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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

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:

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.

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.