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Potentiation of cryopreserved rat adipose-derived multipotent mesenchymal stromal cells by BMP-12 *in vitro* for the treatment of tendinopathy

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ABSTRACT

Bone morphogenetic proteins (BMP), which are part of TGF-b superfamily, have a stimulating effect on bone formation, as well as the proliferation of tenocytes and fibroblasts, which form the tendon tissue.

*The **PURPOSE** of the study was to determine the effect of BMP-12 on the morphofunctional characteristics of cryopreserved rat adipose tissue-derived multipotent mesenchymal stromal cells (AT-MMSCs) and to investigate the effects of the use of AT-MMSCs pre-cultured with BMP-12 on the tendinopathy model.*

***MATERIALS AND METHODS.** MMSCs were obtained from the adipose tissue of rats. Cryopreservation was carried out under the protection of 10 % DMSO with the addition of 20 % fetal bovine serum at a cooling rate of 1 °C/min to -80 °C and subsequent transfer to liquid nitrogen. When culturing AT-MMSCs, the BMP-12 was added at the concentration of 50 ng/mL. The ability of cells to proliferation (by MTT-test) and the synthesis of collagen I and III types were evaluated. Rats with modeled degenerative-dystrophic injury to the Achilles tendons were locally administered with $0.25 \cdot 10^6$ AT-MMSCs or AT-MMSCs+BMP-12 in the defect zone. The animals injected with saline provided control group. On the 21st day after the treatment, a histological, immunofluorescence and biomechanical studies were performed.*

***RESULTS.** The application of BMP-12 results in the decrease in proliferation of AT-MMSCs along with an increase in the relative number of cells that synthesize collagen I and III types relative to AT-MMSCs cultivated under standard conditions. The injection of AT-MMSCs promotes the activation of regenerative processes in damaged tendons. The use of AT-MMSCs+BMP-12 accelerates the histological structure, strength, and the content of collagen I and III types in the Achilles tendons of animals with degenerative-dystrophic damage compared to the cells without BMP-12.*

***CONCLUSIONS** BMP-12 can be used as a stimulating agent for the tenogenic differentiation of AT-MMSCs before transplantation into the damaged tendon tissue.*

***KEY WORDS:** adipose-derived multipotent mesenchymal stromal cells; BMP-12; collagen; tendinopathy; cell therapy*

The pathologies of the tendons are quite common and represent complex clinical cases in orthopedics, mainly because they are poorly treated and require long-term rehabilitation [1, 2]. Such damages include tendinosis, tendinitis and paratendinitis, which have traumatic, inflammatory or degenerative etiology. Existing therapies do not provide a successful long-term effect, and damaged tendons do not fully restore their structure and function [3-6].

The involvement of the biological component in modern therapy of orthopedic disorders is represented by the use of growth factors, stem cells, natural biomaterials and genes alone or in combination *in situ* [7-9]. Multipotent mesenchymal stromal cells (MMSCs), which are isolated from many sources: bone marrow, adipose tissue, skin, cord blood, and others, are considered the most effective in regenerative medicine [10, 11]. MSCs can promote regeneration not only through direct cell differentiation, but also through secretion of growth factors, which are among the most important multifunctional molecular families involved in the repair of damaged tissues [10-14].

Today, understanding the biology of the tendons is far behind the understanding of other skeletal muscle tissues, which complicates the development of new therapies for the treatment of tendinopathy. In recent years,

in vitro and *in vivo* models have been developed to validate the possibility of directed differentiation of cells in the tenogenic direction with the involvement of growth and transcription factors and their subsequent use to stimulate regenerative processes in damaged tendons. Some cytokines, including bone morphogenetic proteins (BMP), transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF), are used for the reconstruction of the tendon tissue [15-19]. However, not all of the above-mentioned factors can contribute to tenogenic differentiation.

BMP, bone morphogenetic proteins, which are part of TGF-b superfamily, have a stimulating effect on bone formation, as well as the proliferation of tenocytes and fibroblasts, the constituents of the tendon tissue. Among these cytokines, BMP-12, also called growth and differentiation factor 7 (GDF-7), showed the highest ability to stimulate tendon recovery both *in vivo* and *in vitro* [15]. The study performed by Inada et al. [16] showed that the cultivation of myoblasts with BMP-12 resulted in a decrease in the expression of myosin with the formation of multinucleated myotubes. The inhibitory effect of BMP-12 on myogenic differentiation was similar to that of BMP-2, although it had lower activity. The effect of BMP-12 on MMSCs is multidirectional, which depends on the source of stem cells. Most studies showed that this factor contributes to the tenogenic differentiation of MMSCs and has a dose-dependent effect [17-19]. A deeper understanding of the functioning of the tendons at the tissue and cellular levels, in combination with the clinical application of modern molecular and cellular tools, can contribute to the development of effective therapeutic agents that are specific to the tendons.

The use of cultivation and cryopreservation technologies allows the stock of stem and specialized cells to be obtained, long-term stored at low temperatures, easy transported and thawed immediately before the use. Therefore, the study of the characteristics of cryopreserved cultures is an important trend in modern biotechnology. In previous studies, we investigated the morphofunctional characteristics of cryopreserved MMSCs obtained from the bone marrow, adipose and tendon tissues, and found that adipose tissue-derived MMSCs (AT-MMSCs) had a greater ability to colony formation, proliferate, and have the same ability to direct differentiation compared with MMSCs from bone marrow [20].

The aim of this study was to determine the effect of BMP-12 on the morphofunctional characteristics of cryopreserved rat adipose-derived multipotent mesenchymal stromal cells and to investigate the effects of the use of AT-MMSCs pre-cultured with BMP-12 on the model of tendinopathy.

MATERIALS AND METHODS

Obtaining and culturing of AT-MMSCs. The primary suspension of adipose tissue cells was obtained from biopsies of the omentum of 3-months old outbreed male rats weighting 250 ± 25 g (n = 5) by their enzymatic digestion. For this, tissue samples (75 ± 3 mg) were washed with Hanks balanced salt solution HBSS (*PAA*, Austria) containing 150 μg/mL gentamicin (*Farmak*, Ukraine) and incubated in collagenase type II at a concentration of 1.5 mg/mL (*PanEco*, Russia) at 37 °C for 18 hours. Cells were isolated from tissue samples by resuspending followed by centrifugation at 840g for 3 min. The culture medium was added to the pellet and seeded at the density $1 \cdot 10^4$ cells/cm^2 into 25 cm^2 culture flask (*PAA*, Austria). The concentration of cells was counted in Goryaev chamber under a light microscope.

The complete culture medium contained IMDM medium (*PAA*, Austria), 10 % fetal bovine serum (*HyClone*, USA), 150 μg/mL kanamycin (*Farmak*, Ukraine) and 5 μg/mL amphotericin B (*PAA*, Austria). Nutrient medium was changed every 3 days. Standard culture conditions at 37 °C in an atmosphere of 5 % CO_2 with an incubator (*Sanyo*, Japan) were used. Upon reaching the monolayer, cells culture was subcultured using 0.25 % trypsin-EDTA solution in a ratio of 1:1. The cultures of the AT-AT-MMSCs of the 2nd passage were used in this study.

Cryopreservation of MMSCs. Cryopreservation of AT-MMSCs cultures was carried out under protection of 10 % DMSO (*PanEco*, Russia) with 20 % fetal bovine serum. The cryoprotectant solution was prepared using a complete culture medium and the cell suspensions were transferred in a 1 ml cryovial (*Nunc*, USA). The cooling rate was 1 °C/min to -80 °C, followed by transfer in liquid nitrogen [21]. Samples were stored in cryostorage for 2 months. The thawing was carried out in a water bath at 40 °C until a liquid phase appeared. The washing of the cryoprotectant was carried out by adding HBSS in a ratio of 1:9 followed by centrifugation at 840xg for 5 minutes. When cultivating thawed AT-MMSCs, the same conditions were used as for the initial culture.

The use of BMP-12 for the cultivation of AT-MMSCs. To determine the effect of the recombinant protein BMP-12/GDF-7 (*Sigma-Aldrich*, USA, cat. #SPR4572-20UG), AT-MMSCs were cultured for 12 days (group AT-MMSCs+BMP12) in the complete nutrient medium with the addition of 50 ng/mL BMP-12 [22-24], which was changed every 3 days. Cultivations were performed using 25 cm^2 culture flasks and 6-well plates (*PAA*, Austria) depending on the purpose of the experiment. AT-MMSCs, cultivated under similar conditions without the BMP-12 were as a control group.

The effects of BMP-12 on morphofunctional characteristics of AT-MMSCs. In the studied cultures, with and without BMP12, the number of viable cells was determined at terms of 1, 3, 7, 12 days by MTT-test [25]. The measurement of the optical density of the formazan solution in the supernatant was performed on KFK-2-UHL42 photometer at a wavelength of 540 nm. A culture medium without cells was used as a control. On the 12th day, the cells were fixed in a 4 % solution of paraformaldehyde followed by azure-II and eosin staining by Romanovsky-Giemsa.

The staining of AT-MMSCs cultures on collagen types I and III was performed using primary mouse anti-collagen type I monoclonal antibody (*Sigma-Aldrich*, USA, cat. #C2456), in the dilution 1:2000, conjugated with CF^{TM}488A (*Sigma-Aldrich*, USA, cat. #SCJ4600014), and rabbit anti-rat collagen type III polyclonal antibody (*Millipore*, USA cat. #AB757P) in the dilution 1:80 with secondary goat anti-rabbit IgG Alexa Fluor 647 antibodies (*Millipore*, USA, cat #AP187SA6), respectively. The cultures were additionally stained with DAPI (*Sigma*, USA) at a concentration of 1 μg/mL for 30 min to visualize cell nuclei. Fluorescence microscopy was performed using a LSM 510 Meta Confocal Scanning Microscope (*Carl Zeiss*, Germany).

Modelling of degenerative-dystrophic damage of Achilles tendons. For male rats (age 12-13 weeks, weighting 200 ± 25 g, n = 45), we modeled experimental tendinopathy by injections of 0.03 mL Diprospan (*Schering-Plow Central East AG*, Switzerland) into both Achilles tendons every 7 days for 3 weeks (4 injections in total) [26, 27].

Cell transplantation. After 12 days of cultivation with or without BMP-12, AT-MMSCs were subcultured using 0.25 % trypsin-EDTA solution in a 1:1 ratio. The washing from the medium was carried out by adding HBSS in a ratio of 1:9 followed by centrifugation at 840xg for 5 min. Saline was added to the pellet and resuspended.

One week after the last injection of Diprospan, the animals were injected locally: control group (n = 15) – 0.025 ml saline; experimental group 1 (n = 15) – $0.25 \cdot 10^6$ AT-MMSCs; experimental group 2 (n = 15) – $0.25 \cdot 10^6$ AT-MMSCs+BMP12. Cell suspension was injected into both Achilles tendons of experimental animals with tendinopathy, for 0.25 cm from the tuber calcanei. On the 21st day after the treatment, the animals of the control and experimental groups were euthanized using asphyxia in the CO_2 atmosphere. For the histological, immunofluorescence and biomechanical examination, the tendons were cut along with the attachment to the heel bone.

Evaluation of the cell therapy effectiveness. The obtained Achilles tendons for histological examination were fixed in 10 % formalin solution and after dehydration and degreasing in acetone and ethanol with increasing concentration were mounted into celloidin. We obtained histological sections in the sagittal projection, which were stained with hematoxylin and eosin. The density of cellular elements in histological preparations was determined as the mean of the number of nuclei per area of the tendon section (0.102 mm^2), followed by a recalculation per 1 mm^2.

The evaluation of the content of collagen type I and III was performed on 7-μm thick cryosections, stained according to the procedure described above for AT-MMSCs cultures. In the presence of autofluorescence, it was quenched with a 0.3 M glycine (PAA, Austria) for 20 minutes followed by repeated microscopy of sections. The relative area of the positive staining for Type I and Type III collagen was measured using the Axiovision Real.4.7 software and calculated its percentage as the ratio of the fluorescence area to the total area of the tendon incision, which was taken as 100 %.

Determination of tendons strength was carried out by measuring the destructive load at their tension – F (MPa) and determined by the formula: F = N /S, where N – the load applied before the rupture; S – the area of the tendon (S = π • a • b, where π = 3,14; a – the height; b – the width of the cross section) [28].

All manipulations with animals were carried out in accordance with the requirements of bioethics and international principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, as well as with the accordance of the «General Principles of Animal Experiments», approved by the II National Congress on Bioethics (Kyiv, 2004)

Statistical analysis. The normality of the distribution was determined by the skewness and the kurtosis. In the normal distribution of variables, the significance of differences between groups was estimated using Student's t-test and presented as M ± m. The critical value of the significance level was assumed to be p = 0.05. Data analysis was performed using Microsoft Excel (*Microsoft*, USA) and Statistika 8 (*StatSoft Inc.*, USA) software.

RESULTS AND DISCUSSION

Effects of BMP-12 on morphofunctional characteristics of AT-MMSCs. The results of the MTT test are presented in **Fig. 1.** At initial cultivation (1-3 days), proliferative activity of cells did not differ significantly in all investigated samples. On the 7th and 12th days in the cultures of AT-MMSCs in the presence of BMP-12, the proliferation index was 1.47 and 1.8 times lower, respectively, compared to the corresponding indices in AT-MMSCs cultivated under standard conditions.

The effect of BMP-12 on morphological characteristics and the ability of AT-MMSCs to produce collagen types I and III are shown in **Fig. 2.** AT-

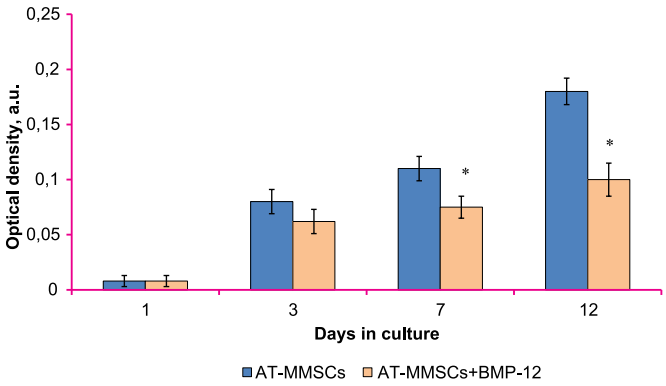

Fig. 1. The optical density of the formazan solution in the supernatant according to the MTT-test as a marker of proliferation in the dynamics of cultivation AT-MMSCs with BMP-12 (M ± m, n = 15).

*Note: * – the difference is significant compared to control (p < 0.05).*

MMSCs during cultivation were represented by sail-shaped, star-shaped and spindle-shaped cells (**Fig. 2 A**). In control group, the relative number of AT-MMSCs that produced type I collagen was 63.4 ± 2.5 % and collagen type III – 45.2 ± 3.1 % (**Fig. 2B, C**). Morphological changes in AT-MMSCs under the impact of BMP-12 were observed from the 5-6th day of cultivation, which was manifested in the predominance of spindle-shaped cells, as compared to control group (**Fig. 2D**). In the AT-MMSCs+BMP-12 samples, the relative number of cells which synthesized collagen types I and III amounted to 83.1 ± 3.7 % and 87.2 ± 3.1 % respectively, which was 1.3 and 1.9 times higher than the corresponding value of AT-MMSCs, cultivated under standard conditions (**Fig. 2 E, F**).

Thus, the addition of BMP-12 to the culture medium leads to a decrease in proliferative activity and activation of synthetic processes in MMSCs, indicating changes in the functional status of these cells under BMP-12 impact and indirectly confirming the possibility of directed tenogenic differentiation of AT-MMSCs.

The effects of AT-MMSCs and AT-MMSCs+BMP-12 therapy on the Achilles tendons reparation at tendinopathy. An analysis of the results of histological examination of Achilles tendons in control animals showed

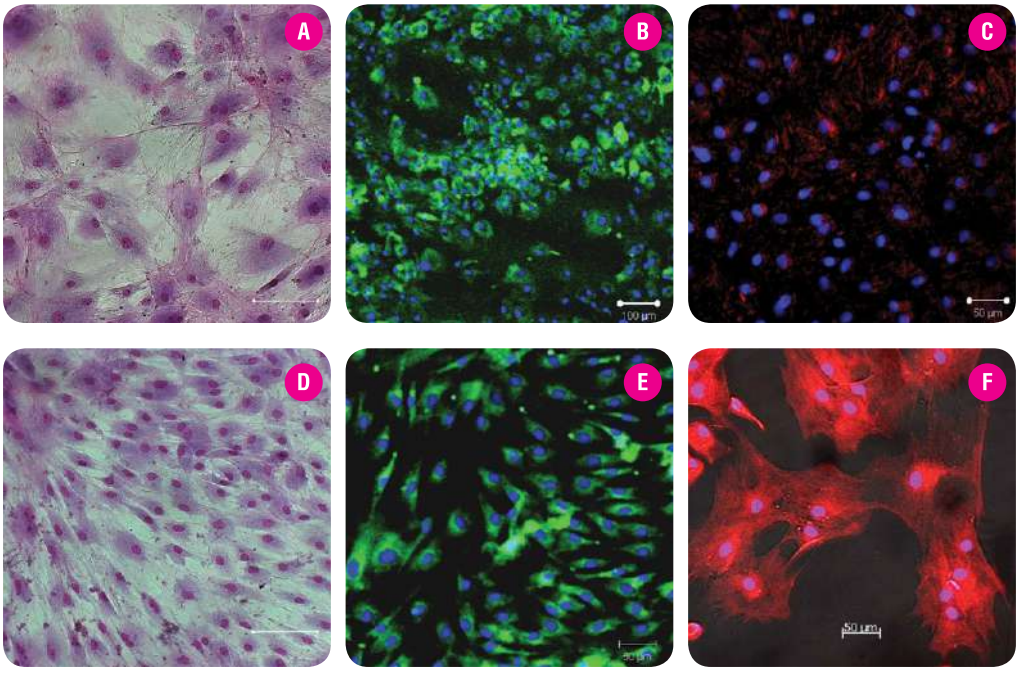

Fig. 2. Microphotographs of AT-MMSCs (**A-C**) and AT-MMSCs+BMP-12 (**D-F**) cultures, the 12th day of cultivation. Light microscopy (**A, D**), azure II and eosin staining. Fluorescent microscopy, immunocytochemical staining for collagen type I (**B, E** – green) and for collagen type III (**C, F** – red), nuclei stained DAPI (blue).

that there were pathological changes that had a manifestation in the disorganization of tendon fibers and their irregular staining (**Fig. 3A**). The luminescence of collagen types I and III were not intense and had a disorganized localization (**Fig. 3B, C**). The relative area of fluorescence of the tissue, positively stained for collagen type I, was 22.6 ± 5.1 % and for collagen type III – 18.1 ± 3.2 %. The ratio of collagens I/III was 1.25. The obtained data testify to the presence of degenerative-dystrophic processes in the tendons of animals in the control group.

In the Achilles tendons of the animals treated with AT-MMSCs, an increase in the number and intensity of staining of cells in the site of the pathological changes was observed compared to control group (**Fig. 3 D**). In the structure of tendon fibers, we observed improved clarity of contours, reduced waviness, regular localization of collagen types I and III production sites and their intensive fluorescence (**Fig. 3 E, F**). The relative area of the tendons to be positively stained for collagen type I was 51.5 ± 6.1 % and for collagen type III – 26.7 ± 2.1 %. The ratio of collagens I/III was 1.93, which was 1.5 times higher than that of the control group.

After the injection of cells of AT-MMSCs+BMP-12 group in the damaged areas, an increase in the number of cells, a decrease in waviness and a disorganization of tendon fibers that had regular, intense staining for collagen type I and III in the periphery and center of the sections was noted (**Fig. 3 H-I**). The relative area of the fluorescence of the tendon tissue positively stained for collagen type I was 59.7 ± 5.8 % and for collagen type III – 28.2 ± 3.13 %. The ratio of collagens I/III was 2.12, that is, it was 1.7 times higher than index in the control group.

According to the literature, type I collagen provides the tissue framework strength, and collagen type III provides its elasticity. Based on the results of the study of qualitative proportion and relative area of the sites of the collagen production in the zone of the degenerative and dystrophic process of the Achilles tendons in the animals of the control and experimental groups, it can be concluded that the therapy with both AT-MMSCs and AT-MMSCs+BMP-12 activates collagen synthesis in tendon tissue.

The next stage of the work was the morphometric study of histological sections of the tendons of animals with tendinopathy after the treatment with AT-MMSCs and AT-MMSCs+BMP-12 (**Fig. 4**). In the control animals, a low density of cells in the tendon tissue was observed. In animals with the injection of AT-MMSCs, this index was 1.8 times higher in comparison with control group. In animals with the injection of AT-MMSCs+BMP12, the density of cells in the tendons tissue was 2.6 times higher relative to control. It should be noted that in the sections of animals with AT-MMSCs+BMP-12 treatment this index was higher in comparison with AT-MMSCs as well. These changes in Achilles tendons of animals after cell therapy characterize high proliferative activity of cells in the sites of injury.

To confirmation the detected qualitative changes, additionally, measurements of the Achilles tendon strength for the rapture were performed.

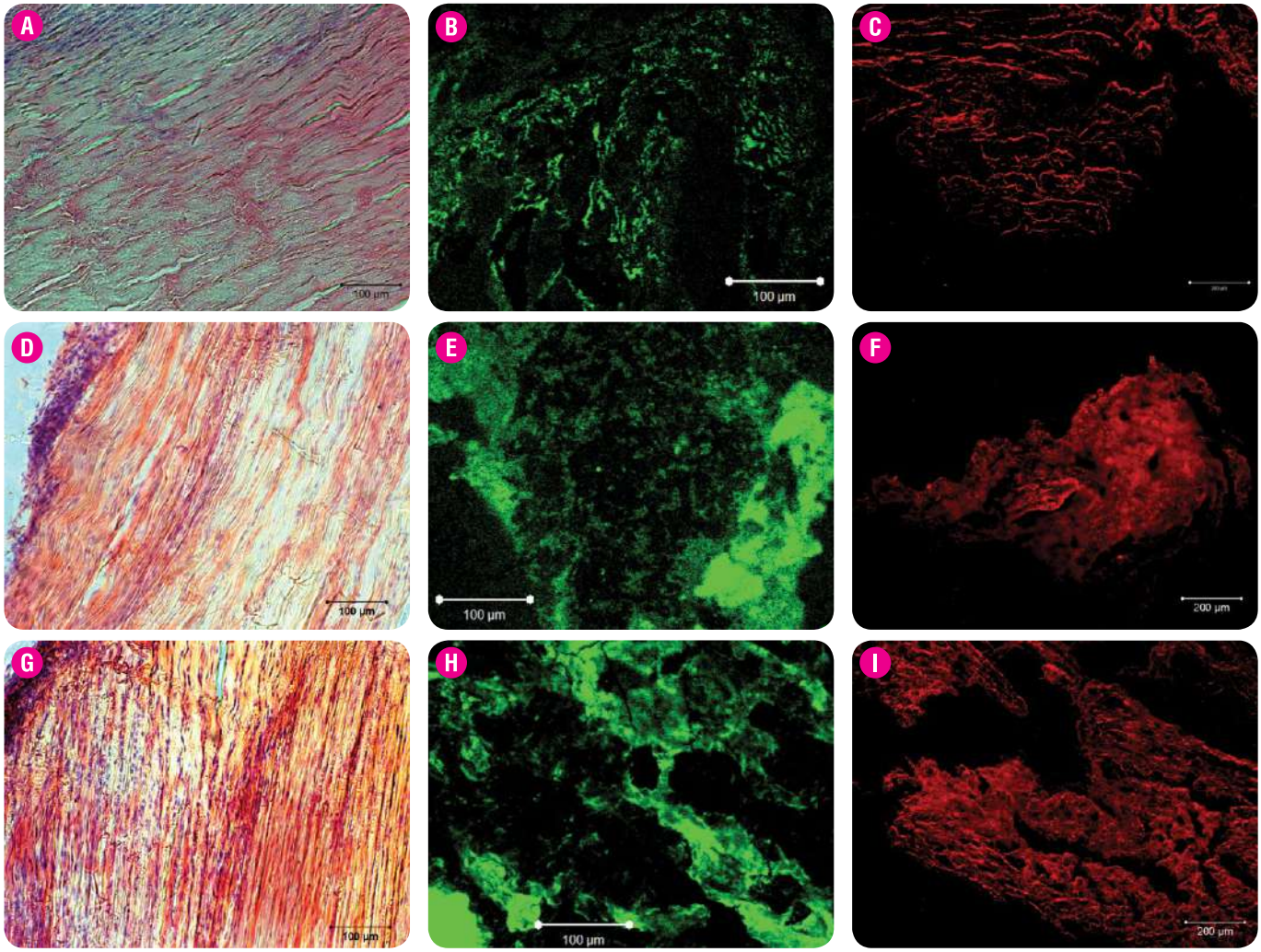

Fig. 3. Microphotographs of Achilles tendons in rats, 21 days after the injection of cells. Control (**A-C**), AT-MMSCs (**D-F**) and AT-MMSCs+BMP-12 (**G-I**) groups. Light microscopy (**A, D, G**), hematoxylin and eosin staining. Fluorescence microscopy, immunohistochemical staining for collagen type I (**B, E, H** – green) and collagen type III (**C, F, I** – red).

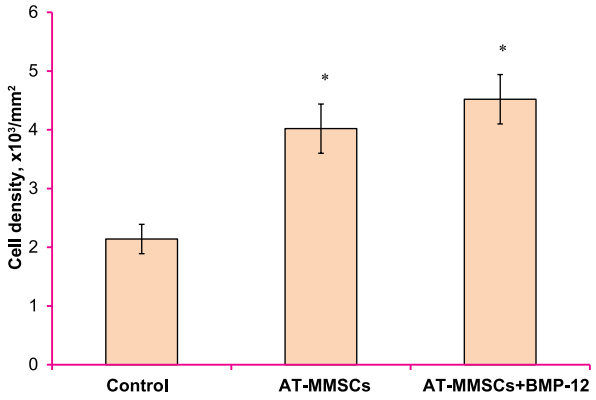

Fig. 4. Cell density on histological sections of rat Achilles tendons on the 21st day after the treatment with AT-MMSCs (M ± m, n = 15).

*Note: * – the difference is significant compared to control (p < 0.05).*

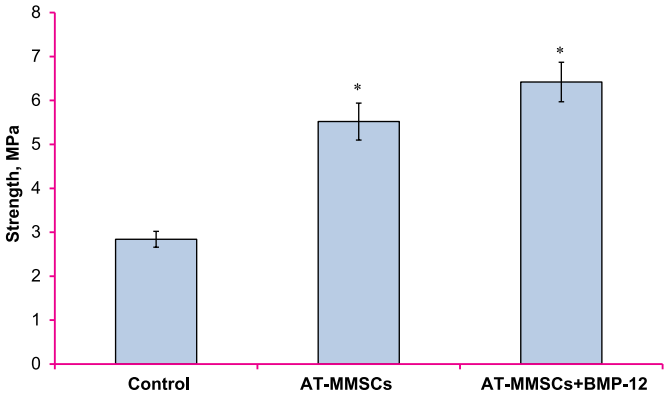

Fig. 5. The strength of the Achilles tendons for the rupture of rats on the 21st days after the treatment with AT-MMSCs (M ± m, n = 15).

*Note: * – the difference is significant compared to control (p < 0.05).*

The obtained data according to the general trend corresponded to the above results of histological, morphometric and immunofluorescence studies (**Fig. 5**). The strength index of the tendons in the group treated with AT-MMSCs was 1.9 times higher, and in the group with the administration of AT-MMSCs+BMP-12 – 2.4 times higher compared with the control group.

Potential use of growth factors and differentiation in cell therapy requires a preliminary study of their effects on cellular characteristics such as morphology, proliferation and synthetic processes. The results obtained in our work have shown that the use of BMP-12 in the culture of AT-MMSCs inhibited proliferation and increased the synthetic activity of cells. It is well known that the processes of differentiation in such tissues as bones, muscles, cartilage or tendons are associated with a significant decrease in the proliferative activity of stem cells.

In the study Violini S. et al. have shown that the use of BMP-12 stimulates the tenogenic differentiation of bone marrow MMSCs, which was manifested in increasing the expression of tendon tissue markers, including tenomodulin and decorin [22]. However, it should be noted that the effect of BMP on cell proliferation varies according to cell types and different types of this factor [29, 30]. According to a number of authors [25], the use of BMP-12 did not affect the migration activity of the MMSCs and led to the activation of tenogenic differentiation, secretory activity, and decreased immunomodulatory potential, which is an important point for substantiating the method of cell administration for therapeutic purposes.

Experimental data of histological, morphometric, immunofluorescence and biomechanical research presented in our study showed that the injection of AT-MMSCs and AT-MMSCs cultured with BMP-12 into the Achilles tendon with degenerative and dystrophic changes contributed to the normalization of the structural and functional organization of tendon tissue, production of collagen types I and III and had a positive effect on the strength index of tendons compared to the control group. The demonstrated difference between the control and experimental groups indicates the ability of AT-MMSCs to improve the recovery processes in the affected tendons. It should be noted that the intensity of the regenerative and reparative processes under the impact of the AT-MMSCs was less pronounced in comparison with the AT-MMSCs cultured with BMP-12. However, there remains a question about the long-term effects of cell therapy, in particular, the duration of inhibition of the degeneration and dystrophy as well as the possible needs for repeated application of cells.

CONCLUSION

The application of BMP-12 in the culture of cryopreserved AT-MMSCs decreased their proliferative activity and increased the production of collagen types I and III. Local administration of AT-MMSCs and AT-MMSCs cultured with BMP-12 stimulates the reparation and regeneration in damaged tendons in animals with tendinopathy. The application of AT-MMSCs cultured with BMP-12 compared with non-treated MMSCs is more effective in restoring the histological structure, strength, the content of collagen types I and III, and their proportion in the rat Achilles tendons with the model of destructive and degenerative injury. The results of our study can be used for development and validation of new treatments of degenerative and dystrophic lesions of tendons for clinical application.

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