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Healing Potential of Mesenchymal Stem Cells Cultured on a Collagen-Based Scaffold for Skin Regeneration

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ABSTRACT

Background: Wound healing of burned skin remains a major goal in public health. Previous reports showed that the bone marrow stem cells were potent in keratinization and vascularization of full thickness skin wounds. **Methods**: In this study, mesenchymal stem cells were derived from rat adipose tissues and characterized by flowcytometry. Staining methods were used to evaluate their differentiation ability. A collagen-chitosan scaffold was prepared by freeze-drying method and crosslinked by carbodiimide-based crosslinker. **Results:** The results of immunecytochemistry and PCR experiments confirmed the adipose-derived stem cells (ASC) in differentiation to the keratinocytes under the treatment of keratinocyte growth factor. The isolated ASC were seeded on the scaffolds and implanted at the prepared wounds. The scaffolds without cells were considered as a control and implanted on the other side of the rat. Histopathological analyses confirmed the formation of new tissue on the scaffold-cell side after 14 days with the formation of dermis and epidermis. **Conclusion**: These results indicated the capacity of ASC in differentiation to keratinocytes and also wound healing *in vivo. Iran. Biomed. J. 16 (2): 68-76, 2012*

Keywords: Tissue engineering, Keratinocytes, Mesenchymal stem cells

INTRODUCTION

The skin as the largest organ in human is believed to be a protective barrier against the variety of microorganisms or harmful conditions [1]. Burn is a skin injury caused by different factors such as heat, electricity, radiations or abrasive chemicals [2]. Although significant progress has been made in the development of tissue-engineered construct for the replacement of lost skin, there is no ideal substitute in the market [3]. Collagen, hyaluronan, amnion, polylactide, polyglycolide and polybuthylene terephthalate are various materials utilized in skin tissue-engineered products [4]. Collagen is found abundantly in connective tissues of mammals and could be isolated from different sources such as Baltic cod, catfish, grass carp, bovine tendon or recombinant systems [5-9]. The main source is dependent on many factors such as cost, application or availability. Porcine products cannot be used for religious reasons and fish-based collagens are not cost effective. On the other hand, transferring infective agents from animals to human such as madcow is not fully confirmed [10]. Therefore, the bovine skin has been preferred as a main collagen sources in the artificial skin production. In several clinically approved products such as Integra®, Matriderm® or Terudermis®, bovine collagen is used as the basic material for wound management [11]. In order to obtain the appropriate skin construct with desired biologically properties, many researches are still conducted. The blend of collagen with other compositions such as chitosan and hyaluronic acid or its modification with different crosslinking agents are being investigated [12-15]. In spite of the advances in burn management, researches continue to find the appropriate way on the both economical and biological performance [16]. It has been well accepted that fibroblast and keratinocyte play an important role in skin regeneration and enhance the healing of wounds through implantation [17].

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The insufficient number of autogenic cells limits their usage. Besides, the ethical and safety problems should be considered for allogenic cells in skin wound repair [18]. For these reasons, stem cells draw much more attention in transplantation procedures.In the previous published work, an *in vitro* investigation was performed on keratinocytes and fibroblasts as potential source for skin grafts. The cells were seeded on a biocompatible scaffold based on collagen-chitosan. In order to increase the biostability, the structure was chemically crosslinked by glutaraldehyde [19]. In this study, mesenchymal stem cells were isolated from adipose tissue, cultured on the same scaffold with a little modification and implanted on burned skin. The differentiation capability of the isolated stem cells and also wound healing potential of this structure was assessed *in vivo*.

MATERIALS AND METHODS

Collagen was isolated from calf skin using salt extraction method to remove non-collagenous materials. The skin slices were solved by acetic acid solution at pH<3 and a salt solution (sodium chloride) used for collagen precipitation at neutral pH. The precipitated collagen fibrils were obtained by ultracentrifugation. Chitosan (medium viscosity, Aldrich, USA), 1-ethyl-3-3-dimethylaminopropyl-carbodiimide (EDC), N-hydroxysuccinimide, Glutaral-dehyde and acetic acid (all from Merck, Germany) were used as received.

Scaffold preparation. The solutions of isolated collagen (2%) and chitosan (2%) in acid acetic (0.5 M) were prepared and mixed with the ratio of 9:1. This solution was kept at -20°C for 24 hours and transferred to freeze-dryer (UniEquip, Unicryo, Germany) for 48 hours.Afterwards, the sample was immersed in a solution of crosslinking agent containing EDC and N-hydroxysuccinimide (5 mM in 70% ethanol) at 4°C for 24 hours. Finally, the sample was rinsed in deionized water for 24 hours, dried at 37°C for 3 days and sterilized by ultraviolet.

Stem cell isolation. Adipose stem cells were isolated from Wistarrats, obtained from Pasteur Institute of Iran. Anesthesia was induced with an intraperitoneal injection of ketamine (85 mg/kg) and xylazine (15 mg/kg). Adipose tissue was harvested from the upper part of the intestine with an incision. This tissue was chopped to the small pieces and digested in an incubator with 0.02 mg/ml collagenase type I (Sigma, USA) for 1 hour. The suspension was centrifuged at 200 ×g for 5 minutes and the cell pellet was separated. The sample adipose-derived stem cells (ASC) was transferred to the culture medium consisted of DMEM (Gibco, Scotland) supplemented with 10% FBS (Seromed, Germany), 100 U/mL penicillin and 100µg/mL streptomycin (Sigma, USA) in a humidified incubator (37°C, 5% CO_2). After 24 hours, non-adhered cells were removed and new culture medium was added.

Stem cell characterization. After three cell passages, the cells were characterized by flowcytometry using antibody CD markers. FITC anti-mouse/rat CD90.1 (0.5 µl), FITC mouse IgG2a isotype control (0.5 µl), FITC anti-rat CD45.2 (1 µl), FITC mouse IgG1 isotype control (1 µl), affinity purified mouse IgG1 isotype control (1 µl), PE donkey F(ab')2 fragment anti-mouse IgG (0.5 µl) were supplied from eBioscience (UK) and FITC anti-rat CD44H (1 µl) and purified mouse anti-rat CD73 (0.5 µl) supplied from BD PharMingen (USA). For each experiment, 5×10^5 cells were centrifuged and separated. An amount of 100 µl FBS (95%) and PBS (5%) was added and homogenized slowly. The CD markers were added according to the manufacture's protocols and incubated in dark for 1 hour.

Differentiation potential of the isolated cells. Adipogenic differentiation medium was made by DMEM/Ham's F12, FBS (10%), dexamethasone (1 µM), IBMX (500 µM), indomethacin (60 µM) and insulin (5 µg) (all from Sigma, Germany). After 21 days, the oily droplets could be observed. The cells were fixed in 4% formaldehyde solution, rinsed three times in deionized water and stained with 500 µl of Oil Red O (Merck, Germany) at room temperature for 15 minutes. The osteogenic medium was consisted of DMEM/Ham's F12, FBS (10%), dexamethasone (0.1 µM) and ascorbate-2-phosphate (50 µM) (both from Sigma, Germany). After 21 days, the mineralized cells were rinsed three times with PBS and fixed with 4% formaldehyde solution. The solution of Alizarin red (Sigma, USA) was added for 30 minutes following washing with sodium chloride solution (0.1%, Merck).

Scanning electron microscopy (SEM). The number of 4×10^4 cells per 50 µlof culture medium was cultured on a sample (4×4 mm^2) and incubated at 37ºC, 5% CO_2. After 3 hours, the culture medium was added to cover the sample surface. At the end of the culture (after 3 days), the cells were fixed with 4% glutaraldehyde solution. In order to observe the stem cell morphology by SEM, samples were dehydrated in graded alcohols (50, 70, 80, 85, 90, 95, and 100%), sputter-coated with gold, and viewed using a scanning electron microscope (XL-30, Philips, Netherland) at accelerating voltage of 20 keV.

Chemical differentiation of stem cells. Each group of isolated cells was treated with the chemical factors for keratinocyte differentiation [20, 21]. The DMEM/Ham'sF12 medium was supplanted with FBS (10%), penicillin (100 U/mL), streptomycin (100 µg/mL), insulin (5 µg/ml, Sigma,USA), hydrocortisone (0.5 µg/ml, Sigma,USA), $CaCl_2$ (1.5 mM, Merck, Germany), epithelial Growth factor (10 ng/ml, ICN Biochemicals, USA, cat # 1544571) and keratinocyte growth factor (10 ng/ml, Peprotech,cat. no.: 100-19). Immunocytochemistry was performed using mouse anti-human involucrin, mouse anti-human Pan-Keratin and anti-mouse Ig-FITC (all from Abcam, USA). The cells were fixed in 4% formaldehyde solution for 20 minutes and permeabilized with Triton X-100 (Sigma, USA). Unspecific bindings were blocked using 1% bovine serum albumin (BSA, Sigma) in PBST (phosphate buffer saline with Tween 20) for 30 min. The cells were incubated in the primary antibody solution (1% BSA in PBST) for 1 hour, then in the secondary antibody solution (1% BSA in PBST) in the dark at room temperature for 1 hour. The samples were observed by aAxioskop fluorescence microscope (Zeiss, Germany).

Reverse transcription polymerize chain reaction (RT-PCR). The isolated stem cells were cultured at a density of 5×10^3 cells on a 6-well plate for two weeks with changing mediums every three days. Total RNA was extracted from cultured cells using RNeasyMiniKit (QIAGEN, 74104) according to the manufacturer's protocols. The concentration of RNA was determined usingPicodrop at 260 nm with the concentration of 1.94µg/ml. The cDNA strand was synthesized using reaction mixture consisting of RNA (1 µg), PCR buffer (4 µl, 15×), dNTPs (2 µl, 20 mM, Roche, Germany), random hexamer (1 µl 10 pmol/ µl, N6; Roche, Germany), deionized water (2 µl) and reverse transcriptase (1 µl, 200 U/ µl; Fermentas, Russia). The mixture with total amount of 20 µl was incubated at 42°C for 45 min, followed by 90°C for 5 min. The specific primers were involucrin (5'AGTGCCAGTGACTGTTCCAGCT 3', 5'TGGC TGCTGGTGGTGCTCAC3'), cytokeratin 18 (5'AGCG CAGCCAGCGTCTATGC3', 5'CCACCCATTCCCG CCAGGC3') and β-actin (5'AGCCATGTACGTAGCC ATCCA3', 5'TCTCCGGAGTCCATCACAATG3') with the marker of GeneRuler™ 50 bp DNA ladder. The PCR mixture was prepared with the addition of 2.5 µl of 10× PCR buffer, 1 µl of 25 mM $MgCl_2$, 1.5 µldNTPs (10 mM), 0.5 µl of each primer (10 pmol/ µl), and 0.1 µl of Taq-DNA polymerase (5 U/ µl, CinnaGen, Iran) to 3 µl of cDNA. The thermal cycler (Techne, England) was set to apply 37 amplification cycles at 92°C for 30 s, 62°C for 30 s, 72°C for 1 min and finally 72°C for 10 min. The visualization of PCR products was performed by running agarose gel (2%) electrophoresis containing ethidium bromide.

In vivo experiments. *In vivo* experiments were performed on 10Wistar rats, obtained from Pasteur Institute of Iran. Anesthesia was induced according to the previous mentioned protocols (cell isolation section). Burned wounds were prepared using a piece of brass alloy with the surface area of 40 × 20 mm^2 which was warmed in boiled water. In order to make a full thickness wound (3^{rd} degree burn),the burning piece was applied to the shaved and cleaned skin of the back of each rat for 25 seconds (two wounds for each rat). After 10 days, the animals were anesthetized and the scars were removed. The wounds were rinsed with physiological saline and the scaffold (without cells as a control) or cell-scaffold construct implanted. The sites of experiments were covered with sterilized gas and fastened with a biocompatible adhesive. Acetaminophen, hydroxyzine and diazepam were supplemented in drinking water of the animals for pain relief. The animals were sacrificed after 14 days to assess the skin regeneration.

Histological staining. Samples were fixed in neutral buffered formalin (10%), embedded in paraffin, and observed histologically by hematoxylin-eosinstaining [22].

RESULTS

Stem cell identification. Figure 1 shows the results of stem cell identification by flowcytometry. CD45 is the marker for hematopoietic cells and not expressed in ASC.CD44 expression (17%) is lower than expression of CD90 and CD73. According to other studies, the expression of CD44 marker is more important than the amount of expression [23]. Therefore, the isolated cells have the potential activity of the mesenchymal stem cells. The main problem with bone marrow stem cells (BMSC) is the lower proliferation potential of these cells in compare with ASC which may need to aspirate much more bone marrow. This is considered as a big challenge for human clinical application. On the other hand, achieving adipose tissue in human is much easier than bone marrow. Besides, the differentiation potential of both sources is the same. For these reasons, adipose tissues were considered in this study for *in vivo* experiments.

Stem cell differentiation. The osteogenic and adipogenic differentiation ability of isolated stem cells can be observed in Fig. 2. The cells were stained with alizarin red for the mineralized depositions (red colors, Fig. 2b) and Oil Red O for the adipose expressions (red

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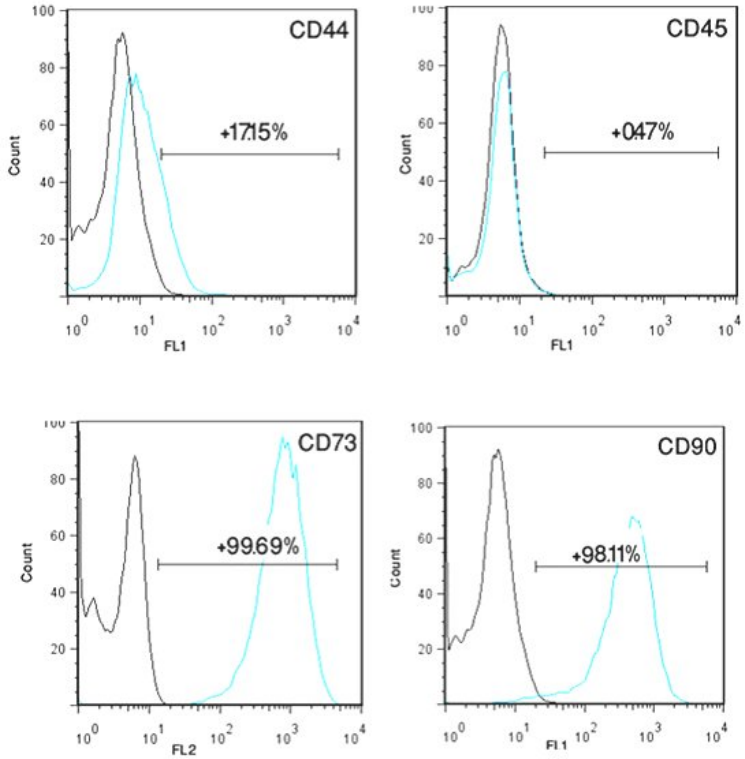

Fig. 1. Flowcytometry assay of adipose-derived stem cells. The expression of CD44, CD90 and CD73 and also no expression of CD45 demonstrate the presence of mesenchymal stem cells.

colors, Fig. 2d) that proves the ability of isolated cells for differentiation. Kashani et al. also showed the same results in the previous study for bone marrow-derived stem cells [24]. Microscopic observations showed that after 14 days of isolation, ASChad spindle and fibroblast-like morphology which was different from the paving stone and epithelial-like appearance (Fig. 3a and 3b). The presence of keratinocyte specific antibodies (cytokeratin and involucrin), investigated by immunofluorescence staining, can be observed in Figure 3, which indicates the higher production of involucrin and pancreatin for the 3D culture versus 2D (Fig. 3c, 3d, 3e and 3f). ASCcultured on 3D scaffoldsshowed higher protein secretion, which may confirm the advantage of 3D over 2D culture. Gene expression results for differentiated ASCcultured after 14 days of treatment with growth factors are shown in Figure 4. The expression of cytokeratin and involucrin by the ASCcultured on 3D scaffolds is higher than ASCcultured on tissue culture plate. These genes were not expressed by untreated ASC (Fig. 4g and 4h). In Figure 5, the collagen-chitosan scaffold, prepared by freeze-drying method, can be observed. The morphology and attachment of the differentiated keratinocytes cultured on the scaffold have been illustrated in Figure 6. As shown in Figures 5 and 6, there is a porous structure with the average size of 100 micrometer according to the SEM image bar. The porosity of the sample was evaluated by density method and estimated about 71 percent.

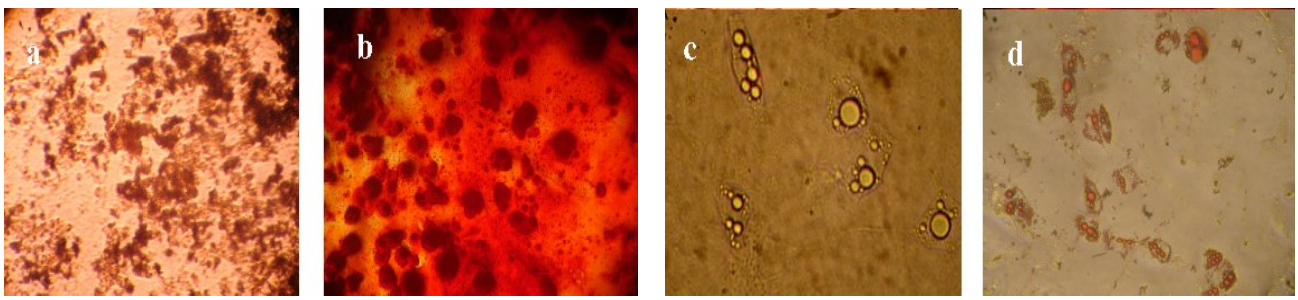

Fig. 2. Osteogenic and adipogenic differentiation of adipose-derived stem cells. **(a)** mineralized part, **(b)** alizarin red staining, **(c)** oil drops and **(d)** Oil Red O staining (magnification ×20).

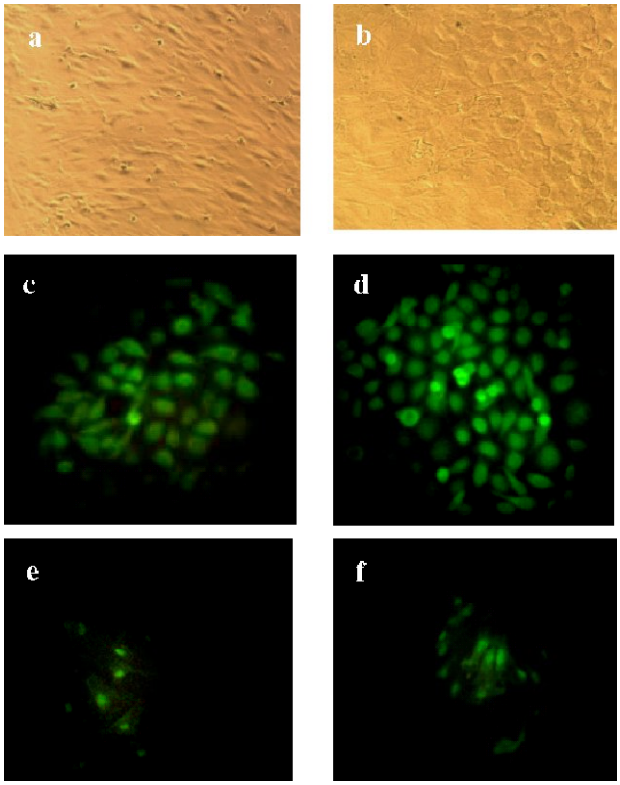

Fig. 3. Adipose-derived stem cells (ASC) differentiation with chemical factors. **(a)** ASC morphology after 14 days (×20), **(b)** chemical treatment with paving stone morphology (×20), **(c)** involucrin and **(d)** pancreatin antibody fluorescence staining observation for ASC differentiation cultured on scaffold (×40), **(e)** Involucrin and **(f)** Pancreatin antibody fluorescence staining observation for ASC differentiation cultured on tissue culture plate (×40).

In vivo results. The procedure of wound preparation, closure and healing has been shown in Figure 7. The scaffolds are effectively helpful in wound healing and cell seeding can speed up this process. The experiments confirmed that the wounds were completely healed using cell-scaffold construct. The difference between amount of healing in control and cell-scaffold samples were completely observable after 14 days. Histological observation was performed using hematoxylin-eosin staining procedure in order to compare normal, burned, implanted, and healed skins (Fig. 8). In normal skin, epidermal layer is observed as a compact and dark layer with the corny layer at its surface. Dermal layer has located under the epidermal layer consisted of cells, blood vessels and collagen fibers. In the burned skin (Fig. 8c and 8d), there is no sign of epidermal layer. A thick compact layer of cells can be observed as a newly formed epidermis in the implanted skin with a little infiltration of epidermis. In healed skin, the epidermal and dermal layers seem to be in a normal condition. In comparison with a normal skin, natural level of collagen production can be seen in dermal layer. Also, epidermis layer is much thicker in healed skin than normal skin. The quantifying results of skin regeneration with the comparison of sample and control sides are observed in Table 3. There was no sign of epidermal layer in the control side after 14 days, while in 50% of the sample side, the epidermal layer was observed.

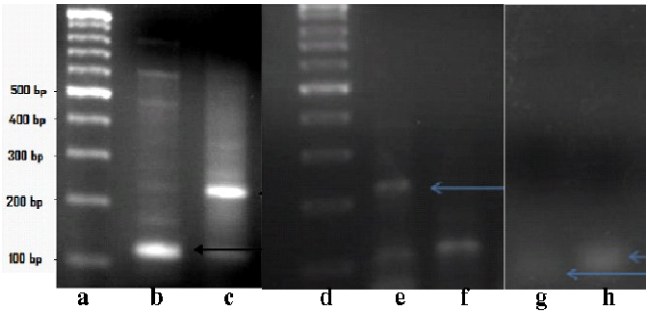

Fig. 4. RT-PCR analysis. The expression of cytokeratin and involucrin by adipose-derived stem cells (ASC) cultured on 3D scaffolds is higher than tissue culture plate. **(a)** and **(d)** are markers, **(b)** and **(c)** expression of cytokeratin and involucrin by ASC cultured on scaffolds, **(e)** and **(f)** expression of cytokeratin and involucrin by ASC cultured on tissue culture plate after 14 days treatment with growth factors, **(g)** and **(h)** expression without treatment with growth factor. Arrows show the difference between the intensities.

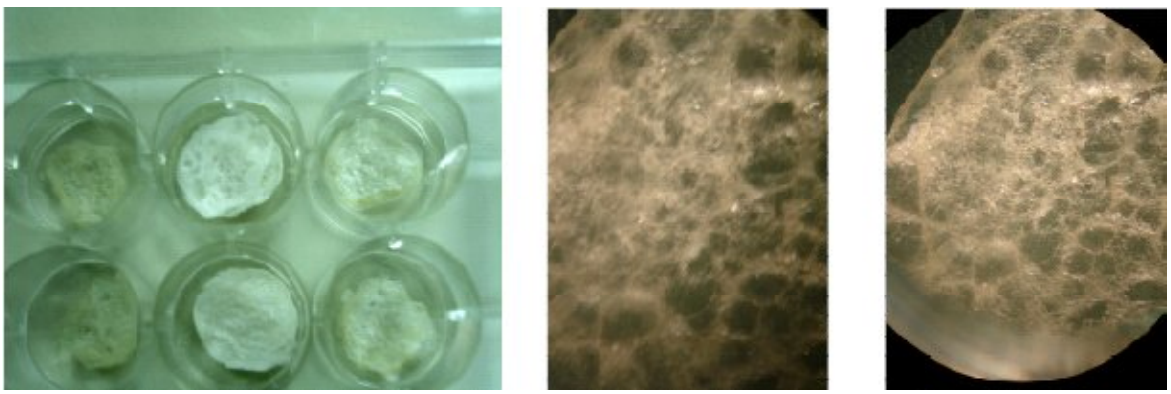

Fig. 5. Collagen-chitosan scaffolds prepared by freeze-drying method. The optical micrographs indicate the presence porosity in the scaffold structure, which is suitable for 3D cell culture.

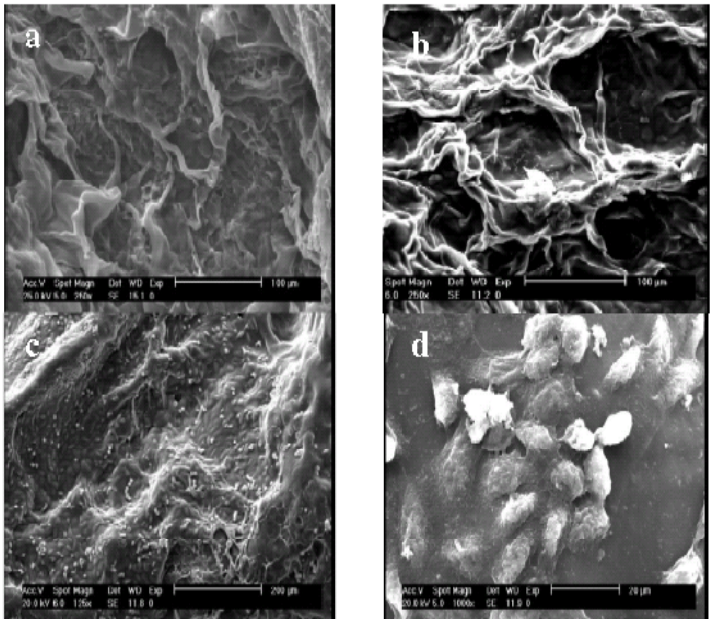

Fig. 6. The scanning electron microscopy images of the scaffold and cultured cells. Images of the **(a)** surface morphology of the scaffold, **(b)** cross section of the scaffold, **(c)** and **(d)** adipose-derived stem cells, cultured on scaffold differentiated to keratinocytes after 3 days.

DISCUSSION

In this research, isolated mesenchymal stem cells were cultured on a collagen-chitosan scaffold in order to repair a full thickness wound in rats. Transplantation of autologous fibroblasts or keratinocytes has been reported with or without scaffold in skin regeneration studies [25, 26].

The proliferation rate of these cells is an age-related process, which is reduced in older patients. Therefore, finding the other source of cell turns much more attention toward mesenchymal stem cells. Liu and coworkers [27] used collagen-glycosaminoglycan composition as a scaffold in their research. It has been shown that the isolated BMSC are potent in healing, keratinization and vascularization of a full thickness skin wounds. Other researchers also investigated the healing potential of BMSC in skin regeneration by using collagen-glycosaminoglycan scaffold [28]. In a recent research, significant healing of skin wounds was reported for BMSC as compared to the allogenic neonatal dermal fibroblasts [29]. The healing was evaluated in the case of re-epithelialization speed, the thickness of the regenerated epidermis, and the number of fibroblasts [30]. However, the isolation of BMSC in medical practice is much more complicated than adipose tissues, which can be easily derived from liposuction procedure [31]. Therefore, the capability of adipose-derived mesenchymal stem cells in differentiation to keratinocytes was successfully investigated to legitimize the use of these cells. According to our previous study, co culture of fibroblasts and keratinocytes provide some the essential growth factors for dermal and epidermal regeneration [19]. Therefore, direct transplantation of

Table 1. The quantifying results of skin regeneration and the comparison of sample and control sides.

Epidermal formation	Control side dermis formation	*U.S. (cm^2)	Epidermis formation	Sample side dermis formation	*U.S. (cm^2)	Rat number
--	√	1.0 × 1.5	--	√	0.2 × 0.5	1
--	√	1.0 1.3	√	√	0.2 × 0.7	2
--	√	0.8 × 1.2	--	√	0.2 × 0.5	3
--	√	0.5 × 1.4	--	√	0.3 × 0.5	4
--	√	1.0 × 1.2	√	√	0.4 × 0.4	5
--	√	1.2 × 1.5	√	√	0.3 × 0.3	6
--	√	0.9 × 1.5	√	√	0.5 × 0.5	7
--	√	1.0 × 1.3	--	√	0.2 × 0.7	8
--	√	1.0 × 1.0	--	√	0.2 × 0.5	9
--	√	1.0 × 1.0	√	√	0.2 × 0.8	10

*U.S., unhealed surface after 14 days of culture

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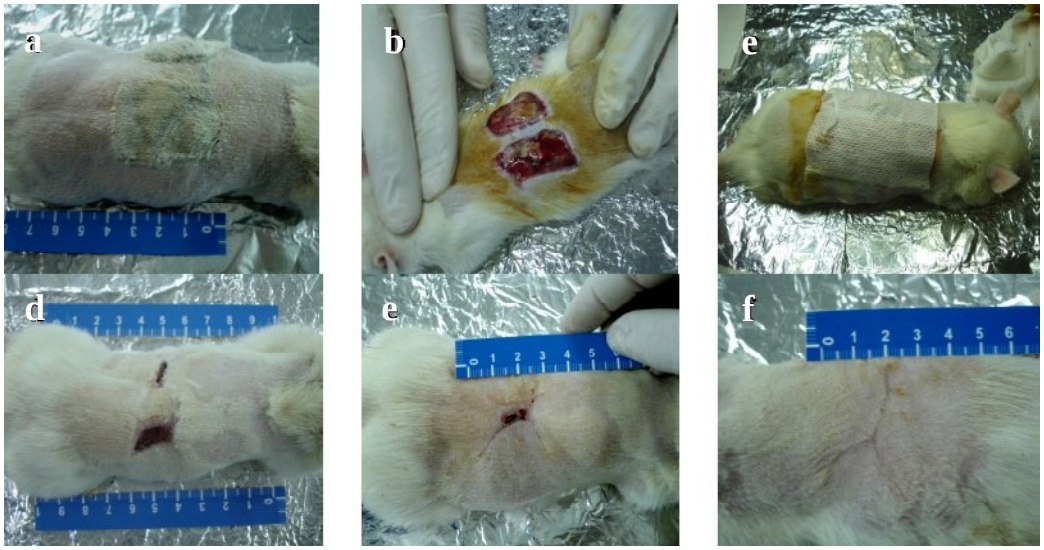

Fig. 7. The images of the wound preparation and healing progress. (**a** and **b**) wound preparation procedure on the back of the rat skin, **(c)** wound coverage, (**d** and **e**) the comparison between wound healed with (0.2 × 0.5 cm^2 unhealed) and without (1 × 1.5 cm^2 unhealed) scaffold after 14 and 18 days, **(f)** wound completely healed using cell-scaffold construct.

mesenchymal stem cells may also be admissible, because surrounding intact tissues *in vivo* supply the needed growth factor for different cell layers in skin, which cannot be created *in vitro*.

The lyophilized collagen-chitosan scaffolds were crosslinked by EDC as a zero length crosslinker. It has been shown that carbodiimidecrosslinkers can be used as an alternative to glutaraldehyde with lower cell toxicity and higher compatibility with collagen molecules [32-34]. On the other hand, the physico-mechanical properties that achieved with EDC crosslinker are also desirable for skin substitution [35-38]. The scaffold does not experience a mechanical stress higher than its yield strength after implantation and its degradation occurs during skin regeneration.

The cell-scaffold constructs were implanted to assess their ability in skin regeneration. The experiments showed that burning scars with diameter less than 15 mm were healed spontaneously before 5 days. Therefore, larger scars with rectangular form of 20 × 40 mm^2 were applied for this test, although some researchers created small trauma with 10-mm diameter by exposing hot water to the rat skin [39]. The pathological observations were proved that healing started from the edge of the trauma at the skin-scaffold interfaces. The presence of the cells on the samples speeds up this healing and the difference between two samples could be clearly detected at the end of day 4.

In this research, adipocyte mesenchymal stem cells were isolated and characterized. These stem cells were cultured on a collagen-chitosan scaffold and implanted on skin wound of rats. The histopathological analyses confirmed the formation of new tissue after 14 days with the formation of dermis and epidermis. These results indicate the capacity of ASC in differentiation to keratinocytes *in vivo* and also wound healing.

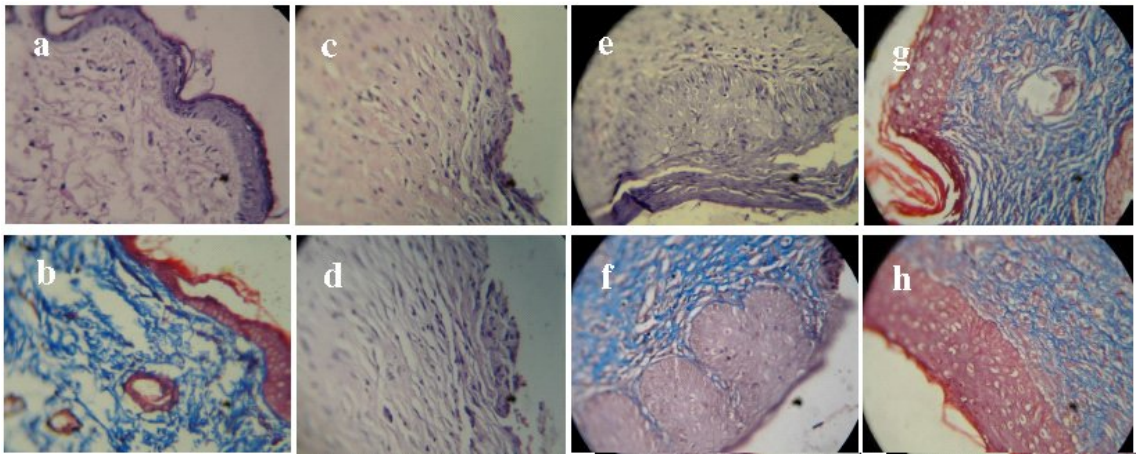

Fig. 8. The histopathology results of the samples staining with hematoxylin and eosin indicating the epidermal and dermal layers. (**a** and **b)** normal skin, (**c** and **d**) burned skin, (**e** and **f**) implanted skin, (**g** and **h**) healed skin (×100).

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