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MELATONIN-INDUCED GLYCOSAMINOGLYCANS AUGMENTATION IN MYOCARDIUM REMOTE TO INFARCTION

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Elevated levels of collagen as well as transient increases of glycosaminoglycans (GAG) have been shown in the myocardium remote to the infarction. The aim of the study is to observe the effect of melatonin on the accumulation of collagen and GAG in the left ventricle wall, remote to the infarction. A second aim is to determine whether the effect of the pineal indole is mediated by the membrane melatonin receptors of heart fibroblasts. Rats with myocardial infarction induced by ligation of the left coronary artery were treated with melatonin at a dose of 60 µg/100 g b.w. or vehicle (2% ethanol in 0.9%NaCl). The results were compared with an untreated control. In the second part of the study, the fibroblasts from the non-infarcted part of myocardium were isolated and cultured. Melatonin at a range of concentrations from 10^{-8} M to 10^{-6} M was applied to the fibroblast cultures. In the final part of the study, the influence of luzindole (10^{-6} M), the melatonin membrane receptor inhibitor, on melatonin-induced GAG augmentation was investigated. Both collagen and GAG content were measured in the experiment. Melatonin elevated GAG content in the myocardium remote to the infarcted heart. Collagen level was not changed by pineal indoleamine. Fibroblasts isolated from the myocardium varied in shape from fusiform to spindle-shaped. Moreover, the pineal hormone (10^{-7}M and 10^{-6}M) increased GAG accumulation in the fibroblast culture. Luzindole inhibited melatonin-induced elevation of GAG content at 10^{-6}M. Melatonin increased GAG content in the myocardium remote to infarction. This effect was dependent on the direct influence of the pineal indole on the heart fibroblasts. The melatonin-induced GAG elevation is blocked by luzindole, the melatonin membrane receptors inhibitor, indicating a direct effect of this indole.

Key words: *melatonin, cell culture, glycosaminoglycans, collagen, connective tissue, luzindole, myocardial infarction, pineal gland, repair*

INTRODUCTION

Occlusion of the coronary artery causes necrosis of the cells in the ischemic area. This process is followed by repair, leading to scar formation. However, myocardial infarction also causes heart remodeling, which expands the infarct thus contributing to the dilation of the necrotic area and changes of the ventricle geometry (1). This process potentializes heart failure (2). The status of the extracellular matrix in the healing area influences the mechanical properties of the scar and is an important determinant of the expansion of the myocardial infarction (3). In the areas remote to infarction, a rebuilding of the extracellular matrix is observed. Consequently, due to domination of matrix metalloproteinase (MMP) over the tissue inhibitors of matrix metalloproteinase (TIMPS), destruction of the extracellular scaffold is followed by the slippage of cardiomyocytes and enlargement of the chamber (4).

However, interstitial fibrosis has been observed in areas remote to the ischemia of the myocardium. As a result, increased accumulation and better crosslinking of type I collagen has been reported in the interstitium (5). Moreover, increased deposition of glycosaminoglycans (GAG) has been seen in the myocardium of the hearts of rats affected by myocardial infarction (6). The transient elevation of GAG was seen to rise during the 3rd week after infarction, peak during the 6th week and decrease during the 12th week after coronary artery ligation. However, these changes in GAG concentration were not observed in the skin (6). Excessive accumulation of collagen in the non-infarcted part of the myocardium increases myocardium stiffness, and not only decreases its compliance, but also participates in structural changes to the chamber geometry. Fibroblasts or myofibroblasts are the sources of the extracellular matrix in both the myocardial infarction scar and the remote region. The fibroblasts in the non-infarcted part of the heart increase synthesis of collagen and are more adherent to collagen type IV. Contrary to the cells isolated from the myocardial infarction scar, the proliferation of remote fibroblasts was not found to increase (7).

The accumulation of connective tissue remains under the regulatory influence of the pineal gland: melatonin was found to increase collagen content in the myocardial infarction scar (8, 9). The most effective dose of the pineal indoleamine was evaluated as 60 µg/100 g b.w.; this dose having an effect equal to

endogenous melatonin secretion (9). However, physiological doses of the pineal hormone did not influence GAG level in the myocardial infarction scar. Only pharmacological doses of melatonin decreased GAG level in the scar. This effect was accompanied by reduction of the oxidative stress markers (8).

An earlier study has demonstrated that melatonin exerts a regulatory influence on both collagen and glycosaminoglycan accumulation in the myocardial infarction (8, 9) scar as well as in the subcutaneous wound model (10). The final effect of the pineal indoleamine is dependent on the applied dose as well as the target tissue. The present study is aimed at explaining whether melatonin possesses a regulatory influence on connective tissue matrix accumulation in the non-infarcted part of the left ventricle. Moreover, the aim of the study is further extended at clarifying whether the effects observed *in vivo* depend on the direct influence of melatonin on the fibroblasts and to verify whether melatonin membrane receptors are involved in that process.

MATERIALS AND METHODS

Animals

Thirty seven male Wistar rats (weighing 290+30 g) were kept at a temperature of 22°C with free access to commercial food pellets (LSM, Bacutil, Poland), as well as tap water *ad libitum*. A regulated light dark cycle (LD=12:12) was applied. The light was turned on at 07:00h. The experiments were approved by Local Commission of Ethics no 9 in Lodz (47/LB379/2007).

Study design

The experiment was divided into two parts: *in vitro* and *in vivo*.

In the *in vitro* part, the 21 rats were divided into 3 groups containing 7 animals each:

Group 1 - non-treated control (CTR);

Group 2 - animals treated with a vehicle of 2% ethanol solution in 0.9% NaCl, at a dose of 0.1 ml/100 g b.w. (V);

Group 3 - rats treated with melatonin (Sigma, St. Louis, USA) at a dose of 60 µg/100 g b.w. (MLT).

The animals in groups 2 and 3 were injected subcutaneously once daily between 5.00 and 6.00 PM. After ligation of the coronary artery, the rats were treated for 28 days. On the last day of the experiment, the samples were collected for biochemical analysis.

In vitro experiments were performed on 16 rats and were divided into two parts containing 8 rats each. The animals served as cell donors were not treated. On the 28th day after ligation of the coronary artery, the cells were isolated from the scar and then cultured. Cells obtained from each rat were first divided into groups: control cells (CTR), cells administered with a melatonin solvent 0.005% DMSO (Sigma, St. Louis, USA) (DMSO), and cells treated with different concentrations of melatonin 10^{-6}M (MLT-6), 10^{-7}M (MLT-7) and 10^{-8}M (MLT-8).

The second part was performed on the cells obtained from the other 8 animals. The cells were divided into the following groups: control (CTR), 0.005% DMSO treated cells (DMSO), a group administered with melatonin at a concentration of 10^{-7}M (MLT-7) or cell treated with melatonin (10^{-7}M) and N-acetyl-2-benzyltryptamine (luzindole, Tocris, Ellisville, MO, USA) at a concentration of 10^{-6}M (MLT-Lu), (11). The last group was treated with luzindole alone 10^{-6}M (Lu).

Left coronary artery ligation

For ligation of the left coronary artery, the Seyle method with some modifications (8, 9) was used. After pentobarbital anesthesia, animals were ventilated with positive pressure through a tube inserted into the trachea. The tube was attached to a small animal respirator. After opening the chest, the heart was brought outside and the left coronary artery was ligated with nylon suture 6/0. The ligation was performed half way along an imaginary line between the closest point of insertion of the left auricular appendage and the left margin of the pulmonary cone. The heart was put gently into the chest and the thoracic incision was sutured. The linear wound of the skin was closed with 4–5 silk sutures. The tube was taken out of the trachea. On the 28th day of the experiment, the animals were euthanized, the hearts were divided into the left ventricle, right ventricle, scar and septum and the weights of each portion were measured.

Cell cultures

Under aseptic conditions, walls of the left ventricle, remote to myocardial infarction were collected for isolation of the cells and then stored in RPMI1640 medium with gentamycin (25 µg/ml) and fungizone (2.5 µg/ml). The samples were minced and then incubated for 3 hours in 0.1% collagenase solution (37°C, 5% CO_2). After centrifugation (5 min, 1000 rpm) the cells were washed with DMEM containing 10% fetal calf serum. The tissue was centrifuged again (5 min, 1000 rpm), the cells were placed on dishes and the adherent cells were incubated in a humidified atmosphere of 5% CO_2, at a temperature of 37°C. The non-adherent cells were washed out after 2 hours. When the confluent growth was obtained the cells were trypsinized and passaged. For setting up the new culture, cells at the density of 8×10^3 cm^2 were applied.

The tests were performed on cells after the first or second passages. For experimental purposes, the cells were grown in medium composed of DMEM with 3% of calf serum, gentamycin (25 µg/ml) and fungizone (2.5 µg/ml). The medium was changed every day. The number of total and necrotic (stained with trypan blue) cells were counted in a Burker chamber on the 6th day of the experiment. The GAG content in the cultures was measured on the 6th day of the experiment. The cells were identified by electron microscopy (12).

Determination of collagen

Collagen was evaluated by the Woessner method (13). The scars were cut into small pieces and vacuum-dried at 60°C. Then, the samples were hydrolyzed with 6 N HCl (3 ml/sample) at 110°C for 24 hours and, all the hydrolizates were evaporated to dryness in a water bath. Three mililiters of redistilled water was added to each tube to dissolve the precipitate and the samples were neutralized with 1 N NaOH. After neutralization, the samples were diluted to 10 ml with redistilled water and 0.5 ml samples were taken from the tube and diluted with redistilled water to 2 ml final volume for further analysis. To oxidize hydroxyproline to pyrrole, 1.25 ml chloramine T in citrate buffer (pH=6) was added and the samples were shaken for 5 min and incubated for 20 min at 20°C. Excess chloramine T was removed by 1.25 ml of 3.15 M perchloric acid. Then, the samples were incubated with 1 ml of 20% p-dimethylaminobenzaldehyde in a 60°C water bath for 20 min. The optical density was measured at 560 nm on a spectrophotometer.

Determination of glycosaminoglycans

For evaluation of total GAG in the samples, the 1,9-dimethylmethylene blue (DMMB) assay was used according to Farndale *et al*. (14). After homogenization, the residue of the samples was dried to constant weight at 60°C. A mixture composed of 0.75 M NaOH and 50 mM sodium borate was

added to 50 mg of dry sample and incubated for 1 hour in a water bath (73°C). The pH was neutralized with 6 M HCl to approximately 7.0. Then, to precipitate proteins, 72 µl of 100% TCA was added to each sample. After centrifugation of the samples (6000 rpm for 30 min) 6 ml of 100% ethanol was added to the supernatant. The samples were kept in a refrigerator (–20°C) overnight to precipitate GAG and then they were centrifuged again (12,500 rpm for 30 min). The precipitate containing GAG was dissolved again in redistilled water. DMMB-reagent (composed of 51 mM DMMB, 45 mM glycine, and 41 mM NaCl adjusted to pH 3.0 with 1 M HCl) at a volume of 1.2 ml was added to 50 µl of the sample. The absorbance was measured at 525 nm on a spectrophotometer.

Electron microscopy preparation

Initially cell suspensions were centrifuged in 2.5% glutaraldehyde in cacodylic buffer at pH 7.4. The pellets were washed with 0.13 M cacodylic buffer and then afterfixed in 2% OsO_4 for two hours. After rinsing in 0.13 M cacodylic buffer, cells were dehydrated in increasing concentrations of alcohol solutions and additionally, in propylene oxide. Specimens were embedded in araldite synthetic resin. Thin sections, 60 nm thick, were placed on a 300-mesh copper grid and contrasted with lead citrate and uranyl acetate. The cells were examined and photographed under a Philips EM301 electron microscope at 5700–25000 × magnification.

Statistical analysis

For statistical analysis, ANOVA test was used. Statistical differences between the groups were evaluated by the NIR test. The minimal level of significance was $p<0.05$.

RESULTS

The rats with myocardial infarction were in good condition and symptoms of heart failure (dyspnea, fatigue, lung edema or congestion, hepatomegaly, ascites) were not seen during the experiment. The size of the myocardial infarction scar was similar in all investigated groups.

Melatonin (*Fig. 1*) applied at a dose of 60 µg/100 g b.w. (MLT) increased the glycosaminoglycan level in the myocardium of the left ventricle remote to myocardial infarction compared with the intact control ($p<0.002$) (CTR) and rats treated with vehicle (V), the melatonin solvent ($p<0.004$). On the other hand, melatonin at the same dose did not change the total collagen level in the myocardium remote to myocardial infarction (*Fig. 2*).

The fibroblasts were isolated from the myocardium remote to the myocardial infarction scar 28 days after coronary artery occlusion. The cells were spread on the Petri dish. Obtained cells had a variety of shapes from stellate to fusiform or spindle. (*Fig. 3*). In case of the most cells investigated in electron microscope, morphological aspects of high metabolic activity were observed. They were stellate in shapes, with many cytoplasmic processes different in size. Nuclei of cells had different diameter. Their nuclear envelopes were sometimes highly folded with deep invaginations. They were euchromatic and contained 1–2 transcriptionaly active nucleoli with highly folded and well visible nucleololemma. Heterochromatin form only thin marginal zone near nuclear envelope. Rough endoplasmic reticulum was well developed with a long interconnecting cisternae that contained middle dense, homogenous substance or, rarely, thin and short fibrils which could be procollagen particles (*Fig. 4*). Thin and long fibrils, formed large complexes were also visible in cytoplasm (*Fig. 5*). Near plasma membrane other type of fibrils were visible. They were well organized parallely, and formed thin bundles. Their morphology and localization suggest that they are actin fibers (*Fig. 6*). Within the cytoplasm two main types of granules and vesicles were observed. Those, connected with secretory activity were larger; contained middle dense, fibrous content. Some of them were in contact with cell membrane because of secretion of their content. Other type of granules was diverse in size and electron density. They contain dense, irregular, sometimes myelin like membranous structures. These features indicate that this type of granules should be indicated as autosomes or secondary lysosomes containing fragments of mitochondria or membranes of endoplasmic reticulum. Different in length and diameter fibrils were connected with extracellular face of cellular membrane. They could be an effect of polymerization of tropocollagen in extracellular space (*Fig. 5*). All those finding suggest that examined cultured cells could be treated as fibroblasts.

The concentration of glycosaminoglycans in both the intact control and DMSO treated cells was unchanged. Melatonin applied at concentrations of 10^{-6}M ($p<0.003$) and 10^{-7}M ($p<0.008$) significantly increased glycosaminoglycan content in the cultures compared with both intact controls and DMSO-treated fibroblasts. On the other hand, lower concentration of the pineal hormone (10^{-8}M) did not significantly influence the GAG level in the cultures of fibroblasts (*Fig. 7*). In the second part of the study, the effect of luzindole, the melatonin membrane receptor inhibitor, was investigated. Melatonin at a concentration of 10^{-7}M ($p<0.004$) was found to increase the glycosaminoglycan concentration in the culture comparing with

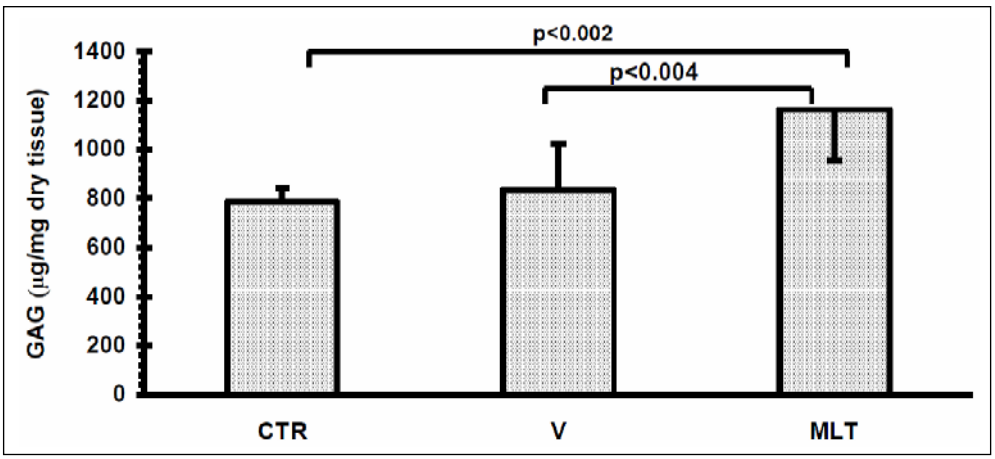

Fig. 1. Glycosaminoglycan content in the muscle of the free wall of the left ventricle remote to myocardial infarction: in intact controls (CTR), rats treated with vehicle (V) the solvent for melatonin and animals administered with melatonin at a dose of 60 µg/100 g b.w. (MLT). Each value expresses a mean of six-seven samples ± standard deviation (S.D.).

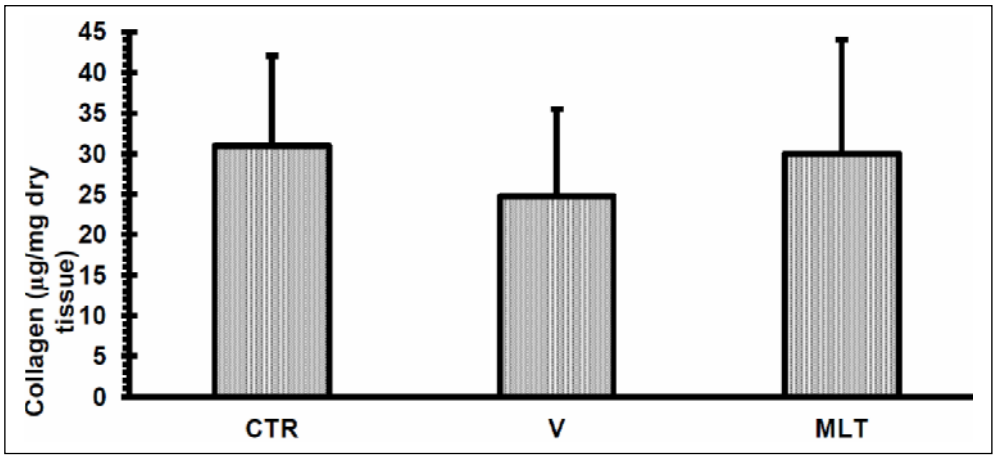

Fig. 2. Total collagen content in the muscle of the free wall of the left ventricle remote to myocardial infarction: in intact controls (CTR), rats treated with vehicle (V) the solvent for melatonin and animals administered with melatonin at a dose of 60 µg/100 g b.w. (MLT). Each value expresses a mean of six-seven samples ± standard deviation (S.D.).

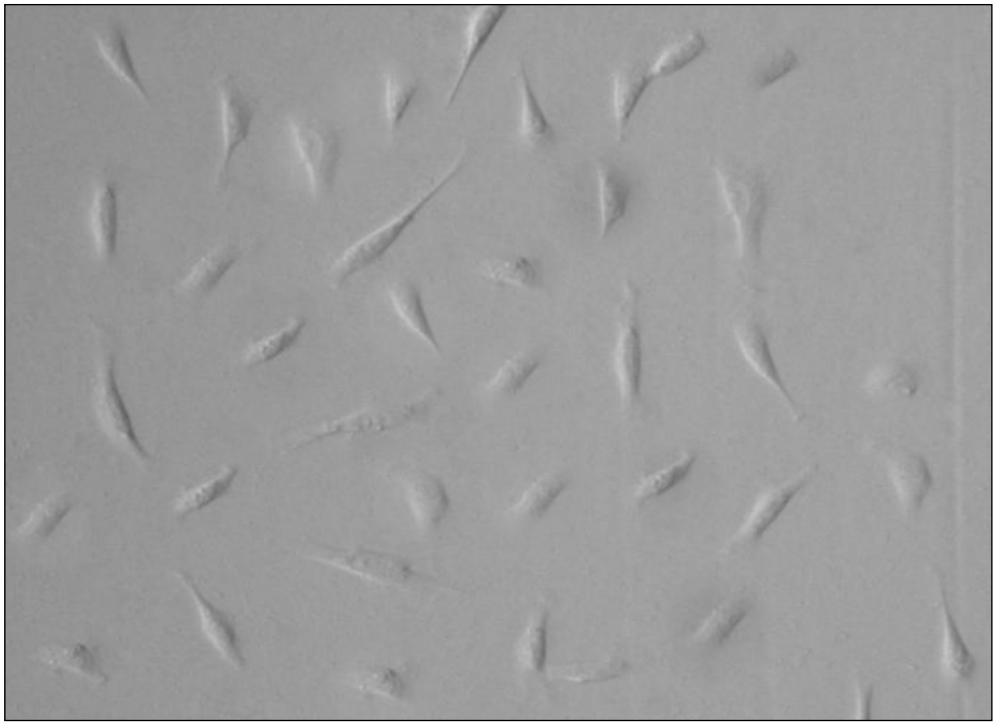

Fig. 3. Cultured fibroblasts isolated from the scar of the infarcted heart of different shapes, with number of cytoplasmic processes. Integrated modulation contrast, magnification 100×.

the control and DMSO administered cells. Luzindole (10^{-6}M) application to the melatonin treated cells diminished the effect of melatonin and the level of GAG in this group was the same as in the controls or DMSO-treated cells. The content of GAG in this group was significantly lower than in the melatonin (10^{-7}M) alone treated cells ($p<0.03$). As luzindole (10^{-7}M) administered alone to the cell cultures did not significantly change the level of GAG, the GAG content in this group was similar to that found in the controls and DMSO incubated cells (*Fig. 5*).

DISCUSSION

The present study shows for the first time the regulatory influence of melatonin on GAG accumulation in the area of the myocardium remote to myocardial infarction. The effect of melatonin applied at the doses ranging from 3 µg/100 g to 300 µg/100 g b.w. was investigated earlier (9). The pineal indole injected at the doses of both 30 µg/100 g b.w. and 60 µg/100 g b.w.) increased collagen content in the scar but only one dose of melatonin (60 µg/100 g b.w.) reversed the effects of both pharmacological and surgical pinealectomies. The applied dose of the pineal hormone (60 µg/100 g b.w.) has been previously proven to promote collagen accumulation most effectivelly in the scar of the myocardial infarction (9). Our present study confirms the accuracy of the dose on the regulation of GAG content in the myocardium (*Fig. 1*).

Previously, pharmacological doses of melatonin (0.3 mg/100 g b.w. – 3 mg/100 g b.w.) were observed to reduce GAG content in the scar of the myocardial infarction (8). This effect was not dependent on the direct influence of melatonin on myofibroblasts synthesizing GAG and isolated from the scar of myocardial infarction (8). Melatonin may exert its action indirectly *via* modification of the regulatory systems: nervous (15), immune (16) and endocrine (17). Administration of melatonin (10^{-6}M and 10^{-7}M) to the fibroblasts isolated from myocardium showed that the pineal indole increased the GAG content of the culture (*Fig. 4*). This fact suggests that effect observed *in vivo* (*Fig. 1*) was dependent on the direct influence of melatonin on cells synthesizing the extracellular matrix. These results were supported by previous studies carried out on the myofibroblasts isolated from the myocardial infarction scar showing that

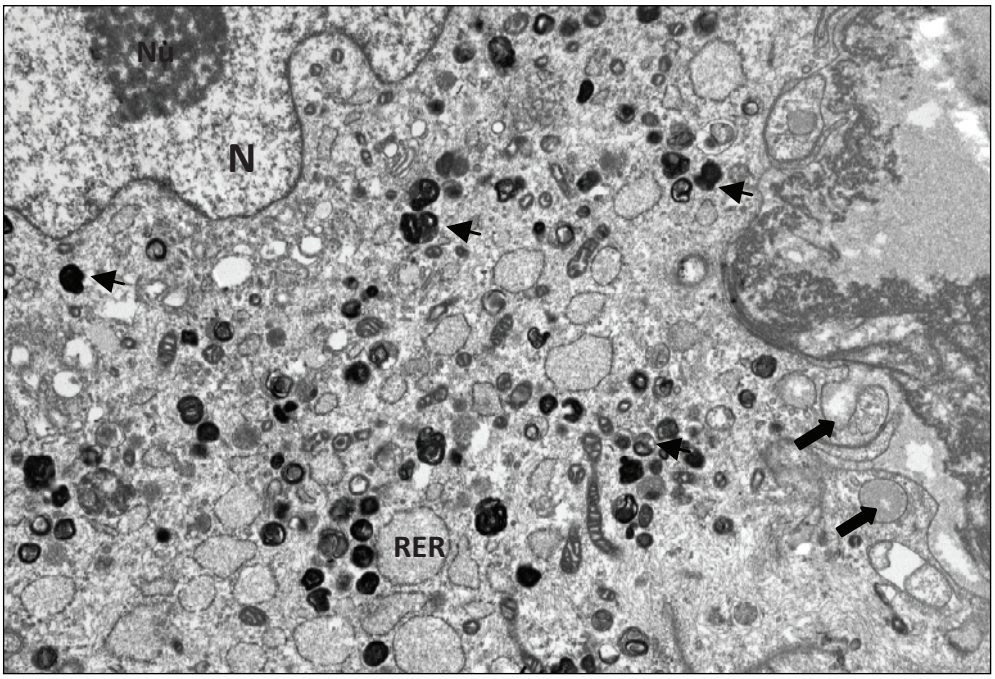

Fig. 4. Fibroblast. Euchromatic nucleus with folded nuclear envelope (N) and transcriptionally active nucleolus (Nu). Well developed cisterns of endoplasmic reticulum (RER), secreting vacuoles (arrows) and endosomes (arrow heads) visible in cytoplasm. Magnification: 15,000×.

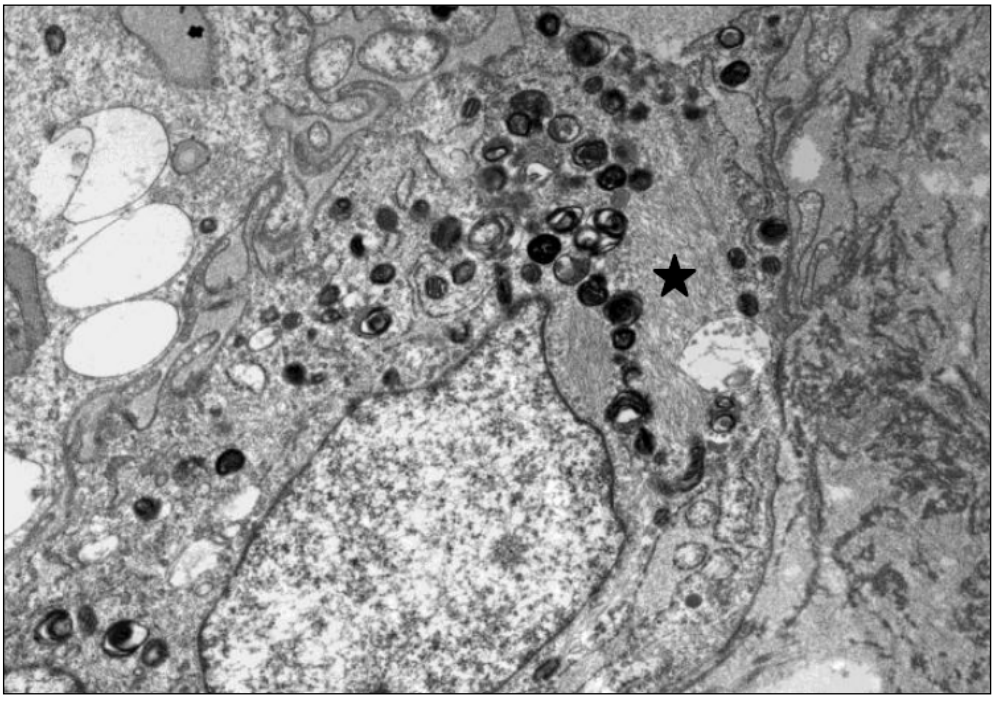

Fig. 5. Large intercellular complex of fibrils (asterisk). Magnification 15,000×.

melatonin (10^{-7}M) elevated GAG accumulation in the cultures (8). Melatonin (10^{-7}M) also increased the collagen content of myofibroblast cultures derived from the scar of the myocardial infarction (9) as well as cells composed of fibroblasts and myofibroblasts isolated from the granulation tissue of the wound (11). Melatonin at higher concentrations from 50 to 100 μM increased synthesis of collagen type I in cultures of both normal human bone cells and human osteoblast cells (18).

Four cellular mechanisms of melatonin action are suggested: activation of melatonin membrane receptors (MT1 and MT2) (19), binding to calmodulin (20) or retinoid nuclear receptors (21) and influence on the level of reactive oxygen species (22). Our study shows that luzindole negates the melatonin-induced augmentation of GAG in the fibroblast cultures derived from the myocardium remote to the area of infarction (*Fig. 8*). This observation is supported by the results of our previous studies showing that collagen content accumulation by both myofibroblasts derived from the subcutaneous wound model, and myofibroblasts of the myocardium infarction scar, is inhibited by luzindole (11). Luzindole was found to diminish the stimulatory effect of melatonin on the lipid metabolism in murine fibroblasts (23) and block the melatonin-induced expression of both Cu/Zn-superoxide dismutase and glutathione reductase in corneal human fibroblasts (24). All our observations suggest that the melatonin membrane receptors have some form of involvement in the control of extracellular matrix accumulation (*Fig. 5*). However, this hypothesis shoud be further investigated.

Domininguez-Rodriguez *et al.* found reduced nocturnal levels of melatonin in patients with acute myocardial infarction.

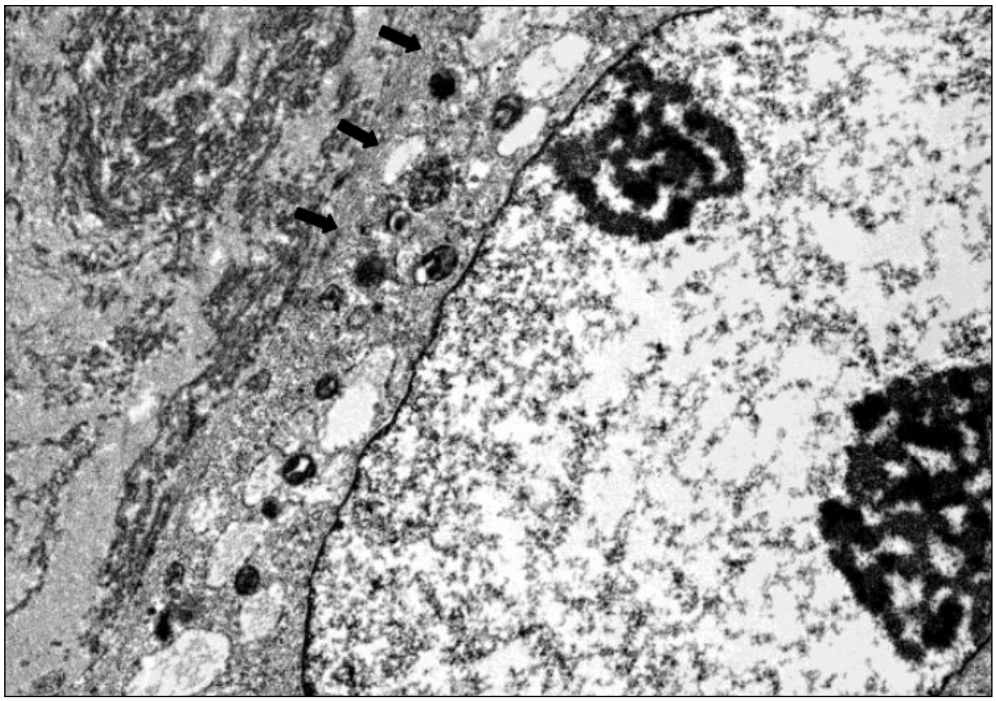

Fig. 6. Highly organized actin fibers near cytoplasmic membrane (arrows). Magnification 20,000×.

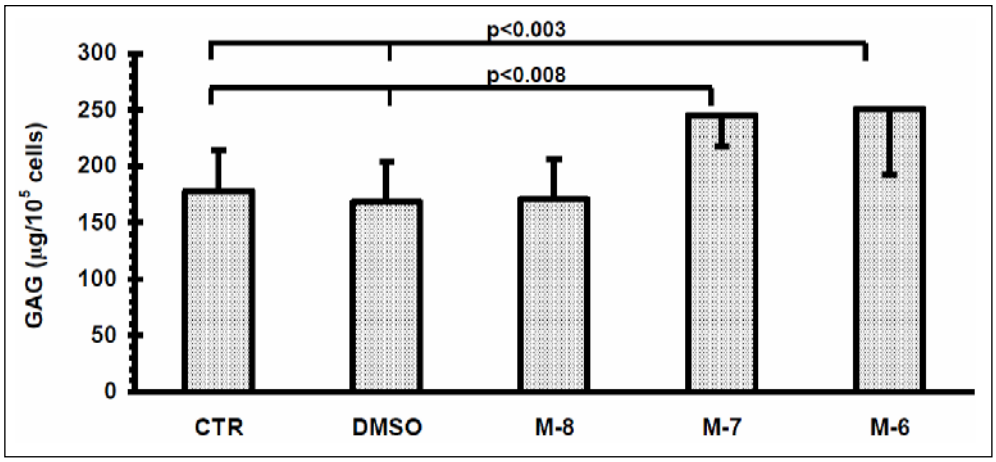

Fig. 7. Glycosaminoglycan level (µg/10^5 cells) in cultures isolated from the left ventricle free wall remote to myocardial infarction scar in control cells (CTR), cells incubated with DMSO (DMSO), or melatonin at concentrations of 10^{-6}M (MLT-6), 10^{-7}M (MLT-7) and 10^{-8}M (MLT-8). Each value expresses a mean of seven-eight samples ± standard deviation (S.D.).

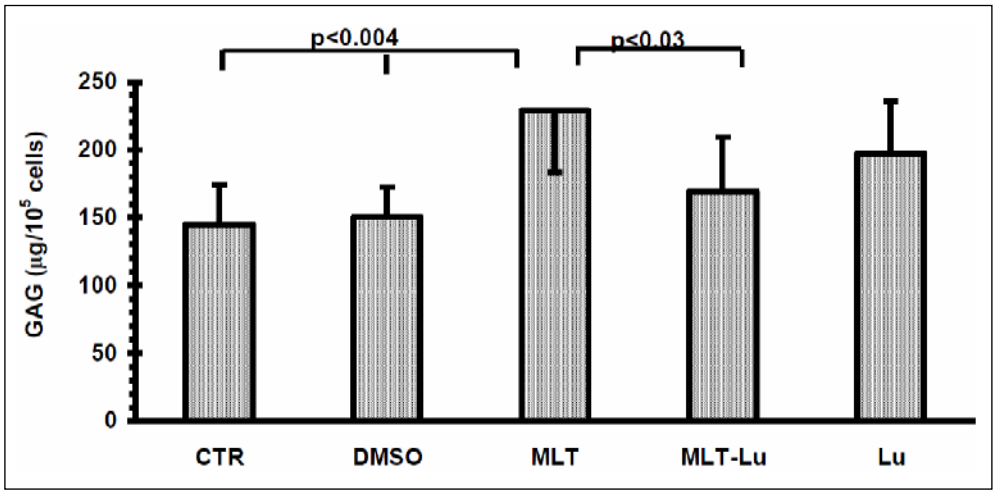

Fig. 8. Glycosaminoglycans level (±µg/10^5 cells) in cultures isolated from the left ventricle free wall remote to myocardial infarction scar in control cells (CTR), cells incubated with DMSO (DMSO), or melatonin at concentration of 10^{-7}M (MLT), melatonin (10^{-7}M) with luzindole at concentration of 10^{-6}M (MLT-Lu), and luzindole alone (10^{-6}M; Lu). Each value expresses the mean of seven-eight samples ± standard deviation (S.D.).

Furthermore the light/dark differences of melatonin concentration in serum were lower in groups of patients with myocardial infarction compared with healthy volunteers (25). A low level of melatonin in patients with acute myocardial infarction is regarded as an independent predictor of the left ventricle remodeling (26). Thus, in patients suffering from myocardial infarction, a low level of melatonin was linked with remodeling of the left ventricle. This effect was not observed in

patients affected by myocardial infarction with higher levels of the pineal hormone (26). However, increased melatonin levels was seen one day after myocardial infarction in rats in both the left ventricle and serum; these changes were linked with increased arylalkylamine N-acetyltransferase level in the pineal gland comparing to the animals with sham myocardial infarction. In addition, rats with myocardial infarction were reported to demonstrate changes of the melatonin membrane receptor expression pattern (27). The role of the pineal gland in the remodeling of the heart after myocardial infarction remains to be elucidated.

Our experiments have shown that melatonin in animals with myocardial infarction may have an influence on the GAG level in the myocardium. The influence of excessive GAG accumulation in the myocardium on the heart function is not clear. Hyaluronic acid hydrogel injection to the heart wall attenuates the remodeling process, reduces left ventricle volume and improves cardiac output (28). Fomovski and coworkers speculate that proteoglycans may have an influence on the heart wall mechanics by regulating water content in the tissue (29). Furthermore, GAG mainly heparin and dermatan sulphate inhibit the synthesis of metal-catalyzed reactive oxygen species and stops lipid peroxidation (30, 31). The results from our laboratory as well as results from other laboratories strongly indicate that melatonin may exert a cardioprotective effect by inhibiting oxidative stress (30, 32) and remodeling changes, as well as improving the mechanics of the heart by increasing the GAG content in the myocardium. Moreover, reduction of the pineal hormone level in patients with myocardial infarction may retard the cardioprotective effect of GAG. This hypothesis should be further investigated.

Cells isolated from myocardium remote to infarction were identified as fibroblasts. They express features of high metabolic activity and variety of shapes from stellate to fusiform. These results are in aegreement with the studies of van den Borne and coworkers (33) who has not found myofibroblasts in myocardium remote to ishemia 4 weeks after infarction. However, 12 weeks after infarction, the myofibroblasts have been found in the heart remote to infarction (33).

The present study has shown that GAG accumulation in the myocardium of the infarcted heart is augmented by melatonin. This effect is dependent on the direct influence of the pineal indole on the fibroblast responsible for GAG synthesis. Moreover, the action of melatonin on GAG deposition is blocked by luzindole, a melatonin membrane receptor (MT1 or MT2) inhibitor. The results presented herein are important for understanding the mechanism of changes in the myocardium during heart remodeling after myocardial infarction and also highlight a new target for antiremodeling therapy.

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Conflict of interests: None declared.

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