

## Preparation of skin tissue engineering scaffold based on Adipose-derived Tissue

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### Abstract

**Introduction:** In clinical tissue engineering, scaffolds play a critical role in the formation of the appropriate physical, chemical and biological environment. It seems that extracellular matrix-based materials such as adipose supports the cellular functions like adhesion, growth or differentiation.

**Objective:** In this study, human-derived adipose tissue was modified with different chemically crosslinking methods including carbodiimide, aldehyde and isocyanate reagents to fabricate skin tissue engineered scaffolds.

**Material and Methods:** In this study, biological-based scaffolds were fabricated through modification of liposuction tissues and crosslinked by different chemical agents including glutaraldehyde (GLA), hexamethylene diisocyanate (HMDI) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The chemically formed bands were characterized by Fourier Transform Infrared spectroscopy (FTIR) and mechanical properties analyzed by standard compression testing. Adipose-derived mesenchymal stem cells (ADSCs) were isolated and cultured on the samples.

**Result:** The MTT assay and microscopy observations of cultured adipose-derived stem cells confirmed the biological performance of these scaffolds in vitro. The degradation results on phosphate buffer saline also showed that crosslinking with 10 mM concentration of EDC preserve the scaffold integrity for 2 months. Real-time PCR observations confirmed that ADSCs were

**Conclusion:** According to the results, adipose-derived scaffolds could find broad clinical applications in tissue engineering for skin regenerations in deep burns or plastic surgeries.

**Keyword:** Skin tissue engineering; Adipose-derived stem cells; Human adipose tissue; Extra-cellular matrix

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### 1. Introduction

A diverse set of bioactive molecules exist in the structure of the extra-cellular matrix (ECM) encourages the researchers to investigate the use of these components for tissue regeneration [1, 2]. In

addition to the supportive and protective roles of ECM, it can provide regulatory signals through the reciprocal actions by its structural molecules [3]. These signals affect the main function of the cells including growth or tissue formation [4].



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According to the tissue engineering concepts, artificial ECMs (scaffolds) should be utilized to support the cells in the constructive remodeling of missing tissues [5, 6]. These cell supporting structures can be composed of naturally occurring materials to facilitate the attachment, proliferation, and differentiation of cells [7, 8]. Also, bio-mimicking plans have been proposed to design scaffolds based on naturally derived components [9]. Decellularization of whole tissue or incorporation of a fraction of ECM proteins are considered as the main strategies to imitate the natural biochemical cues [10-12]. Indeed, to establish a normally functioning tissue, the right cell at the right time should be cultured on the proper scaffold [13].

Three-dimensional biological scaffolds derived from non-autologous sources can be made of allogeneic or xenogeneic extracellular matrix [14]. One of the main problems using these scaffolds involves the risk of immunogenicity due to the presence of antigenic molecules to the hosts [15].

As a result, finding the supplemental autogenic sources of tissues for substitution of own donor is a promising way that draws many attentions. Adipose tissues can be easily removed by a cosmetic liposuction surgery from different sites of the human body such as abdomen [16]. Several studies have been conducted on the process of aspirated fat as porous tissue engineered scaffolds [17, 18]. Recellularisation of these templates with autologous stem cells or differentiated cells induces the reconstruction of injured tissues [19, 20].

Multipotent mesenchymal stem cells (MSCs) can be isolated from adipose tissue (as used in this study) and other tissues such as bone marrow, or umbilical cord [21]. Numerous preclinical studies support the safety and efficacy of the adipose-derived mesenchymal stem cells (ADMSCs) as favorable cells for regenerative medicine due to the easily harvesting and low donor-site morbidity [22, 23]. The combinations of ADMSCs with various types of scaffolds have been proposed for different medical treatments such as orthopedic, plastic or maxillofacial surgeries [24]. Physical and mechanical stability of adipose tissue limits its application in tissue engineering. Therefore, in this study, human-derived adipose tissue was

modified with different chemically crosslinking methods including carbodiimide, aldehyde and isocyanate reagents to fabricate skin tissue engineered scaffolds. The physical-chemical properties as well as biological responses of the cultured MSCs to the optimized structure were evaluated.

## 2. Materials and Methods

### 2.1. Sample preparation

Human adipose tissue was obtained via liposuction with informed consent from three healthy female donors who had undergone liposuction at the Farmanie surgery clinic (Tehran, Iran). The adipose tissue was washed several times with distilled water to remove blood components and cut into the small pieces. Distilled water was added to the adipose tissue, and the tissue/water mixture was homogenized at 12000 rpm for two hours at room temperature using a homogenizer (SilentCrusher M, Heidolph). The tissue suspension was centrifuged at 3000 g for 5 min to separate the oil-containing layer. This viscous suspension was treated with NaCl (1M, Merck, Germany) for 2 hours at 37°C under shaking and centrifuged at 200 g for 5 min at 4°C. After removing superfluous oil component, the residue was rinsed with distilled water, kept at 4°C for 24 hours and incubated in sodium dodecyl sulfate (0.5%, Sigma, Germany) for 1 hour at 25°C. The suspension was washed with distilled water containing RNase (1000 unit/ gram sample, Sigma) and DNase (1000 unit/ gram sample, Sigma) for 2 hours at 37°C. The isolated tissue products were shaped and dried using a freeze dryer (Christ, Germany).

### 2.2. Crosslinking reactions

Three different crosslinking systems based on aldehyde, carbodiimide and isocyanate chemistry were utilized. Glutaraldehyde (50 mM, Merck, Germany) in de-ionized water, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 5, 25 and 50 mM, Sigma) with N-hydroxysuccinimide (NHS, 50 mM, Sigma) in 70% ethanol and hexamethylene diisocyanate (HMDI, 5, 25 and 50 mM, Sigma) in dimethyl sulfoxide (DMSO, Sigma) were prepared. The cylindrical shape samples with a diameter of 6 mm and a height of 10 mm were soaked in each

crosslinking solution at 25°C. After 24 hours, the samples were washed three times in de-ionized water and dried by lyophilization.

### 2.3. FTIR spectroscopy

The samples were cut into small pieces and mixed with KBr grain spectroscopy grade to make transparent tablets. FTIR spectroscopy (Nicolet 6700 FT-IR spectrometer) was utilized over the wave number region of 400–4000 cm<sup>-1</sup> at a resolution of 2 cm<sup>-1</sup>.

### 2.4. Water uptake measurement

The water uptake measurements were performed based on ASTM- D570 instruction. Each specimen was immersed in de-ionized water at 37°C and regularly weighing at different time intervals to equilibrate. The percent of water uptake was calculated according to the following equation:

$$\text{Water Uptake} = \frac{W_t - W_o}{W_o} \times 100$$

where  $W_t$  is the weight of the sample at time  $t$  and  $W_o$  is the initial mass.

### 2.5. Mechanical properties determination

The cylindrical samples (diameter of 5 mm and height of 10 mm) were immersed in phosphate buffer saline (PBS) for 24 hours to reach the equilibrium condition. Compressive strength was determined to evaluate the mechanical properties of the specimens, according to ASTM standard and procedure. These tests were performed utilizing a dynamic servohydraulic testing machine (Zwick/Roell, Z050, Germany). The rate of movement was fixed for all samples at 0.5 mm/min

### 2.6. Mesenchymal stem cell culture

Human adipose stem cells (ASC, passage three) were obtained from the National Cell Bank of Iran (Pasteur Institute of Iran). The cells were cultured in Dulbecco's Modified Eagle's (DMEM)/ Ham's F12 Medium (GIBCO, Scotland) supplemented with 10% FBS (Seromed, Germany), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma, USA) in a humidified incubator (37°C, 5% CO<sub>2</sub>). The differentiation potential of ASCs was determined by

the cultivation of the cells in adipogenic, osteogenic and keratinogenic mediums for 21 days according to the previously published report [25].

The cells were treated with a medium containing dexamethasone (Dex, 1 µM, Sigma), 3-isobutyl-1-methylxanthine (IBMX, 500 µM, Sigma), Indomethacin (60 µM, Sigma) and Insulin (5 µg, Sigma) for adipogenic differentiation with the emergence of oily droplets. Osteogenic differentiation was conducted by addition of dexamethasone (0.1 µM, Sigma) and ascorbate 2 phosphate (50 µM, Sigma) to the medium. After 21 days, the cells were fixed in formaldehyde (4%, Merck) solution and stained with alizarin red solution (2%, Sigma) for 45 minutes following washing with sodium chloride solution (0.1%, Merck) to confirm the expression of calcium by differentiated cells.

Keratinogenic medium was prepared by adding hydrocortisone (0.5 µg/ml, Sigma), CaCl<sub>2</sub> (1.5 mM, Merck), epithelial growth factor (EGF, 10 ng/ml, ICN, cat no:1544571) and keratinocyte growth factor (KGF, 10 ng/ml, Peprotech, cat no:100-19). The cells were fixed by 4% paraformaldehyde solution, permeabilized in triton X-100 (0.01 %, Sigma) and visualized by fluorescence microscopy (Axioscope. Ziess, Germany) after staining with mouse monoclonal anti-human involucrin and anti-mouse Igg-FITC (Abcam, USA) [25].

### 2.7. Cytotoxicity evaluation

The extraction procedure based on ISO 10993-12 was performed on UV-sterilized samples by adding 1 ml of culture medium (DMEM+10% FBS) to each specimen with 6cm<sup>2</sup> surface areas and kept at 37°C for 7 days. The same medium without sample was considered as a negative control.

The number of 1×10<sup>4</sup> adipose stem cells per well were cultured in a 96 well plate (Greiner, Germany). After 24 hours, the culture medium was replaced by the samples extracts (100 µlit) and incubated for the next 24 hours. The medium for each well was subsequently removed following the addition of 100µlit of MTT solution (0.5 mg/ml, Sigma) and 5 hours incubation at 37°C. The absorbance of each well was measured at 575 nm after dissolving the purple crystal by isopropanol (100 µlit, Merck). The optical density

(OD) was recorded on ELISA reader (ICN, Switzerland) at 545 nm and normalized to the control OD.

## 2.8. Microscopy observation

The number of  $5 \times 10^4$  cells was cultured on the optimized sample (based on toxicity and mechanical measurements) in a non-treated centrifuged tube plate for 7 weeks. The cells were fixed in formalin (10%), embedded in paraffin, and observed histologically by hematoxylin- eosin (H&E) staining. The same procedure on the cultivation of cells was performed for scanning electron microscopy observation. Karnovsky fixation solution was utilized for cell fixation and graded alcohols (50, 70, 80, 85, 90, 95, and 100%) for dehydration process [26]. The sample was sputter-coated with gold and visualized using SEM (Tscan, Czech).

## 2.9. Reverse transcription polymerize chain reaction

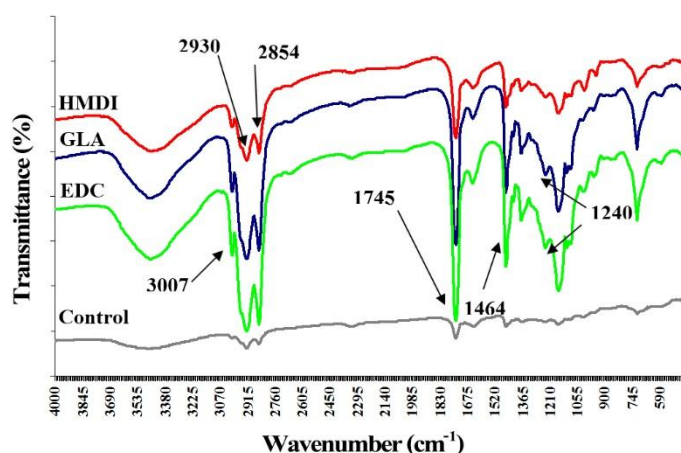
The effect of adipose-derived scaffold on gene expression profiles of the ADMSCs during the treatment with different differentiation mediums were analyzed by real-time PCR. The number of  $1 \times 10^3$  cells/well in 6 well plates were seeded and incubated for a period of 14 days with the medium exchange at day 7. Experimental groups consist of different culture mediums (adipogenic, osteogenic and keratinogenic) with or without scaffolds. The total RNA was extracted by RNeasy MiniKit (QIAGEN, 74104) based on the manufacturer's instructions and quantified using Thermo scientific NanoDrop®. QuantiTect Reverse Transcription Kit (QIAGEN, Gaithersburg, MD, USA) was utilized for cDNA synthesis. The real-time PCR was performed in an ABI 7300 real-time PCR system (Applied Biosystems) with SYBR Green PCR master mix (Applied Biosystems) and specific primer sequences including GAPDH, Collagen I, Involucrin, Keratin X, Osteocalcin, and PPR $\gamma$ .

## 3. Results and Discussion

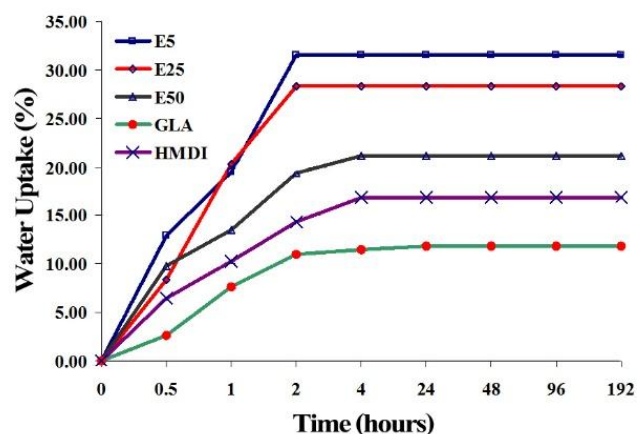
### 3.1. FTIR spectroscopy

Figure 1 shows the FTIR spectra of the processed adipose tissue before and after crosslinking. The

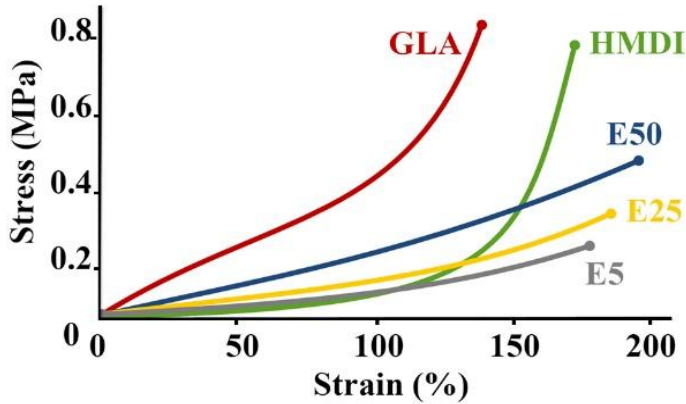
peaks at  $3007 \text{ cm}^{-1}$ ,  $1745 \text{ cm}^{-1}$ , and  $1464 \text{ cm}^{-1}$  represent stretching vibration of CH in lipids, C-O stretch in esters and bending vibrations in lipids, respectively. Moreover,  $2930 \text{ cm}^{-1}$  and  $2854 \text{ cm}^{-1}$  can be attributed to the fundamental vibrational modes of the lipid-rich materials [27, 28]. The characteristic peak of isocyanate (NCO) at  $2270 \text{ cm}^{-1}$  has been eliminated in the HMDI crosslinked sample which confirms the consumption of these groups in the reaction with samples. It is difficult to prove the crosslinking reaction because of the overlapping of the bonds C=N and C=O (stretching in proteins) at  $1650 \text{ cm}^{-1}$ . However, the characteristic peak of the C-N groups (at  $1240 \text{ cm}^{-1}$ ) is intensified after crosslinking reactions by GLA and EDC.



**Figure 1.** FTIR spectra of the processed adipose tissue (control) and crosslinked with EDC, GLA and HMDI.



**Figure 2.** Water uptake measurements for the samples with different crosslinking agents. EDC 5 mM and GLA samples showed the highest and lowest amount of water absorption, respectively.



**Figure 3.** Compressive mechanical properties of the samples with different crosslinking agents. E5 shows the lowest mechanical strength.

### 3.2. Physical-Mechanical properties

Figure 2 indicates the water uptake percent for different samples with different cross-linking agents. The samples cross-linked with EDC showed a higher range of water absorption. It can be seen that the GLA cross-linked sample demonstrates the lowest amount of water uptake.

The compressive strength of the samples with different cross-linking agents were compared in figure 3. GLA, and HMDI showed higher strength (0.8 MPa) than the EDC cross-linked samples (0.4 MPa). This range of strength (for all samples) can be considered for non-load bearing tissues such as skin, adipose or nerve tissues.

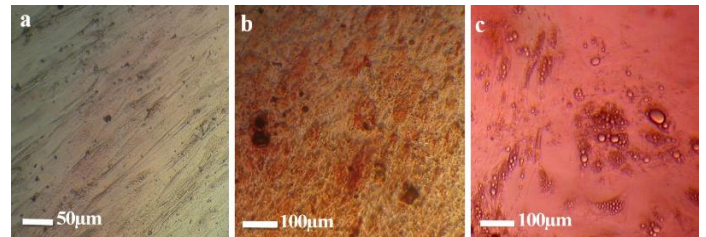
### 3.3. Biological evaluations

Figure 4 shows the differentiation potential of human adipose-derived stem cells treated with the osteogenic and adipogenic medium. The red colors after staining the cells with alizarin red confirm the presence of calcium depositions (figure 4b).

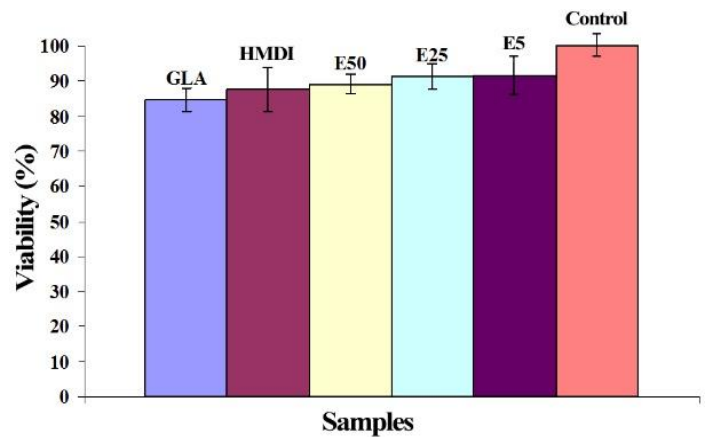
Also, the existence of oil droplets proves the adipose differentiation of ADSCs (figure 4c).

Cytotoxicity of the crosslinked samples were evaluated using MTT assay. GLA and HMDI crosslinked samples showed lower viability percent than EDC ones (figure 5). This can be due to the remaining small amount of cross-linking agents in the scaffold structure which is harmful to in vivo applications.

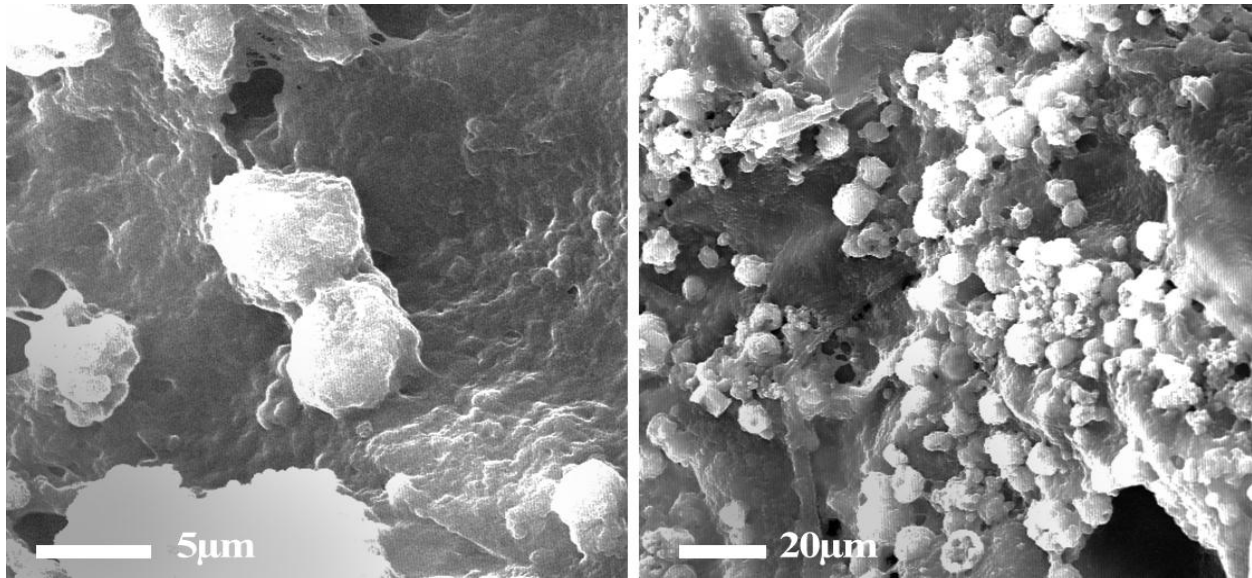
EDC/NHS as zero-length cross-linking proceeds through the formation of amide bonds between carboxyl groups and amine residues on the protein chains [29]. These are well believed as non-toxic crosslinking agents at low dosages to fabricate stable scaffolds [30]. Therefore, based on the lower toxicity as well as higher water uptake than GLA and HMDI crosslinked and also higher strength among the EDC crosslinked samples, E50 (EDC 50mM) was selected as the optimized specimen. Figure 6 illustrates the attachment of human ADSCs cultured on the E50 with the spherical morphology.



**Figure 4.** Evaluation of the differential potential of human ADSCs. a) negative control for osteogenesis with no sign of alizarin red staining, b) alizarin red staining for calcium detection, c) oil drops after adipose differentiation.



**Figure 5.** Evaluation of cell viability of the samples with different crosslinkers



**Figure 6.** Spherical morphology of human adipose-derived stem cells cultured on E50

### 3.4. Gene expression

Native adipose tissue contains a potentially abundant source of ECM including different types of collagen (I, III or IV), laminin or growth factors. Biochemical properties of the processed ECM in the form of the scaffold may support a superior tissue remodeling outcome [17]. By-products of the scaffold degradation could change the gene expression profile of the cultured cells. Peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ) as the master regulators of adipogenic differentiation play a crucial role in the control of adipogenic gene expression [31].

Brown et al., stained the adipose-derived scaffolds with Adipo-red to prove the adipogenicity of the ADSCs when seeded on these scaffolds and cultured in adipogenic medium (Zen-bio Inc.) [17]. Flynn also confirmed the adipogenic potential of decellularized adipose tissue through the expression of PPAR- $\gamma$  using RT-PCR [32].

Multilineage differentiation activity of mesenchymal stem cells has been extensively discussed, and differentiation towards a specific lineage can be induced by special stimulatory factors [33]. Evaluation of the keratinogenic potential of the adipose-derived scaffolds was considered as the main purpose of this research. Therefore, three different culture medium including keratinogenic, adipogenic and osteogenic mediums were prepared to evaluate

the effect of adipose-derived scaffold on the differentiation of MSCs. The scaffold could induce keratinogenic differentiation with decreasing in Collagen type I and increasing in keratin X expressions (Figure 7). The same outcome was observed using keratinogenic medium while involucrin expression also increased. Moreover, gene expression results demonstrated that adipose-derived scaffold could cause an elevation in the expression of involucrin for three different differentiation mediums (Figure 7). The high-level expression of involucrin in keratinocytes is believed to be a sign of mature phenotype [34].

Endogenous expression of involucrin in cells can be enhanced by treatment of dexamethasone [35]. In contrast, it was reported that proliferation and differentiation response of keratinocytes could be delayed by using dexamethasone [36]. For this reason, the effect of dexamethasone on the activity of adipose-derived scaffolds was separately evaluated due to the presence of dexamethasone in both adipogenic and osteogenic mediums. High-level expression of involucrin in both osteogenic and adipogenic treated samples can be explained by the function of dexamethasone (figure 7). The expression of involucrin ADSCs treated with adipose-derived scaffold and keratinogenic medium can be observed in figure 8.

#### 4. Conclusion

Clinical applicability of autologous human adipose tissue-derived stem cells is widely under the investigation for tissue engineering and cell-based therapies purposes. In this research, the regenerative potential of adipose-derived stem cells cultured on adipose-derived scaffolds was evaluated. The scaffolds were chemically modified with different crosslinking agents and characterized from physical-chemical as well as biological points of view. The ADSCs cultured on the optimized sample were treated with three inductive medium including keratinogenic, adipogenic and osteogenic to specify the influence of each medium on ADSCs differentiation to keratinocyte. The inductive role of adipose-derived scaffold for involucrin expression, as mature keratinocyte marker, was proved using real-time-PCR. Taken together adipose-derived scaffolds could find broad clinical applications in tissue engineering for skin regenerations in deep burns or plastic surgeries.

#### Conflict of interest

The authors declare that they have no conflict of interests.

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