Original Article

Mesenchymal Stem Cell-derived Exosomes Improved Healing of Cutaneous Wound in a Rat Model

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Abstract

Introduction: Wounds of skin are common injuries causing familial burdens. Exosomes received attention as a cell-free therapy. **Aim:** Studying the role of exosomes (derived from mesenchymal stromal cells [MSCs]) on healing of cutaneous wound in adult male Wistar rats. **Materials and Methods:** Five weaned rats were used for exosome isolation. Exosomes were isolated (from bone marrow-MSCs) through ultracentrifugation. They were then characterized using a transmission electron microscope. The study was conducted on 42 adult male rats. They were divided into Group I (control group); Group II (spontaneous wound healing) and Group III (exosomes treated group): 24 h after generation of skin wound rats received a single intravenous injection of 1 mL phosphate buffer saline containing purified exosomes. Skin specimens were collected after 14 days and 21 days. Skin specimens were subjected to proper histological techniques. **Results:** Administration of exosomes decreased pain experienced by rats, improved wound healing, enhanced epidermal reepithelization and the regeneration of skin appendages, significantly increased epidermal cell proliferation and lead to better organization of newly formed collagen. **Conclusions:** Intravenous injection of exosomes was effective in accelerating cutaneous wound healing in adult rats.

Keywords: Collagen, exosomes, proliferating cell nuclear antigen, skin wound, stem cells, wound healing

Introduction

Annually, over 100 million people develop scars because of trauma or even surgeries. These scars usually lead to discomfort and pain. Scars are also associated with disfigurement and depression. It may be also associated with limitation of movement, and permanent disability. Surgery and laser therapy are examples of therapeutic measures for scarring. However, they have several limitations, that restrict their wide clinical application.[1,2]

Studies showed that the beneficial effects of stem cells therapy in wound healing occur mainly via paracrine mechanisms. It was reported that exosomes play the main role in this mechanism.[3] Therefore, attentions of recent researchers have been attracted to using exosomes derived from mesenchymal stromal cells (MSCs)-as a novel mechanism through which cell-to-cell communication occurs.[2]

Exosomes are membrane-bound vesicles with a diameter ranging from 30 to 150 nm. They represent an essential medium for communication between cells with many proteins and micro-RNAs (miRNAs) inside them. Recent studies demonstrated that exosomes enhanced tissue repair because of their advantages of high stability, homing effect, and absence of immune rejection.[4,5]

Aim of the work

Study the possible role of exosomes (derived from MSCs) in accelerating the healing of cutaneous wound in adult male albino rats.

Materials and Methods

Isolation and culture of bone marrow-mesenchymal stromal cells

Five young weaned male albino rats, weighing about 70 g and aged about 4 weeks, were used to isolate bone

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marrow (BM)-MSCs. Femurs were collected from rats, and isolation and culture of BM-MSCs were performed at the Stem Cell Lab, Histology Department, Faculty of Medicine, Ain Shams University.[6,7]

Isolation and characterization of exosomes

The isolation of exosomes

After three to five passages of culturing BM-MSCs, when tissue culture flasks reached 70%–80% confluence, conditioned media were collected and centrifugated. The collected media was centrifugated, using a cooling centrifuge, at >1000 ×g for 10 min at 4°C to remove residual cells. Then, the pellet was discarded, and the supernatant was transferred to a new centrifuge tube. Samples were then centrifugated, using a cooling centrifuge, at 2000–5000 ×g at 4°C for 20 min to remove dead cells and large debris. Then, the pellet was discarded, and the supernatant was transferred to a new centrifuge tube. Residual cellular debris in supernatant was removed by passing through 0.22 µm filter on top of a sterile bottle. The filtered sample was then transferred to an ultracentrifuge tube/s and was ultra-centrifugated at 100,000 ×g for 1 h. The supernatant was discarded and the pellet collected contained the small EVs. The final collected pellet (that contained the small extracellular vesicles) was then resuspended in 100 µm phosphate buffer saline (PBS) and was stored at −80 till usage.[8] Ultracentrifugation was done at the National Research Center, Cairo using a Cooling centrifuge (up to 5000 ×g) (Centurion Scientific K2015 R Centrifuge, UK). Ultracentrifuge (100,000 ×g) (Sorval-MTx150, USA).

Characterization of exosomes

It was done using transmission electron microscopy (TEM), JEOL-1010; 80kv, (at The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt). This was done by placing 3.5 µL of exosomes concentrate on a formvar/carbon-coated grid. The grid was left for 60 s. Blotting with filter paper was then done to remove the excess liquid. About 3.5 µL of 1% phosphotungstic acid was pipetted and put on the grid. The grid was left for 15 s. Blotting with filter paper was done to remove the excess liquid. Then sections were examined and photographed by TEM.[9]

Animals

This study was conducted on 42 adult male albino Wistar rats with an average weight of 200 g. Rats were housed in plastic cages. During the experiment, rats were given tap water and standard rat diet. Rats were maintained under suitable conditions (of light and temperature). The experiment was carried out in the Medical Research Center Institute (MASRI), Faculty of Medicine Ain Shams University.

Ethical approval

All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Permission of Ethical approval was obtained from the Ethical committee (FMASU REC) of animal research, (Faculty of Medicine, Ain Shams University) with Federal Wide Assurance No. FWA 00017585.

Animal groups

After 7-day acclimatization period, adult rats were divided randomly into three groups, 14 rats each.

Group I (control group)

Rats were anesthetized (by intraperitoneal injection of 40 mg/kg pentobarbital sodium).[10] The hair on the back was removed. After 24 h, rats received a single intravenous injection of 1 ml PBS (Lonza, 17-516f, USA) in the tail vein. Then, rats were equally divided into two subgroups: *Subgroups Ia* and *Ib* where rats were sacrificed after 14 days and 21 days, respectively.

Group II (spontaneous wound healing)

Included rats that were anesthetized, and hair removal was performed as in group I. Rats were then subjected to full-thickness skin wound.[11,12] On the 2nd day after surgery, rats were given an intravenous injection of 1 mL PBS in the tail vein. Rats were then divided into *Subgroups IIa* and *IIb* where rats were sacrificed after 14 days and 21 days, respectively, and skin specimens were then taken including wound area in the middle.

Group III (treated skin wound with exosomes)

Included rats that were anesthetized and subjected to full-thickness skin wound as in Group II. On the 2nd day after surgery, rats received a single intravenous injection of 1 mL PBS in the tail vein containing purified exosome concentrate collected from the conditioned medium of one million MSCs (1 mg/mL MSCs derived exosomes). Then rats were equally divided into: *Subgroups IIIa* and *IIIb* where rats were sacrificed after 14 days and 21 days, respectively, and skin specimens were taken including wound area in the middle.

Procedure of full-thickness skin wound and postoperative care

Rats of groups II and III were anesthetized using pentobarbital sodium at a dose of 40 mg/kg.[10] The hair on the back was removed first with scissors and then, through the application of One® depilatory cream (Eva Cosmetics, Egypt). The skin of the back was then sterilized with betadine and 70% ethanol. Full thickness wound (approximately 1.0 cm × 1.0 cm) was aseptically generated in the back (in the midline, 2 cm behind the head) using scissors and scalpel [Figure 1a and b]. The wound was bandaged with Vaseline gauze. Then, the Vaseline gauze was kept in place by medical adhesive tape (Pharmaplast, Egypt).[11,12] Rats from all groups received topical neomycin/bacitracin antibiotics spray (Medical Union Pharmaceuticals, Egypt) for 7 days. Rats also received systemic antibiotics (amoxicillin/flucloxacillin) (EIPICO, Egypt) and analgesics (diclofenac 3.5 mg/k) (Novartis, Switzerland) for 3 days.[13] In Groups II and III, the Vaseline gauze was changed daily, in complete aseptic conditions, till the 7th day.

Gross examination of the wound

Skin wound was observed grossly and photographed. Both the length and width of the wound were also measured using

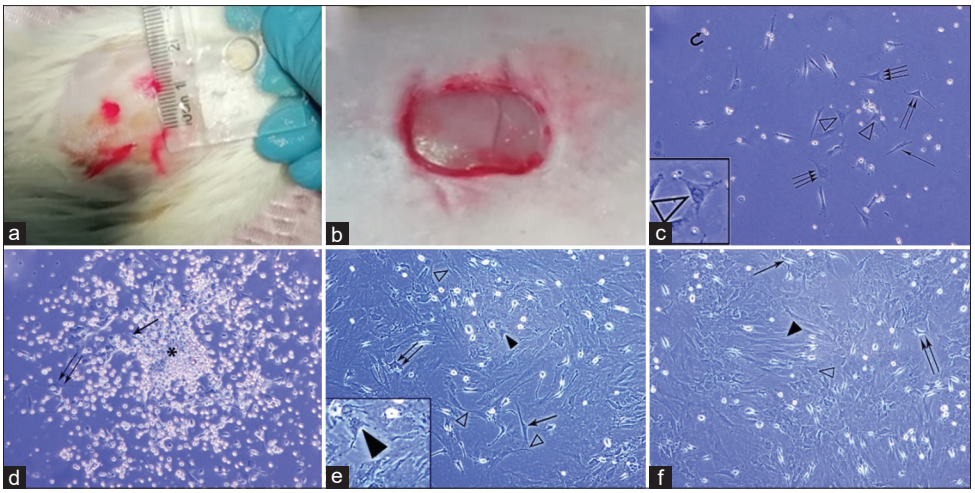


Figure 1: (a) The dorsal surface of rats after hair removal. Red dots represent where the wound will be generated (about 1 cm × 1 cm) (b) Full-thickness skin wound. (c-f) Phase contrast photomicrographs of rat bone marrow mesenchymal stromal cells ×200, insets ×400 (c) Day 3 showing spindle-shaped (↑), triangular (↑↑), and star-shaped cells (↑↑↑). Cells with rounded vesicular nuclei and granular cytoplasm (Δ). Nonadherent hematopoietic stem cells appear rounded and refractile (curved arrow). (d) day 5 showing a colony of proliferating stem cells (*). Note: Spindle (↑) and star-shaped cells (↑↑). (e) Day 7 showing spindle-shaped (↑) and star-shaped cells (↑↑) whose processes are interdigitating with each other (Δ). Some cells show granular cytoplasm and round vesicular nuclei (▲). (f) Day 12 showing spindle-shaped (↑), triangular (↑↑), and star-shaped cells (Δ) with long interdigitating cytoplasmic processes (▲)

a conventional metric ruler. These were done to all rats from Groups II and III on days 7, 14, and day 21.

Analyzing the nociceptive behavior of rats

The nociceptive behavior experienced by rats was observed throughout the study in all rats of Groups II and III according to Sotocinal *et al*.,[14] using Rat Grimace Scale (RGS). They used rat`s facial expressions to analyze the subjective intensity of pain in rats. The RGS is composed of 4 action units (Orbital Tightening, nose/cheek flattening, ear changes, and whisker change). They assigned three scores for each of these units: 0 (A sent), 1 (Moderate), and 2 (obvious). Hence, in this study, each action unit was scored accordingly and an average score of (0–2) was calculated.

Sample collection

At 14 days and 21 days, rats were sacrificed after ether inhalation anesthesia. The skin of the back including the wound area and surrounding normal skin was harvested from all rats.

Preparation of tissue

Skin samples were fixed (in 10% formalin) for 5 days. Specimens were then processed till obtaining of paraffin blocks.[15] Serial sections were cut and subjected to proper techniques (Hematoxylin and eosin [H and E], Masson's trichrome and periodic acid–Schiff [PAS]). In addition, paraffin sections were also subjected to immunohistochemical study using anti-proliferating cell nuclear antigen (PCNA) for detection of proliferating cells in the epidermis and dermis, (the kit was supplied by Santa Cruz Biotechnology Dallas Texas USA Catalog number: proliferating cell nuclear antigen [PCNA] Antibody [F-2]: Sc-25280).[15] Positive control was a skin section from the control group. Paraffin sections of the skin from all groups were cut at 5 m thickness on positively charged glass slides. They were incubated in 42°C oven for 24 h. Sections were deparaffinized in xylene. They were washed in 100% ethyl alcohol. Then they were rehydrated in descending grades of alcohol. Sections were washed two times in the buffer. Then were incubated in 5% normal horse serum block to reduce unspecific background staining. Sections were washed four times in the buffer for 5 min each. Slides were incubated in 3% hydrogen peroxide block for 10 min, to reduce nonspecific background staining due to endogenous peroxidase. Then, they were washed four times in the buffer for 5 min each. Antigen retrieval was done by putting the slides in citrate buffer pH6 in the microwave for 3 min. Two drops of ready-to-use primary antibody (diluted at 1:1000 for 30 min) were applied to each section then they were incubated for 1 h at room temperature. Sections were washed four times in the buffer for 5 min each. Two drops of biotylinated secondary antibody were added and then sections were incubated for 10 min at room temperature. Sections were washed four times in the buffer for 5 min each. Two drops of HRP Polymer were added and sections were incubated for 15 min at room temperature. Sections were washed four times in the buffer for 5 min each. One drop (40 µ) of DAB Plus Chromogen was added to 2 mL of DAB Plus Substrate. They were mixed by swirling and were applied to sections for 3 min at room temperature. Then sections were incubated for 5 min. Sections were washed four times in distilled water. Then, they were counterstained with Mayer's hematoxylin for 2 min. Sections were then dehydrated in ascending grades of alcohol, cleared, and mounted by Digital Picture Exchange (DPX). For negative control, the same steps were done except for the primary antibody which was replaced with PBS. Positive PCNA reaction showed a brown nuclear reaction, while no reaction was detected in the negative control run.

Morphometric and statistical study

The following was quantitated to measure the mean of:

1. Wound size (length and width) was measured in rats of groups II and III with a conventional metric ruler on days 7, 14, and 21
2. The thickness of the epidermis at the edge and center of the wound (×20)
3. Dermal thickness at the center of the wound (×10)
4. Area percentage of collagen fibers (×20) (in Masson's trichrome sections)
5. Number of PCNA-positive nuclei per high power field was measured in the epidermis (×40).

Measurements for parameters 2, 3, 4, and 5 were done using an image analyzer Leica Q win V.3 program (Wetzlar, Germany) in the Department of Histology, Faculty of Medicine, Ain Shams University. Measurements were taken from five nonoverlapping fields from all animals.

Statistical analysis

The mean value and the standard deviation (SD) were calculated in different groups using SPSS statistical program version 21 (IBM Inc., Chicago, Illinois, USA). Comparison between studied groups was performed using:

a. Student's *t*-test in analyzing the size of wound area
b. One-way analysis of variance with *post hoc* test in analyzing the other parameters.

Values were presented as mean ± SD. The probability of chance (*P* value) was used to determine the significance of data, ($P < 0.05$ is significant and $P > 0.05$ is nonsignificant).

Results

Morphology of rat bone marrow-derived mesenchymal stromal cells in primary culture using inverted phase contrast microscope

Primary culture on day 3 showed some cultured stem cells adherent to the bottom of the culture flasks [Figure 1c]. On day 5, an apparent increased number of attached cells was noticed [Figure 1d]. On day 7, more increase in the number of attached cells was noticed than on day 5. The cells reached nearly 70% confluence and showed variable shapes [Figure 1e]. On day 12 of culturing the stem cells, the adherent cells reached about 95% confluence [Figure 1f]. At this stage, subculturing and passaging of stem cells were done for isolation of exosomes after 3–5 passages.

Characterization of exosomes by transmission electron microscopy

The shape and size of exosomes were analyzed using TEM using phosphotungstic acid as a staining agent. Exosomes appeared as membrane-bounded electron-lucent vesicles with a mean diameter of 41.7 nm [Figure 2].

General behavior of the rats

Rats of spontaneous wound healing (Group II) showed nociceptive behaviors (score "1" using RGS). This nociceptive behavior lasted for 7 days after wound generation despite using analgesics. Rats of treated skin wound with exosomes (Group III) showed nociceptive behaviors (score "1" using RGS) for only 4 days.

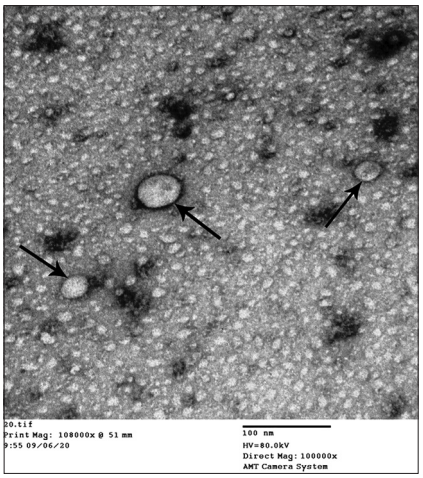


Figure 2: A transmission electron micrograph showing exosomes (↑) of different diameters. They have rounded to oval shape. Notice: Phosphotungstic acid is concentrated on the membrane of exosomes. Transmission electron microscopy ×100.000

Mortality rate

Two rats from Group II died during the experiment. No mortality was observed in the control group (Group I) or wound group treated with exosomes (Group III).

Gross examination of the wound

Gross examination of the wound was done on days 7, 14, and 21 [Figure 3]. In the spontaneous wound healing group (Group II), the wound showed no signs of inflammation on days 7, 14, and 21. The size of the wound was about 1.0 cm × 1.0 cm on day 0. On day 7, it was almost of the same size. On day 14, it reached approximately <1.0 cm × 1.0 cm and about half the original size on day 21. In Group III (treated skin wound with exosomes) the wound showed no signs of inflammation on days 7, 14, and 21. Regarding the size of the wound, it was approximately 1.0 cm × 1.0 cm on day 0. An apparent decrease in the size of the wound was noticed on days 7, 14, and 21 in comparison with corresponding days in group II [Figure 3 and Histogram 1].

Histological and immunohistochemical results

Skin sections from rats of control subgroups (Ia and Ib), showed similar results by using different histological techniques.

H and E-stained sections from control rats showed the skin formed of epidermis and dermis. The epidermis consisted of stratified squamous keratinized epithelium resting on a wavy basement membrane. The deepest layer of epidermis was stratum basale which was formed of a single layer of small cuboidal cells resting on a wavey basement membrane. The cells had basophilic cytoplasm and rounded vesicular nuclei. Stratum spinosum consisted of many layers of polyhedral cells with acidophilic cytoplasm and central rounded vesicular

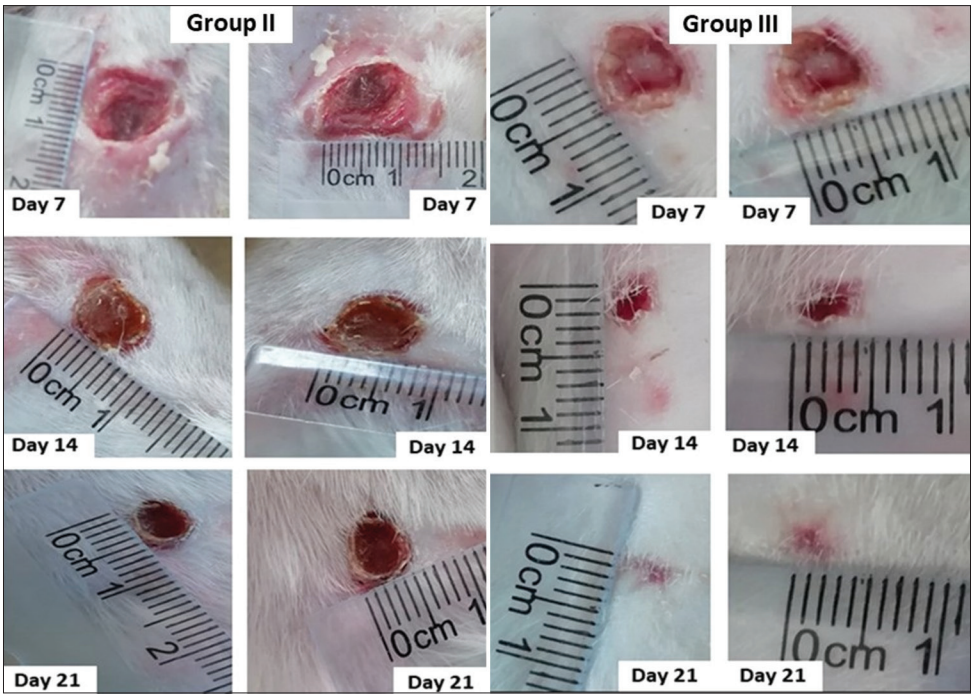
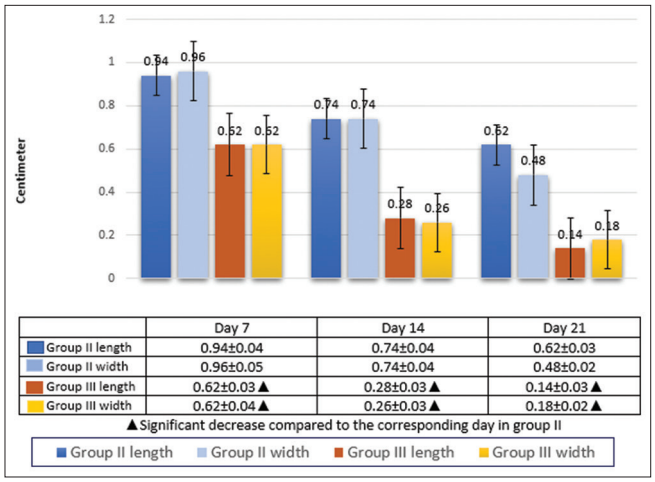


Figure 3: Photographs of skin wound in Groups II and III on days 7, 14, and 21. Notice the decrease in wound size in Group III compared to corresponding day in Group II



Histogram 1: The mean wound size (length and width) in groups II and III

nuclei. Stratum granulosum was formed of two to three layers of flat cells with numerous basophilic granules. Finally, the superficial stratum corneum appeared as noncellular acidophilic scales. The underlying dermis was formed of superficial papillary and deep reticular layers. Deep to the dermis, the hypodermis was seen formed of adipose tissue arranged in lobules separated by connective tissue septa. The papillary layer of the dermis was formed of loose well-vascularized cellular connective tissue. The deep reticular dermis was less cellular than the papillary dermis. It was formed of thick layers of dense irregular collagen bundles [Figure 4a and b]. The edge and the center of the wound area in subgroup IIa showed the newly formed epidermis formed of few layers of flattened cells. An apparent decrease in cellularity and vascularity was noticed in the papillary dermis [Figure 4c and d]. In subgroup IIb, the newly formed epidermis was formed of undifferentiated layers of keratinocytes resting on the ill-defined basement membrane. The absence of hair follicles and sebaceous glands was still noticed in the underlying dermis [Figure 4e and f]. With administration of exposomes in subgroup IIIa, down growth of the epidermis into the underlying dermis was frequently noticed although no skin appendages could be detected. The epidermis appeared more differentiated in comparison to subgroup IIa. Moreover, multiple layers of epidermal cells were seen migrating from the edge to cover the center of the wound. However, the separation between the new epidermis and dermis was frequently observed [Figure 5a-c]. In subgroup IIIb, the newly formed epidermis was thinner than that of subgroup IIIa and more differentiated when compared to subgroup IIb. New hair follicles and sebaceous glands were also detected [Figure 5d-f].

Masson's trichrome-stained sections of control subgroups showed the papillary dermis, containing fine collagen fibers. While deeper in the reticular dermis, short thick collagen bundles were seen [Figure 6a and b]. In subgroup IIa, the dermis at the wound area contained a high amount of reddish interfibrillar matrix with few disorganized collagen fibers in between [Figure 7a and b]. In subgroup IIb, an apparent increased content of collagen fibers was seen with an inapparent reddish interfibrillar matrix in-between as compared to subgroup IIa. In the reticular dermis, collagen bundles were relatively thicker but were still disorganized, and widely separated [Figure 7c and d]. In subgroup IIIa, the edge of the wound showed better organization of collagen

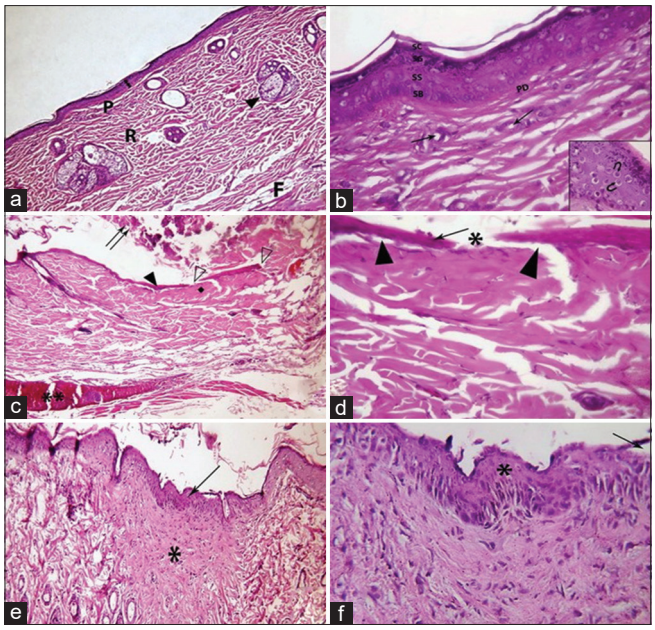


Figure 4: Hematoxylin and eosin-stained skin sections: (a and b) *group I*: (a) The epidermis (↕) consists of stratified squamous keratinized epithelium. The dermis is formed of papillary layer (P), and deep reticular layer (R), containing sebaceous glands (▲). Note: Fat cells (F) in the hypodermis. (b) Stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. Papillary dermis (PD) is formed of loose well vascularized cellular connective tissue. Small blood vessels are seen (↑). Inset: Showing clear Langerhans cells with rounded deeply stained nuclei and poorly stained cytoplasm (curved arrow). (c and d) *subgroup IIa:* (c) Epidermal re-epithelialization (▲) is seen covering most of the wound except few small areas (Δ). Necrotic tissue and crust (↑↑) are seen. The underlying dermis (♦) shows absence of skin appendages. Congested area (**) is seen deep in the hypodermis. (d) The epidermis at the center of wound is formed of few layers of flattened cells (↑) with flat nuclei and acidophilic cytoplasm. Separation (▲) between newly formed dermis and the regenerated epidermis is seen. Notice: Small area is not covered with epidermis (*). An apparent decrease cellularity and vascularity is seen in the PD. (e and f) *subgroup IIb:* (e) The wound site (*) is covered with thick, new epidermis (↑) formed of stratified squamous keratinized epithelium. Notice absence of hair follicles and sebaceous glands in the dermis at the wound site (*) (f) the wound is covered with undifferentiated layers of keratinocytes (*) with indistinct basement membrane. Separation of the most superficial layers of epidermis is seen (↑). (H and E, [a, c and e, ×100], [b, d and f, ×400])

with an inapparent reddish interfibrillar matrix compared to subgroup IIa [Figure 8a and b]. In subgroup IIIb, better organization of collagen in the wound area was noticed compared to subgroup IIb. Cellularity, vascularity, and extravasation of red blood corpuscles were infrequently noticed in comparison with Group IIIa [Figure 8c and d].

PAS-sections of control subgroups showed the epidermis resting on a well-defined continuous PAS-positive basement membrane [Figure 9a]. In subgroups IIa and IIb, the newly formed epidermis was seen resting on an ill-defined basement membrane [Figure 9b and c, respectively]. In subgroup IIIa, areas with ill-defined basement membranes were sometimes noticed [Figure 9d]. While in subgroup IIIb, a well-defined PAS-positive basement membrane was seen under the newly formed epidermis [Figure 9e].

Examination of PCNA sections in the control subgroups showed strong positive PCNA reaction in the nuclei of most cells of stratum basale, few cells of stratum spinosum, in the basal fusiform cells in the sebaceous glands as well as the outer root sheath [Figure 10a and b]. In subgroup IIa, mild positive PCNA reaction was noticed in some flattened cells in the new epidermis as well as in few nuclei in the papillary dermis [Figure 10c]. In subgroup IIb, increased number of PCNA-positive nuclei was noticed in the basal layer of the epidermis and the papillary dermis compared to subgroup IIa [Figure 10d]. In subgroup IIIa, a positive PCNA reaction was seen in all layers of the newly formed epidermis as well as in the papillary dermis [Figure 10e]. In subgroup IIIb, positive PCNA reaction was seen in many cells of the basal cell layer and prickle cell layer as well as the outer root sheath of hair follicles. Furthermore, there was an apparent increase in the number of PCNA-positive cells in the papillary dermis [Figure 10f].

Histomorphometric results and statistical analysis

Statistical results showed nonsignificant differences between control subgroups in all measured parameters.

The mean wound size by gross examination

Statistical results of the mean wound size (both length and width) on days 7, 14, and 21, showed a significant decrease in group III compared to the corresponding day in Group II [Histogram 1 and Table 1].

The mean epidermal thickness (at edge and center) [Table 1]

A significant decrease was noticed at both the edge and center of the wound in *subgroup IIa* compared to control subgroups, whereas *subgroup IIb* showed a significant increase compared to control subgroups and subgroup IIa. Regarding *subgroup IIIa*, a significant increase – at the edge of the wound – was observed compared to control subgroups and subgroup IIa. On the other hand, a significant decrease in the mean epidermal thickness was noticed at the center of the wound compared to control subgroups. Moreover, *subgroup IIIb* revealed a significant decrease at the edge of the wound and a nonsignificant change at the center of the wound compared to subgroup IIIa. Moreover, *subgroup IIIb* revealed a significant decrease at the edge and center of the wound compared to control subgroups and subgroup IIb.

The mean dermal thickness [Table 1]

A significant decrease in *subgroups IIa* and *IIb* was noticed compared to control subgroups. While a significant increase was noticed in *subgroup IIb* compared to subgroup IIa. Regarding *subgroups IIIa* and *IIIb*, there was a significant increase compared to subgroups IIa and IIb, respectively. A significant increase was also noticed compared to control subgroups.

The mean area percentage of collagen fibers [Table 1]

A significant decrease was noticed in *subgroups IIa and IIb* compared to control subgroups, whereas a significant increase

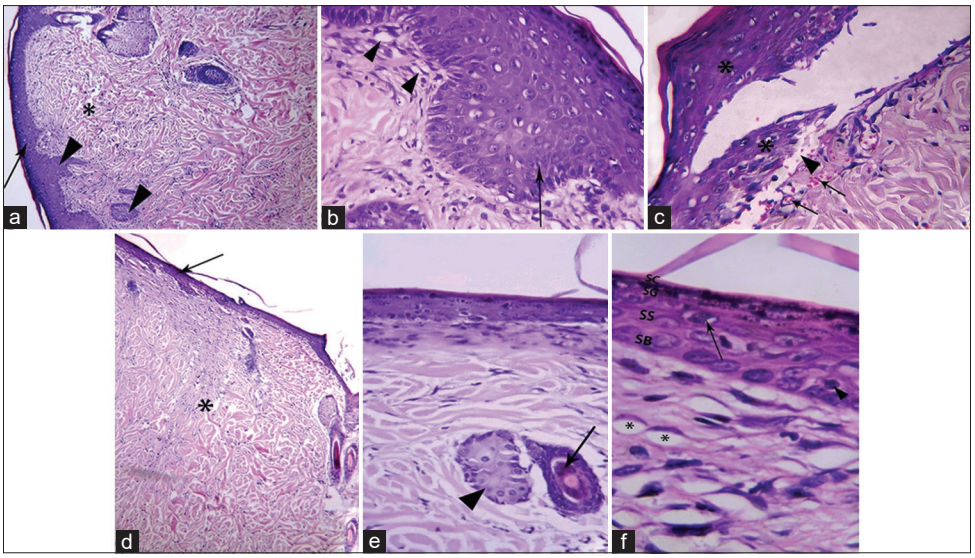


Figure 5: Hematoxylin and eosin-stained skin sections. (a-c) *subgroup IIIa:* (a) the edge of the wound (*) is covered with thick, new epidermis (↑). Down growth of the epidermis (▲) into the underlying dermis is seen with absence of skin appendages. (b) The newly formed epidermis at the edge of the wound is formed of thick layer of stratified squamous keratinized epithelium (↑). Notice numerous blood vessels (▲) and high cellularity of the papillary dermis. (c) Multiple layers of epidermal cells cover the center of the wound (*) with separation between the new epidermis and dermis (▲). Notice extravasation of red blood cells (↑) and high cellularity of the dermis. (d-f) *subgroup IIIb:* (d) The wound (*) completely covered by thin epidermis (↑). Notice sebaceous gland and hair follicle in adjacent normal skin. (e) A newly formed hair follicle (↑) and sebaceous gland (▲). (f) A differentiated epidermis is formed of stratum basale, stratum spinosum, stratum granulosum (SG), and stratum corneum, with the presence of clear cells (▲) and mitotic figures (↑). Notice basophilic keratohyalin granules in cells of SG. Notice cellular dermis and blood vessels (*) in the papillary dermis (a and d, ×100), (b, c, e and f, ×400)

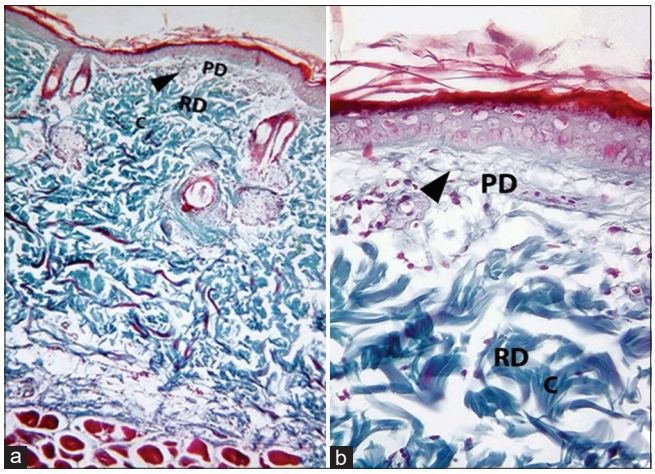


Figure 6: (a and b) Photomicrographs of Masson's trichrome-stained sections of the control group (Group I). The papillary dermis containing fine interlacing collagen fibers (▲). The reticular dermis contains thick short wavy collagen bundles (C) (a, ×100), (b, ×400)

was observed in *subgroup IIb* compared to subgroup IIa. Regarding *subgroups IIIa* and *IIIb*, there was a significant increase compared to subgroups IIa and IIb, respectively, and a nonsignificant change compared to control subgroups.

The mean number of positive proliferating cell nuclear antigen nuclei in the epidermis [Table 1]

A significant decrease was noticed in the epidermis in *subgroups IIa,* compared to control subgroups. While a significant increase was detected in *subgroup IIb* compared to control subgroups and subgroup IIa. Regarding *subgroup IIIa*, it showed a significant increase compared to control subgroups and subgroup IIa. Moreover, *subgroup IIIb* showed a significant decrease compared to both subgroups IIIa and IIb.

Discussion

The skin protects our body from the external environment. Hence, the goal of wound healing is to restore skin function.[16] Recently, exosomes are considered a new emerging treatment for tissue repair. Their efficacy was reported in the regeneration of skin, nerve, blood vessels, and bone.[17]

In the current study, exosomes were administered intravenously. It was reported that intravenous injection of exosomes promoted wound healing better than local injection. They added that the injection itself can disrupt the wound healing process while when exosomes are injected intravenously, they are recruited to the wound area through homing process.[18]

Throughout the current study, the general behavior of rats was observed. Rats in group III showed nociceptive behavior for a shorter duration than the rats in group II. This could suggest the role of exosomes in relieving pain. Although the effects of exosomes on pain are still under research, Zhang *et al*., found that MSCs-derived exosomes had a significant role in suppressing the pain as well as reducing the expression of genes associated with pain as Substance P and nerve growth factor.[19] Moreover, studies of He *et al*., demonstrated that exosomes played a significant role in alleviating pain on

Table 1: The mean epidermal and dermal thickness (μm), the mean area percentage (%) of collagen fibers and the mean number of epidermal proliferating cell nuclear antigen positive nuclei in different groups

Variable	Group I (control group)		Group II (spontaneous wound healing group)		Group III (exosome treated group)	
	Subgroup Ia (14 days)	Subgroup Ib (21 days)	Subgroup IIa (14 days)	Subgroup IIb (21 days)	Subgroup IIIa (14 days)	Subgroup IIIb (21 days)
Mean epidermal thickness						
Edge	39.22±5.02	38.56±4.92	23.94±3.04*	55.28±5.76*	69.92±9.67*	32.61±2.06*
Center	39.22±5.02	38.56±4.92	11.97±1.94*	54.16±6.37*	28.62±2.06▲	29.52±2.86▲
Mean dermal thickness	818.44±59.58	820.52±54.39	265.9±7.75*	500.26±70.41*	942.94±59.91▲	969.42±59.11▲
Mean area percentage of collagen fibers	26.41±1.56	27.01±2.3	11.54±1.28*	20.17±1.41*	29.77±5.56▪	28.94±3.05▪
Mean number of PCNA positive nuclei	37.17±2.32	38.2±3.4	29.0±4.74*	60.6±3.5•	63.0±5.1•	42.5±4.23*

*Significant change compared to all subgroups, ▲Significant change compared to subgroups Ia, Ib, IIa and IIb, ▪Significant change compared to subgroups IIa and IIb, •Significant change compared to subgroups Ia, Ib, IIa and IIIb. PCNA: Proliferating cell nuclear antigen

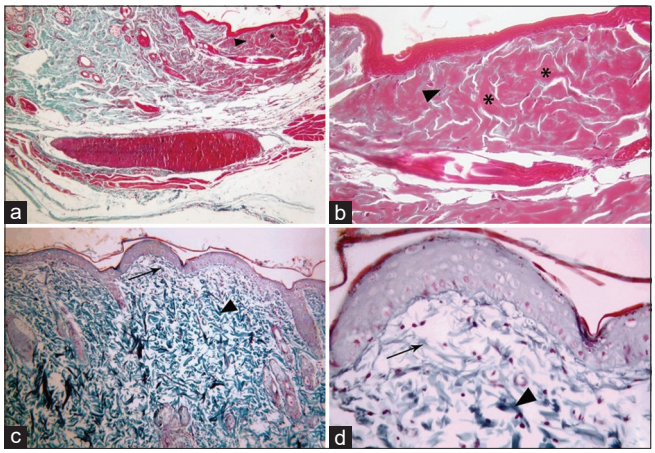


Figure 7: Masson's trichrome-stained sections. (a and b) *subgroup IIa*: The dermis contains high amount of reddish interfibrillar matrix (*) with few collagen fibers in between (▲). (c and d) *subgroup IIb:* Fine dispersed, fragmented collagen fibers (↑) in the papillary layer with relatively thicker, disorganized, widely separated collagen bundles (▲) in the reticular dermis (a and c, ×100), (b and d, ×400)

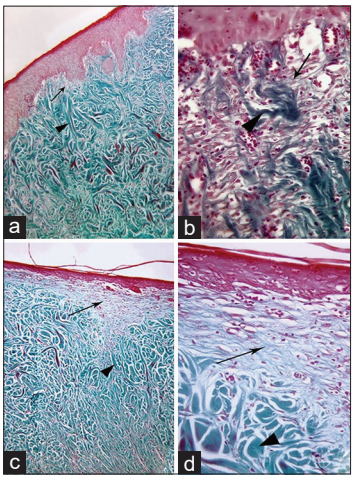


Figure 8: Masson's trichrome-stained sections. (a and b) *subgroup IIIa:* (a) at the edge of wound, collagen fibers fill the whole dermis. Fine collagen fibers (↑) in papillary dermis and thick, wavy collagen bundles (▲) in reticular dermis are seen. (b) The center of the wound contains disorganized fine collagen fibers (↑) and thick collagen bundles (▲) in both papillary and reticular layers. Notice high vascularity, extravasation of red blood cells, and high cellularity of the dermis. (c and d) *subgroup IIIb:* Fine collagen fibers below the epidermis in the papillary dermis (↑). Thick, wavy collagen bundles are seen in the reticular dermis (▲) (a and c, ×100), (b and d, ×400)

the clinical and biochemical levels.[20] It was reported that pain reduction caused by exosomes could be linked to their anti-inflammatory effects.[21] A similar observation was noticed by He *et al*., who reported that exosomes had a significant effect in reducing the levels of inducible nitric oxide synthase which is an inflammatory marker related to pain.[20]

In the current study, gross examination of skin wounds was conducted with measuring the wound size on days 7, 14, and 21. Group III showed faster healing, with a significant decrease in wound size when compared to the corresponding timeline in spontaneous wound healing Group II. This was confirmed by the current histo-morphometric results. This could suggest that MSCs-derived exosomes could accelerate wound closure. Similar results were observed by other researchers.[22,23] Regarding the mechanism of exosome-mediated wound healing, some authors reported that the cargo of exosomes (mRNA, miRNA, and various pro-angiogenic and anti-apoptotic factors) are found to mediate wound healing.[24] This occurs through the transfer of cargo which then modifies the activity of target cells.[25] It was reported that in skin wound, miRNAs produced in exosomes might be the cause of enhanced proliferation and migration of cells. They could target the corresponding mRNAs, and therefore, regulate the expression of proteins that increase the proliferation and migration of cells and might significantly increase wound healing.[23]

Furthermore, the faster healing process in the exosomes-treated group was explained by some authors who attributed this to the release of cytokines from exosomes. Cytokines promote growth factor secretion, decrease the inflammation, and reduce the capillary permeability, leading eventually to better tissue repair.[22] It was reported that there are two types of macrophages present during the healing process of skin wounds. M1 is a pro-inflammatory type and M2 is

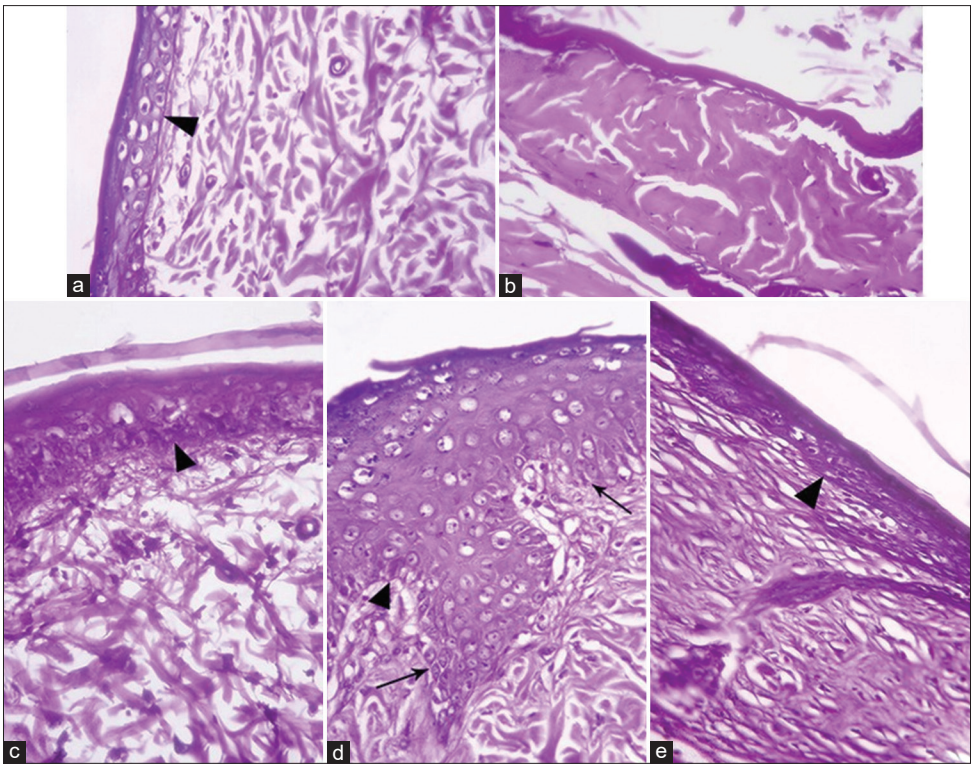


Figure 9: Periodic Acid Schiff (PAS)-stained sections of different groups ×400. (a) Group I, (b) Subgroup IIa, (c) Subgroup IIb, (d) Subgroup IIIa, (e) Subgroup IIIb. Well-defined PAS-positive basement membrane (▲), ill-defined basement membrane (↑)

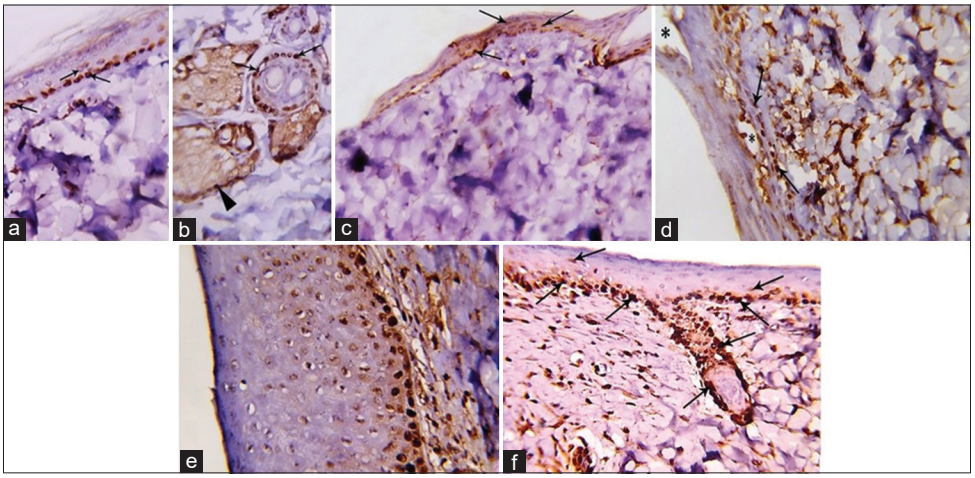


Figure 10: Photomicrographs of proliferating cell nuclear antigen (PCNA)-stained sections of different groups ×400. (a and b) *Group I:* (a) strong positive PCNA reaction (↑) in most cells of stratum basale and few cells of stratum spinosum. (b) In the outer root sheath of hair follicles (↑) as well as the basal fusiform cells of sebaceous glands (▲). (c) *subgroup IIa:* Mild positive PCNA reaction (↑). (d) *Subgroup IIb:* Positive PCNA reaction (↑) in basal layer of the epidermis. An apparent increase in the number of PCNA positive cells is observed in the papillary dermis. Separation between the layers of the epidermis (*) is seen. (e) *Subgroup IIIa:* Positive PCNA reaction is seen in all layers of the newly formed epidermis. Notice an apparent increase in the number of PCNA positive cells in the papillary dermis. (f) *Subgroup IIIb*: Positive PCNA reaction (↑) is seen in many cells of the stratum basale and stratum spinosum as well as in outer root sheath of a newly formed hair follicle. Notice an apparent increase in the number of PCNA-positive cells in the papillary dermis

an anti-inflammatory type. Recent evidence revealed that exosomes resulted in the switching of macrophages to the anti-inflammatory type.[26]

In the current study, in subgroup IIa, the beginning of re-epithelialization was noticed through the appearance of flattened cells covering most of the wound. Such results followed the explanation made by some authors who reported that keratinocytes during wound healing underwent some morphological changes as they became more flattened and elongated with long ruffling cytoplasmic projections.[27]

In subgroup IIb, although the newly formed epidermis was seen covering the whole wound area, it was formed of undifferentiated layers of keratinocytes resting on an ill-defined basement membrane with separation of the most superficial layers. Some authors reported that the differentiation of keratinocytes begins 2–3 weeks postwounding, taking a long duration, up to 2 years.[28] The differentiation of the epidermis is determined by the development of the usual five typical epidermal layers, forming the cornified envelope as the final step of such a complex process.[29] In contrast, in the current study, in subgroup IIIa, a thick layer of the stratified squamous keratinized epithelium was seen covering the wound edge and was seen migrating toward the wound's center. In subgroup IIIb, a thin layer of epidermis was seen. The mean thickness of the newly formed epidermis significantly decreased when compared to subgroup IIIa. It was more differentiated compared to subgroup IIb and was formed of the typical layers of the epidermis. This suggested that MSCs-derived exosomes could have a role in accelerating the process of re-epithelialization and promoting epidermal cell differentiation that resulted in enhancing wound healing.

The mechanisms of re-epithelialization in skin wounds treated with exosomes were discussed by many authors. they reported that exosomes induce keratinocyte migration and significantly increase the skin`s cell proliferation, promoting the re-epithelialization process and accelerating wound healing in the treated group compared to those in the untreated group.[18,26,30] In exosomes treated group of the current study, the thickness of newly formed epidermis at 21 days significantly decreased when compared to that on day 14. Some authors reported that the thickness of newly formed epidermis decreased with time postwounding as keratinocytes showed more differentiation, in which excess cells were removed by apoptosis, progressing from the edges of the wound inward.[31]

Regarding regeneration of skin appendages in the current study, the absence of hair follicles and sebaceous glands was noticed in subgroups IIa and IIb. Subgroup IIIa showed downgrowth of the epidermis without the formation of hair follicles. While in subgroup IIIb, the formation of hair follicles as well as sebaceous glands was noticed. This suggested that exosomes could have a role in the cutaneous regeneration, forming new hair follicles and sebaceous glands. This agreed with the results of El-Tookhy *et al*.[22]

The down growth of the epidermis in subgroups IIIa and IIIb might reflect the beginning of the formation of hair follicles. This was supported by the opinion of Ito *et al*.,[32] who reported that hair follicle regeneration started with the formation of epithelial placode (which is a cluster of epidermal cells), overlying a dermal condensate. The epidermal cells in epithelial placodes proliferated, moved downward, and engulfed the dermal condensate, through mesenchymal-epithelial interactions, eventually forming a mature hair follicle. Other authors reported that exosomes carry various growth factors that are essential for hair growth.[30]

In the current study, it was evident that group III showed an apparent increased number of newly formed blood vessels at the wound site when compared to those in Group II. This suggested that exosomes played a role in inducing angiogenesis during skin wound healing. Angiogenesis is an important step in cutaneous wound healing and tissue repair as it provides oxygen supply to the wound site to support the proliferation of migrated cells during the proliferative phase.[18,30] It was also reported that pro-angiogenic miRNAs, were found in large quantities in human MSC-derived exosomes, significantly inducing angiogenesis.[18]

In the current study, using PAS reaction, group II showed an inapparent basement membrane. In subgroup IIIa, the basement membrane was well-defined in most areas with some areas of ill-defined basement membrane while complete restoration of basement membrane was evident in subgroup IIIb. It was reported that keratinocytes in wound healing start to synthesize components of basement membrane during their migration over the wound bed. The restoration of basement membrane occurs at multiple sites of the wound at the same time, till complete restoration occurs 4 weeks postwounding.[33] This suggested that MSCs-derived exosomes could enhance the regeneration of the basement membrane by accelerating the process of re-epithelialization.

Masson`s trichrome stained sections of subgroup IIIa showed a significant increase in the mean area percentage of collagen compared to subgroup IIa, with better organization of collagen and inapparent reddish interfibrillar matrix. While subgroup IIIb showed better organization of collagen with a significant increase in the mean area percentage of collagen in comparison to subgroup IIb. This suggested that MSCs-derived exosomes could promote the synthesis and organization of collagen during wound healing. This agreed with the findings of El-Tookhy *et al*.[22]

In the current study, the presence of high amount of interfibrillar matrix was noticed on day 14 in subgroup IIa. This was in accordance with the results of Doillon *et al*.,[34] who reported that during wound healing, there was a high interfibrillar matrix in-between the formed collagen fibers, and such interfibrillar matrix progressively decreased with time postwounding.[34] It was reported that during wound healing, insufficient or excessive extracellular matrix formation can disrupt the process of healing, causing failure of wound healing or leading to scar formation. It was found that exosomes could regulate the re-synthesis of extracellular matrix, promoting its regeneration and therefore, playing a role in advancing wound healing.[26] It was reported that MSCs-derived exosomes could induce migration and proliferation of dermal fibroblasts as well as promoting their synthesis of collagen, elastin, and fibronectin.[26,30] It was reported that the mRNA and miRNA carried by exosomes could promote the proliferation of fibroblasts with enhancing collagen synthesis.[24]

In addition, exosomes were found to regulate the synthesis of collagen at different stages of wound healing, increasing the

production of type I early in wound healing, while at a late stage, they were found to inhibit collagen synthesis, leading to the reduction of scar formation.[26] This follows the histological changes of healing of soft tissue wounds, in which collagen deposition is important in the early stage of healing while in the late stage, remodeling is more important.[4] The accumulation of myofibroblasts in the wound areas leads to the formation of scar. Exosomes were found to prevent scar formation in skin wounds through inhibiting the differentiation of fibroblasts into myofibroblasts.[26,30]

In the current study, immunohistochemical technique for PCNA was performed. It was reported that PCNA is a marker for keratinocytes' proliferation.[35] In the current study, in subgroup IIIa, a significant increase in mean number of PCNA-positive nuclei was seen in the epidermis when compared to subgroup IIa with an apparent increase in the positive nuclei of the papillary dermis. While in subgroup IIIb, positive nuclear PCNA reaction was seen in many cells of the epidermis, the outer root sheath, as well as in the papillary dermis. These findings suggested that MSC-derived exosomes could have a role in increasing the proliferation of both epidermal and dermal cells, which enhanced the healing of skin wounds eventually. This came in agreement with the results of Zhang *et al*.,[36] It was found that exosomes enhanced proliferation of cells in the skin including epidermal keratinocytes and dermal fibroblasts, as indicated by increasing their expression of PCNA.[26,37]

Exosomes carry various growth factors in their cargo which promotes cell proliferation.[30] Moreover, mRNA and miRNA carried by exosomes promote cells' proliferation, either keratinocytes or dermal fibroblasts.[23,24]

Conclusions

From the current study, it was concluded that intravenous injection of exosomes (derived from BM mesenchymal stem cells) enhanced cutaneous wound healing in adult rats. Exosomes resulted in decreased pain experienced by rats, improved wound healing, and enhanced epidermal reepithelization. Exosomes also enhanced the regeneration of skin appendages and lead to better organization of newly formed collagen.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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