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Burn wound healing effect of mesenchymal stem cells seeded into collagen-chitosan in animal model

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Abstract: Skin burn healing is still a major issue and treatments aim to accelerate the burn healing process to minimize the associated complications. Despite the fact that a wide range of wound dressings have been designed, few of them act well and sufficiently in repairing and protecting the burn wounds. This study aimed to investigate the accelerating effect of mesenchymal stem cells (MSCs) seeded collagen-chitosan hydrogel on burn wound healing in rats. In this in vitro study, adipose tissue was obtained from 10 patients undergoing liposuction surgery and MSCs were isolated and identified. Cell viability was measured by MTT assay method. The cells were examined for apoptosis and expression of surface markers using flow cytometry. Burn wounds were created on the back of rats and the animals were divided into: control group (n = 6), and groups treated with MSCs (n = 6), treated with collagen-chitosan hydrogel (n = 6), and treated with MSCs seeded collagen-chitosan hydrogel. The healing process of burn wounds was evaluated quantitatively and qualitatively on days 7, 14 and 21 after treatment. Data were analyzed using ANOVA. The wound closure, and skin re-epithelialization, vascularization, collagen synthesis, thickness, density and elasticity at the transplant site were highly promoted by MSCs seeded collagen-chitosan hydrogel than MSCs or collagen-chitosan hydrogel alone ($P < 0.001$). Dressing of burn wounds with MSCs seeded collagen-chitosan hydrogels has significant potential to accelerate the process of burn wound healing.

Keywords: Mesenchymal stem cells, Collagen-chitosan hydrogel, Burn wound healing, Elasticity, Rat

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1 Introduction

The burn wound is a type of damage to the skin or tissue caused by heat, electricity, chemicals, friction, or radiation (Ma et al., 2011). Burns are the fourth most common trauma worldwide, causing the destruction of skin cells. Serious consequences can accompany and even lead to death. There are different types of burn wounds and they need to be treated differently depending on the degree of burn (Sen et al., 2016). Statistics show that most burns are small and can be treated on an outpatient basis, but when the burn is severe, there is a need for special care in medical centers, and sometimes a person may need surgery, physiotherapy and rehabilitation. Every year, 300,000 people die from burns (Jahromi et al., 2018). Although extensive burns can cause death, new treatments developed since the 1960s have significantly reduced burn deaths, especially in children and adolescents. There have been significant recent studies on the effects of stem cell transplantation on wound healing, but research in this area is still of interest to scientists and there are many challenges in this area (Hassanshahi et al., 2019).

Wound healing, the biological process is complex, including stages of hemostasis, inflammation, proliferation and reconstruction caused by biological reactions and molecular cell migration, cell proliferation and induced extracellular matrix synthesis (Ghieh et al., 2015; Wang et al., 2018). Mesenchymal stem cells (MSCs) have been reported to accelerate the wound healing process. MSCs are pluripotent cells that have the property of self-renewability, and therefore, are an ideal source for use in cell therapy. Preliminary studies have shown that stem cells may cause epithelial proliferation, granulation tissue formation, and new angiogenesis to lead to rapid closure (Hassan et al., 2014).

Hydrogels are a hydrophilic and biocompatible three-dimensional network with a porous structure that can hold up to 95% of water and can be used for tissue regeneration due to its extracellular matrix structure. In addition to creating a moist environment for migrating cells, these hydrogel dressings have the ability to absorb wound secretions and keep the wound moist, which reduces the patient's pain (Dhaliwal and Lopez, 2018). Collagen is normally present in the extracellular matrix and is a suitable substrate for cell attachment, survival, proliferation and differentiation. Collagen strength is mostly due to the abundance of proline and hydroxyproline (Ge et al., 2020). Chitosan, another commonly used hydrogel, is a linear, natural polysaccharide derived from the distillation of chitin. Chitosan has desirable properties such as non-toxicity, biocompatibility, cell adhesion, anti-inflammatory properties, antimicrobial effect and activity, which makes it a suitable and effective polymer in wound healing areas (Patrulea et al., 2015). Due to all these properties, the use of collagen and chitosan hydrogels along with MSCs can be a suitable method for healing of burn wounds. Although the potential of MSCs for wound healing has been demonstrated, plausible adverse effects caused by MSCs transplantation to wound area, is still a serious concern. In this regard, evidence suggests that MSCs stimulate tumor growth and cancer metastasis (León-Moreno et al., 2020).

Despite many studies on the effect of MSCs with chitosan collagen hydrogel on wound healing, there is still no accurate and sufficient information in this regard. The use of xenogeneic stem cells has received much attention in human research. In this method, human stem cells are transplanted into animal models and the behavior of the cells inside the recipient animal is

evaluated (Chang et al., 2018). Considering all these features, using MSCs seeded collagen-chitosan hydrogel can be a useful strategy for promoting burn wound repair.

2 Materials and Methods

2. 1. Isolation of MSCs

MSCs were isolated from human adipose tissue according to previous studies (Nie et al., 2011). Informed written consent for sample collection was obtained from 10 patients (aged 30 to 58 years) undergoing lipoabdominoplasty. Adipose tissue was obtained using liposuction technique and transferred a sterile tube. PBS and antibiotics (400 units/ml penicillin and 200/ lg/ ml streptomycin (GIBCO (USA)) were added. The solution was centrifuged (1200 RPM and 5 min). The supernatant was discarded. Collagenase (0.1 mg /ml) A (Sigma-Aldrich(USA)), plus 0.5x CnT-GAB10 antibiotic/ antimycotic was added to cell pellet and the solution incubated for 2 h. The enzyme activity was neutralized by adding an equal volume of 10% culture medium (DMEM) (GIBCO (USA)) and the solution was centrifuged (1200RPM and 5min). The supernatant was drained and high glucose DMEM medium containing 10% serum (FBS) (GIBCO (USA)) was added to the cell pellet and the cells were transferred to a culture flask.

2. 2. Characterization of MSCs

According to previous studies (Klar et al., 2017; Tettamanti et al., 2004), immunocytochemistry staining was used to identify the isolated MSCs. The cells were grown on coverslips for 24h and then fixed in acetone for 30 min at -20 °C. After blocking with 3% H2O2 in 1% Sodium Azide PBS for 30 min and several washes with PBS and again blocking with 1.5% goat serum for 60 min at room temperature, cells were stained with primary mouse monoclonal anti-vimentin antibodies (Anti-Vimentin antibody [RV202]-Cytoskeleton Marker (ab8978)) in dilution of 1:100. The cells were washed three times with PBS to remove the excess antibody and were incubated with secondary goat antimouse Ig G antibody (Abcam (United Kingdom)), Goat Anti-Mouse IgG H&L (HRP) preadsorbed (ab7068), diluted1:50) and subsequently washed with PBS. After adding DAB (3,3'-Diaminobenzidine) (Sigma-Aldrich), cells were counterstained with hematoxylin (Thermo Fisher Scientific Co., Ltd (Shanghai, China)). The coverslips were dehydrated and mounted onto microscopic slides. Obtained slides were observed using fluorescence microscope (Nikon Eclipse TE200).

2. 3. Evaluation of MSCs viability

To study the viability of the cells, Annexin-V and 7-AAD (BD Biosciences) were used according to the manufacturer's instructions. Briefly, cells were cultured, detached and washed and incubated with PE-conjugated Annexin-V and 7-AAD in Annexin-V-binding buffer at r/t for 15 min. The percentage of live and dead cells were analyzed in a Beckman Coulter Navios flow cytometer.

2. 4. 3-D culture of MSCs in collagen-chitosan hydrogel

MSCs were cultivated according to a previously established technique (Koukaeyan et al.,

2021). Briefly, collagen was dissolved in a Falcon tube containing 25 mL of sterile deionized water and shaken for a few minutes for further solubility. 16 mL of PBS buffer and 8 mL of HEPES buffer were then poured into Falcon 50 tube and combined. Both Falcon tubes were placed in a human containing zero-degree ice for a few minutes, and finally the contents of the two tubes were combined and transferred to the desired container and placed at room temperature, thus preparing collagen hydrogel. 10 ml of acetic acid (0.17 M) was dissolved in 100 ml distilled water. Chitosan (1.0 g, Aladdin) was added to resulting acetic acid solution and was vigorously stirred. Aqueous NaOH solution was added into the mixture for crosslinking of chitosan solution. The solution was centrifuged (5000 rpm for 2 min) to isolate the chitosan hydrogel. The hydrogel was dehydrated using acetone solution and sterilized by UV irradiation. The morphology of the hydrogel was examined and photographed using an Olympus BX61 Research Slide Scanner microscope. A 1:1 ratio was used to prepare collagen-chitosan hydrogel.

2. 5. Hydrogel cytotoxicity assessment

MTT test was used to evaluate the toxicity of hydrogels for cells. For this purpose, hydrogels were prepared in 96-well plates and MSCs with a density of 10^5 per well were cultured. After 48 hours, the supernatant was drained and 100 µl of DMSO was poured on each well and incubated for 5 hours. DMSO was then drained and MTT solution was poured onto the cells for 10 minutes and read at wavelength 545 nm by Elisa Reader. Live/Dead staining was also used to check the viability of the cells in hydrogel according to a previous study (Nie et al., 2011). Briefly, after 1 and 7 days of culture, 1 mM calcein AM was applied to the samples for 1 hour of incubation, followed by 1 g/mL PI (propidium iodide) for 5 minutes at 37 °C. Finally, fluorescence microscopy was used to take fluorescent images (IX51, Olympus, Japan).

2. 6. Animal experiments

24 male Wistar rats weighing between 200-220 g were purchased from Pasteur institute (Tehran, Iran). Food and water were freely available to the animals. Animals were kept on a 12 h light/dark cycle which would start at 8 am in the temperature range of 20-26 °C and humidity range of 45-70% for one week. Wounded rats were divided into 7 groups including control group (treated with normal saline) and experimental group (treated with hydrogel containing adipose tissue-derived MSCs). The animals were anesthetized by intraperitoneal injection of 2 mg ketamine and 0.2 mg xylazine and their backs were moistened and completely shaved. The shaved area was disinfected with chlorhexidine gluconate. A heated sterile biopsy punch was used to create a full thickness burn wound with the diameter of 8 mm at the dorsal supracostal region on both sides of the midline. The wound area was sterilized by gauze and washed by normal saline. The dressings were fixed on the wound using Vaseline gauze and transparent adhesive tape. Images of burn wound area were captured on days 0, 7, 14 and 21 after wound establishment using a digital camera (S9+, Samsung, south Korea). The wound area was analyzed used using Image J software (Version 1.50i).

2. 7. Histological examination

The rats were sacrificed by spinal cord destruction method on post-treatment days 7, 14 and

21. The wound bed and the surrounding intact skin (2 cm diameter) containing dermis and hypodermis were excised. Harvested specimens were fixed with 10% formalin and were stained with H&E staining (Thermo Fisher Scientific Co., Ltd (Shanghai, China)) for histological analysis. Fixed and stained tissue slides were photographed using the digital camera (Olympus IX 70).

2. 8. Biometric analysis

Thickness of skin and also skin density were measured using a 75-MHz ultrasound probe (digital ultrasound imaging system DUB SkinScanner75, tpm taberna pro medicum GmbH, Germany) on days 7, 14 and 21 after treatment. The elasticity of wound area skin (net elasticity (R2), gross elasticity (R5) and deformation after recovery (R7)) of anesthetized rats was evaluated using a Cutometer® Dual MPA 580 (Courage & Khazaka electronic GmbH, Germany) in post-treatment days 7, 14 and 21.

2. 9. Statistical analysis

All data were expressed as means ± SD and analyzed by student's t-test and one-way ANOVA with Fisher adjustment ((SPSS® (version 20.0; IBM)). Difference between groups was analyzed by Tukey test. $P < 0.05$ was considered statistically significant in all analyses.

3 Results and Discussions

3. 1. Microstructure and morphology of collagen-chitosan

The color of hydrogel was milky and had a relative transparency (Figure 1A). The microstructure observation of the samples revealed a mildly rough surface with a sponge-like composition and appeared to be smooth and uniform (Figure 1B).

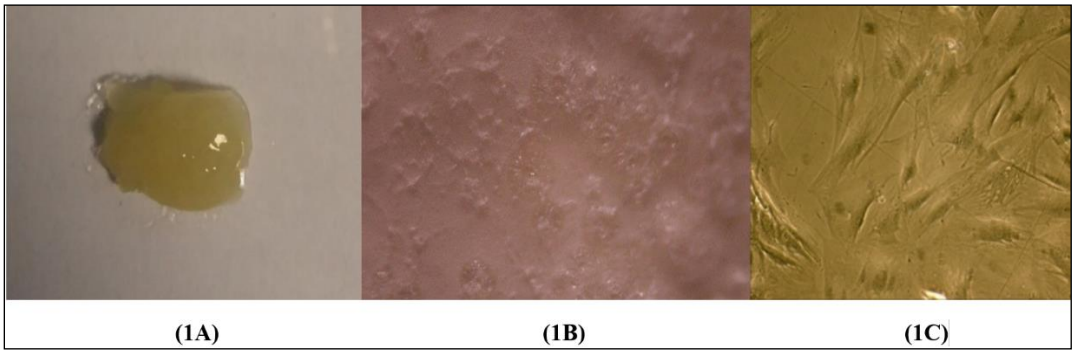


Figure 1: Digital images of the hydrogel samples (1A) and microstructure of the collagen-chitosan (1B). The isolated adipose derived cells stained with indirect immunocytochemistry (400 x magnification) (1C).

3. 2. Immunocytochemical analysis

In microscopic observation of the human adipose derived MSCs stained with ICC method, MSCs were brown in appearance and the cells showed a high expression of vimentin (Figure 1C).

3. 3. MSCs viability

The results from flow cytometry showed a high percentage of viable MSCs (89.2%) on the day of isolation (Figure 2A).

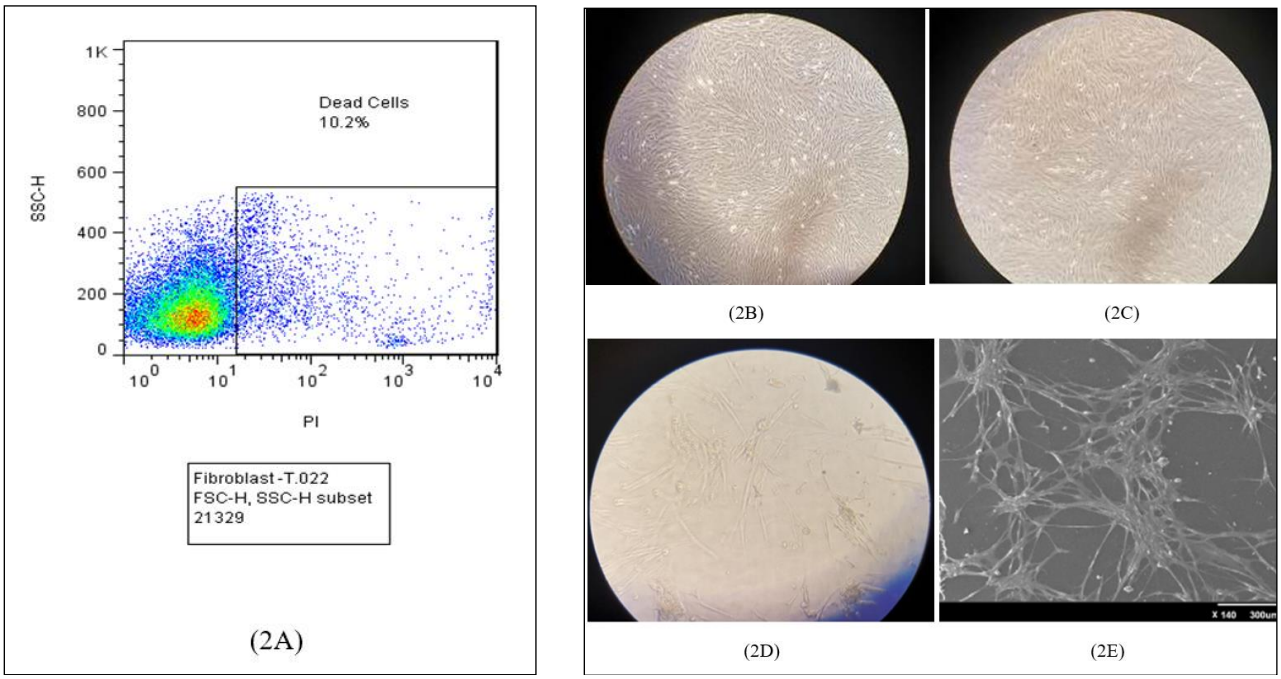


Figure 2: Viability of MSCs isolated from human adipose tissue on the day of isolation (2A). MSCs in passage 3 (2B) and passage 4 (2C) of cell culture. Microscopic image of MSCs cultured on collagen-chitosan disc under inverted microscope observation (2D), and image of MSCs cultured on collagen- chitosan, obtained from scanning electron microscope (SEM) (2E) (×140; scale bar: 300 μm).

The results obtained from cell count by hematocytometer revealed that each of 75% confluence cell culture flasks (passage 3 and 4) had approximately 2×10^6 number of cells.

3. 4. 3-D culture of MSCs in collagen-chitosan hydrogel

After 24 h 3-dimensional culture of MSCs in collagen-chitosan discs, using an inverted microscope and also a scanning electron microscope, spindle-shaped MSCs cells were observed which migrated and attached to the surface of collagen-chitosan polymer. These cells also penetrated well into the collagen-chitosan discs. Microscopic observations also showed good attachment and distribution of the cells in collagen-chitosan discs (Figure 2D & 2E).

3. 5. Cytotoxicity of hydrogel

No significant difference was observed between the viability of MSCs cultured on collagen-chitosan and control untreated cells ($P = 0.273$), according to which, the hydrogel did not show significant cytotoxic effect on MSCs. Results obtained from PI/AO cell viability assay also showed a high viability (98%) in MSCs cultured on collagen-chitosan.

3. 6. Wound closure assessment

Macroscopic observation (figure 3) and measurement of wound area using ImageJ software

showed an accelerated wound healing process in treatment groups compared to control group on days 7, 14 and 21 post-treatment. Animals treated with collagen-chitosan hydrogel + MSCs had significantly lower wound area than other groups on day 14 post-treatment ($P < 0.001$), which was confirmed by Image J assisted wound area measurement (Figure 8); however, there was not significant difference in wound area between the group treated with collagen-chitosan hydrogel + MSCs and groups treated with hydrogel or MSCs on days 7 and 21 post-treatment (figure 4).

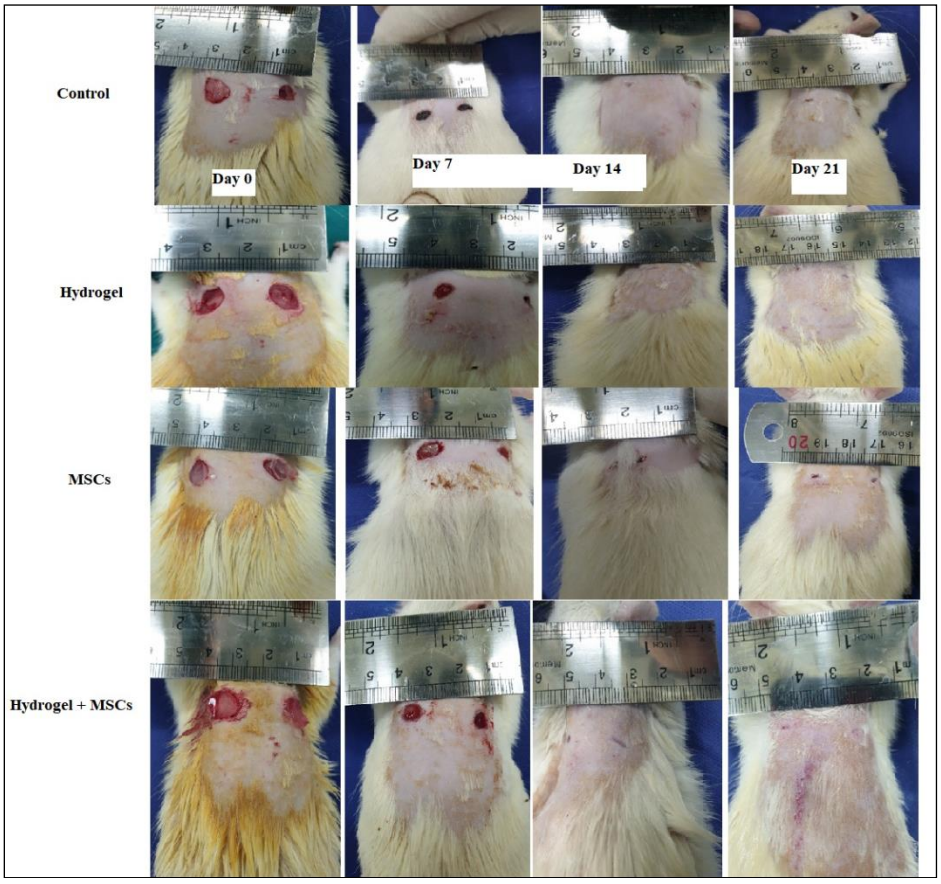


Figure 3: Wound closure on days 0, 7, 14 and 21 in control and experimental rats.

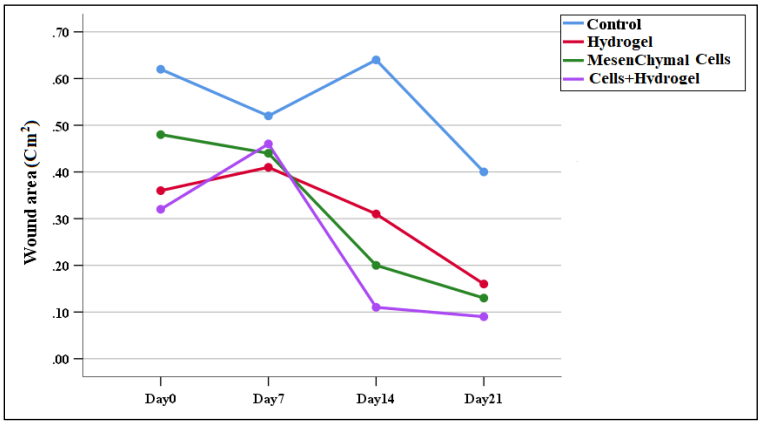


Figure 4: Wound area on days 0, 7, 14 and 21 in control and experimental rats (calculated by ImageJ).

3. 7. Microscopic examination of the wound

Microscopic examination of skin tissue samples showed that the number of neutrophils infiltrated to the wound site increased significantly in all groups and was almost the same in all 3 intervals of the study, indicating that the wound has stimulated immune system, which was followed by inflammatory reaction leading to increased neutrophils count at the wound site. On day 21 post-treatment, the number of macrophages decreased in all groups, however, the macrophage cells were significantly lower in group treated with hydrogel + MSCs than other groups. Epithelial tissue started to from during first week post-treatment and continued to expand during weeks 2 and 3 after operation. The epithelial formation was higher in group treated with hydrogel + MSCs than other groups on days 7, 14 and 21 post-treatment (figure 5).

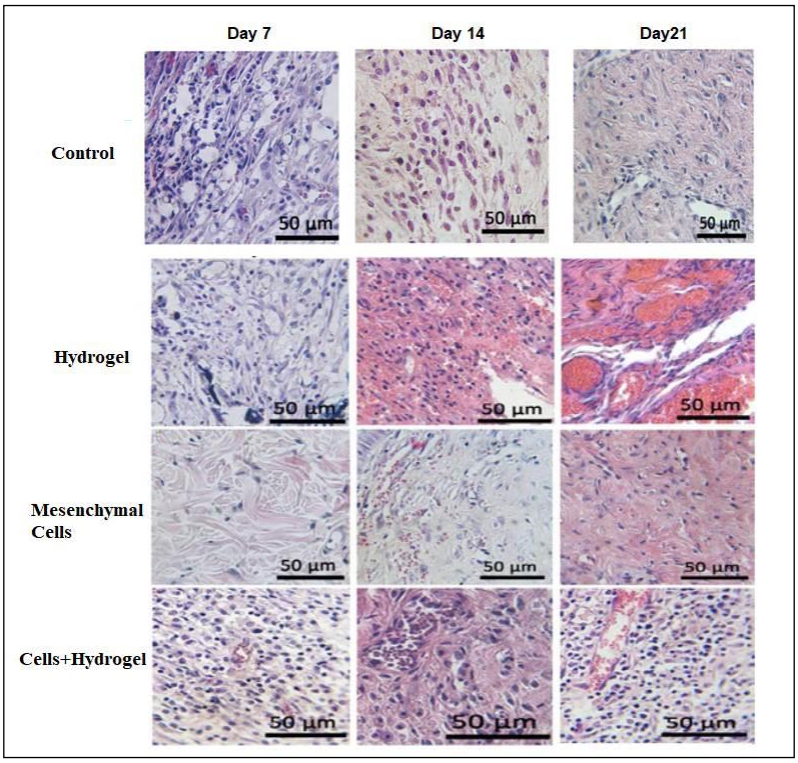
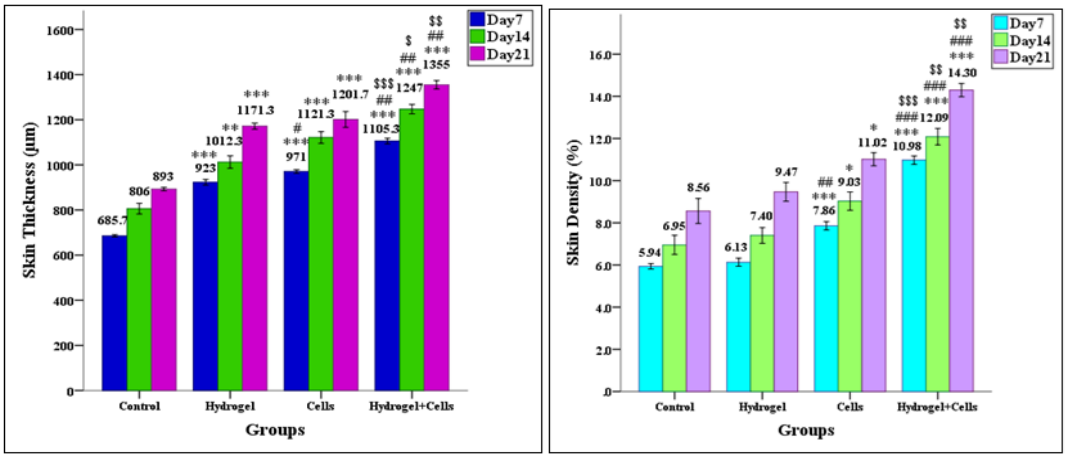


Figure 5: Histological sections of harvested wound area specimens on days 7, 14 and 21 in control and experimental groups.

3. 8. Biometric analysis

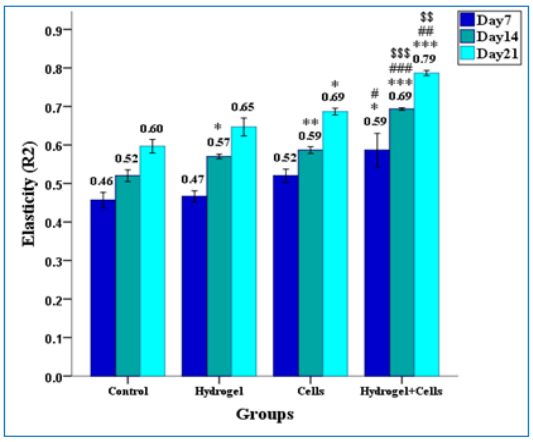
The thickness of the skin (epidermis + dermis) was significantly more in group treated with hydrogel + MSCs than control group and also in the groups treated with hydrogel, and MSCs on days 7, 14 and 21 after treatment (figure 6A). The skin (epidermis + dermis) density was significantly more in group treated with collagen-chitosan + MSCs than control group and the groups treated with hydrogel, and cells on days 14 and 21 after treatment (figure11). On day 7, skin density was significantly more in rats treated with collagen + cells than control group (and not other groups) (figure 6B). Skin gross elasticity (R2) and net elasticity (R5) were higher in group treated with "hydrogel + cells" on days 14 and 21 after treatment than control group and groups treated with cells, and hydrogel. On day 7 after treatment, R2 did not show significant

change in group treated with "hydrogel + cells" compared with other groups; however, R5 was higher compared to control group and group treated with hydrogel. In group treated with "hydrogel + cells", recovery after deformation (R7) parameter was higher on day 21 after treatment than all other groups and on day 14 was higher than only control group (figure 6C, 6D and 6E).

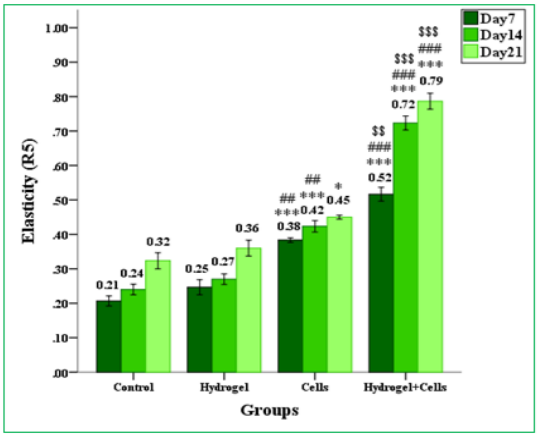


6A

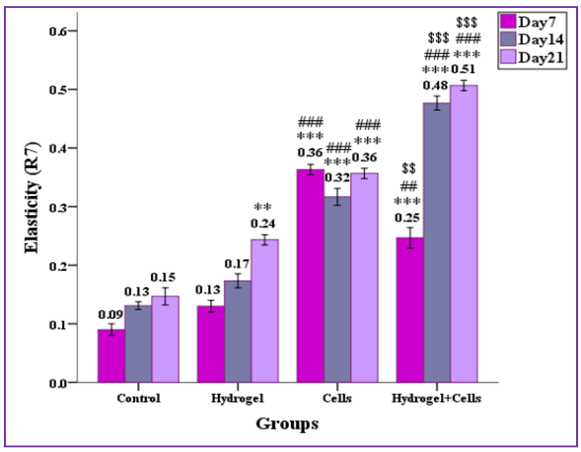
6B



6C



6D



6E

Figure 6: (6A): Skin thickness in control group and groups treated with hydrogel, cells, and "hydrogel + cells". *, # and $ represent a statistically significant difference compared to control group, and groups treated with hydrogel, and cells, respectively (***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$, ###: $P < 0.001$, $$$: $P < 0.001$). **(6B):** Skin density in

control group and groups treated with hydrogel, cells, and "hydrogel + cells". *, # and $ represent a statistically significant difference compared to control group, and groups treated with hydrogel, and cells, respectively (***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$, ###: $P < 0.001$, ##: $P < 0.01$, $$$: $P < 0.001$, $: $P < 0.05$). **(6C):** Skin elasticity (R2: gross elasticity) in control group and groups treated with hydrogel, cells, and "hydrogel + cells". *, # and $ represent a statistically significant difference compared to control group, and groups treated with hydrogel, and cells, respectively (***: $P < 0.001$, ###: P < 0.001, $$$: $P < 0.001$, $$: $P < 0.01$). **(6D):** Skin elasticity (R5: net elasticity) in control group and groups treated with hydrogel, cells, and "hydrogel + cells". *, # and $ represent a statistically significant difference compared to control group, and groups treated with hydrogel, and cells, respectively (***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$, ###: $P < 0.001$, ##: $P < 0.01$, $$$: $P < 0.001$). **(6E):** Skin elasticity (R7: recovery after deformation) in control group and groups treated with hydrogel, cells, and "hydrogel + cells".*, # and $ represent a statistically significant difference compared to control group, and groups treated with hydrogel, and cells, respectively (**: $P < 0.01$, *: $P < 0.05$, ##: P<0.01, $: $P < 0.05$).

Obtained results from ultra-sound imaging showed that skin thickness and density was higher on day 21 after treatment in animals with burns treated with "hydrogel + MSCs" than other groups (figure 7).

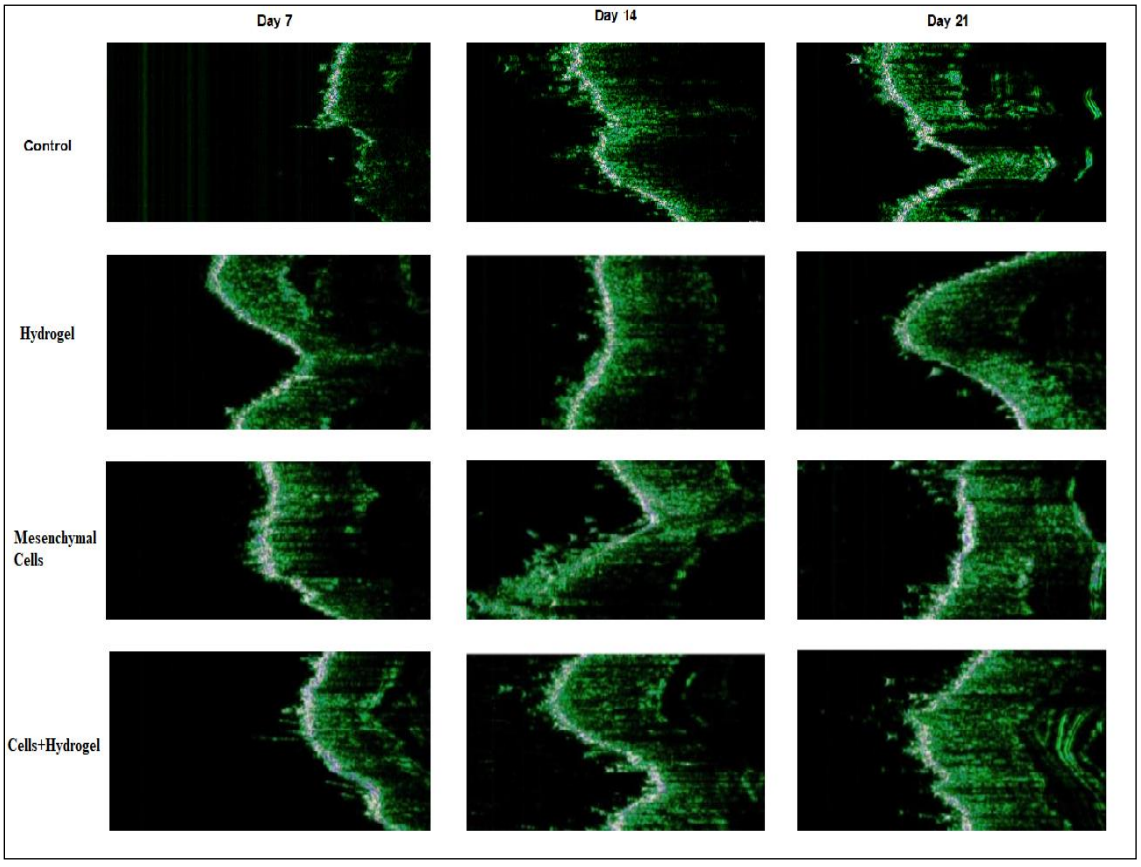


Figure 7: Ultra-sound imaging for measurement of density and thickness of wound area skin using a 75 MHz ultrasound probe on days 7, 14 and 21 after treatment.

Although many studies have shown that adipose-derived MSCs can be used to repair burn wounds, the healing effect of these cells on skin wounds, especially burn wounds, is still one of the most challenging research topics (Fernandes-Cunha et al., 2019). The studies concerned with MSCs effects on burn wound healing are aiming to show whether these cells are able to

repair burn wounds at the right time and by what tissue mechanisms they perform this repair (Yan et al., 2020). The results of this study show that dressing burn wound with MSCs seeded collagen-chitosan hydrogel promoted skin reepithelialization, vascularization, collagen synthesis, thickness, density and elasticity at the transplant site and led to faster wound healing process than MSCs or collagen-chitosan hydrogel alone. However, in conventional burn wound healing pathways, the healing process takes a long time (Ouyang et al., 2018). In line with the findings of this study, it has been shown that MSCs have a beneficial effect on skin wound healing through rapid epithelialization and effective contraction in the wound area (Pop et al., 2015; Azari et al., 2011). Umbilical cord derived MSCs have also been reported to accelerate skin wound healing in diabetic rat (Han et al., 2019). However, MSCs seeded hydrogels may play a more significant role in wound repair than MSCs alone. Acellular amnion (as scaffold) seeded with adipose-derived MSCs has been reported to result in faster wound healing and better histopathology characteristic than MSCs alone (Fatemi et al., 2014). In a study it has been clearly shown that the treatment of burn wound with the human umbilical cord derived MSCs seeded in chitosan-collagen hydrogel led to shortened healing period and restricted inflammation (Zhou et al., 2019). Collagen-chitosan hydrogel has been suggested to be a suitable scaffold to transfer cells to target tissues. Research has shown that this type of hydrogel does not damage cells and can transport cells safely to target tissue (Li et al., 2021).

We have shown in the present study that MSCs seeded collagen-chitosan hydrogel increases collagen synthesis, which was followed by more increase in skin thickness, density and elasticity than MSCs alone. During wound healing, adipose tissue derived stem cells have a great ability in migration to be recruited rapidly into wounded sites added to their differentiation towards dermal fibroblasts (which secret collagen), endothelial cells, and keratinocytes leading to accelerated wound healing process. MSCs have been reported to release bioactive molecules that have anti-apoptotic properties and to increase angiogenesis. They have high potency to induce the expression of collagen-producing genes and thus be effective in wound healing (Mazini et al., 2020). Adipose-derived MSCs induce the tissue cell proliferation and stimulate the secretion of growth and proliferation factors in the target tissue (Tettamanti et al., 2004; Lee et al., 2016; Mazini et al., 2020), resulting in faster increasing of skin thickness, density and elasticity.

Collagen-chitosan hydrogel can provide a suitable environment for MSCs, allowing them to obtain nutrients and perform gas exchange (Wang et al., 2021), leading to higher cell viability and functioning, which results in rapid migration of MSCs to wounded site and accelerated healing process.

4 Conclusion

Dressing of burn wound with MSCs seeded collagen-chitosan hydrogel accelerates the process of wound healing faster than MSCs alone by inducing more increase in skin reepithelialization, vascularization, collagen synthesis, thickness, density and elasticity at the transplant site than MSCs alone.

Acknowledgments

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Conflict of interests

The authors state that there are no conflicts of interests regarding the publication of this article.

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