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Analyzing the Effectiveness of Adipose Tissue Stem Cell and Microvesicle Therapy in Premature Skin Aging Caused by Chronic Exposure to Ultraviolet Radiation

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Abstract
Adipose-derived mesenchymal stem cells (ADSCs) and microvesicles (MVs) isolated from ADSCs are promising therapeutic agents for various pathological and physiological skin conditions. Investigate the effects of ADSC and MV therapy on skin regeneration under photoaging conditions in vivo. CD-1 mice were exposed to a course of UV radiation exposure for 6 weeks. After 4 weeks, the animals were injected by multiple intradermal punctures with ADSCs, MVs, or PBS. To analyze the effectiveness of ADSCs and MVs, changes in the microcirculation within the dorsal skin of the mice was assessed using laser Doppler flowmetry. Morphometric and morphological assessment of histological changes were also performed. No differences in skin perfusion were identified at 4 weeks post-injection of ADSCs and MVs. However, histological analysis showed treatment with ADSCs and MVs both led to a decrease in UV-mediated epidermal thickening and improved organization of the dermal layer. ADSC- and ADSC-MV-based therapy acts to prevent skin damage caused by UV photoaging.

Keywords Photoaging · Skin mice · UV radiation · Adipose-derived mesenchymal stem cells · Microvesicles

1 Introduction

The skin is an important and complex barrier, which performs five primary functions: prevention of dehydration, passive participation in the regulation of gas diffusion, gradient maintenance, body temperature regulation and other types of sensing/body sensitivity [1]. One of the most important functions of the skin is in creating a barrier that protects the body from ultraviolet radiation (UV), a known mediator of skin aging and damage (photoaging) and a cause of DNA mutation and tumor initiation [2, 3]. Photoaging is a manifestation of dryness, peeling, pigmentation disorders, and the formation of deep wrinkles after chronic exposure of skin to UV light [4–6]. Exposure to UV causes a thickening of the epidermis and cellular atypism [7], primarily through the suppression of structural protein procollagen type I synthesis within the connective tissues; this causes the appearance of dermal fibroblasts. In the process, collagenase expression is stimulated, causing degradation of collagen and a reduction in the skin's elasticity [2, 7–9]. These phenomena not only cause a cosmetic defect in skin but can also affect mental well-being and physical health [10]. Currently, many different drugs are been used to treat UV photoaging. However, the use of these drugs is associated with significant side effects, and their effectiveness on the skin is usually temporary or absent [4, 11, 12]. Therefore, there is an urgent need for new, safer, and effective therapeutic methods.

Adipose-derived mesenchymal stem cells (ADSCs) are a promising cellular therapy in regenerative medicine. ADSCs share the same properties as stem cells isolated from the bone marrow, but have the advantage of being more accessible [13, 14]. ADSCs also circumvent the ethical issues associated with the use of embryonic stem cells. Previous studies have shown that ADSCs have positive effects on skin regeneration, such as improved skin survival during autoplasty and antioxidant and whitening effects on hyperpigmented skin, as well as

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stimulating skin regeneration undergoing photoaging [15–17]. These effects may potentially be in part due to the ability of ADSCs to increase transcription of procollagen type I [6, 18]. However, ADSCs are not able to penetrate into the deeper layers of the skin and are inefficient when infused intravenously [19, 20]. Therefore, the use ADSCs is currently limited.

Stem cells are known to secrete a variety of cytokines such as platelet growth factor, transforming growth factor, and major fibroblast growth factor, all of which contribute to the proliferation of fibroblast in the dermis [21]. In addition to cytokines, stem cells also secrete a large number of microvesicles (MVs) [22]. MVs are vesicular structures shed by outward blebbing of the plasma membrane. They are released by many different cell types and contain bioactive molecules such as proteins, lipids, transcription factors, and RNAs. Several studies have shown the critical role of vesicles as mediators of intercellular communication between stem cells and other cell types including damaged cells [23–25]. ADSC-MVs have been demonstrated to have similar functions to ADSCs when used therapeutically, such as repairing damaged tissues and suppressing inflammatory reactions [22]. Use of MVs derived from ADSCs also has significant benefits compared with the use of the original cells. For example, MVs do not contain a nucleus; therefore, they are not able to divide and form a tumor [26, 27]. MVs as well contain a cargo of potential regulatory components; they are also smaller and potentially more easily delivered [26, 28]. Intravenous delivery of MVs has already been shown to deliver positive improvement of the regenerative processes in the skin [20]. In this study, we used an in vivo model to investigate the therapeutic effect of ADSCs and MVs on the skin when undergoing premature aging caused by exposure to chronic UV light.

2 Materials and Methods

Experiments were conducted using 46 CD-1 mice (6 weeks old) with a starting weight of 20–30 g (GMBH "Nursery RAMTN," Moscow, Russia). All animals were acclimatized for 2 weeks before starting the experiment. Animals were kept under standard vivarium conditions in the day/night mode (12 h light/12 h dark), with free access to feed and water. Mice were kept and used for experimental procedures in accordance with the rules accepted by the Kazan Federal University and approved by the Local Ethical Committee (Permit Number 15 of 28.03.2019). Animals were used in accordance with international bioethical standards defined by the *International Guiding Principles for Biomedical Research Involving Animals* (2012), the EU directive 2010/63/EC, and the 3Rs principles.

Animals were randomly divided into 5 groups: (1) mice exposed to UV radiation for 6 weeks (group UV*, $n = 9$); (2) mice exposed to UV radiation received PBS as a placebo (group UV + PBS, $n = 9$); (3) mice exposed to UV radiation and treated with ADSCs (group UV + ADSCs, $n = 9$); (4) mice exposed to UV radiation and treated with MVs isolated from ADSCs (group UV + MV, $n = 11$); (5) the controls were intact animals (group intact, $n = 8$). Depilation was carried out once a week in order to get rid of the dorsal hair area on the experimental animal's skin.

2.1 ADSC Derivation and Culture

ADSCs were derived from the inguinal fold subcutaneous adipose tissue of a healthy female mouse. Adipose tissue enzymatic disaggregation was achieved by incubation with type 1 collagenase (Biolot, Russia) at 37 °C with agitation for 1 h. Pre- and post-incubation, the tissue was washed three times with DPBS solution (Dulbecco's Phosphate-Buffered Saline, Paneco, Russia) containing 5% of penicillin and 5% of streptomycin and centrifugation at 100 g for 5 min at room temperature. The cell pellet was resuspended and plated in culture in αMEM (Alpha Minimum Essential Medium, Invitrogen, USA) with 10% of FBS (Fetal Bovine Serum, Sigma, USA). Cultures were passaged and expanded in αMEM with 10% of FBS, 2 mM of L-glutamine (Sigma, USA), and penicillin and streptomycin (100 U/ml; 100 μg/ml) (Sigma, USA) using a MCO-15AC incubator (Sanyo, Japan) at 5% CO_2 and 37 °C. In the experiment, we used ADSCs that reached 3–4 passages.

2.2 MV Extraction

MVs were isolated from ADSC culture using the established methods of Gomzikova et al. [29]. Briefly, when cultures reached 90% confluency, the growth medium was removed and cells dissociated with 0.25% trypsin-EDTA solution (PanEco, Russia). Following dissociation, cells were washed in PBS and resuspended in DMEM without FBS (1×10^6 ADSCs/ml), containing 10 μg/ml cytochlozin B (Sigma-Aldrich, USA), and incubated for 30 min at 37 °C in a humid atmosphere containing 5% CO_2. After 10 min, cells were centrifuged at 100*g* for 10 min and the supernatant removed. The supernatant was further centrifuged at 100*g* for 10 min and then at 2000*g* for 15 min to precipitate the MVs. The supernatant was discarded and the MV pellet resuspended in PBS (1×10^6 MVs (equal in biomass 1×10^6 ADSCs) in 500 μl PBS).

2.3 Irradiation

The light source used to mimic photo aging was a fluorescent UVB lamp model PHILIPS TL/12 40W RS (MEDICAL-NETHERLANDS). The lamp emits radiation in the range of 270 to 400 nm with a peak emission at 313 nm. The dose of irradiation used was 4.14 J/cm^2 at a distance of 20 cm from the

lamp. Mice were treated for 5 consecutive days per week for 20 min/day in week 1, 30 min/day in week 2, and 40 min/day for weeks 3 to 6.

2.4 Doppler Flowmetry

Changes in the blood microcirculation within the dorsal skin was performed prior to the start of the experiment and after 6 weeks of UV irradiation (4 weeks after therapy) for groups intact, UV and UV + ADSCs, UV + MV, and UV + PBS groups. The density of the capillary system was visualized by volume of blood flow using Easy-LDI laser Doppler flowmetry (AIMAGO, Switzerland) in real time. Data processing was done using Easy LDI Studio software.

2.5 ADSC- and MV-Derived Therapy

After exposure of the mice to UV, a resting interval of 4 weeks was maintained. Thereafter, the dorsal skin was treated with multiple intradermal punctures using an insulin syringe. A total of 1 × 106 ADSCs resuspended in 500 μl PBS were injected in UV + ADSCs mice ($n = 9$); the UV + MV group was injected with MVs in an amount equal to the protein concentration of 1 × 106 ADSCs resuspended in 500 μl PBS. Control mice (UV + PBS) were injected with 500 μl PBS only. Microinjections were performed evenly over the entire dorsal skin; the volume of each injection was 20–25 μl. The input modes of solution were the same in each group.

2.6 Histology and Morphometry

Four weeks after injection of ADSCs or MVs (6 weeks post-initiation of UV treatment), the dorsal skin was dissected as a full-layer tissue. Skin tissue samples from mice in each group were obtained and fixed in 4% paraformaldehyde for 12 h then embedded in paraffin and sliced into 5 μm sections on a rotary microtome Microm HM 355 S (Thermo Scientific, USA). The sections were mounted on glass slides, dewaxed, rehydrated with distilled water, and stained with hematoxylin and eosin (BioVitrum, Russia). Sections were also stained with Heidenhain's azocarmine (BioVitrum, Russia). Sections were observed under a light microscope to determine the thickness of the epidermis and dermis and details of the skin morphology [30].

2.7 Statistical Analysis

Statistical analysis was performed using GraphPad InStat 3.10 software. One-way analysis of variance or Student's t test was used for determination of significant differences between groups. It was considered that $P < 0.05$ indicates a statistically significant difference.

3 Results

3.1 Doppler Flowmetry

Chronic exposure of the dorsal skin to UV radiation for 6 weeks increased the dorsal skin perfusion by 24.2% compared with the unexposed intact group (Intact 165.36 ± 10.72 apu and UV* 205.38 ± 13.48 apu). Four weeks post-injection of ADSCs or MVs and after 6 weeks of UV exposure, perfusion significantly decreased in groups UV + PBS, UV + ADSCs, and UV + MVs compared with UV* group which received no intervention. However, there was no significant differences in skin perfusion observed between UV + ADSCs and UV + MVs groups in comparison with the UV + PBS control treatment group (UV + ADSCs 153.76 ± 13.39 apu, UV +MVs 161.33 ± 9.1 apu, and UV + PBS 138.6 ± 10.78 apu) (Fig. 1).

3.2 Morphometric Analysis

Epidermis Thickness In the UV + PBS, UV + ADSCs, and UV + MV mice groups, which were exposed to UV radiation for 6 weeks and received an injected treatment of PBS, the epidermis thickness was significantly higher than in the intact group (no UV and no injected treatment). Of all groups exposed to UV, only UV + ADSC-treated animals demonstrated a reduction in epidermis thickness as a result of the injected ADSC treatment, although this did reach the same thickness measured in unexposed (no UV) animals (intact 17.28 ± 0.35 μm, UV + PBS 21.69 ± 0.48 μm; UV + MV 21.76 ± 0.44 μm; UV + ADSCs 20.42 ± 0.36 μm, $P < 0.05$) (Fig. 2).

Dermis Thickness After 6 weeks of UV exposure, the thickness of the dermis in the UV + PBS group was significantly higher than in the intact (no UV exposure) group (325.95 ±

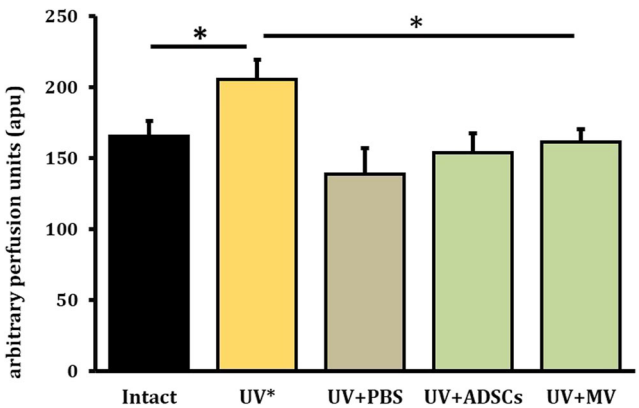

Fig. 1 Perfusion indicator analysis of dorsal skin. The density of the capillary system of the dorsal skin was visualized by the volume of blood flow using Easy-LDI laser Doppler flowmetry in real time. Perfusion parameters displayed as absolute perfusion units (apu). *Significant difference between groups ($P < 0.05$) as determined by Student's t test

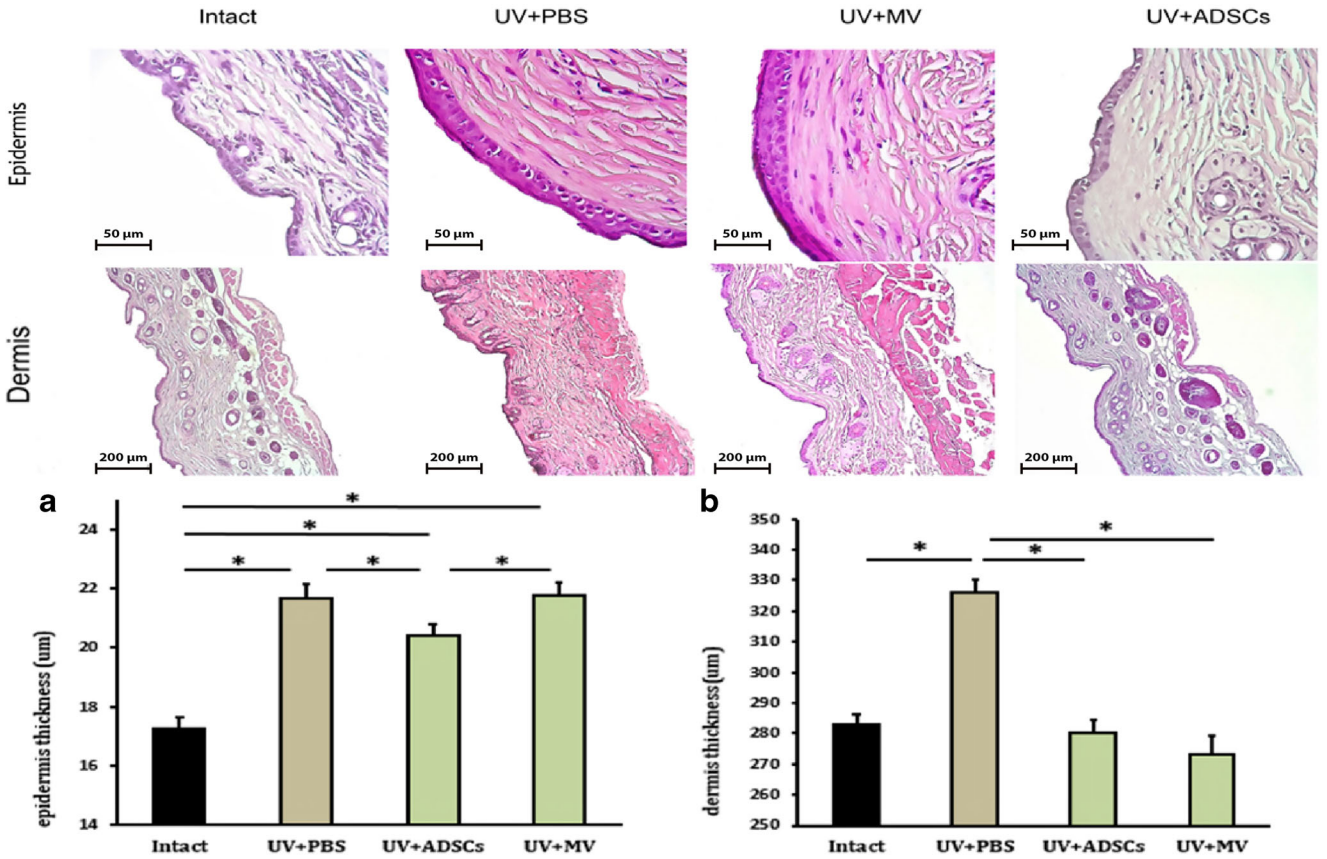

Fig. 2 Morphometric analysis of the dorsal skin. Slices of histological samples of the skin were stained with hematoxylin and eosin staining and analyzed by light microscopy at × 100 and × 400 magnification. In groups of mice exposed to UV radiation for 6 weeks and treated with ADSCs, MV, and PBS as a placebo, the epidermis thickness significantly exceeds the intact (none UV exposed) group. However, these values were significantly lower in UV + ADSCs than in other experimental groups. The thickness of the dermis in the UV + PBS group was significantly higher than in the intact group and in the UV + ADSCs and UV + MVs groups; for these latter two groups, the indices did not differ from those of intact animals. **a** Epidermis thickness indicators; **b** dermis thickness indicators. *Statistically significant difference between groups ($P < 0.05$) as determined by Student's *t* test

4.17 μm and 282.88 ± 3.21 μm, respectively). However, the indices of the treated groups UV + ADSCs and UV + MV groups did not differ from the mice in the intact group (UV + ADSCs 280.17 ± 4.2; UV + MV 273.34 ± 5.82) (Fig. 2), demonstrating a clear effect of the injected ADSC and MV treatment on maintaining a normal dermis thickness despite UV exposure.

3.3 Morphological Analysis

To evaluate the protective effects of the use of ADSC and MV therapy for skin lesions caused by photoaging, morphological changes within the skin were determined by histological analysis. In comparison with the intact (no UV exposure) skin, the UV + PBS group showed areas with pronounced epidermal thickening, characterized by an increase in the thickness of the stratum corneum and rows of keratinocyte of the spinous layers.

Also, disorganization and decreased density of dermis collagen fibers both indicate the destructive effects of UV radiation on the skin. Animals treated with ADSCs and MVs showed an improvement in the organization of the dermal layer and an increase in the density of fibroblasts. The collagen fibers in treated mice were arranged more densely and were more organized, similar to that seen in the intact (no UV exposure) group.

4 Discussion

ADSCs are promising therapeutic agents for skin regeneration. Recent work by other authors demonstrated that the therapeutic effect of stem cells is partially mediated by extracellular vesicles, which include exosomes and MVs [31]. In this study, we evaluated the regenerative effects of ADSCs and MVs on skin regeneration under photoaging in vivo experiment.

Although, the specific mechanisms of ADSCs on skin restoration are still unknown, it is believed that ADSCs can stimulate skin regeneration using the following mechanisms: (1)

ADSCs transplanted into the skin secrete cell growth factors through paracrine mechanisms, which stimulate the restoration of fibroblasts; (2) ADSCs provide tissue with antioxidants and free radical scavengers [32]. Acceleration of angiogenesis is considered to be another explanation for the protective effect of ADSCs against skin aging. Wang H et al. showed that ADSCs can induce angiogenesis by secreting VEGF through skin aging caused by administration of D-galactose in nude mice [32]. In an experiment by Ren et al., injection of microvesicles isolated from human fat cells into the wound area of BALB/c mice significantly increased neovascularization, reepithelization, and deposition of collagen and led to accelerated wound closure [31].

In this study, changes in skin vascularization by photoaging were assessed using laser Doppler flowmetry. Irradiation of mice with ultraviolet light for 6 weeks showed an increase in the perfusion of the dorsal skin as compared with the intact control. These changes caused by irradiation were associated with inflammatory reactions, which caused increased blood flow and skin vasodilation [33].

Changes in skin microcirculation caused by acute exposure to ultraviolet radiation are reported to disappear within a few days. In this study, animals were treated with ADSCs, MVs, and PBS (as placebo) and their perfusion was analyzed after 4 weeks; the perfusion indices decreased to values similar to that of the intact (none UV treated) group. However, no significant differences between ADSC, MV, and PBS treatments were detected. The absence of differences in the vascularization indices in the experimental groups may be due to the disruptive effects of skin pigmentation resulting from irradiation affecting the measurement depth and subsequent accuracy of the laser Doppler flowmetry [34].

Ultraviolet radiation leads to an increase in the thickness of the epidermis, hyperkeratosis, affecting the phenotype of the skin structure, directly affecting the keratinocytes of the epidermis [35]. A few hours after exposure to ultraviolet radiation, epidermal keratinocytes begin to rapidly proliferate, which leads to their accumulation and an increase in the thickness of the epidermis [36] Using histological evaluation methods, we observed that in the group of mice treated with ADSC therapy, the UV-induced thickening of the epidermal layer was significantly lower than in the group of mice treated with UV + PBS as placebo. However, this observation was not found in the group of mice treated with MVs as therapy. We assume that active mesenchymal stem cells have a longer therapeutic effect than microvesicles, which have a short-term effect.

It was identified that exposure to ultraviolet radiation increases the dermis thickness, which is associated with the formation of edema [33]. This data shows that in groups of animals treated with ADSCs and MVs, the dermis thickness was significantly lower compared with the UV group treated with PBS as a placebo and was equal to the mice in the intact group. We link this to the ability of mesenchymal stem cells to suppress inflammation mainly through paracrine signaling. Liu et al. showed that wounds treated with MSCs have lower numbers of inflammatory cells and proinflammatory cytokines, such as IL-1 and TNFα upon exposure to proinflammatory cytokines, including IFN-γ, TNFα, IL-1α, and IL-1β. The immunosuppressive phenotype of MSCs becomes activated, and they begin to express chemokines and inducible nitric oxide synthase (iNOS), which suppress T cell responsiveness to inflammation [37, 38].

Our data shows that ADSCs and MVs restore histological abnormalities when injected in the skin of the mice with photoaging caused by chronic exposure to UV radiation. This maybe perhaps through the release of cytokines and growth factors. ADSCs promote proliferation of fibroblasts, which stimulate the production of collagen and elastic fibers, thereby restoring the structure of the extracellular matrix damaged by ultraviolet radiation.

The results confirm positive effects of ADSCs and MVs on the protection of skin from photoaging. However, further studies are needed to determine the exact molecular mechanisms underlying the restoration of the skin.

5 Conclusion

Our data demonstrates that ADSCs and MVs can restore histological abnormalities when injected into the skin of mice with photoaging caused by chronic exposure to UV radiation. This is potentially through the release of cytokines and growth factors mediated by MVs. ADSCs promote proliferation of fibroblasts, which stimulate the production of collagen and elastic fibers, thereby restoring the structure of the extracellular matrix damaged by UV radiation. These results demonstrate the positive effects of ADSCs and MVs on skin affected by photoaging, opening new avenues to understand the molecular mechanisms underpinning skin repair and identifying novel and refined treatment options.

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Compliance with Ethical Standards

Conflict of Interest None.

Research Involving Humans and Animals Statement Animals were kept and used for experimental procedures in accordance with the rules accepted by Kazan Federal University and approved by the Local Ethical Committee (Permit Number 15 of 28.03.2019). Animals were used in accordance with international bioethical standards defined by the International guiding principles for biomedical research involving animals (2012), the EU directive 2010/63/EC and the 3Rs principles.

Informed Consent None.

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