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Effects of transplantation of preconditioned mesenchymal stem cells seeded on a biomimetic 3D scaffold

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Aim. The study aims to determine the effects of transplantation of preconditioned human umbilical cord mesenchymal stem cells (hUC-MSC) seeded on a biomimetic 3D scaffold. **Methods.** Porous collagen 3D scaffolds were prepared by freeze-drying collagen type I solutions. hUC-MSC were isolated by the explant method. The hUC-MSC were preconditioned with H_2O_2 or LPS for 24 hours, and the cells were seeded onto 3D collagen scaffolds. The effects of MSC preconditioning were studied on the xenogenic transplantation model. The scaffolds isolated five days after implantation were analyzed histologically and by the PCR-based detection of human-specific sequences in genomic DNA extracted from animal tissues. **Results.** The developed porous collagen scaffolds were able to support the adhesion and growth of hUC-MSC *in vitro*. The *in vivo* pro-angiogenic activity was mostly pronounced in hUC-MSC preconditioned with hydrogen peroxide compared to both native and LPS preconditioned hUC-MSC. The data from PCR analysis demonstrated prolonged retention of human DNA in the samples isolated from animals implanted with hUC-MSC preconditioned by low doses of hydrogen peroxide. That might suggest the prolonged survival of the cells in scaffolds upon implantation. **Conclusions.** hUC-MSC preconditioning with low doses of hydrogen peroxide increases their therapeutic potential.

K e y w o r d s: MSC, preconditioning, biomimetic 3D scaffolds, angiogenesis

Introduction

Mesenchymal stem cells (MSC) are the most widely used multipotent stem cells in regenerative therapy. For the last 30 years, they passed from laboratory studies to clinical trials.

Numerous clinical trials utilizing MSC for the treatment of various human diseases are conducted worldwide [1, 2]. However, despite the substantial progress in the field, only ten MSC-based therapeutics were approved for market entry by regulatory authorities worldwide [2]. The results of the preclinical and clinical trials indicated that the main problems, which impeded the successful realization of the therapeutic potential of MSC, were the transient retention within the target tissue due to the quick mechanical washout of the cells from the administration zone by the blood and lymph fluid and poor graft viability; ischemic and pro-inflammatory microenvironment, unfavorable for the cells survival and proliferation; a destructive influence of the oxidative stress, and induction of the anoikis due to the loss of the cells contact with the extracellular matrix [3]. To overcome these obstacles, several strategies for the enhancement of the MSC therapeutic effects are being developed. The MSC preconditioning during multiplication *ex vivo* and utilization of the 3D carriers for the MSC implantation are among the most promising ones. The MSC preconditioning is considered cultivation under the decreased concentration of oxygen (hypoxia), incubation with pharmacological/chemical agents or tropic factors/cytokines, gene modification, and preconditioning with physical factors [4]. The use of 3D scaffolds increases the MSC survival upon implantation by providing temporary mechanical support to the cells, thus preventing anoikis and preserving the cells and the products secreted by MSC, facilitating the prolongation of the therapeutic effects. Noteworthy, that in our previous studies, we demonstrated that the implantation of the human umbilical cord mesenchymal stem cells (hUC-MSC) on a 3D collagen scaffold increased the survival of the cells within the host organism even in the case of the xenogeneic transplantation [5]. Therefore the combination of these two approaches, namely preconditioning and use of 3D scaffolds, may substantially increase the therapeutic effects of hUC-MSC.

Thus, this study aimed to determine the effects of transplantation of preconditioned human umbilical cord mesenchymal stem cells seeded on the biomimetic 3D scaffold.

Materials and Methods

hUC-MSC Isolation and Cell Culture. hUC-MSC were isolated from umbilical cords of healthy donors (39–40 weeks gestation) by the explant method described earlier [6]. According to the minimal criteria for defining multipotent mesenchymal stromal cells, the CD90, CD73, CD105, CD34 and CD45 surface markers expression was verified on the second passage by FACS analysis (BD FACS Aria, USA), using the FITC Mouse Anti-Human CD90 (#561969), APC Mouse anti-Human CD73 (#560847), PerCP-Cy™5.5 Mouse anti-Human CD105 (#560819), FITC Mouse Anti-Human CD45(#555482), APC Mouse Anti-Human CD34 (#555824) as described in [7]. The ability of the cells to undergo osteogenic, adipogenic, and chondrogenic differentiation was assessed previously [7]. After the cultivation on the 3D collagen scaffolds, the cells were analysed for retention of expression of CD90, CD73, CD105.

Collagen scaffold preparation. The porous collagen scaffolds were produced from a solution of bovine atelocollagen in acetic acid

using a freeze-drying technique described previously [8]. Briefly, the solution of type I atelocollagen, isolated from a bovine tendon, of 20 mg/mL in 0.5 M acetic acid was prepared. The collagen solution was then frozen in glass Petri dishes (10 cm in diameter) at –40 °C and held for 18 h using a freeze-dryer. Under vacuum, the frozen suspensions were subsequently sublimed at –40 °C to +22 °C for 24 h. The atelocollagen scaffolds were stored at –20 °C for future use. Prior to usage, the scaffolds were brought to +22 °C, cut into rectangles under the sterile laminar hood, and sterilized by UV exposure for 40 min. The scaffolds were equilibrated in 0.1 M HEPES pH 8.0 solution at +4 °C overnight. Afterward, HEPES solution was changed with DMEM/high glucose medium containing 10 % (vol/vol) of FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The scaffolds were equilibrated for 48 hours, +37 °C, 5 % CO_2 before cell seeding.

Confocal Laser Scanning Microscopy (CLSM). Confocal laser scanning microscopy (CLSM) analysis was performed using LSM 510 META Confocal system with Inverted Axiovert 200 M with Carl Zeiss Objectives (Carl Zeiss, Germany). 24 and 144 hours after hUC-MSCs seeding on the 3D collagen scaffold, the samples were fixed in 4 % paraformaldehyde in PBS for 24 h, +4 °C. The staining was performed with propidium iodide (PI).

hUC-MSC preconditioning. Short-term exposure to a low concentration of H_2O_2 was applied to obtain H_2O_2-preconditioned hUC-MSC. Briefly, hUC-MSC were exposed to 30 μM of H_2O_2 for 24 hours, 37 °C, 5 % CO_2. Short-term exposure to a low concentration of LPS (*E. Coli* O111:B4, Sigma-Aldrich, USA) was applied to obtain LPS-preconditioned hUC-MSC. Briefly, hUC-MSCs were exposed to 100 ng/mL of LPS for 24 hours, 37 °C, 5 % CO_2. Non-preconditioned hUC-MSC, obtained from the same donor, were cultured in parallel for the same number of passages. Twenty-four hours later, the culture medium was removed, and hUC-MSC were washed three times with PBS, pH 7.4. After preconditioning, $1x10^5$ cells were seeded on 0.5x0.5x0.2 cm porous scaffold. The cell-seeded scaffolds were transferred into a fresh complete culture medium and incubated at +37 °C, 5 % CO_2 for 18 hours. Before implantation, scaffolds with cells were washed three times in sterile PBS pH 7.4.

3D scaffold implantation and histological staining. The experiments were conducted on male mice of BALB/c line (breeding of IMBG, NAS of Ukraine), 2 months old. All the manipulations with animals were performed using sedative and anesthetic preparations in compliance with the requirements of veterinary legislation. The subcutaneous implantations of developed 3D scaffolds were performed on the dorsal region of BALB/c mice by small skin incisions (0.8–1 cm). The scaffolds were resected 5 days after the surgeries. The scaffolds with the adjacent tissue were fixed in 10 % formalin and dehydrated through increasing ethanol concentrations (70 % to 96 %) and dioxane. The samples were immersed in paraffin and used to prepare paraffin blocks. 10 μm paraffin sections were prepared on a sledge microtome. The sections were stained by hematoxylin and eosin. For the morphometric analysis of the histological elements, the images of sequential sections of each scaffold were obtained. In total,

15 sections per scaffold were analysed. The number of vessels was counted by analyzing 25 fields of view (with a total surface area = 5134,275 μm^2 or 205,371 μm^2/field of view) on a section of the collagen matrix according to [9].

Isolation of DNA. Genomic DNA from the scaffolds was isolated, as previously described [10]. The DNA concentration and purity were determined using the NanoDrop 2000 UV-Vis spectrophotometer (ThermoFisher Scientific, USA).

PCR. To detect transplanted human cells in mice, the polymerase chain reaction analysis was performed using primers to amplify human-specific 850bp fragment of the alpha-satellite DNA on human chromosome 17 [11]. PCR was performed as described before [5]. Each reaction contained 100 ng of genomic DNA template in 20 μl of the reaction mixture. DNA from cultured hUC-MSC served as a positive control, and DNA from an untreated animal was used as a negative control. Oligonucleotides were used for PCR as described by Warburton *et al.* [12]:

Cr17_1a f (5'-GGG ATA ATT TCA GCT GAC TAA ACA G-3 ') 15..39

Cr17_2b r (5'TTC CGT TTA GTT AGG TGC AGT TAT C-3 ' 867..891.

The amplification was performed on DNA-amplifier Tercik (DNA-technologies, RF) using the following scheme: 95 ºC, 3 min; one cycle at: 94 ˚C, 1 min, 58 ºC, 1 min; 72 ºC, 1 min, followed by 34 cycles: at 94 ˚C for 0.5 min, 58.5˚C for 0.5 min, 72˚C for 0.5 min, and 72˚C for 5 min.

Separation of amplification products was performed in 1.2 % agarose gel in TAE buffer with subsequent visualization under ultraviolet light after ethidium bromide staining. GeneRuler 1000 bp DNA Ladder (ThermoScientific, USA) was used as a PCR product fragment length marker.

Statistical data analysis. Statistical significances were determined with the use of GraphPad Prism software, Version 8.0.1. Comparisons between multiple groups were evaluated via one-way analysis of variance (ANOVA) followed by Tukey's test. For all tests, $p < 0.05$ was considered significant.

Results and Discussion

Over the past years, transplantation of autologous or allogenic MSC has been demonstrated to have profound therapeutic effects in the treatment of pathologies of different genesis, both in experimental and clinical studies. However, many clinical trials revealed reduced efficiency of MSCs-based therapeutics compared to that demonstrated in experimental studies. Many notable efforts have been made to optimize the efficiency of MSC delivery systems aiming at the increase of the cell's survival and retention within the damaged tissue [13]. After transplantation, MSC are exposed to a new microenvironment *in vivo* that can lead to irreversible cell changes, often resulting in cell death. Transplantation of MSC seeded onto 3D scaffolds developed by different techniques (3D printing, leaching, melt molding, freeze-drying, *etc.*) from natural and synthetic polymers allows stabilizing of the cells and prolonging their survival in the host organism.

In the present work, we used the porous 3D collagen scaffolds, developed using freeze-drying technique described in our previous study [8]. The technique allowed obtaining the

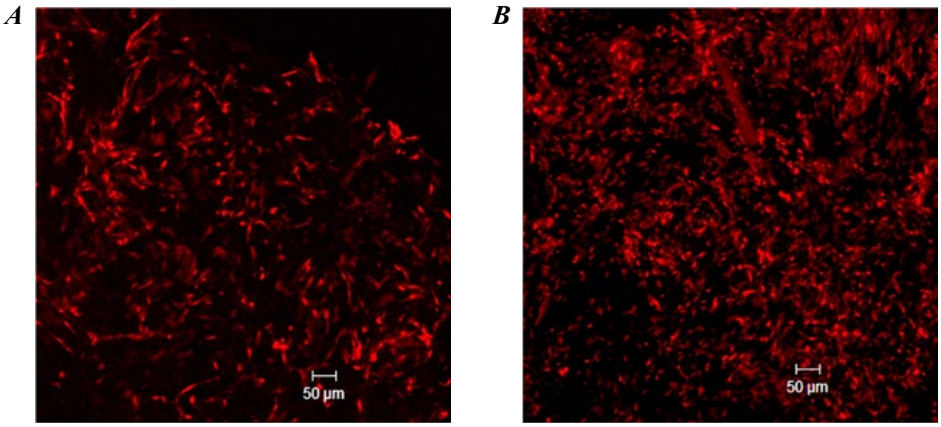
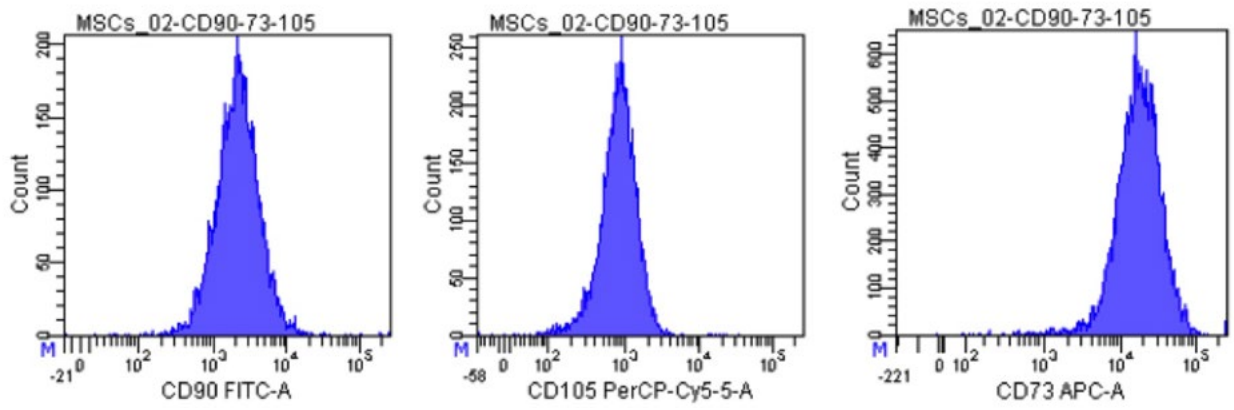


Fig. 1. Representative confocal laser scanning microscopy images of hUC-MSC seeded on the 3D collagen scaffold (24 (***A***) and 144 (***B***) hours past the cell seeding, the samples were fixed with 4 % paraformaldehyde solution and stained with PI (red signal). Scale bar — 50 µm.

scaffolds that had an average pore diameter of 117±57 µm. The internal microstructure of the scaffolds consisted of multi-layered porous sheets with an average distance between the layers of 91±50 µm. The scaffolds were capable of supporting the adhesion and growth of HEK293 cells. However, despite the presence of the RGD domains on the collagen-based scaffolds that mediates the attachment of the cells [14], the properties of the scaffold (i.e., stiffness, topography, surface hydrophobicity, and charge, *etc.*) can substantially influence the properties of the MSCs [15]. Thus we investigated the ability of developed 3D collagen scaffolds to support hUC-MSC adhesion and proliferation. Our results suggest that hUC-MSC are attached to the scaffolds (Fig. 1). The cells were actively proliferating as an increase in cell numbers was observed by confocal laser scanning microscopy six days after seeding, as compared to the 1 day.

The results of FACS analysis of the MSC characteristic CD expression (CD90, CD73, CD105: $CD90^+$ — 97.9±0.9 %, $CD73^+$ — 97.6±1.5 %, $CD105^+$ — 99.2±0.6 %, $CD90^+CD73^+$ — 98.1±1.6 % from that 97.9±1.6 $CD90^+CD73^+CD105^+$, (n = 3)) demonstrated that the cells retain the immunophenotype, characteristic of hUC-MSC, after cultivation on 3D collagen scaffolds for 6 days (Fig. 2).

Preconditioning of MSC by exposure to different physical, chemical/pharmacological agents and various cytokines/chemokines is considered a promising strategy to increase the therapeutic efficiency of the cells. Aiming at the increased survival of transplanted MSCs, their resistance to oxidative stress, and enhancement of anti-inflammatory properties, we selected two factors for cell preconditioning: hydrogen peroxide and bacterial lipopolysaccharide (LPS). Preconditioning with each of the selected factors was proven to enhance the anti-inflammatory properties of the MSC. As demonstrated previously, preconditioning of hUC-MSC with low doses of H_2O_2 significantly increases their anti-inflammatory properties [16] and survival [17, 18]. LPS preconditioning of MSC has been demonstrated to promote the ability of the latter to modulate the balance of macrophages through the up-regulation of the expression of the anti-inflam-



Population	#Events	%Parent
All Events	14,187	####
cells	9,818	69.2
dd-SSC	7,845	79.9
dd-FSC	6,944	88.5
CD90	6,904	99.4
CD105	6,802	98.0
CD73	6,924	99.7
CD90+73+	6,902	99.4
CD90+73+105+	6,769	98.1

Fig. 2. Flow cytometric characterization of hUC-MSC after 6-days cultivation of 3D collagen scaffold. The figures are representative of 3 independent experiments.

matory cytokines and the promotion of M2 macrophage activation [19].

Besides the pro-inflammatory microenvironment, another major obstacle to successful transplantation, particularly when injecting the MSCs into ischemic tissue or transplantation of multi-layered 3D constructs, is insufficient vascularization of the tissue or graft. Preconditioning of MSC with non-toxic concentrations of hydrogen peroxide was demonstrated to upregulate the accumulation of hypoxia-inducible factor-1α (HIF-1α) — one of the key regulator factors of angiogenesis by the cells [20]. Also, there is an evidence that MSC induced the synthesis of VEGF in H9c2(2–1) myoblasts after preconditioning with LPS [21]. So both factors, selected for the preconditioning, may stimulate MSC to facilitate the vascularisation of the graft.

Thus, the primary endpoints in this study were the difference in pro-angiogenic effects *in vivo* of the hUC-MSC seeded on the biomimetic collagen 3D scaffold and the survival of the cells after transplantation in the xenogeneic system.

To test the effects of hUC-MSC preconditioned for 24 hours with 30 μM of H_2O_2 (group 4) or with 100 ng/mL of LPS (group 3), the cells were seeded onto 3D collagen scaffolds and implanted subcutaneously into immunocompetent BALB/c mice. The scaffolds seeded with unmodified hUC-MSC (group 2) and without cells (group 1) were used as comparison groups. The scaffolds were resected

from the implantation site 5 days later and processed for histological analysis.

The histological analysis of the samples from group 1 implanted with empty scaffolds revealed the absence of the inflammatory reaction to the implant, the lack of implant engraftment into surrounding tissues, and the absence of any manifestation of angiogenesis (Fig. 3, A1). The scaffold colonization by the host cells was observed only on the periphery of the implant. The cell populations were mainly represented by segmented and rod-shaped neutrophils, eosinophils, lymphocytes, and single fibroblasts (Fig. 3, A2). The cells were almost absent in the central part of the implant.

The analysis of the samples from group 2, implanted with hUC-MSC seeded scaffolds, revealed partial implant engraftment into surrounding tissues. A significant number of vessels at the formation stage were detected (Fig. 3, B1). The formation of the vessel wall by pericytes was observed within the pores of the scaffold (Fig. 3, B2). The cells were detected not only on the periphery of the scaffold as in group 1 but also those that migrated evenly throughout the matrix: lymphocytes, segmented neutrophils, eosinophils, single fibroblasts, *etc.*

The samples from the group implanted with LPS-preconditioned hUC-MSC seeded scaffolds (group 3) showed the implant engraftment into surrounding tissues. Inflammatory reactions and necrotic processes were not observed, and there were no vessels and their initial stages of formation compared to group 2 (Fig. 3, C1). The migration of the cells into the scaffold was observed, but it was more profound compared to the group with unmodified hUC-MSC. The cell population was represented by fibroblasts, rod-shaped neutrophils, macrophages (Fig. 3, C2).

In group 4, implanted with hydrogen peroxide preconditioned hUC-MSC seeded on the 3D collagen scaffold, complete engraftment of the implanted scaffold was observed. The results of the histological analysis of the samples provided proof of active angiogenesis (Fig. 3, D1). The inflammatory necrotic processes were absent and fully formed blood vessels were visible. A profound migration of various cell types throughout the matrix in the collagen network is noted: fibroblasts, eosinophils, rod-shaped and segment neutrophils, and a large number of fibroblast-like cells, most likely hUC-MSC (Fig. 3, D2).

Histological morphometric measurements revealed a significant difference in the average blood vessel counts in implants seeded with hydrogen peroxide preconditioned hUC-MSC (group 4) compared to the implants from other groups (Fig. 4).

Thus, it could be concluded that the proangiogenic activity was most pronounced in hUC-MSC preconditioned with hydrogen peroxide. A large number of blood vessels, both formed and at the initial stages of vessel formation were observed in the samples from this group. The increased proangiogenic activity can be attributed to the upregulation of HIF-1α accumulation in MSC preconditioning with non-toxic concentrations of hydrogen peroxide, which in turn leads to the increase of the production of angiogenic cytokines: VEGF, stromal-derived factor 1, placental growth factor, angiopoietin 1, angiopoietin 2, and platelet-derived growth factor (PDGF) [22].

The presence of the hUC-MSC within the scaffolds from this group can be the proof of better survival of the cells after short-term

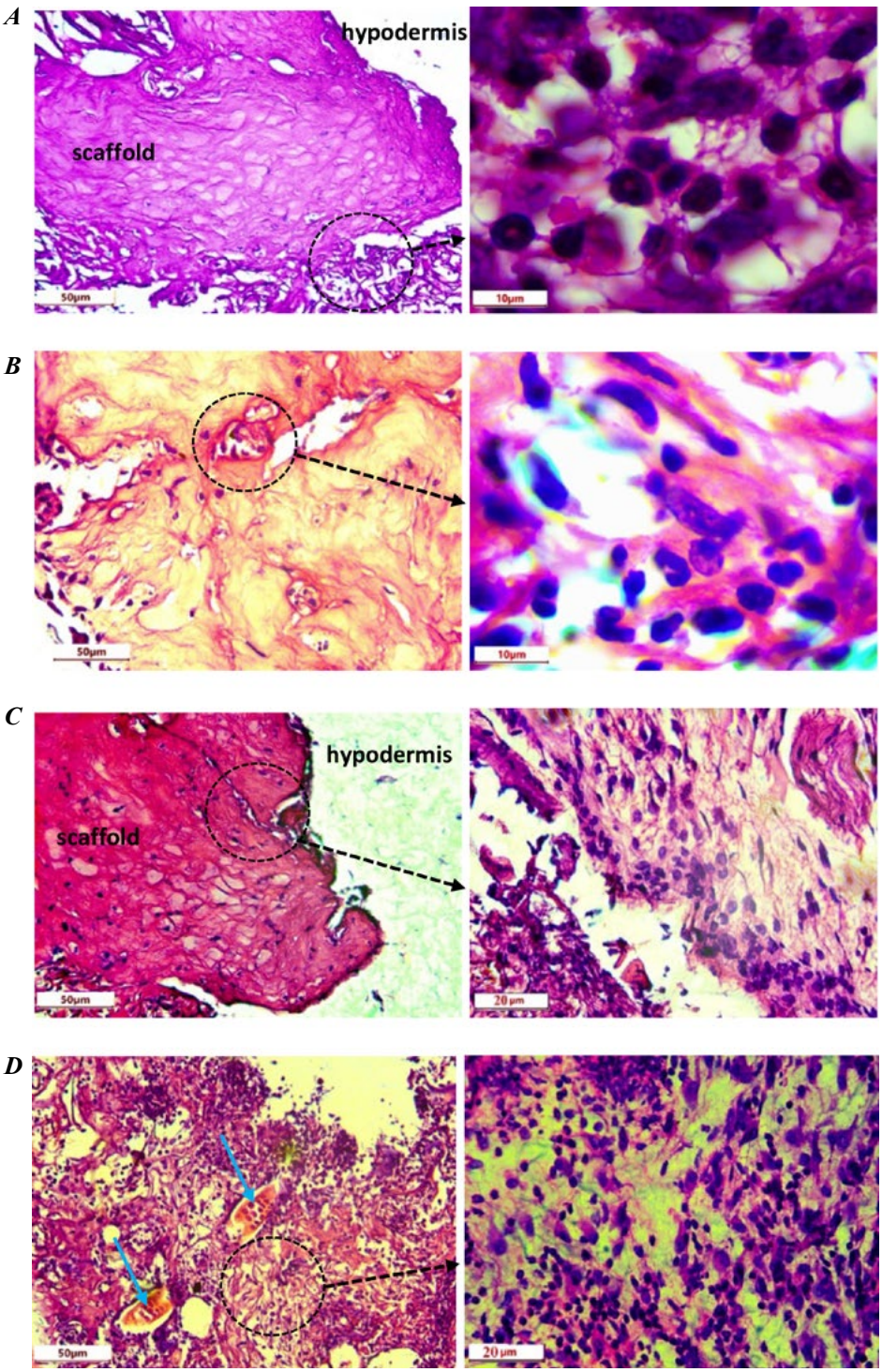


Fig. 3. Histological analysis of hUC-MSC-seeded 3D collagen scaffolds after 5 days of implantation in BALB/c mice. ***A*** — group 1, implanted with empty 3D collagen scaffolds; ***B*** — group 2, implanted with 3D collagen scaffolds seeded with native hUC-MSC; ***C*** — group 3, implanted with 3D collagen scaffolds seeded with LPS preconditioned hUC-MSC; ***D*** — group 4, implanted with 3D collagen scaffolds seeded with hydrogen peroxide preconditioned hUC-MSC. Ehrlich's haematoxylin-eosin staining. Blood vessels formed in collagen scaffold implanted under the skin of a mouse are indicated by blue arrows.

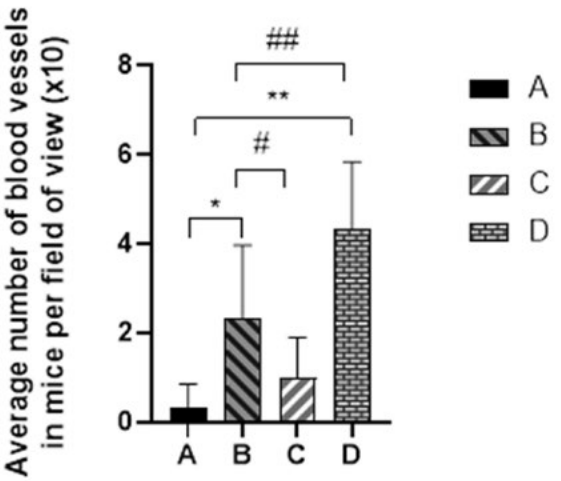


Fig. 4. Effect of 5-days implanted hUC-MSC-seeded 3D collagen scaffolds on the blood vessel development in BALB/c mice. The surface area of one field of view (with 10x objective) was 205.4 μm^2. ***A*** — group 1, implanted with empty 3D collagen scaffolds; ***B*** — group 2, implanted with 3D collagen scaffolds seeded with native hUC-MSC; *C* — group 3, implanted with 3D collagen scaffolds seeded with LPS preconditioned hUC-MSC; *D* — group 4, implanted with 3D collagen scaffolds seeded with hydrogen peroxide preconditioned hUC-MSC. * $p < 0.05$ — (***A***) group 1 vs. (***B***) group 2; ** $p < 0.05$ — (***A***) group 1 vs. (***D***) group 4; $^{\#}p < 0.05$ — (***B***) group 2 vs. (***C***) group 3;$^{\#\#}p < 0.05$ — (***B***) group 2 vs. (***D***) group 4.

preconditioning with low doses of hydrogen peroxide.

To further study the retention of hUC-MSC within the implanted scaffolds, we determined the presence of the human-specific alpha-satellite DNA by PCR analysis. The method has high sensitivity due to the highly repetitive alpha-satellite sequence. The sensitivity of the PCR was approximately one human cell per 10^5 of murine cells [5]. As seen from the results, alpha-satellite sequences of human DNA were detected only in the samples obtained from animals in group 4 (Fig. 5). The samples obtained from animals in group 1–3 were negative for human DNA.

Thus, the data of PCR analysis demonstrated the prolonged retention of the human DNA in samples of 3D collagen scaffolds seeded with hUC-MSC preconditioned by low-dose hydrogen peroxide, that might indicate the prolonged survival of the cells *in vivo* in a xenogeneic system. On the contrary, preconditioning with LPS did not demonstrate any pro-angiogenic activity or increase in the survival of the cells *in vivo*.

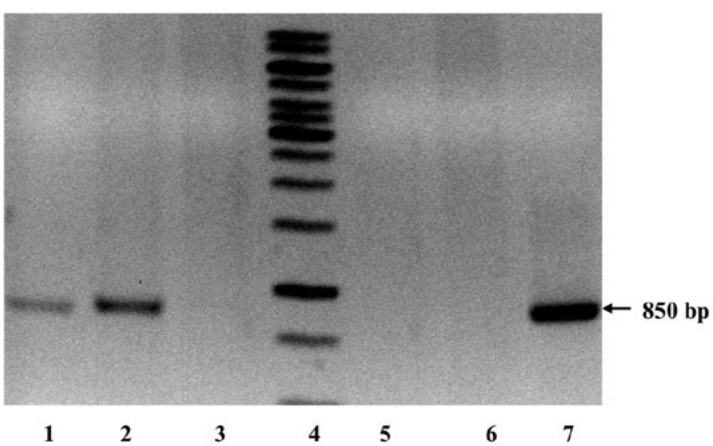


Fig. 5. The PCR results for the presence of human alpha-satellite DNA in sections of implanted 3D collagen scaffold seeded with hUC-MSC (the scaffolds isolated 5 days after subcutaneous implantation into BALB/c mice). *1* — positive control (isolated from human MSC- 500 pg); *2* — DNA, isolated from scaffolds of group 4; *3* — DNA, isolated from scaffolds of group 3; *4* — molecular weight marker, 1000 bp DNA Ladder (ThermoFisher, USA); *5* — DNA, isolated from scaffolds of group 2; *6* — DNA, isolated from the scaffolds of group 1; *7* — positive control (DNA of hUC-MSC — 5 ng). The amount of DNA in the reaction mixture — 100 ng.

Conclusions

The data described in the present article allow us to conclude that preconditioning of hUC-MSC with low doses of hydrogen peroxide seeded on the extracellular matrix-mimicking (biomimetic) 3D scaffold increases pro-angiogenic properties of the cells and seems to increase the survival of the engrafted cells. This facilitates the engraftment of the transplant into surrounding host tissues, enhance the resistance of MSC to the unfavourable environment and prolong their survival within the damaged tissue, which can potentially lead to the increase of their therapeutic potential.

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Ефекти трансплантації прекондиціонованих мезехімальних стовбурових клітин на біоміметичному 3D скефолді

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Мета. Метою дослідження було вивчення ефектів трансплантації прекондиціонованих мезенхімальних стовбурових клітин пуповини людини (МСК) на біоміметичному 3D скефолді. **Методи.** Пористі колагенові 3D скефолди отримували шляхом ліофільної сублімаційної сушки розчину колагену I типу. МСК пупкового канатику виділяли методом експлантів. МСК прекондиціонували обробкою розчинами перекису водню чи ліпополісахариду протягом 24 годин, після чого клітини висівали на 3D колагеновий скефолд. Ефекти прекондиціонованих МСК вивчали на моделі ксеногенної трансплантації. Для проведення гістологічного аналізу та для проведення ПЛР-аналізу наявності специфічних для людини послідовностей в геномній ДНК, ізольованій з тканин тварин, скефолди видаляли на п'яту добу після імплантації. **Результати.** Отримані пористі колагенові скефолди підтримували адгезію та ріст МСК пуповини людини *in vitro*. Проангіогенна активність *in vivo* була найбільш вираженою у випадку імплантації МСК прекондиціонованих перекисом водню порівняно із немодифікованими та прекондиціонованими ЛПС МСК пуповини людини. Дані ПЛР аналізу продемонстрували більш тривале збереження ДНК людини у зразках, отриманих від тварин, яким було імплантовано МСК пуповини, прекондиціоновані низькими дозами перекису водню. Це може вказувати на більш тривале виживання цих клітин у скефолді після імплантації. **Висновки.** Прекондиціонування МСК пуповини людини низькими дозами пероксиду водню підвищує їх терапевтичний потенціал.

Ключові слова: МСК, прекондиціонування, біоміметичний 3D скефолд, ангіогенез

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