6-24-2024

Adipose-Derived Mesenchymal Stem Cells and Their Derived Epidermal Progenitor Cells Conditioned Media Ameliorate Skin Aging in Rats

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Tissue Eng Regen Med
https://doi.org/10.1007/s13770-024-00643-3

Online ISSN 2212-5469

ORIGINAL ARTICLE

Adipose-Derived Mesenchymal Stem Cells and Their Derived Epidermal Progenitor Cells Conditioned Media Ameliorate Skin Aging in Rats

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Received: 2 January 2024 / Revised: 18 March 2024 / Accepted: 20 March 2024

Abstract

BACKGROUND: Skin alterations are among the most prominent signs of aging, and they arise from both intrinsic and extrinsic factors that interact and mutually influence one another. The use of D-galactose as an aging model in animals has been widely employed in anti-aging research. Adipose tissue-derived mesenchymal stem cells (Ad-MSCs) are particularly promising for skin anti-aging therapy due to their capacity for effective re-epithelization and secretion of various growth factors essential for skin regeneration. Accordingly, we aimed to examine the potential utility of Ad-MSCs as a therapy for skin anti-aging.

METHODS: In this study, we isolated and characterized adipose-derived mesenchymal stem cells (Ad-MSCs) from the epididymal fat of male *Sprague Dawley* rats. We assessed the *in vitro* differentiation of Ad-MSCs into epidermal progenitor cells (EPCs) using ascorbic acid and hydrocoritsone. Additionally, we induced skin aging in female *Sprague Dawley* rats via daily intradermal injection of D-galactose over a period of 8 weeks. Then we evaluated the therapeutic potential of intradermal transplantation of Ad-MSCs and conditioned media (CM) derived from differentiated EPCs in the D-galactose-induced aging rats. Morphological assessments, antioxidant assays, and histopathological examinations were performed to investigate the effects of the treatments.

RESULTS: Our findings revealed the significant capability of Ad-MSCs to differentiate into EPCs. Notably, compared to the group that received CM treatment, the Ad-MSCs-treated group exhibited a marked improvement in morphological appearance, antioxidant levels and histological features.

CONCLUSIONS: These results underscore the effectiveness of Ad-MSCs in restoring skin aging as a potential therapy for skin aging.

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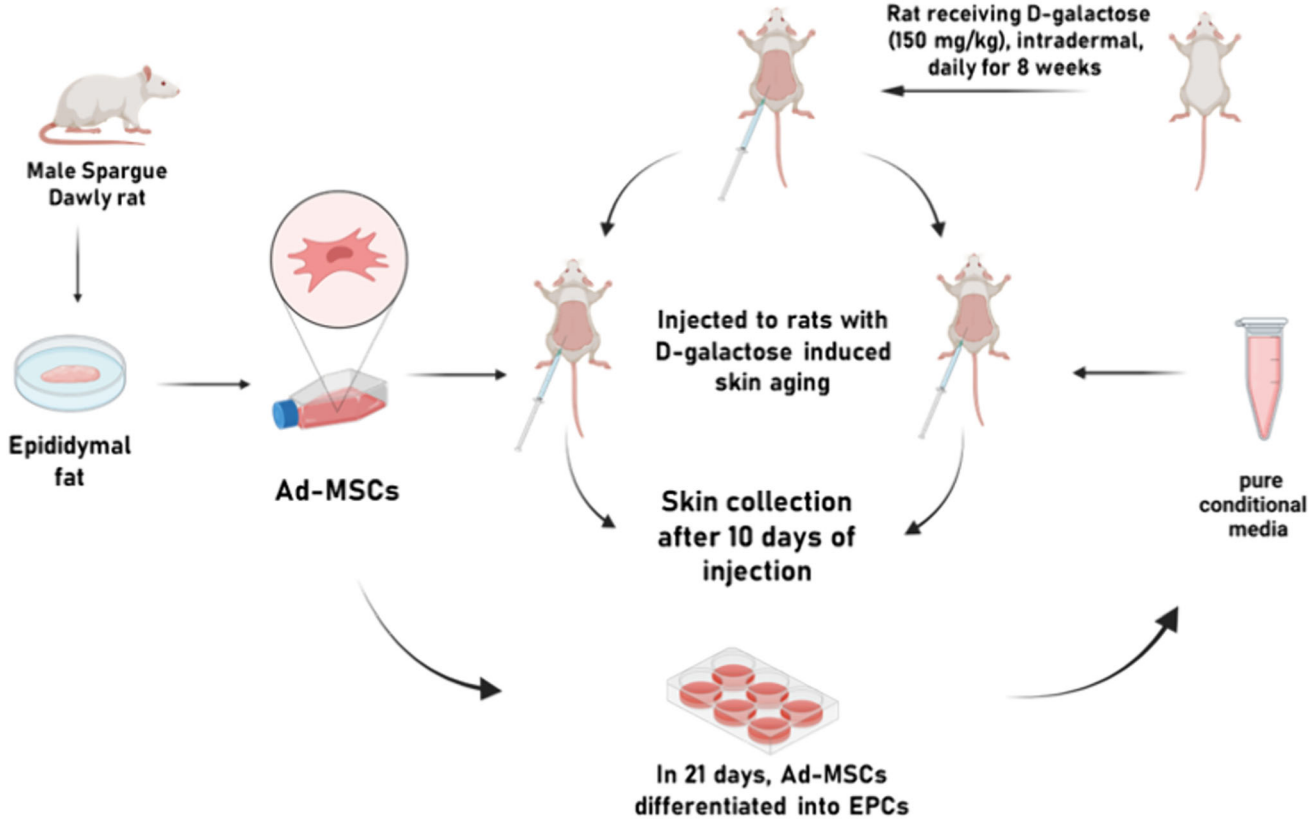
Published online: 24 June 2024

GRAPHICAL ABSTRACT



Keywords Skin aging · D-galactose · Adipose tissue · Mesenchymal stem cells · Epidermal progenitor cells · Regenerative medicine

1 Introduction

Skin aging comprises a part of natural human that manifests and follows diverse pathways in various cells, tissues and organs throughout time [1]. It's described as a process in which both intrinsic and extrinsic factors lead progressively to alter the structural integrity and physiological function of the skin. The intrinsic structural changes occur as natural consequence of ageing while extrinsic factors arise as photo damage caused by prolonged or repetitive ultraviolet exposure [2].

Associated risk factors of skin aging are categorized into non-modifiable and modifiable factors. Age and gender are examples of non-modifiable risk variables that are associated with intrinsic skin aging and cannot be adjusted. However, smoking and sun exposure are examples of modifiable risk factors which could be modified by treatments and changes in lifestyle that have an impact on extrinsic skin aging [1, 3, 4].

The three fundamental structural components of the dermis, collagen, elastin, and glycosaminoglycan have been the focal point of the majority of anti-aging research and attempts for aesthetic anti-aging techniques relevant to the skin [5]. The skin anti-aging techniques are classified as follows, cosmetological care, topical agents, invasive procedures, and systemic agents where they employed to reverse the dermal and epidermal signs associated with photo- and chronological aging [1].

Recently, mesenchymal stem cells (MSCs) are becoming increasingly acknowledged as therapeutic agents for skin regeneration and rejuvenation [6]. MSCs are multipotent adult stem cells (ASCs) [6, 7] which can be derived from a variety of sources, including bone marrow, umbilical cord blood and adipose tissue [8]. Various research studies revealed that MSCs derived from adipose depots can be appropriately used to treat a wide range of skin diseases. They can enhance skin regeneration following injuries and those associated with skin aging [7, 9–11]. Adipose-derived mesenchymal stem cells (Ad-MSCs) have acquired prominence in skin anti-aging therapy due to their effective re-epithelization as well as generation of various growth factors that required for skin regeneration [7].

Furthermore, ASCs has shown their efficiency to ameliorate photo-aging wrinkles and stimulate collagen production and epidermal thickness in photo-aged fibroblasts [12].

There has been a great deal of debate on how significant it is of elevated blood glucose levels as an "aging accelerator" for the skin [13]. Glycation is a non-enzymatic process that occurs between free amine groups, such as those found in proteins, and reducing sugars such as glucose. This process eventually results in the development of advanced glycation end products which may be responsible for covalent bonding between macromolecules. Glycation is particularly widespread in tissues with slow turnover rates of macromolecular structures and is thus expected to have a key role in aging [14].

Galactose is a glucose Carbon-4 (C-4) epimer that interacts with glucose to generate the disaccharide lactose [15]. There is evidence that D-galactose (D-gal) is a more glycating agent than glucose and can cause oxidative stress [16]. D-gal has been shown to cause aging-like effects in experimental animals [17, 18]. Indeed, the use of D-gal for animal aging models has been widely used in anti-aging research since the early 1990s [16].

In this regard, the current study focused on investigating the capabilities of Ad-MSCs to differentiate into epidermal progenitor cells (EPCs) *in vitro*. Moreover, the therapeutic potential of intradermal transplantation of Ad-MSCs and conditioned media (CM) derived from differentiated EPCs into the D-gal-induced skin-aging rats were assessed *in vivo*.

2 Material and methods

2.1 Isolation, culture and characterization of Ad-MSCs

All of the experiments and procedures were carried out in accordance with the guidelines and were approved by the ethical committee of the Faculty of Pharmacy, The British University In Egypt, Cairo, Egypt (EX:2317). Ten male *Sprague–Dawley* rats weighing 250–300 g were housed in the laboratory animal center in temperature-controlled rooms (20–22 °C) under 12 h light/dark cycle with free access to food and water. Two rats were used per isolation. The rats were anaesthetized by intraperitoneal injection of ketamine hydrochloride (33 mg/kg body weight) and xylazine hydrochloride (13 mg/kg body weight) freshly prepared as described previously [19] then they were euthanized by cervical dislocation.

All isolation procedures were carried out as described previously with few modifications [20, 21]. Under aseptic conditions, epididymal fat was obtained from the rat and minced into pieces. A washing process with phosphate buffer saline (PBS) was induced, and the fat tissue was digested by 0.1% collagenase for 45 min at 37 °C till an almost homogenous solution was formed. After centrifugation, the stromal vascular fraction (SVF) was obtained. The SVF was suspended in 1% bovine serum albumin (BSA), and the content was centrifuged, then the pellets obtained were suspended in a Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) media (Lonza, Basel, Switzerland), 1 × of 200 mM L-Glutamine (Gibco, Thermo Fisher, Grand Island, NY, USA), 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher), and 1 × antibiotic/antifungal solution containing 100 U/ml Penicillin, 100 µg/ml Streptomycin and 0.25 µg/ml of Amphotericin B (Gibco, Thermo Fisher) and incubated for culture and expansion at 37 °C with 5% CO_2 and saturated humidity. This initial passage was referred to as "passage 0". Upon reaching 85–90% cell confluency, cells were washed, and trypsin- (EDTA) (Lonza, Switzerland) solution was used where the cells were harvested, counted, and passaged into new flasks. Immune phenotypic and multi-differentiation assay assessments were used to characterize the isolated Ad-MSCs [22]. Flow-cytometry was used to phenotypically characterize the cultured cells for the expression of MSC markers as CD90, CD105, and expression of hematopoietic marker as CD34. Using CD90 monoclonal antibody labeled with fluoroisothiocyanate (FITC) or anti-CD105 and CD34 phycoerythrin (PE) labeled (Stem cells Technologies, Vancover, Canada). Subsequently, their multi-differentiation characteristics using the MSC functional identification kit (R&D systems Inc., Minneapolis, MN, USA) established their differentiation into adipocytes, osteocytes, and chondrocytes.

2.2 *In vitro*, differentiation of Ad-MSCs into epidermal progenitor cells (EPCs)

Differentiation potential of Ad-MSCs into EPCs was performed as previously described [23]. Initially, Ad-MSCs at P3-P4 were cultivated in 10% FBS-supplemented DMEM-

Table 1 Sequence of primers used for the RT-qPCR

Genes	Forward primer 3′-5′	Reverse primer 5′-3′
Cyt-14	TGAACGAGATGAGGGACCAG	TGCAGTTCTATCTCCAGGCC
Vimentin	AGGCCCAGATTCAGGAACAG	ACTTCGCAGGTGAGTGACT
β-actin	TGGAGAAGATTTGGCACCAC	AACACAGCCTGGATGGCTAC

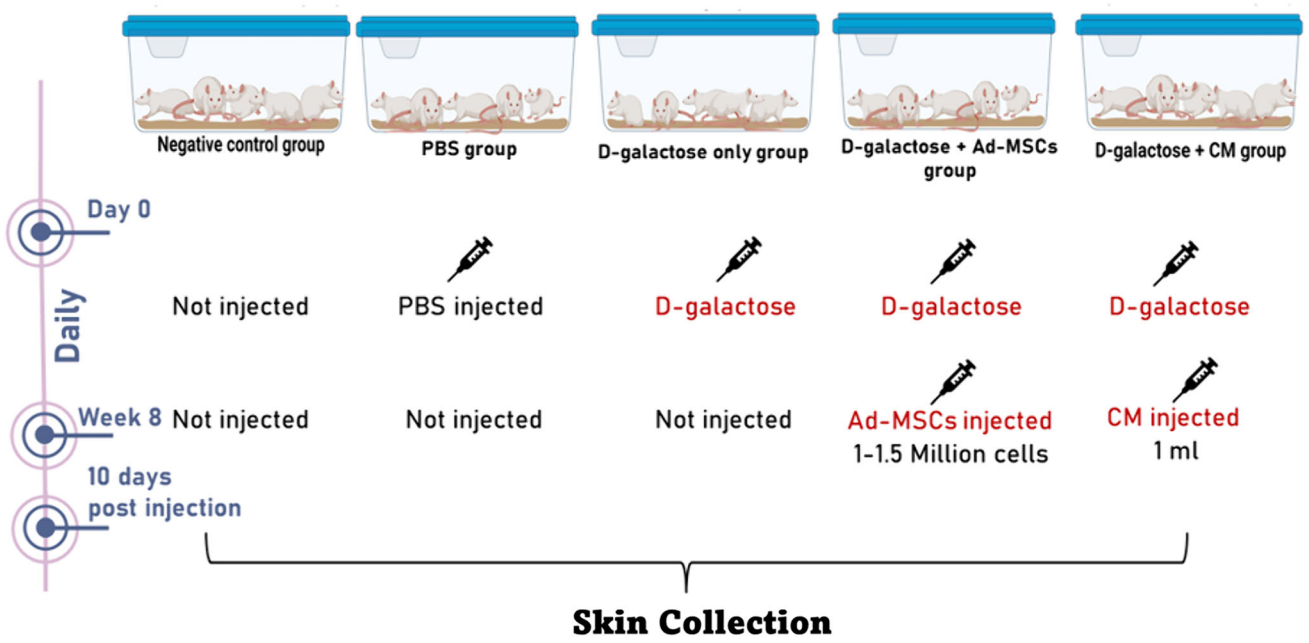


Fig. 1 A schematic presentation for the experimental design of the *in vivo* assay

F12 until 80–90% confluence and further seeded at a density of about 1×10^6 cells into six-well plates then induced with differentiating culture media. Differentiating media containing 10% FBS-supplemented DMEM/F-12 media, 1 × of 200 mM L-Glutamine, 1 × antibiotic/antifungal, 0.3 µM ascorbic acid (Sigma Aldrich, Boston, MA, USA), and 0.5 ug/ml hydrocortisone (Sigma Aldrich) and then cultured for 21 days to differentiate into EPCs. As a control, Ad-MSCs were cultured in 10% FBS-containing DMEM-F12 without any differentiation factors. On day 21 of the complete differentiation, when the cells attained a rounded or polygonal shape, the expressions of keratinocyte marker cytokeratin 14 (Cyt-14), and the mesenchymal marker vimentin were examined using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

2.3 RNA extraction, cDNA-synthesis, and q-RT-PCR

Total RNA (both control and differentiated cells) were isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). According to manufacture instruction, the cells were lysed by 1ml of trizol. 200 µLs of chloroform (Fisher scientific, Waltham, MA USA) were added into lysed samples. Following centrifugation, RNA collected exclusively from the aqueous phase. 600 µLs of 100% isopropanol (Sigma Aldrich) were added to the collected aqueous phase and centrifuged at 12,000 × *g* for 30 min at 4 °C, then washed with 1 mL 80% ethanol followed by centrifugation 5 min at 9600 × *g* at 4 °C. After drying, the concentration and purity of total RNA were determined by measuring the optical density (OD) at 260 nm and a 260/280 nm ratio, respectively. Complementary DNA (cDNA) was synthesized from a total of 0.5 µg RNA according to the manufacturer's instructions of the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) after RNA free DNAse treatment as per manufacturer's instructions (Thermo Scientific, USA). For the analysis of differentiating genes, primers used in this study are shown in the (Table 1), the qRT-PCR reaction was carried out for each target gene using a Maxima Sybr green Master Mix (Thermo Fisher) according to the manufacturer's instruction. The Step One Plus RT-PCR system (Applied Biosystems, Foster City, CA, USA) was employed for all qRT-PCR experiments, along with the default program and dissociation curve. The $2^{(-\Delta\Delta Ct)}$ method was used to calculate relative RNA expression, with β-actin serving as a housekeeping gene.

2.4 D-galactose (D-gal)-induced aging model and experimental design

D-gal has been reported to bring on aging-like effects in experimental animals [17, 18] where they have been carried out to investigate D-gal-induced skin aging [16, 24, 25]. A total of 30 female *Sprague Dawley* rats weighing 250–300 g were provided by Laboratory Animal facility, Faculty of Pharmacy, The British University in Egypt, Cairo, Egypt. Five groups of rats (6 rats each) were randomly selected. Rats with fasting blood glucose (FBG) above 110 mg/dl were excluded.

Table 2 Scoring assessment of skin aging lesion of the different groups

	Epidermis	Upper dermis			Deep dermis		
		Pilosebaceous units	Collagen fibers	Fibrous bands	Hair follicles	Collagen fibers	Fibrous bands
Control	0	0	0	0	0	0	0
PBS	0	+	+	0	0	+	0
D-gal	++	++	++	++	++	++	+
D-gal+Ad-MSCs	0	0	+	0	0	0	0
D-gal+CM	++	+	+	+	0	+	+

Epidermis: 0: Regular/intact+: Atrophied++: Irregular atrophied

Pilosebaceous units: 0: Average+: scattered atrophied++: Markedly atrophied

Hair follicles: 0: Average+: scattered atrophied++: Markedly atrophied

Collagen fibers: 0: Average+: scattered thick++: excess thick

Fibrous bands: 0: No+: thin fibrous bands++: thick fibrous bands

For eight weeks, 18 rats (3 groups) were intradermally injected daily with D-gal (150 mg/kg) at the specific shaved side of dorsum's rat, while another 6 rats (PBS group) was intradermally injected daily with PBS only. The injection locations of the 4 groups were then noted. On the other hand, a group of animals (6 rats) were neither injected with D-gal nor PBS to be served as negative control group. Experimental design of the *in vivo* study is summarized in (Fig. 1). Everyday observation and free access to food and water were provided for all animals.

2.5 Transplantation of Ad-MSCs and CM into d-gal induced rats

2.5.1 Preparation of Ad-MSCs and CM

Ad-MSCs at P3-P4 were used after being passaged using trypsin–EDTA (Gibco, Grand Island, NY, USA) then washed and counted using hemocytometer. About 1–1.5 $\times$ 10^6 of isolated Ad-MSCs in 1 ml PBS were used for intradermally transplantation. Additionally, CM derived from EPCs were obtained by firstly, discarding the differentiated media at D21 and washed with PBS, then replaced with a media containing only DMEM/F-12, and 1 $\times$ antibiotic/antifungal. The EPCs with a fresh media were incubated and cultured for another 2 days. The CM were collected and centrifuged at 1500 rpm (300 $\times$ *g*) for 10 min to discard any cell debris. 1 ml of CM was prepared for intradermally transplantation into each rat.

2.5.2 Transplantation process

Rats induced with D-gal were randomly divided into three groups as follows: D-gal only non-treated group (Positive control group), D-gal+Ad-MSCs treated group, and a D-gal+CM treated group. D-gal+Ad-MSCs treated rats received 1–1.5 $\times$ 10^6 Ad-MSCs in 1 mL of PBS, while D-gal+CM treated group received 1 ml of CM. After the intradermally transplantation, all five groups of rats were housed for another 10 days at same condition as mentioned earlier.

At the end of the treatment, all rats in five groups were anesthetized and euthanized by cervical dislocation and skin tissue was immediately snap-frozen for anti-oxidant assay examination, while another part of skin kept at 10% PBS buffered formalin for histopathological assessment.

2.5.3 Antioxidant assay, histopathological and histochemical staining examination

Two sets of measurements were made on all excised skin tissues. Antioxidant enzyme level of superoxide dismutase (SOD) and level of malondialdehyde (MDA) were measured in addition to the histological investigation.

Reactive oxygen species (ROS) has a significant role in the pathophysiology of various diseases, including several skin diseases [26, 27]. SOD is an antioxidant enzyme. On the other hand, MDA, a byproduct of lipid peroxidation, is known to be an oxidative stress marker which is an outstanding indicator of free radical-mediated damage and oxidative stress [28]. For antioxidant assay, skin tissue samples were cut into approximately 0.5 g and then homogenized in 10 volumes of PBS. Homogenate tissue was incubated at tissue rotator for 30 min followed by spun at 13,000 $\times$ *g* for 15 min. The supernatant was collected for SOD and MDA measurement using commercial Elisa kit (Rat Superoxide Dismutase (SOD) ELISA assay, BioSource, San Diego, CA, USA); Rat Malondialdehyde (MDA) ELISA assay, BioSource).

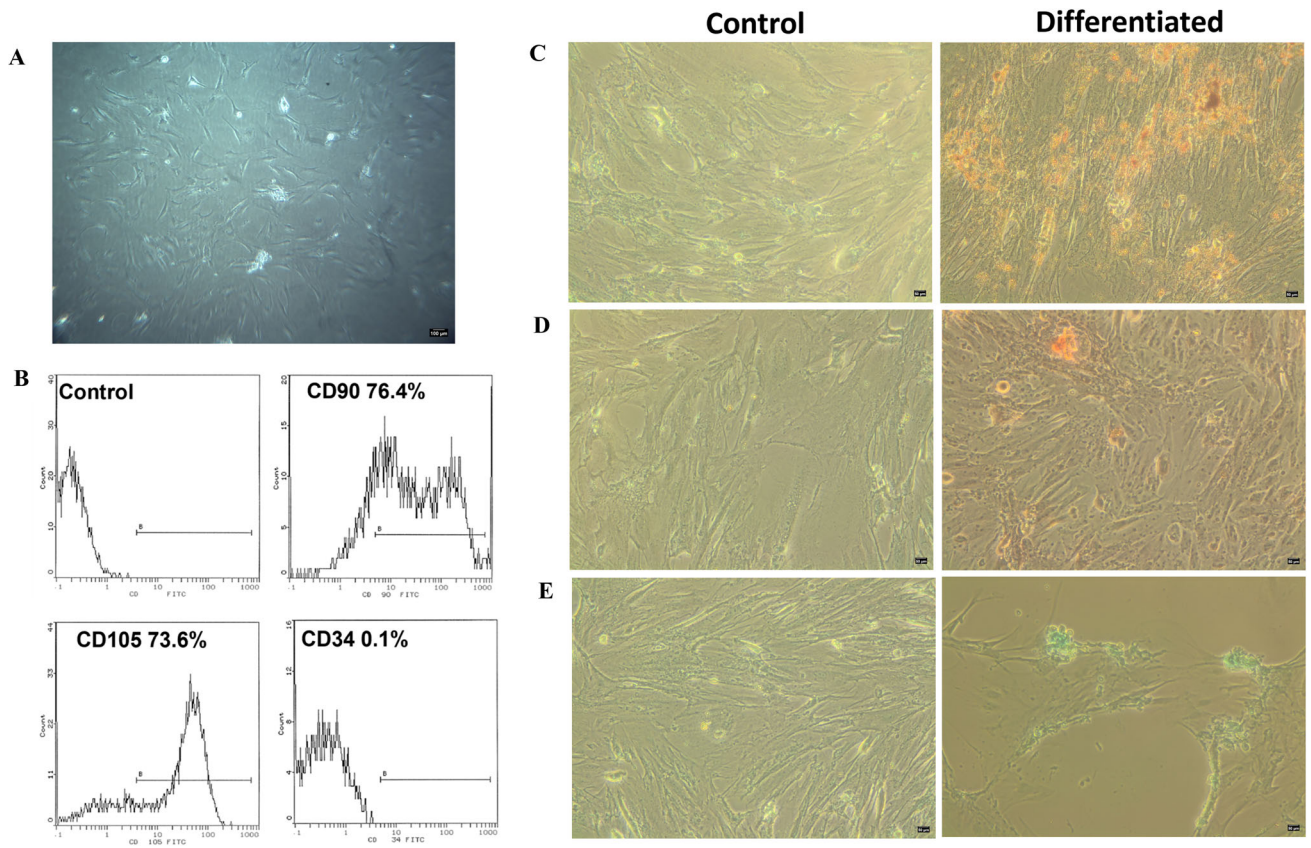


Fig. 2 Characterization of Ad-MSCs **A** Phase contrast images of cultured Ad-MSCs; showing a homogenous population of fibroblast-like shaped cells (Day7, P0) (at magnification 10x). **B** Flow cytometric analyses of surface MSCs marker expression CD90 (89.93%), CD105 (39.21%), and CD73 (88.51%). Multi-differentiation assay; **C** Right panels: adipogenic differentiation of Ad-MSCs (lipids in cells were stained with Oil Red O Stain). Left panels: negative noninduced controls. **D** Right panels: osteocytes stained with alizarin red. Left panels: negative noninduced controls **E** Right panels: chondrocytes stained with alcain blue. Left panels: negative noninduced controls (at magnification 10x)

For histological examination, the cut dorsal skin tissues of the scarified animals were preserved in 10% PBS buffered formalin for 48 h. Tissues were fixed in paraffin, and the paraffin blocks were sectioned at 2m before stained with hematoxylin and eosin using conventional procedures. The severity of skin aging lesion was determined based on three features which are upper dermis including the epidermis and the pilosebaceous units; the deep dermis including hair follicles; and the fibers including collagen fibers and fibrous bands. An arbitrary score of (0) was given for the normal parameters of the skin in the control group. Then, assessment for other groups were done based on this control base line and a (+) values were given for pathological changes in a horizontal manner as shown in Table 2. Histologic scoring was evaluated via blinded randomized examination by two independent researchers.

Elastic fibers of the dorsal skin tissues of the studied groups were visualized by using histochemical Shikata's Orcein stain kit (Clini-Tech, Guildford, UK) according to manufacturer's instruction, Tissue blocks were deparaffined, situated in potassium permanganate solution and displaced in 1% oxalic acid then immersed in Shikata's Orcein stain and dehydrated in graded alcohols and cleared in xylene.

All stained sections were examined under light microscopy and were assigned to each microscopic field at 400X magnification. The microscopic examination of stained sections was performed by LABOMED Fluorescence

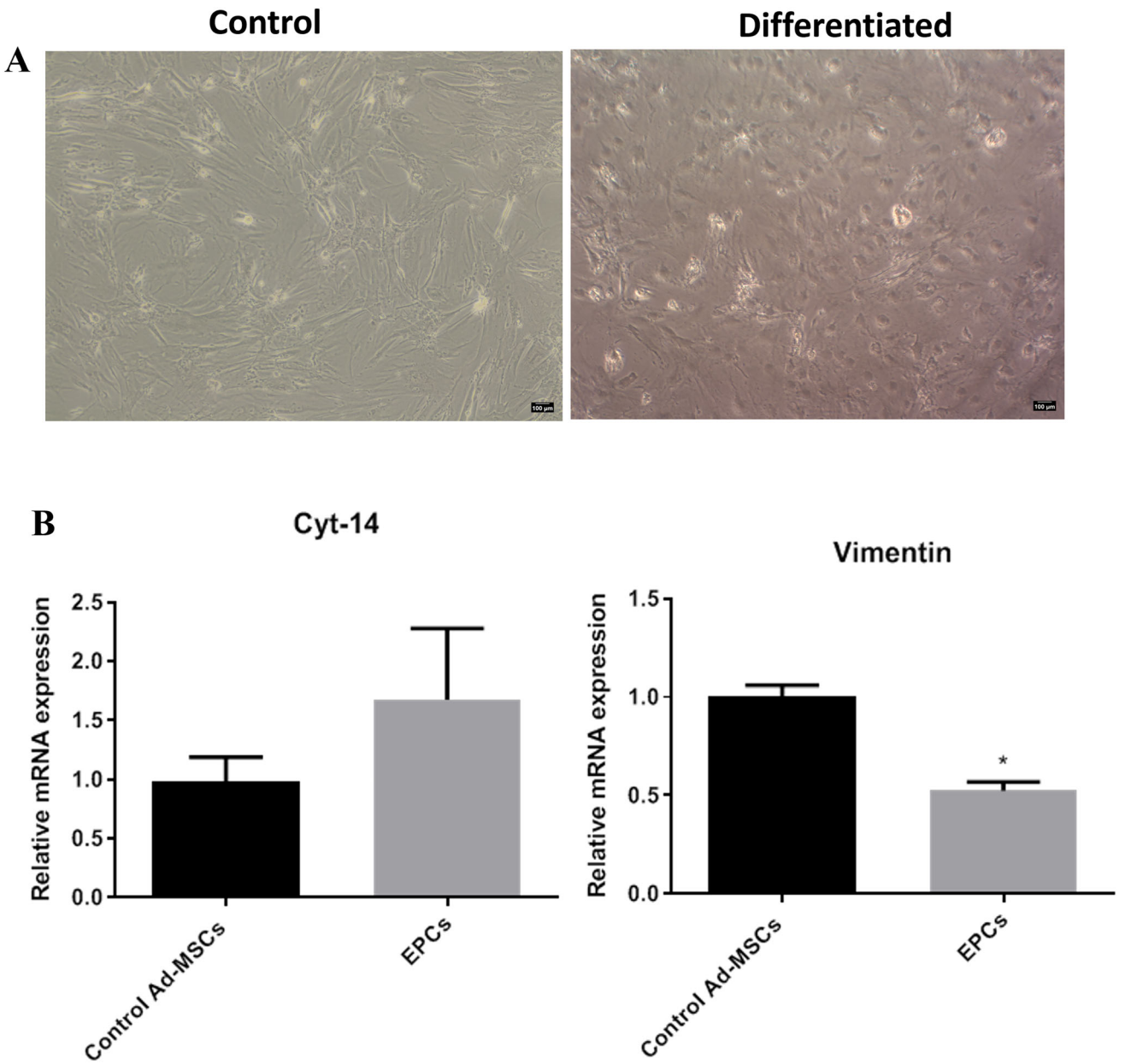


Fig. 3 Ad-MSCs transdifferentiate into keratinocytes (EPCs). **A** Left panel: Phase contrast images of isolated Ad-MSCs (non-induced control) showing fibroblast-like shaped cells. Right panel: cluster of polygonal cobblestone shape like cells. **B** Expression levels of mRNAs of EPCs specific markers. Left panel: Up regulation of cytokeratin-14 and down regulation of vimentin (right panel), ($n = 3$). *: significant different from Control non-induced (Ad-MSCs) at $p < 0.05$

microscope LX400, and LABOMED camera software, (Labomed, Los angelos, CA, USA).

2.6 Data analysis

All data are expressed as mean±standard error of the mean (SE). In the biochemical assays and qRT-PCR, one-way analysis of variance (ANOVA) followed by Tukey post hoc test were applied to evaluate statistically significant differences between the treatment groups. All means were considered significantly different with p value < 0.05. All figures and statistical analyses were prepared using Graph Pad Prism (GraphPad software version 6, San Diego, CA, USA).

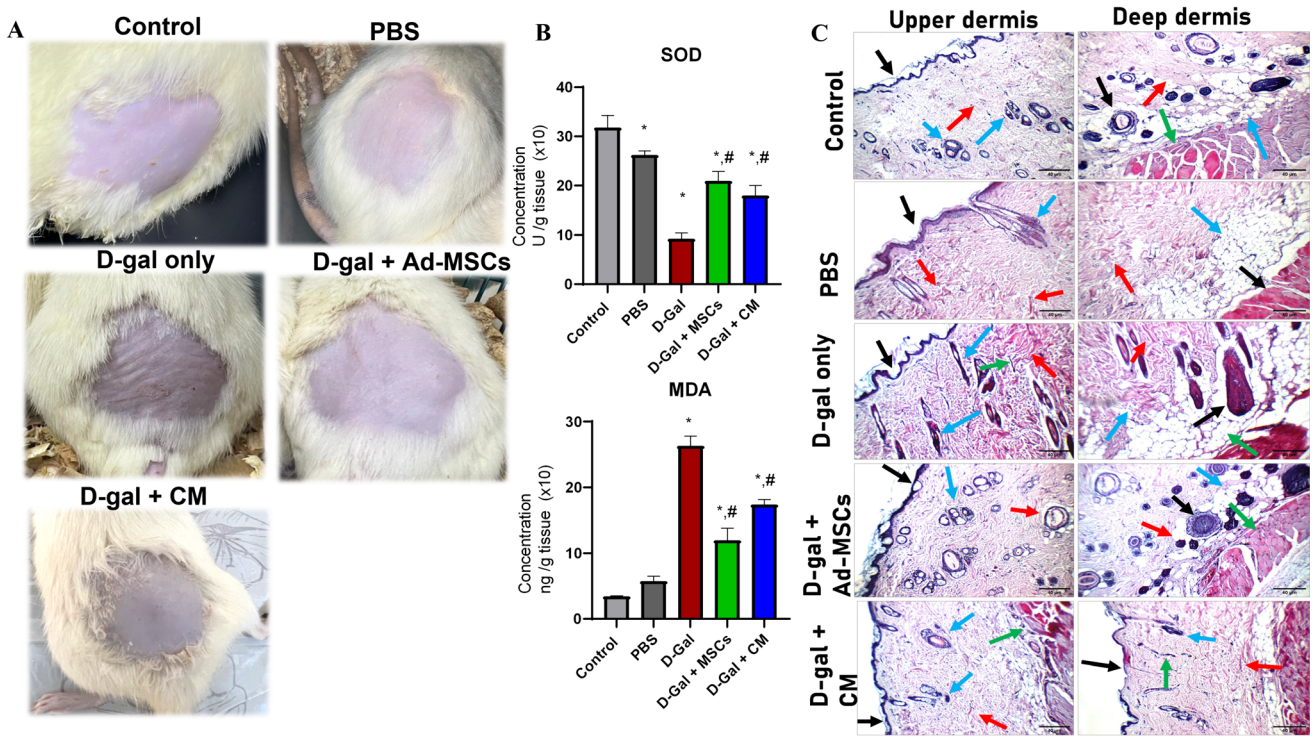


Fig. 4 Transplantation of Ad-MSCs and CM derived from EPCs into D-gal induced rat. **A** Morphological observation of the dorsum shaved skin of transplanted rats after 10 days of treatment. **B** Upper panel: Superoxide dismutase (SOD) and lower panel: Malondialdehyde (MDA) expression level of different study groups ($n = 3$). **C** Histopathological examination of skin of different study groups showing upper epidermis and deep dermis (at (H& X 200). (Black arrow: Epidermis, Red arrow: pilosebaceous units, Blue arrow: Collagen, Green arrow: average muscles). *: significant different from Control at $p < 0.05$. #: significant different from D-gal only at $p < 0.05$

3 Results

3.1 Culture and characterization of Ad-MSCs

MSCs are multipotent stem cells that are characterized based on their ability to adhere to plastic, expression of cell surface markers such as CD90, CD105 and CD34 and ability to differentiate into adipocytes, osteoblasts, and chondrocytes [9, 20, 22]. Isolated Ad-MSCs expanded, cultured, and displayed a homogenous population of a characteristic fibroblast-like appearance cells (Fig. 2A). Flow cytometry assessments were used to confirm and quantify the expression of MSCs surface markers which revealing that the majority of isolated Ad-MSCs express MSC markers (CD90+76.4%, CD105+73.6%) and almost lacked the expression of hematopoietic marker (CD34+-cells: 0.1%) (Fig. 2B). Furthermore, the isolated Ad-MSCs showed their potential toward multilineage differentiation assay (Fig. 2C–E). Oil red-O labeling of intracellular lipid droplets demonstrated Ad-MSC adipogenesis (Fig. 2C). The production of calcium oxalates on differentiated Ad-MSCs was readily apparent using alizarin staining (Fig. 2D). Chondrogenic differentiation was observed through Alcian blue staining of cartilage-specific proteoglycans (Fig. 2E). While the control non-differentiated cells of the three lineage assay preserved their fibroblast like shape. These findings validated the cells' characterization of the isolated Ad-MSCs.

3.2 Differentiation of Ad-MSC into keratinocyte lineage (EPCs)

In order to determine if the isolated Ad-MSCs had the capacity to trans-differentiate into keratinocyte-like cells, we assessed their morphological change after 21 days of the differentiation protocol in addition to their EPCs markers expression assessment. In this regard, isolated Ad-MSCs gradually lost their spindle-like shape morphology and began to proliferate into a cluster of polygonal cobblestone-shaped cells after 18 days of cultivation (Fig. 3A). To investigate the proliferation and trans-differentiation of Ad-MSCs into EPCs, the expression levels of proliferation-related markers of keratinocytes including cyt-14 and vimentin were measured which are originally distributed in the skin where they are used to identify and isolate specific populations of epidermal stem cells [29–32]. The expression level of Cyt-14 was substantially increased when compared to the control (non-differentiated

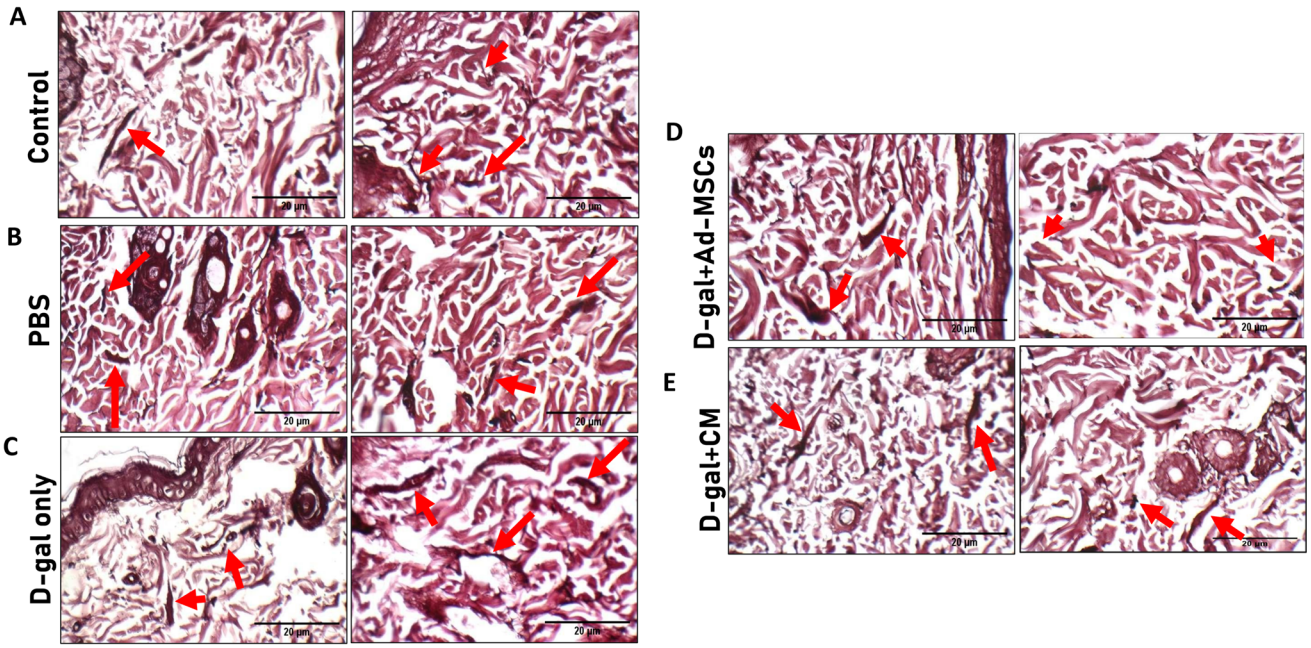


Fig. 5 Histochemical Orcein staining of elastic fiber of different study group. **A** Control group's skin showed average intact thin long elastic fibers in upper and deep dermis. **B** PBS injected group showing scattered intact thick short elastic fibers in upper and deep dermis **C** The D-gal group's skin showing excess fragmented thick elastic fibers in upper and deep dermis. **D** The D-Gal Ad-MSCs group showing skin showed excess intact thick short elastic fibers in upper and deep dermis. **E** The D-Gal+CM group's skin showing excess fragmented thick elastic fibers in upper and deep dermis at 200X magnification, Red arrow: stained dark brownish purple of elastic fibers. *PBS* Rats received PBS only, D-Gal only: rats received D-galactose only, D-Gal+Ad-MSCs: rats received D-galactose and Ad-MSCs, D-Gal+CM: rats received D-galactose and conditioned media from differentiated epidermal progenitors of Ad-MSCs

Ad-MSCs) but it didn't fulfill a significant level ((Control): 1.05 ± 0.21, (EPCs): 1.66 ± 0.60). However, a significant down regulation of vimentin was observed which is a mesenchymal marker [33] ((Control): 1 ± 0.05, (EPCs): 0.56 ± 0.04, *p value* < 0.05) (Fig. 3B). Thus, the isolated Ad-MSCs revealed their multipotent differentiation capabilities that possessed *in vitro* via transition into epithelial phenotype with loss of the mesenchymal marker vimentin.

3.3 Effect of Ad-MSCs on morphology and antioxidant enzyme activity

MSC treatments have been reported to significantly decrease reactive ROS levels in animal models [34–36]. Most often, MSC therapies that reduce oxidative stress markers are associated with functional recovery as well as positive outcomes in animal models [37].

For the morphological examination, the control group preserved their smooth and normal color of skin with no any sign of wrinkles, while PBS group became lesser elastic that predictably due to the daily injection exposure. D-gal only group begun gradually to exhibit less elastic tinged color of skin with prominent wrinkles. However, the skin condition of D-gal+Ad-MSCs group was improved by which the dryness state was minimized, skin color was enhanced, and wrinkles were greatly reduced. Moreover, D-gal+CM group showed also a great improvement in dryness, color and wrinkles of skin (Fig. 4A). In order to verify that Ad-MSCs have a protective effect on the skin through antioxidant action, the levels of SOD and MDA were determined. The D-gal only group as expected expressed significant decline on the level of SOD and an elevation expression level of MDA in comparing to control non-injected group. Meanwhile, the treated group (D-gal+Ad-MSCs) showed significant improvement on the SOD level (*p value* < 0.05) (SOD level; (Control): 31.76 ± 1.45 U/gm tissue, (PBS): 26.2 ± 0.49 U/gm tissue, (D-gal only): 9.2 ± 0.70 U/gm tissue, (D-gal+Ad-MSCs): 20.9 ± 1.12 U/gm tissue, (D-gal+CM): 17.9 ± 1.1 U/gm tissue) and decreased MDA expression levels than the (D-gal+CM) treated group (*p value* < 0.05) (MDA level; (Control): 3.4 ± 0.05 nmol/gm tissue, (PBS): 5.7 ± 0.45 nmol/gm tissue, (D-gal only): 26.3 ± 0.87 nmol/gm tissue, (D-gal+Ad-MSCs): 11.9 ± 1.05 nmol/gm tissue, (D-gal+CM): 17.3 ± 0.43 nmol/gm tissue) when both treated groups were compared to the D-gal only group (Fig. 4B).

3.4 Histopathological and histochemical staining observation

Histopathological examination results showed significant changes on skin appendages within injected and treated

groups (Fig. 4C), while the scoring evaluation of the skin aging lesion of the groups was clarified in the (Table 2). The skin of the control group showed intact epidermis, upper dermis with average pilosebaceous units and average collagen, deep dermis with average hair follicles and average collagen, average muscles, and average subcutis. While PBS group, the skin showed intact regular epidermis, upper dermis with scattered fibrotic pilosebaceous units and scattered peri-adnexal thick collagen, deep dermis showed average hair follicles and scattered thick collagen, average muscles, and average subcutis. On the other hand, D-gal only group showed a skin with irregular atrophied epidermis, upper dermis with markedly fibrotic pilosebaceous units and excess thick collagen with thick fibrous bands, deep dermis showed markedly fibrotic hair follicles and excess thick collagen with thin fibrous bands, average muscles, and average subcutis. Interestingly, D-gal+Ad-MSCs group showed intact skin with regular epidermis, upper dermis with average pilosebaceous units and scattered peri-adnexal thick collagen, deep dermis with average hair follicles and average collagen, average muscles, and average subcutis. Additionally, D-gal+CM group displayed atrophied irregular epidermis, upper dermis with scattered fibrotic pilosebaceous units and scattered peri-adnexal thick collagen with thin fibrous bands, deep dermis with average hair follicles and scattered thick collagen with thin fibrous bands, average muscles, and average subcutis. These finding results indicated that D-gal+Ad-MSCs improved the intact of skin with a significant enhancement in deep dermis including hair follicles, collagen and subcutis.

To provide further evidence of the aging, we employed histochemical staining of the elastic fibers that stained dark brownish purple by orcein stain. As shown in (Fig. 5A), the control group showed the normal average skin intact thin long elastic fibers in upper and deep dermis. While in case of PBS group, the skin appeared as scattered intact thick and short elastic fibers in upper and deep dermis (Fig. 5B). Upon injection of D-gal, Fig. 5C showed that the skin of the D-Gal only group showed excess thick and fragmented elastic fibers in upper and deep dermis. Interestingly, skin of treated group (D-gal+Ad-MSCs) showed restoration of these fibers exemplified by the staining of excess intact thick and short elastic fibers in upper and deep dermis (Fig. 5D). On the other hand, the skin of the treated group (D-gal+CM) showed excess thick and fragmented elastic fibers in upper and deep dermis (Fig. 5E).

4 Discussion

Skin aging is a highly complicated but still not yet fully understood process. Aging can be characterized by the accumulation of various deleterious changes in cells and tissues [3]. The aging process, both intrinsic and extrinsic, is influenced by the formation of free radicals, also known as ROS. Collagen loss is thought to be the most common histology finding in aging skin [5]. Wrinkling and pigmentary alterations are closely related to photo-aging and are regarded the most visible cutaneous signs of the condition [5]. Because the skin is the most obvious organ of aging, researchers are becoming increasingly interested in the physiology and treatment of wrinkles. In this study, the potential effect of Ad-MSCs and conditional media of differentiated EPCs were investigated in attenuating skin-induced aging in rats. Our findings suggest that Ad-MSCs hold promise as a therapeutic avenue for combating skin aging and offer insights into the underlying protective effect on aged-skin *in vivo* in addition their capabilities to trans-differentiate into epidermal progenitor cells *in vitro*.

Over the past 20 years, researchers have focused primarily on investigations that may impede or delay skin aging at the cellular and molecular level [38]. Stem cell transplantation is a promising treatment for skin aging. Adipose-derived stem cells contribute to skin regeneration during aging [39]. They have significant role via producing a variety of growth factors, including basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF) which influence in skin anti-aging therapy [40].

Adipose-derived stem cells are mesenchymal stem cells that originate from abundant adipose tissue that characterized by their ability to adhere on plastic culture flasks, capable of *in vitro* expansion, and capable of differentiating into several cell lineages. When compared to bone marrow-derived MSCs, Ad-MSCs from rich adipose tissue can be acquired using a minimally invasive method, resulting in a large number of cells. As consequently, Adipose derived stem cells hold a great promise for regenerating tissues and organs injury [41]. Accordingly, in the present study, MSCs were isolated from epididymal fat of male *Sprague–Dawley* rat then characterized according to well established criteria by International Society for Cell and Gene therapy (ISCT) [22]. Isolated Ad-MSCs revealed fibroblast-like cells, their ability to differentiate *in vitro* into three mesodermal lineages. Besides, the immunophenotyping analysis result showed that the majority of adherent isolated cells displayed surface antigens expressed in MSCs and lacked of hematopoietic marker.

MSCs have the capacity to differentiate into multiple cell lineages, including keratinocytes [42]. Epidermal differentiation is a complex process in which keratinocytes undergo morphological and biochemical changes over duration of 15–30 days. The pathogenesis of various skin diseases is influenced by abnormal keratinocyte differentiation [43]. Therefore, MSCs can be employed to treat congenital or acquired skin abnormalities [42]. On that account, the present study also highlights the

transdifferentiation potential of the isolated Ad-MSCs into epidermal progenitor cells using differentiated media containing ascorbic acid, and hydrocortisone. A variety of genes expression are getting involved during epidermal differentiation program that contribute to skin barrier formation, such as involucrin, loricrin, filaggrin, and cytokeratins expression [43].

One of a notable finding within the *in vitro* study is the up regulation of keratinocyte specific markers including Cyt-14, and down regulation of vimentin which is in accordance with several studies that have been conducted to investigate keratinocyte differentiation through the analysis of specific marker protein and gene expression [23, 43, 44]. It is worth noting that it was previously reported as they failed to demonstrate the particular expression of suprabasal markers such as cytokeratin 10, involucrin, and filaggrin on MSC-based epidermis including finding only pan-cytokeratin [45] and minimal cytokeratin 10 or filaggrin [46] in MSC stimulated to develop into keratinocytes. However, further confirmation of this differentiation using other parameters and/or protein expression of these parameters is required for more deep mechanistic studies of such differentiation.

Previous studies demonstrated that skin aging induction using D-gal causes an accelerated aging phenotype, as well as alterations in advanced glycation end products levels and the expression levels of senescence markers including SOD and MDA [24, 47]. Upon injection of the D-gal, the skin starts to induce skin changes that are similar to aged skin. First, morphologically, the skin showed prominent wrinkles and darkness of the skin. Furthermore, histopathologically, the skin of the d-galactose injected group showed atrophied irregular epidermis and appearance of thick collagen and thick fibrous bands in both upper and deep dermis. In addition, specific orcein staining of elastic fibers showed that D-gal stimulated the formation of scattered thick fragmented fibers. Interestingly, the initial significant finding of this study is that intradermally transplantation of Ad-MSCs restored the wrinkle formation in the D-gal induced skin-aged rat model, restored the intactness of skin epidermis, decreased the thick collagen/fibers bands in upper and deep dermis and restored the thin intact elastic fibers of the skin indicating the abilities of the Ad-MSCs to ameliorate skin aging. On the other hand, the treated group with conditioned media derived from EPCs revealed an improvement in the morphological appearance, histological examination of skin appendages along with SOD, MDA level. Therefore, the anti-aging properties of the conditioned media derived from EPCs - were observed in this study. It has been previously reported that conditioned media derived from EPCs substantially reduced hydrogen peroxide-induced changes in antioxidative enzyme activity, as well as mRNA and protein levels, such as superoxide dismutase, and glutathione peroxidase [23].

The observed improvement in the skin condition is likely attributed to the ability of Ad-MSCs to restore collagen synthesis and inhibits ROS process. These findings align with a previous study demonstrating that MSCs may contribute to the regeneration of skin during aging, in addition to that they can provide an actual beneficial effect in an aging mouse model through glycation suppression, antioxidation, and trophic actions [48]. Likewise on the other study, the skin texture, wrinkles, and dermal thickness were reported to have been improved eight weeks following treatment with Ad-MSCs intradermally [40]. On the other side of clinical trials, autologous fat grafting has been shown to rejuvenate aging skin and increase the amount of peri-ocular and peri-oral skin in recipients with an average age of 50 years [49].

Although our findings provide valuable insights into the potential effect of Ad-MSCs as an anti-aging therapy, several limitations should be acknowledged. Firstly, this study focused on a rat model, and translation to human subjects may necessitate further research. Secondly, the long-term effects and safety profile of Ad-MSC therapy need to be investigated to ensure its clinical applicability. Additionally, the specific mechanisms underlying the therapeutic effects of MSCs in skin aging require further clarification. The limitations were in accordance with a study that explained a significant barrier in cell treatment which is the poor engraftment rate of transplanted cells, which reduces cell therapy efficiency [50]. A viewpoint of another study is that the capacity of the full transplanted adipose derived stem cells to migrate and differentiate into skin cells may be challenging. These cells became apparent to undergo apoptosis within a few days after transplantation [51].

In conclusion, the current study demonstrates that adipose tissue is a niche source for MSCs. Adipose derived MSCs and conditioned media derived from EPCs provide a promise anti-aging strategy which leads to improved skin elasticity, reduced wrinkles, enhanced skin architecture, and reduced oxidative stress in a skin induced aged rat model. These findings suggest that Ad-MSCs and CM of EPCs may hold promise as a therapeutic strategy for mitigating skin-induced aging. Further research is warranted to fully understand the molecular mechanisms underlying the regenerative effects of MSCs and to evaluate their therapeutic potential in human subjects.

Acknowledgements We acknowledge Dr. Sayed Abdel-Raheem, Professor of Histopathology, Faculty of Medicine, Al Azhar University, Cairo, Egypt for performing the histopathological and histology specific stain examination for the skin used in this study.

Author contributions All authors contributed to concept, design, or operational aspects of the study. Badr. OI, Mahmoud. T., and Anter. A., Magdy. I., Chukueggu. M., Khorshid. M., Darwish. M., Farrag. M., Elsayed. M., Amr.Y., Amgad. Y. did the experiments and collected data. Badr. OI, Kamal. MM presented the results and did the statistical analyses. Badr. OI, and Kamal. MM wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Data availability All data is available upon request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical statement This study was performed in line with the guidelines and after the approval of the Ethical Committee of Faculty of Pharmacy, The British University in Egypt (EX:2317).

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