Sankari S, Osterberg S, Sandholm M. Variation of erythrocyte glutathione peroxidase activity in Finn sheep. Res Vet Sci. 1981 Nov;31(3):267-71. PMID: 7342221.

73. Sharma P, Maffulli N. Biology of tendon injury: healing, modeling and remodeling. J Musculoskelet Neuronal Interact. 2006 Apr-Jun;6(2):181-90. PMID: 16849830.

74. Yamaguchi K, Ditsios K, Middleton WD, Hildebolt CF, Galatz LM, Teefey SA. The demographic and morphological features of rotator cuff disease. A comparison of asymptomatic and symptomatic shoulders. J Bone Joint Surg Am. 2006 Aug;88(8):1699-704. doi: 10.2106/JBJS.E.00835.

75. Longo UG, Ronga M, Maffulli N. Acute ruptures of the achilles tendon. Sports Med Arthrosc Rev. 2009 Jun;17(2):127-38. doi: 10.1097/JSA.0b013e3181a3d767.

76. Nelson F, Billinghurst RC, Pidoux I, Reiner A, Langworthy M, McDermott M, Malogne T, Sitler DF, Kilambi NR, Lenczner E, Poole AR. Early post-traumatic osteoarthritis-like changes in human articular cartilage following rupture of the anterior cruciate ligament. Osteoarthritis Cartilage. 2006 Feb;14(2):114-9. doi: 10.1016/j.joca.2005.08.005.

77. Fleming BC, Hulstyn MJ, Oksendahl HL, Fadale PD. Ligament Injury, Reconstruction and Osteoarthritis. Curr Opin Orthop. 2005 Oct;16(5):354-362. doi: 10.1097/01.bco.0000176423.07865.d2.

78. Fleming BC. Biomechanics of the anterior cruciate ligament. J Orthop Sports Phys Ther. 2003 Aug;33(8):A13-5. PMID: 12968863.

79. Woo SL, Livesay GA, Engle C. Biomechanics of the human anterior cruciate ligament. Muscle stabilization and ACL reconstruction. Orthop Rev. 1992 Aug;21(8):935-41. PMID: 1523008.

80. Hill CL, Seo GS, Gale D, Totterman S, Gale ME, Felson DT. Cruciate ligament integrity in osteoarthritis of the knee. Arthritis Rheum. 2005 Mar;52(3):794-9. doi: 10.1002/art.20943.

81. Mullaji AB, Marawar SV, Simha M, Jindal G. Cruciate ligaments in arthritic knees: a histologic study with radiologic correlation. J Arthroplasty. 2008 Jun;23(4):567-72. doi: 10.1016/j.arth.2007.05.024.

82. Asahara H, Inui M, Lotz MK. Tendons and Ligaments: Connecting Developmental Biology to Musculoskeletal Disease Pathogenesis. J Bone Miner Res. 2017 Sep;32(9):1773-1782. doi: 10.1002/jbmr.3199.

83. Birk DE, Trelstad RL. Extracellular compartments in tendon morphogenesis: collagen fibril, bundle, and macroaggregate formation. J Cell Biol. 1986 Jul;103(1):231-40. doi: 10.1083/jcb.103.1.231.

84. Al-Awar A, Kupai K, Veszelka M, Szűcs G, Attieh Z, Murlasits Z, Török S, Pósa A, Varga C. Experimental Diabetes Mellitus in Different Animal Models. J Diabetes Res. 2016;2016:9051426. doi: 10.1155/2016/9051426. Epub 2016 Aug 9. PMID: 27595114.

85. Connizzo BK, Yannascoli SM, Soslowsky LJ. Structure-function relationships of postnatal tendon development: a parallel to healing. Matrix Biol. 2013 Mar 11;32(2):106-16. doi: 10.1016/j.matbio.2013.01.007.

86. Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, Li L, Leet AI, Seo BM, Zhang L, Shi S, Young MF. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nat Med. 2007 Oct;13(10):1219-27. doi: 10.1038/nm1630.

87. Jepsen KJ, Wu F, Peragallo JH, Paul J, Roberts L, Ezura Y, Oldberg A, Birk DE, Chakravarti S. A syndrome of joint laxity and impaired tendon integrity in lumican- and fibromodulin-deficient mice. J Biol Chem. 2002 Sep 20;277(38):35532-40. doi: 10.1074/jbc.M205398200.

88. Kannus P. Structure of the tendon connective tissue. Scand J Med Sci Sports. 2000 Dec;10(6):312-20. doi: 10.1034/j.1600-0838.2000.010006312.x.

89. Docheva D, Hunziker EB, Fässler R, Brandau O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Mol Cell Biol. 2005 Jan;25(2):699-705. doi: 10.1128/MCB.25.2.699-705.2005.

90. Schweitzer R, Zelzer E, Volk T. Connecting muscles to tendons: tendons and musculoskeletal development in flies and vertebrates. Development. 2010 Sep 1;137(17):2807-17. doi: 10.1242/dev.047498.

91. Steinert AF, Kunz M, Prager P, Barthel T, Jakob F, Nöth U, Murray MM, Evans CH, Porter RM. Mesenchymal stem cell characteristics of human anterior cruciate ligament outgrowth cells. Tissue Eng Part A. 2011 May;17(9-10):1375-88. doi: 10.1089/ten.TEA.2010.0413.

92. Alzoubi F, Joseph B, Andersson L. Healing of soft tissue lacerations in diabetic-induced rats. Dent Traumatol. 2017 Dec;33(6):438-443. doi: 10.1111/edt.12372.

93. Wang Y, Graves DT. Keratinocyte Function in Normal and Diabetic Wounds and Modulation by FOXO1. J Diabetes Res. 2020 Oct 28;2020:3714704. doi: 10.1155/2020/3714704.

94. Siqueira MF, Li J, Chehab L, Desta T, Chino T, Krothpali N, Behl Y, Alikhani M, Yang J, Braasch C, Graves DT. Impaired wound healing in mouse models of diabetes is mediated by TNF-alpha dysregulation and associated with enhanced activation of forkhead box O1 (FOXO1). Diabetologia. 2010 Feb;53(2):378-88. doi: 10.1007/s00125-009-1529-y.

95. Al-Mulla F, Leibovich SJ, Francis IM, Bitar MS. Impaired TGF-β signaling and a defect in resolution of inflammation contribute to delayed wound healing in a female rat model of type 2 diabetes. Mol Biosyst. 2011 Nov;7(11):3006-20. doi: 10.1039/c0mb00317d.

96. Behmoaras J, Gil J. Similarities and interplay between senescent cells and macrophages. J Cell Biol. 2021 Feb 1;220(2):e202010162. doi: 10.1083/jcb.202010162.

97. Hippensteel KJ, Johnson J, McCormick J, Klein S. A Comparison of Wound Complications With Surgical Treatment of Achilles Tendon Conditions Using 2 Surgical Approaches. Foot Ankle Orthop. 2019 Feb 4;4(1):2473011418814004. doi: 10.1177/2473011418814004.

98. Kato S, Saito M, Funasaki H, Marumo K. Distinctive collagen maturation process in fibroblasts derived from rabbit anterior cruciate ligament, medial collateral ligament, and patellar tendon in vitro. Knee Surg Sports Traumatol Arthrosc. 2015 May;23(5):1384-92. doi: 10.1007/s00167-013-2773-8.

99. Loots MA, Lamme EN, Mekkes JR, Bos JD, Middelkoop E. Cultured fibroblasts from chronic diabetic wounds on the lower extremity (non-insulin-dependent diabetes mellitus) show disturbed proliferation. Arch Dermatol Res. 1999 Feb-Mar;291(2-3):93-9. doi: 10.1007/s004030050389.

100. Furnary AP, Zerr KJ, Grunkemeier GL, Starr A. Continuous intravenous insulin infusion reduces the incidence of deep sternal wound infection in diabetic patients after cardiac surgical procedures. Ann Thorac Surg. 1999 Feb;67(2):352-60; discussion 360-2. doi: 10.1016/s0003-4975(99)00014-4.

101. Lenguerrand E, Beswick AD, Whitehouse MR, Wylde V, Blom AW. Outcomes following hip and knee replacement in diabetic versus nondiabetic patients and well versus poorly controlled diabetic patients: a prospective cohort study. Acta Orthop. 2018 Aug;89(4):399-405. doi: 10.1080/17453674.2018.1473327.

102. Black E, Vibe-Petersen J, Jorgensen LN, Madsen SM, Agren MS, Holstein PE, Perrild H, Gottrup F. Decrease of collagen deposition in wound repair in type 1 diabetes independent of glycemic control. Arch Surg. 2003 Jan;138(1):34-40. doi: 10.1001/archsurg.138.1.34.

103. Lerman OZ, Galiano RD, Armour M, Levine JP, Gurtner GC. Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia. Am J Pathol. 2003 Jan;162(1):303-12. doi: 10.1016/S0002-9440(10)63821-7.

104. K-C L, Zernicke RF, Barnard RJ, A F-Y L. Response of immature diabetic rat bone-ligament junctions to insulin and exercise. Clin Biomech (Bristol, Avon). 1995 Sep;10(6):331-336. doi: 10.1016/0268-0033(95)00034-i.

105. Vicente A, Bravo-González LA, Navarro JA, Buendía AJ, Camacho-Alonso F. Effects of diabetes on oxidative stress, periodontal ligament fiber orientation, and matrix metalloproteinase 8 and 9 expressions during orthodontic tooth movement. Clin Oral Investig. 2021 Mar;25(3):1383-1394. doi: 10.1007/s00784-020-03446-7.

106. Ko KI, Sculean A, Graves DT. Diabetic wound healing in soft and hard oral tissues. Transl Res. 2021 Oct;236:72-86. doi: 10.1016/j.trsl.2021.05.001.

107. Fahey E, Doyle SL. IL-1 Family Cytokine Regulation of Vascular Permeability and Angiogenesis. Front Immunol. 2019 Jun 25;10:1426. doi: 10.3389/fimmu.2019.01426.

108. Sainson RC, Johnston DA, Chu HC, Holderfield MT, Nakatsu MN, Crampton SP, Davis J, Conn E, Hughes CC. TNF primes endothelial cells for angiogenic sprouting by inducing a tip cell phenotype. Blood. 2008 May 15;111(10):4997-5007. doi: 10.1182/blood-2007-08-108597.

109. Elshabrawy HA, Chen Z, Volin MV, Ravella S, Virupannavar S, Shahrara S. The pathogenic role of angiogenesis in rheumatoid arthritis. Angiogenesis. 2015 Oct;18(4):433-48. doi: 10.1007/s10456-015-9477-2.

110. Jeon HH, Yu Q, Witek L, Lu Y, Zhang T, Stepanchenko O, Son VJ, Spencer E, Oshilaja T, Shin MK, Alawi F, Coelho PG, Graves DT. Clinical application of a FOXO1 inhibitor improves connective tissue healing in a diabetic minipig model. Am J Transl Res. 2021 Feb 15;13(2):781-791. PMID: 33594326.

111. Thulasingam S, Krishnasamy S, Raj C D, Lasch M, Vedantham S, Deindl E. Insulin Treatment Forces Arteriogenesis in Diabetes Mellitus by Upregulation of the Early Growth Response-1 (Egr-1) Pathway in Mice. Int J Mol Sci. 2019 Jul 5;20(13):3320. doi: 10.3390/ijms20133320.

112. Scholz D, Ito W, Fleming I, Deindl E, Sauer A, Wiesnet M, Busse R, Schaper J, Schaper W. Ultrastructure and molecular histology of rabbit hind-limb collateral artery growth (arteriogenesis). Virchows Arch. 2000 Mar;436(3):257-70. doi: 10.1007/s004280050039.

113. Asadian L, Haddadi K, Aarabi M, Zare A. Diabetes Mellitus, a New Risk Factor for Lumbar Spinal Stenosis: A Case-Control Study. Clin Med Insights Endocrinol Diabetes. 2016 May 5;9:1-5. doi: 10.4137/CMED.S39035.

114. Anekstein Y, Smorgick Y, Lotan R, Agar G, Shalmon E, Floman Y, Mirovsky Y. Diabetes mellitus as a risk factor for the development of lumbar spinal stenosis. Isr Med Assoc J. 2010 Jan;12(1):16-20. PMID: 20450123.

115. Lotan R, Oron A, Anekstein Y, Shalmon E, Mirovsky Y. Lumbar stenosis and systemic diseases: is there any relevance? J Spinal Disord Tech. 2008 Jun;21(4):247-51. doi: 10.1097/BSD.0b013e31813707af.

116. Abate M, Schiavone C, Pelotti P, Salini V. Limited joint mobility in diabetes and ageing: recent advances in pathogenesis and therapy. Int J Immunopathol Pharmacol. 2010 Oct-Dec;23(4):997-1003. doi: 10.1177/039463201002300404.

117. Maruf MH, Suzuki A, Hayashi K, Habibi H, Salimi H, Terai H, Tamai K, Hoshino M, Toyoda H, Yamada K, Takahashi S, Ohyama S, Hori Y, Nakamura H. Increased advanced glycation end products in hypertrophied ligamentum flavum of diabetes mellitus patients. Spine J. 2019 Oct;19(10):1739-1745. doi: 10.1016/j.spinee.2019.06.001.

118. Shemesh S, Sidon E, Kaisler E, Sheinis D, Velkes S, Ohana N, Benayahu D. Diabetes mellitus is associated with increased elastin fiber loss in ligamentum flavum of patients with lumbar spinal canal stenosis: results of a pilot histological study. Eur Spine J. 2018 Jul;27(7):1614-1622. doi: 10.1007/s00586-017-5315-0.

119. Lin YC, Li YJ, Rui YF, Dai GC, Shi L, Xu HL, Ni M, Zhao S, Chen H, Wang C, Li G, Teng GJ. The effects of high glucose on tendon-derived stem cells: implications of the pathogenesis of diabetic tendon disorders. Oncotarget. 2017 Mar 14;8(11):17518-17528. doi: 10.18632/oncotarget.15418.

120. Wu YF, Wang HK, Chang HW, Sun J, Sun JS, Chao YH. High glucose alters tendon homeostasis through downregulation of the AMPK/Egr1 pathway. Sci Rep. 2017 Mar 7;7:44199. doi: 10.1038/srep44199.

121. Damerau A, Gaber T, Ohrndorf S, Hoff P. JAK/STAT Activation: A General Mechanism for Bone Development, Homeostasis, and Regeneration. Int J Mol Sci. 2020 Nov 26;21(23):9004. doi: 10.3390/ijms21239004.

122. Oakes SA, Papa FR. The role of endoplasmic reticulum stress in human pathology. Annu Rev Pathol. 2015;10:173-94. doi: 10.1146/annurev-pathol-012513-104649.

123. Cao ZH, Wu Z, Hu C, Zhang M, Wang WZ, Hu XB. Endoplasmic reticulum stress and destruction of pancreatic β cells in type 1 diabetes. Chin Med J (Engl). 2020 Jan 5;133(1):68-73. doi: 10.1097/CM9.0000000000000583.

124. Song X, Li J, Jiao M, Chen Y, Pan K. Effect of endoplasmic reticulum stress-induced apoptosis in the role of periodontitis on vascular calcification in a rat model. J Mol Histol. 2021 Oct;52(5):1097-1104. doi: 10.1007/s10735-021-10015-z.

125. Tan J, Zhou L, Xue P, An Y, Luo L, Zhang R, Wu G, Wang Y, Zhu H, Wang Q. Tumor Necrosis Factor-α Attenuates the Osteogenic Differentiation Capacity of Periodontal Ligament Stem Cells by Activating PERK Signaling. J Periodontol. 2016 Aug;87(8):e159-71. doi: 10.1902/jop.2016.150718.

126. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2009 Jan;32 Suppl 1(Suppl 1):S62-7. doi: 10.2337/dc09-S062.

127. Mergenthaler P, Lindauer U, Dienel GA, Meisel A. Sugar for the brain: the role of glucose in physiological and pathological brain function. Trends Neurosci. 2013 Oct;36(10):587-97. doi: 10.1016/j.tins.2013.07.001.

128. Ekmektzoglou KA, Zografos GC. A concomitant review of the effects of diabetes mellitus and hypothyroidism in wound healing. World J Gastroenterol. 2006 May 7;12(17):2721-9. doi: 10.3748/wjg.v12.i17.2721.

129. Sapra A, Bhandari P. Diabetes Mellitus. [Updated 2020 Jun 7]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK551501/

130. Shi L, Rui YF, Li G, Wang C. Alterations of tendons in diabetes mellitus: what are the current findings? Int Orthop. 2015 Aug;39(8):1465-73. doi: 10.1007/s00264-015-2775-x.

131. Leong NL, Kator JL, Clemens TL, James A, Enamoto-Iwamoto M, Jiang J. Tendon and Ligament Healing and Current Approaches to Tendon and Ligament Regeneration. J Orthop Res. 2020 Jan;38(1):7-12. doi: 10.1002/jor.24475.

132. World Health Organisation WHO. About diabetes. 2014 [cited 2023 May 8]; Available from: https://www.who.int/diabetes/action_online/basics/en/index3.html.

133. Gustafson B, Hedjazifar S, Gogg S, Hammarstedt A, Smith U. Insulin resistance and impaired adipogenesis. Trends Endocrinol Metab. 2015 Apr;26(4):193-200. doi: 10.1016/j.tem.2015.01.006.

134. World Health Organisation WHO. Global report on diabetes. 2016 [cited 2023 May 8]; Available from: https://www.who.int/publications/i/item/9789241565257.

135. Cui A, Li H, Wang D, Zhong J, Chen Y, Lu H. Global, regional prevalence, incidence and risk factors of knee osteoarthritis in population-based studies. EClinicalMedicine. 2020 Nov 26;29-30:100587. doi: 10.1016/j.eclinm.2020.100587.

136. Liao KP, Gunnarsson M, Källberg H, Ding B, Plenge RM, Padyukov L, Karlson EW, Klareskog L, Askling J, Alfredsson L. Specific association of type 1 diabetes mellitus with

anti-cyclic citrullinated peptide-positive rheumatoid arthritis. Arthritis Rheum. 2009 Mar;60(3):653-60. doi: 10.1002/art.24362.

137. Armitage LH, Wallet MA, Mathews CE. Influence of PTPN22 Allotypes on Innate and Adaptive Immune Function in Health and Disease. Front Immunol. 2021 Feb 25;12:636618. doi: 10.3389/fimmu.2021.636618.

138. Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. Circulation. 2006 Aug 8;114(6):597-605. doi: 10.1161/CIRCULATIONAHA.106.621854.

139. Dilworth L, Facey A, Omoruyi F. Diabetes Mellitus and Its Metabolic Complications: The Role of Adipose Tissues. Int J Mol Sci. 2021 Jul 16;22(14):7644. doi: 10.3390/ijms22147644.

140. Cheng L, Wang J, Dai H, Duan Y, An Y, Shi L, Lv Y, Li H, Wang C, Ma Q, Li Y, Li P, Du H, Zhao B. Brown and beige adipose tissue: a novel therapeutic strategy for obesity and type 2 diabetes mellitus. Adipocyte. 2021 Dec;10(1):48-65. doi: 10.1080/21623945.2020.1870060.

141. Daryabor G, Atashzar MR, Kabelitz D, Meri S, Kalantar K. The Effects of Type 2 Diabetes Mellitus on Organ Metabolism and the Immune System. Front Immunol. 2020 Jul 22;11:1582. doi: 10.3389/fimmu.2020.01582.

142. Nahdi AMTA, John A, Raza H. Elucidation of Molecular Mechanisms of Streptozotocin-Induced Oxidative Stress, Apoptosis, and Mitochondrial Dysfunction in Rin-5F Pancreatic β-Cells. Oxid Med Cell Longev. 2017;2017:7054272. doi: 10.1155/2017/7054272.

143. Suman RK, Ray Mohanty I, Borde MK, Maheshwari U, Deshmukh YA. Development of an Experimental Model of Diabetes Co-Existing with Metabolic Syndrome in Rats. Adv Pharmacol Sci. 2016;2016:9463476. doi: 10.1155/2016/9463476.

144. Farid A, Moussa P, Youssef M, Haytham M, Shamy A, Safwat G. Melatonin relieves diabetic complications and regenerates pancreatic beta cells by the reduction in NF-kB expression in streptozotocin induced diabetic rats. Saudi J Biol Sci. 2022 Jul;29(7):103313. doi: 10.1016/j.sjbs.2022.103313.

145. Ahmad Hajam Y, Rai S, Basheer M, Ghosh H, Singh S. Protective Role of Melatonin in Streptozotocin Induced Pancreatic Damages in Diabetic Wistar Rat. Pak J Biol Sci. 2018 Jan;21(9):423-431. doi: 10.3923/pjbs.2018.423.431.

146. Costes S, Boss M, Thomas AP, Matveyenko AV. Activation of Melatonin Signaling Promotes β-Cell Survival and Function. Mol Endocrinol. 2015 May;29(5):682-92. doi: 10.1210/me.2014-1293.

147. Ramracheya RD, Muller DS, Squires PE, Brereton H, Sugden D, Huang GC, Amiel SA, Jones PM, Persaud SJ. Function and expression of melatonin receptors on human pancreatic islets. J Pineal Res. 2008 Apr;44(3):273-9. doi: 10.1111/j.1600-079X.2007.00523.x.

148. McMullan CJ, Schernhammer ES, Rimm EB, Hu FB, Forman JP. Melatonin secretion and the incidence of type 2 diabetes. JAMA. 2013 Apr 3;309(13):1388-96. doi: 10.1001/jama.2013.2710.

149. McMullan CJ, Curhan GC, Schernhammer ES, Forman JP. Association of nocturnal melatonin secretion with insulin resistance in nondiabetic young women. Am J Epidemiol. 2013 Jul 15;178(2):231-8. doi: 10.1093/aje/kws470.

150. Garfinkel D, Zorin M, Wainstein J, Matas Z, Laudon M, Zisapel N. Efficacy and safety of prolonged-release melatonin in insomnia patients with diabetes: a randomized, double-blind, crossover study. Diabetes Metab Syndr Obes. 2011;4:307-13. doi: 10.2147/DMSO.S23904.

151. Bonnefond A, Clément N, Fawcett K, Yengo L, Vaillant E, Guillaume JL, Dechaume A, Payne F, Roussel R, Czernichow S, Hercberg S, Hadjadj S, Balkau B, Marre M, Lantieri O, Langenberg C, Bouatia-Naji N; Meta-Analysis of Glucose and Insulin-Related Traits Consortium (MAGIC); Charpentier G, Vaxillaire M, Rocheleau G, Wareham NJ, Sladek R, McCarthy MI, Dina C, Barroso I, Jockers R, Froguel P. Rare MTNR1B variants impairing melatonin receptor 1B function contribute to type 2 diabetes. Nat Genet. 2012 Jan 29;44(3):297-301. doi: 10.1038/ng.1053.

152. Karamitri A, Plouffe B, Bonnefond A, Chen M, Gallion J, Guillaume JL, Hegron A, Boissel M, Canouil M, Langenberg C, Wareham NJ, Le Gouill C, Lukasheva V, Lichtarge O, Froguel P, Bouvier M, Jockers R. Type 2 diabetes-associated variants of the MT_2 melatonin receptor affect distinct modes of signaling. Sci Signal. 2018 Aug 28;11(545):eaan6622. doi: 10.1126/scisignal.aan6622.

153. Flannick J, Mercader JM, Fuchsberger C, Udler MS, Mahajan A, Wessel J, Teslovich TM, Caulkins L, Koesterer R, Barajas-Olmos F, Blackwell TW, et al. Exome sequencing of 20,791 cases of type 2 diabetes and 24,440 controls. Nature. 2019 Jun;570(7759):71-76. doi: 10.1038/s41586-019-1231-2.

154. Garaulet M, Qian J, Florez JC, Arendt J, Saxena R, Scheer FAJL. Melatonin Effects on Glucose Metabolism: Time To Unlock the Controversy. Trends Endocrinol Metab. 2020 Mar;31(3):192-204. doi: 10.1016/j.tem.2019.11.011.

155. Damasceno DC, Netto AO, Iessi IL, Gallego FQ, Corvino SB, Dallaqua B, Sinzato YK, Bueno A, Calderon IM, Rudge MV. Streptozotocin-induced diabetes models: pathophysiological mechanisms and fetal outcomes. Biomed Res Int. 2014;2014:819065. doi: 10.1155/2014/819065.

156. Johansen JS, Harris AK, Rychly DJ, Ergul A. Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. Cardiovasc Diabetol. 2005 Apr 29;4:5. doi: 10.1186/1475-2840-4-5.

157. Ito F, Sono Y, Ito T. Measurement and Clinical Significance of Lipid Peroxidation as a Biomarker of Oxidative Stress: Oxidative Stress in Diabetes, Atherosclerosis, and Chronic Inflammation. Antioxidants. 2019; 8(3):72. doi: 10.3390/antiox8030072.

158. Volpe CMO, Villar-Delfino PH, Dos Anjos PMF, Nogueira-Machado JA. Cellular death, reactive oxygen species (ROS) and diabetic complications. Cell Death Dis. 2018 Jan 25;9(2):119. doi: 10.1038/s41419-017-0135-z.

159. Gheibi S, Ghasemi A. Insulin secretion: The nitric oxide controversy. EXCLI J. 2020 Sep 8;19:1227-1245. doi: 10.17179/excli2020-2711. PMID: 33088259; PMCID: PMC7573190.

160. Nagareddy PR, Xia Z, MacLeod KM, McNeill JH. N-acetylcysteine prevents nitrosative stress-associated depression of blood pressure and heart rate in streptozotocin diabetic rats. J Cardiovasc Pharmacol. 2006 Apr;47(4):513-20. doi: 10.1097/01.fjc.0000211744.93701.25.

161. Welsh N, Eizirik DL, Sandler S. Nitric oxide and pancreatic beta-cell destruction in insulin dependent diabetes mellitus: don't take NO for an answer. Autoimmunity. 1994;18(4):285-90. doi: 10.3109/08916939409009530.

162. Anggård E. Nitric oxide: mediator, murderer, and medicine. Lancet. 1994 May 14;343(8907):1199-206. doi: 10.1016/s0140-6736(94)92405-8.

163. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007 Jan;87(1):315-424. doi: 10.1152/physrev.00029.2006.

164. Sekizuka H. Uric acid, xanthine oxidase, and vascular damage: potential of xanthine oxidoreductase inhibitors to prevent cardiovascular diseases. Hypertens Res. 2022 May;45(5):772-774. doi: 10.1038/s41440-022-00891-7.

165. Najafi A, Pourfarzam M, Zadhoush F. Oxidant/antioxidant status in Type-2 diabetes mellitus patients with metabolic syndrome. J Res Med Sci. 2021 Jan 28;26:6. doi: 10.4103/jrms.JRMS_249_20.

166. Gandy SE, Buse MG, Crouch RK. Protective role of superoxide dismutase against diabetogenic drugs. J Clin Invest. 1982 Sep;70(3):650-8. doi: 10.1172/jci110659.

167. Attachaipanich T, Chattipakorn SC, Chattipakorn N. Potential Roles of Melatonin in Doxorubicin-Induced Cardiotoxicity: From Cellular Mechanisms to Clinical Application. Pharmaceutics. 2023; 15(3):785. https://doi.org/10.3390/pharmaceutics15030785.

168. Bicer M, Baltaci SB, Patlar S, Mogulkoc R, Baltaci AK. Melatonin has a protective effect against lipid peroxidation in the bone tissue of diabetic rats subjected to acute swimming exercise. Horm Mol Biol Clin Investig. 2018 Mar 16;34(2):/j/hmbci.2018.34.issue-2/hmbci-2017-0079/hmbci-2017-0079.xml. doi: 10.1515/hmbci-2017-0079.

169. Huang CC, Lai CJ, Tsai MH, Wu YC, Chen KT, Jou MJ, Fu PI, Wu CH, Wei IH. Effects of melatonin on the nitric oxide system and protein nitration in the hypobaric hypoxic rat hippocampus. BMC Neurosci. 2015 Oct 6;16:61. doi: 10.1186/s12868-015-0199-6.

170. Sáenz DA, Turjanski AG, Sacca GB, Marti M, Doctorovich F, Sarmiento MI, Estrin DA, Rosenstein RE. Physiological concentrations of melatonin inhibit the nitridergic pathway in the Syrian hamster retina. J Pineal Res. 2002 Aug;33(1):31-6. doi: 10.1034/j.1600-079x.2002.01880.x.

171. Hosseini A, Samadi M, Baeeri M, Rahimifard M, Haghi-Aminjan H. The neuroprotective effects of melatonin against diabetic neuropathy: A systematic review of non-clinical studies. Front Pharmacol. 2022 Aug 31;13:984499. doi: 10.3389/fphar.2022.984499.

172. Storr M, Koppitz P, Sibaev A, Saur D, Kurjak M, Franck H, Schusdziarra V, Allescher HD. Melatonin reduces non-adrenergic, non-cholinergic relaxant neurotransmission by inhibition of nitric oxide synthase activity in the gastrointestinal tract of rodents in vitro. J Pineal Res. 2002 Sep;33(2):101-8. doi: 10.1034/j.1600-079x.2002.02909.x.

173. Ferlazzo N, Andolina G, Cannata A, Costanzo MG, Rizzo V, Currò M, Ientile R, Caccamo D. Is Melatonin the Cornucopia of the 21st Century? Antioxidants. 2020; 9(11):1088. https://doi.org/10.3390/antiox9111088.

174. Lee J, Yoo YM, Lee YH. et al. Melatonin Induces Apoptotic Cell Death in 3T3-L1 Preadipocytes. Mol Biol 54, 204–212 (2020). https://doi.org/10.1134/S0026893320020120.

175. Marcucci G, Domazetovic V, Nediani C, Ruzzolini J, Favre C, Brandi ML. Oxidative Stress and Natural Antioxidants in Osteoporosis: Novel Preventive and Therapeutic Approaches. Antioxidants (Basel). 2023 Feb 3;12(2):373. doi: 10.3390/antiox12020373.

176. Al-Hariri M. Sweet Bones: The Pathogenesis of Bone Alteration in Diabetes. J Diabetes Res. 2016;2016:6969040. doi: 10.1155/2016/6969040.

177. Bicer M, Baltaci SB, Patlar S, Mogulkoc R, Baltaci AK. Melatonin has a protective effect against lipid peroxidation in the bone tissue of diabetic rats subjected to acute swimming exercise. Horm Mol Biol Clin Investig. 2018;34(2):/j/hmbci.2018.34.issue-2/hmbci-2017-0079/hmbci-2017-0079.xml. doi: 10.1515/hmbci-2017-0079.

178. Atalay M, Laaksonen DE, Niskanen L, Uusitupa M, Hänninen O, Sen CK. Altered antioxidant enzyme defences in insulin-dependent diabetic men with increased resting and exercise-induced oxidative stress. Acta Physiol Scand. 1997;161(2):195-201. doi: 10.1046/j.1365-201X.1997.00200.x.

179. Atalay M, Laaksonen DE. Diabetes, oxidative stress and physical exercise. J Sports Sci Med. 2002;1(1):1-14. PMID: 24672266.

180. Turgut M, Uslu S, Uysal A, Yurtseven ME, Ustün H. Changes in vascularity of cartilage endplate of degenerated intervertebral discs in response to melatonin administration in rats. Neurosurg Rev. 2003 May;26(2):133-8. doi: 10.1007/s10143-003-0259-8.

181. Bae WJ, Park JS, Kang SK, Kwon IK, Kim EC. Effects of Melatonin and Its Underlying Mechanism on Ethanol-Stimulated Senescence and Osteoclastic Differentiation in Human Periodontal Ligament Cells and Cementoblasts. Int J Mol Sci. 2018 Jun 12;19(6):1742. doi: 10.3390/ijms19061742.

182. Zhao Y, Shao G, Liu X, Li Z. Assessment of the Therapeutic Potential of Melatonin for the Treatment of Osteoporosis Through a Narrative Review of Its Signaling and Preclinical and Clinical Studies. Front Pharmacol. 2022 May 11;13:866625. doi: 10.3389/fphar.2022.866625.

183. Hara M, Iigo M, Ohtani-Kaneko R, Nakamura N, Suzuki T, Reiter RJ, Hirata K. Administration of melatonin and related indoles prevents exercise-induced cellular

oxidative changes in rats. Biol Signals. 1997 Mar-Apr;6(2):90-100. doi: 10.1159/000109113.

184. França EL, Feliciano ND, Silva KA, Ferrari CK, Honorio-França AC. Modulatory role of melatonin on superoxide release by spleen macrophages isolated from alloxan-induced diabetic rats. Bratisl Lek Listy. 2009;110(9):517-22. PMID: 19827332.

185. Bazyar H, Zare Javid A, Bavi Behbahani H, Moradi F, Moradi Poode B, Amiri P. Consumption of melatonin supplement improves cardiovascular disease risk factors and anthropometric indices in type 2 diabetes mellitus patients: a double-blind, randomized, placebo-controlled trial. Trials. 2021 Mar 25;22(1):231. doi: 10.1186/s13063-021-05174-z.

186. Amiri P, Javid AZ, Moradi L, Haghighat N, Moradi R, Behbahani HB, Zarrin M, Bazyar H. Associations between new and old anthropometric indices with type 2 diabetes mellitus and risk of metabolic complications: a cross-sectional analytical study. J Vasc Bras. 2021 Sep 20;20:e20200236. doi: 10.1590/1677-5449.200236.

187. Bazyar H, Zare Javid A, Zakerkish M, Yousefimanesh HA, Haghighi-Zadeh MH. Effects of melatonin supplementation in patients with type 2 diabetes mellitus and chronic periodontitis under nonsurgical periodontal therapy: A double-blind randomized controlled trial. J Res Med Sci. 2022 Jul 29;27:52. doi: 10.4103/jrms.JRMS_927_19.

188. Dludla PV, Ziqubu K, Mabhida SE, Mazibuko-Mbeje SE, Hanser S, Nkambule BB, Basson AK, Pheiffer C, Tiano L, Kengne AP. Dietary Supplements Potentially Target Plasma Glutathione Levels to Improve Cardiometabolic Health in Patients with Diabetes Mellitus: A Systematic Review of Randomized Clinical Trials. Nutrients. 2023 Feb 14;15(4):944. doi: 10.3390/nu15040944.

189. El-Sayed NS, Elatrebi S, Said R, Ibrahim HF, Omar EM. Potential mechanisms underlying the association between type II diabetes mellitus and cognitive dysfunction in

rats: a link between miRNA-21 and Resveratrol's neuroprotective action. Metab Brain Dis. 2022 Oct;37(7):2375-2388. doi: 10.1007/s11011-022-01035-z.

190. Hu A, Zou H, Chen B, Zhong J. Posttranslational modifications in diabetes: Mechanisms and functions. Rev Endocr Metab Disord. 2022 Oct;23(5):1011-1033. doi: 10.1007/s11154-022-09740-x. Epub 2022 Jun 13.

191. Rehman K, Akash MSH. Mechanism of Generation of Oxidative Stress and Pathophysiology of Type 2 Diabetes Mellitus: How Are They Interlinked? J Cell Biochem. 2017 Nov;118(11):3577-3585. doi: 10.1002/jcb.26097. Epub 2017 May 31.

192. Raygan F, Ostadmohammadi V, Bahmani F, Reiter RJ, Asemi Z. Melatonin administration lowers biomarkers of oxidative stress and cardio-metabolic risk in type 2 diabetic patients with coronary heart disease: A randomized, double-blind, placebo-controlled trial. Clin Nutr. 2019 Feb;38(1):191-196. doi: 10.1016/j.clnu.2017.12.004.

193. Armagan A, Uz E, Yilmaz HR, Soyupek S, Oksay T, Ozcelik N. Effects of melatonin on lipid peroxidation and antioxidant enzymes in streptozotocin-induced diabetic rat testis. Asian J Androl. 2006 Sep;8(5):595-600. doi: 10.1111/j.1745-7262.2006.00177.x.

194. Ghanbari E, Nejati V, Khazaei M. Improvement in Serum Biochemical Alterations and Oxidative Stress of Liver and Pancreas following Use of Royal Jelly in Streptozotocin-Induced Diabetic Rats. Cell J. 2016 Fall;18(3):362-70. doi: 10.22074/cellj.2016.4564.

195. Khorsand M, Akmali M, Akhzari M. Efficacy of melatonin in restoring the antioxidant status in the lens of diabetic rats induced by streptozotocin. J Diabetes Metab Disord. 2019 Dec 2;18(2):543-549. doi: 10.1007/s40200-019-00445-8.

196. Görgün FM, Oztürk Z, Gümüştaş MK, Kökogu E. Melatonin administration affects plasma total sialic acid and lipid peroxidation levels in streptozotocin induced diabetic

rats. J Toxicol Environ Health A. 2002 May 24;65(10):695-700. doi: 10.1080/00984100290071045.

197. Ertik O, Sener G, Yanardag R. The effect of melatonin on glycoprotein levels and oxidative liver injury in experimental diabetes. J Biochem Mol Toxicol. 2023 Mar;37(3):e23268. doi: 10.1002/jbt.23268.

198. Rahbarghazi A, Alamdari KA, Rahbarghazi R, Salehi-Pourmehr H. Co-administration of exercise training and melatonin on the function of diabetic heart tissue: a systematic review and meta-analysis of rodent models. Diabetol Metab Syndr. 2023 Apr 1;15(1):67. doi: 10.1186/s13098-023-01045-6.

199. Andersson AK, Sandler S. Melatonin protects against streptozotocin, but not interleukin-1beta-induced damage of rodent pancreatic beta-cells. J Pineal Res. 2001 Apr;30(3):157-65. doi: 10.1034/j.1600-079x.2001.300304.x.

200. Abdulwahab DA, El-Missiry MA, Shabana S, Othman AI, Amer ME. Melatonin protects the heart and pancreas by improving glucose homeostasis, oxidative stress, inflammation and apoptosis in T2DM-induced rats. Heliyon. 2021;7(3):e06474. doi: 10.1016/j.heliyon.2021.e06474.

201. Mirapoğlu S. L. , Kaymakcı A. , Akın S. , Gültekin F. , Güler E. M. Oxidative Stress and Inflammation Markers in Undescended Testes Patients. Med Records. 2023; 5(1): 29-32. doi: 10.37990/medr.1140268.

202. Grabia M, Socha K, Soroczyńska J, Bossowski A, Markiewicz-Żukowska R. Determinants Related to Oxidative Stress Parameters in Pediatric Patients with Type 1 Diabetes Mellitus. Nutrients. 2023; 15(9):2084. https://doi.org/10.3390/nu15092084.

203. Albazal A, Delshad AA, Roghani M. Melatonin reverses cognitive deficits in streptozotocin-induced type 1 diabetes in the rat through attenuation of oxidative stress

and inflammation. J Chem Neuroanat. 2021 Mar;112:101902. doi: 10.1016/j.jchemneu.2020.101902.

204. Kahya, M. C. & Nazıroğlu, M. Melatonin reduces lens oxidative stress level in STZ-induced diabetic rats through supporting glutathione peroxidase and reduced glutathione values . Journal of Cellular Neuroscience and Oxidative Stress , 2016;8 (2), 588-594 . doi: 10.37212/jcnos.334113.

13. List of tables

14. List of figures

15. Appendixes

Consent issued by Institutional Ethics Committee of Warsaw School of Applied Sciences.

UCHWAŁA NR WAW2/027/2019

z dnia 25 lutego 2019 r.

II Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Warszawie

§ 1

Na podstawie art. 48 ust. 1 pkt. 1[1] ustawy z dnia 15 stycznia 2015r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266), zwanej dalej „ustawą" po rozpatrzeniu wniosku pt.: „Wpływ cukrzycy na regenerację więzadła pobocznego bocznego stawu kolanowego" z dnia 09.01.2019, złożonego przez I Wydział Lekarski, Warszawski Uniwersytet Medyczny, adres: ul. Żwirki i Wigury 61, 02-091, Warszawa, zaplanowanego przez Agnieszkę Cudnoch-Jędrzejewską[2]

przy udziale[3] -

Lokalna Komisja Etyczna:

WYRAŻA ZGODĘ

Na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku.

§ 2

W wyniku rozpatrzenia wniosku o którym mowa w § , Lokalna Komisja Etyczna ustaliła, że:

1. Wniosek należy przypisać do kategorii: [PB6] badania podstawowe: układ mięśniowo-szkieletowy.
2. Najwyższy stopień dotkliwości proponowanych procedur to: dotkliwy.
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków[4]:

Gatunek	Wiek/stadium rozwoju	Liczba
Szczur wędrowny, szczep Sprague Dawley	12-tygodniowe	88

4. Doświadczenia będą przeprowadzane przez: Agnieszka Cudnoch-Jędrzejewska, Robert Wrzesień, Agata Gondek.
5. Doświadczenie będzie przeprowadzane w terminie[5] od 01.03.2019 do 01.03.2022 r.
6. Doświadczenie będzie przeprowadzone w ośrodku[6]: nie dotyczy.
7. Doświadczenie będzie przeprowadzone poza ośrodkiem, w: nie dotyczy.
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez: nie dotyczy.
9. Doświadczenie zostanie/~~nie zostanie~~ poddane ocenie retrospektywnej w terminie do 6 miesięcy od dnia przekazania przez użytkownika dokumentacji, mającej stanowić podstawę dokonania oceny retrospektywnej. Użytkownik jest zobowiązany do przekazania ww. dokumentacji niezwłocznie, tj. w terminie, o którym mowa w art. 52 ust. 2 ustawy.

[1] Niewłaściwy zapis usunąć
[2] imię i nazwisko osoby, która zaplanowała i jest odpowiedzialna za przeprowadzenie doświadczenia
[3] Wypełnić w przypadku dopuszczenia do postępowania organizacji społecznej.
[4] Podać liczbę, szczep/stado, wiek/stadium rozwoju
[5] Nie dłużej niż 5 lat
[6] Podać jeśli jest to inny ośrodek niż użytkownik

§ 3

Uzasadnienie:

Komisja oceniła wniosek zgodnie z kryteriami zawartymi w art. 47.1. ustawy z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266). Po zapoznaniu się z problematyką badawczą przedstawioną we wniosku komisja stwierdza, że przedstawiony projekt spełnia zasady dopuszczenia doświadczeń na zwierzętach.

Na podstawie art. 107 § 4 ustawy z dnia 14 czerwca 1960 r. – Kodeks postępowania administracyjnego z późniejszymi zmianami (Dz. U. z 2017 poz. 1257) odstąpiono od sporządzania uzasadnienia decyzji, gdyż uwzględnia ona w całości żądanie strony.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1.

(Pieczęć lokalnej komisji etycznej)

PRZEWODNICZĄCA

(Podpis Przewodniczącej komisji)

Pouczenie:

Zgodnie z art. 33 ust. 3 i art. 40 ustawy w zw. z art. 127 § 1 i 2 oraz 129 § 2 ustawy z dnia z dnia 14 czerwca 1960 r. Kodeks postępowania administracyjnego (Dz. U. 2017, poz. 1257 – t.j.; dalej KPA) od uchwały Lokalnej Komisji Etycznej strona może wnieść, za jej pośrednictwem, odwołanie do Krajowej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w terminie 14 od dnia doręczenia uchwały.

Na podstawie art. 127a KPA w trakcie biegu terminu do wniesienia odwołania strona może zrzec się prawa do jego wniesienia, co należy uczynić wobec Lokalnej Komisji Etycznej, która wydała uchwałę. Z dniem doręczenia Lokalnej Komisji Etycznej oświadczenia o zrzeczeniu się prawa do wniesienia odwołania przez ostatnią ze stron postępowania, decyzja staje się ostateczna i prawomocna.

Otrzymuje:
1) Użytkownik,
2) Organizacja społeczna dopuszczona do udziału w postępowaniu (jeśli dotyczy)
3) a/a

Użytkownik kopie przekazuje:
- Osoba planująca doświadczenie
- Zespół ds. dobrostanu