ORIGINAL ARTICLE

Burn wound healing effect of neonatal fibroblast cells seeded into chitosan hydrogel in diabetic rats

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Abstract

Background: Diabetic chronic wounds are the most critical clinical issues. Although a wide range of wound dressings have been designed, few of them act sufficiently in repairing and protecting wounds. *Aim and Objectives***:** This study aimed to investigate the burn wound healing effect of neonatal fibroblasts seeded into chitosan hydrogel in diabetic rats. *Material and Methods:* Two burn wounds were created in male Wistar diabetic rats. The rats were divided into groups treated with fibroblast cells, chitosan hydrogel, fibroblast cell cultured chitosan hydrogel (chitosan + fibroblasts group), and control untreated group. Macroscopic and microscopic examination, wound area measurement, measurement of skin density and thickness, and also skin elasticity were applied to evaluate wound healing. The data were analyzed using ANOVA. *Results*: Treatment of diabetic burn wound with chitosan + fibroblasts accelerated healing process by promoting re-epithelialization and reducing inflammatory responses in skin tissue. Skin elasticity, thickness and density were higher in chitosan + fibroblasts group compared with control group 3 weeks after treatment. *Conclusion:* The results of the present study show that dressing the diabetic burn wound with neonatal fibroblasts seeded into chitosan scaffold plays a fundamental role in wound healing process.

Keywords*:* Fibroblast, Chitosan hydrogel, Burn wound healing, Fibroblasts

Introduction

Burn injuries are challenging wounds to treat with a relatively high death rate, particularly in patients with diabetes [1]. Severe burns affect nearly every organ system and are vulnerable to leakage, infection, electrolyte, and protein loss [2-3], leading to significant morbidity and mortality. Early burn wound excision and skin grafting significantly improve the complications caused by severe burn in patients [4]. However, delayed wound healing accompanied by infection and hypertrophic scarring still remains a major challenge in burn research. Diabetic wound care is of great importance [5] and successful cell therapy for burn injuries also is facing great challenges in diabetic animal models or patients with diabetes [6]. Wound healing progress has four fundamental stages: 1-hemostasis phase; 2-inflammatory phase; 3-proliferative phase (angiogenesis, epithelialization, and epithelial cell migration as well as some other mechanisms, occur in this phase); 4-maturation phase [7]. A growing body of evidence shows delayed healing process in diabetic wounds due to impaired angiogenesis, collagen synthesis dysfunction, inhibited cell migration, and extended inflammatory phase [8]. Fibroblasts have been reported to accelerate many aspects of wound healing process *in-vitro* and *in-vivo* [9]. Fibroblasts are dermal stem cells that stimulate cell proliferation, angiogenesis,

cell migration and cell differentiation by producing growth factors, collagen fibers and extracellular matrix [10]. Growth factors secreted by fibroblasts have essential role in morphogenesis of suprabasal keratinocytes and organization of the natural process of keratinocyte differentiation [11], which plays a pivotal role in skin regeneration process. Fibroblasts are also involved in epithelial homeostasis loss, ulcer chronicity and organ fibrosis [12].

Chitosan hydrogel is a bioactive, bacteriostatic, fungistatic, analgesic and oxygen permeable material that is helpful in stabilizing growth factors and modulating differentiation of stem cells for tissue repair [13]. Chitosan also improves angiogenesis in skin tissue [14], resulting in accelerated wound healing process. Cationic nature of chitosan hydrogel improves cell proliferation and differentiation and also amplifies extracellular matrix generation and growth factors production [15]. In recent studies, chitosan hydrogel has been used to deliver a variety of stem cells including mesenchymal stem cells and fibroblast cells [16]. Considering all these features, using chitosan hydrogel with fibroblast cells can be a useful strategy for promoting diabetic wound repair. In this study the burn wound healing effect of foreskin-derived fibroblast cells seeded on chitosan hydrogel was evaluated in diabetic rats.

Material and Methods

Ethics approval

Animal experiments were approved by Azad University Ethic Committee with grant number of GRS, 2020.1SC. All materials were reproduced with permission from other sources

Preparation of the chitosan hydrogel

Ten milliliter (ml) of acetic acid (0.17M) was dissolved in 100 ml distilled water. Chitosan [1.0 gram (g), Aladdin] was added to resulting acetic acid solution and was vigorously stirred. Aqueous sodium hydroxide solution was slowly added into the mixture for crosslinking of chitosan solution. Centrifugation of obtained solution at 5000 revolutions per minute (rpm) for 2 minutes (min) was used to isolate the chitosan hydrogel. The obtained hydrogel was dehydrated using acetone solution and sterilized by ultraviolet (UV) irradiation. The morphology of the hydrogel was examined and photographed using an Olympus BX61 Research Slide Scanner microscope.

Isolation of fibroblasts

Newborn human foreskin tissue obtained following routine circumcision was collected in 0.15 N sodium chloride containing 400 units/ml penicillin and 200 /lg/ml streptomycin (GIBCO (USA)) and stored at 4°C for up to 24 hours (h) before use. The foreskin was cut into small-sized pieces using a scalpel and the pieces of skin were placed in a 15 ml centrifuge tube containing 10 ml of 1× dispase solution [GIBCO(USA)], and 2 × antibiotics/ antimycotics and incubated overnight (~ 16 h) at 4°C in a horizontal orientation. Phosphate-buffered saline (PBS, Xilong Chemical Co., Ltd) was used to wash away excess dispase. The dermis was separated from the epidermis and cut into very small pieces using a scalpel blade. The pieces were transferred into a 50 ml centrifugation tube containing 5 ml of collagenase A (Sigma-Aldrich (USA)), plus 0.5× CnT-GAB10 antibiotic/ antimycotic, and incubated at 37°C for 4-8 h. Five ml Dulbecco's Modified Eagle Medium (DMEM) [GIBCO (USA)] containing fetal bovine serum 10% (FBS) [GIBCO (USA)] was added to dilute the collagenase and the solution was mixed by pipetting up and down, and then passed through 70 micrometer (μm) cell

strainer to obtain a single-cell suspension. The suspension was centrifuged for 5 min, 200 × g at room temperature, and the pellet was re-suspended in 5 ml DMEM and centrifuged for 5 min, 200 × g at room temperature. The supernatant was discarded and the cell pellet was cultured in DMEM plus 10% (v/v) FBS, 1% Glutamax [Thermo Fisher Scientific Co., Ltd (Shanghai, China)], and 1% (v/v) Penicillin-Streptomycin [GIBCO (USA)] and incubated in an atmosphere of 37 C°, 5% CO2, 95% humidity. Culture medium was changed every 2-3 days. The cells were sub-cultured when they reached 70-80% confluence and observed using an inverted microscope (Olympus, IX 70). The cultured cells were used for in vivo experiments after 3-4 passages.

Fibroblast cells characterization and count

Immunocytochemistry staining of obtained cells was performed to confirm the identification of the isolated fibroblast cells. The cells were grown on coverslips for 24 h and then fixed in acetone for 30 min at -20°C. After blocking with 3% H_2O_2 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 IgG antibody [Abcam (United Kingdom)], Goat Anti-Mouse IgG H&L (HRP) pre-adsorbed (ab7068), diluted to 1:50 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 microscope slides. Obtained slides were observed using fluorescence microscope (Nicon Eclipse TE200).

Cell count was conducted by hemocytometer (Fisher Scientific) and Olympus IX 70 light microscope. 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 percentages of live and dead cells were analyzed in a Beckman Coulter Navios flow cytometer.

3-D culture of fibroblasts in chitosan hydrogel

Fibroblast cells were cultivated according to a previously established technique. Briefly, chitosan discs with 5 millimeter (mm) diameter and 2 mm thickness were used. The discs were put into a 96-well plate. Forty microliter (µl) of culture medium containing fibroblast cells was added into each disc. After 2 h, to ensure sufficient cell proliferation, another 100 µl of culture medium was added at 6h post-culture. The discs were transferred into 24-well plates for assurance of existence of adequate growth medium for cell 3-D proliferation. The fibroblast cell cultured chitosan discs were incubated in condition of 5% CO_2 at 37°C. The culture medium was changed every day. The 3-D fibroblast cell culture in hydrogels was observed using the inverted microscope (Olympus, IX 70) and a scanning electronic microscope (SEM, Hitachi, S-3000 N, Japan). After 14 days, chitosan discs cultured with fibroblast cells were used for wound dressing.

Chitosan cytotoxicity assessment

3-[4, 5-dimethylthiazol-2yl]-2, 5 diphenyl-tetrazolium bromide (MTT) assay was used to assess the cytotoxicity of chitosan. Chitosan was added to a 96-well plate and fibroblast cells were cultured into chitosan with concentration of 10^5 cells/ well. After 48 h, 100 µL of the MTT solution (0.5 mg/mL) was added into each well and incubated for 3h. Fifty µL DMSO was added to each well to dissolve formazan crystals. Absorbance was measured at 545 nm using a spectrophotometer (Spectramax 250) and sample cell viability was reported in comparison to control using the following equation:

% viability =100 × Absorbance of sample/ Absorbance of control.

Live/dead staining was also used to check the viability of the cells in chitosan according to a previous study. 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).

Animal experiments

Twenty-four, 3-months male Wistar rats weighing between 200-220 g were purchased from Pasteur Institute (Tehran, Iran). All animal experiments were approved by Azad University Ethic Committee. 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. The rats were divided into 4 groups (n=6 in each group): control (normal saline received group), fibroblast cells treated (fibroblast group), chitosan treated (chitosan group), and chitosan + fibroblast cells treated group (chitosan + fibroblast group). Diabetes was induced by one injection of Streptozotocin (STZ, 40 mg/kg, Aladdin) into peritoneum. After one week of injection, the rats with fasting blood glucose higher than 16.7 mmol/L were considered diabetic. 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 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 using Image J software (Version 1.50i)

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).

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) on post-treatment days 7, 14 and 21.

Statistical analysis

All data were expressed as mean ± 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's test and value of *p* less than 0.05 was considered statistically significant in all analyses.

Results

Microstructure and morphology of chitosan

The color of chitosan hydrogel was milky and had a relative transparency as shown in Figure 1A. The results showed that UV irradiation for 1 hour for sterilizing the powdered and also non-dry samples were sufficient. The microstructure observation of the chitosan samples revealed a mildly rough surface with a sponge-like composition and appeared to be smooth and uniform (Figure 1B).

Immunocytochemical (ICC) analysis

In microscopic observation of the foreskin derived cells stained with ICC method, fibroblast cells were brown in appearance and the cells showed a high expression of vimentin (Figure 2), which shows that there is a large number of fibroblast cells in the isolated cells.

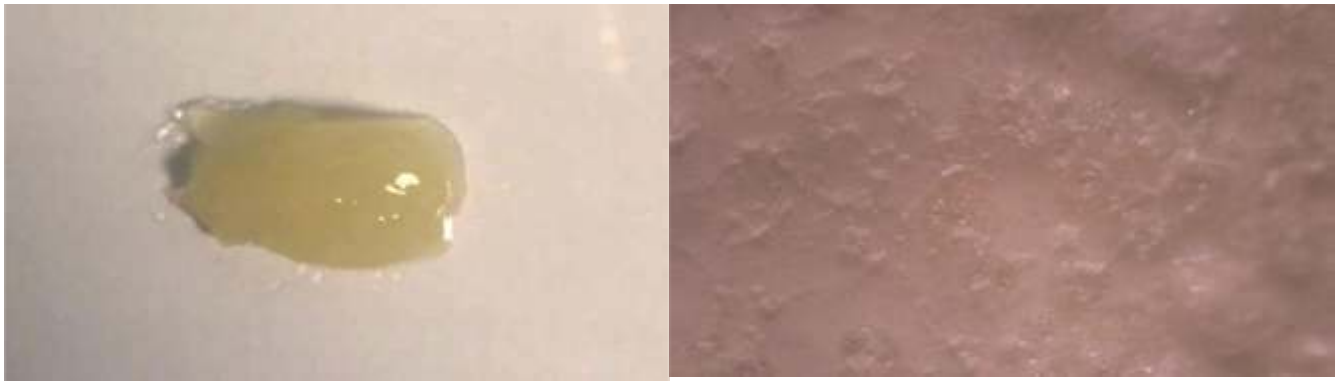

Figure 1: Digital images of the hydrogel samples (Figure 1A) and microstructure of the chitosan (Figure 1B) Immunocytochemical (ICC) analysis

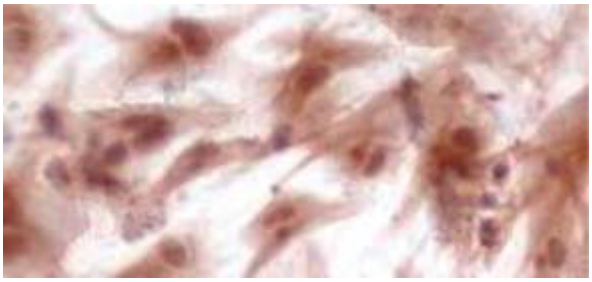

Figure 2: The isolated foreskin-derived cells stained with indirect immunocytochemistry (400 × magnification) Isolated cell viability and cell count

The results from flow cytometry observation showed that a high percentage of fibroblast cells kept viability (89.2%) on the day of isolation (Figure 3). Obtained results represented that the used method was suitable for isolation.

The cell counting of three times and four times sub cultured cell cultures was performed. The results revealed that each of 75% confluence cell culture flasks had approximately 2×10^6 number of cells (Figure 4)

3-D culture of fibroblasts in chitosan hydrogel

After 24 h 3-dimensional culture of fibroblast cells on chitosan discs, using an inverted microscope and also a scanning electron microscope, spindle-shaped fibroblast cells were observed which migrated and attached to the surface of chitosan polymer. These cells also penetrated well into the chitosan discs. Microscopic observations also showed good attachment and distribution of the cells in chitosan discs (Figure 5).

Cytotoxicity of chitosan

No significant difference was observed between the viability of fibroblast cells cultured on chitosan and control untreated cells ($p = 0.273$) (figure 6A), according to which, chitosan did not show significant cytotoxic effect on fibroblast cells. Results obtained from PI/AO cell viability assay also showed a high viability (89%) in fibroblast cells cultured on chitosan (Figure 6B).

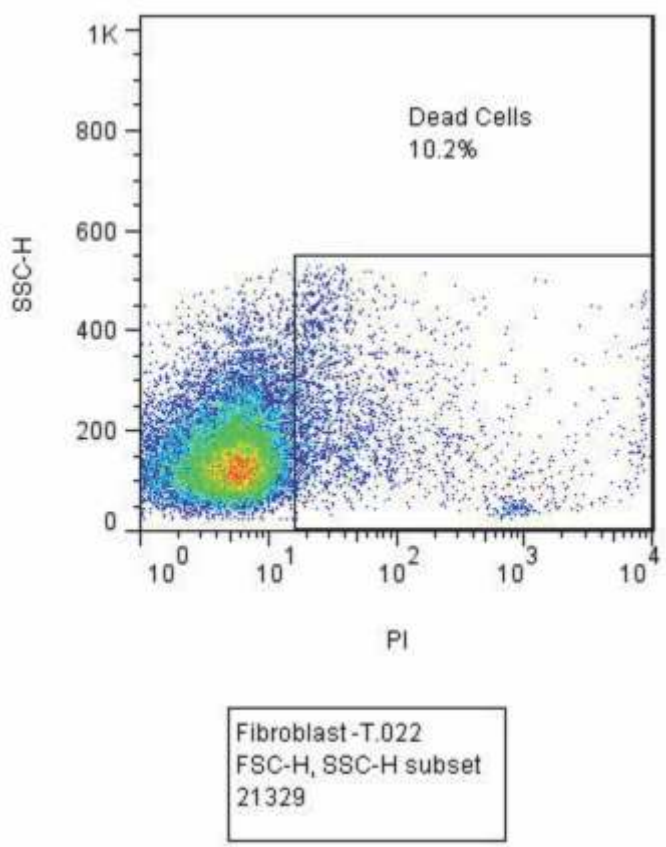

Figure 3: Viability of fibroblast cells isolated from foreskin on the day of isolation

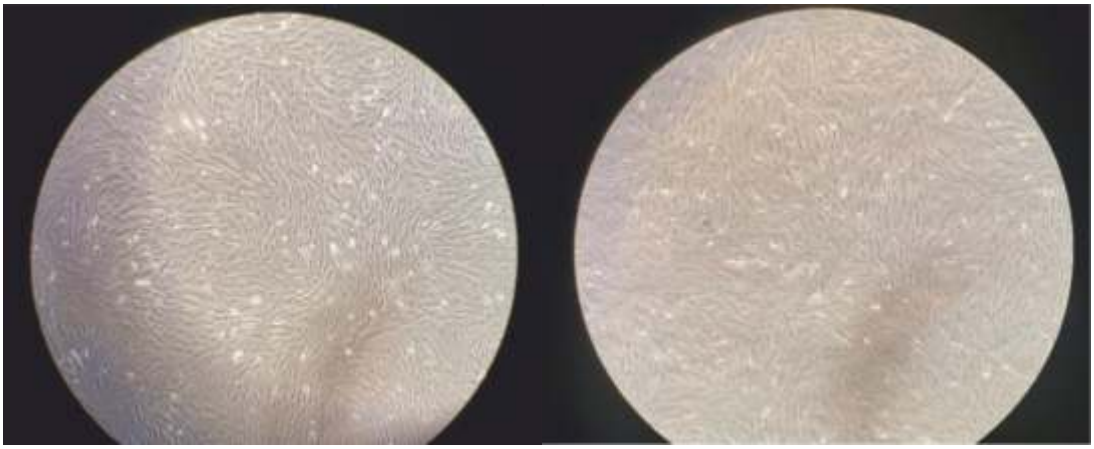

Figure 4: Three times passaged (4A) and 4 times passaged fibroblast cells cultures (4B)

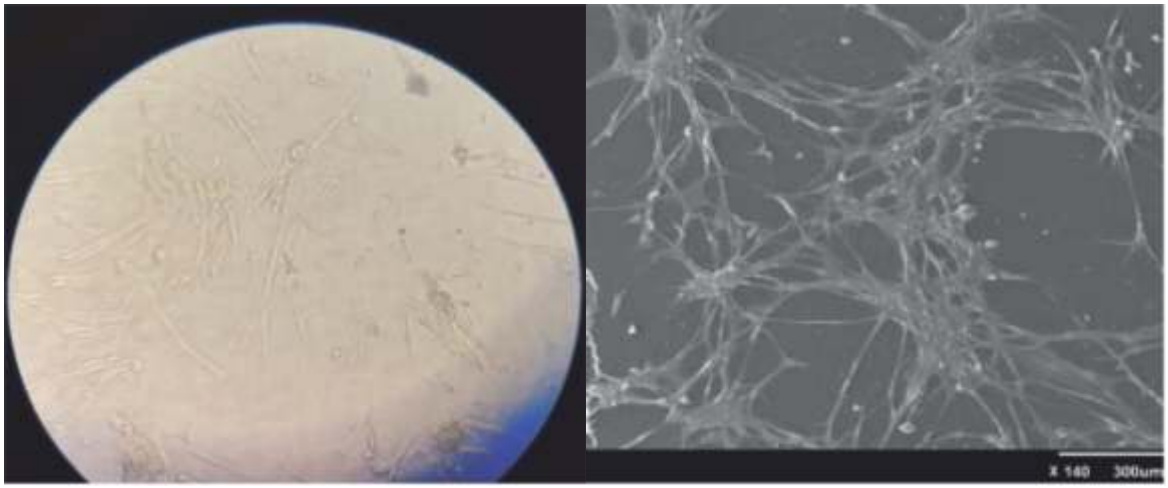

Figure 5: Microscopic image of fibroblast cells cultured on chitosan disc under inverted microscope observation (5A), and image of fibroblast cells cultured on chitosan, obtained from Scanning Electron Microscope (SEM) (5B) (×140; scale bar: 300 µm)

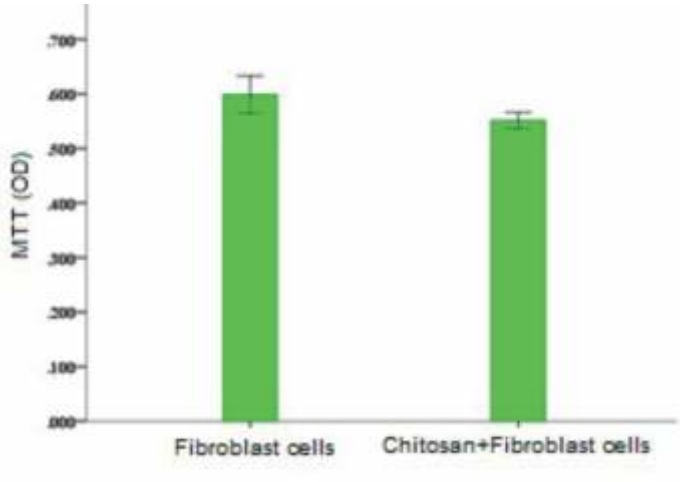

Figure 6A

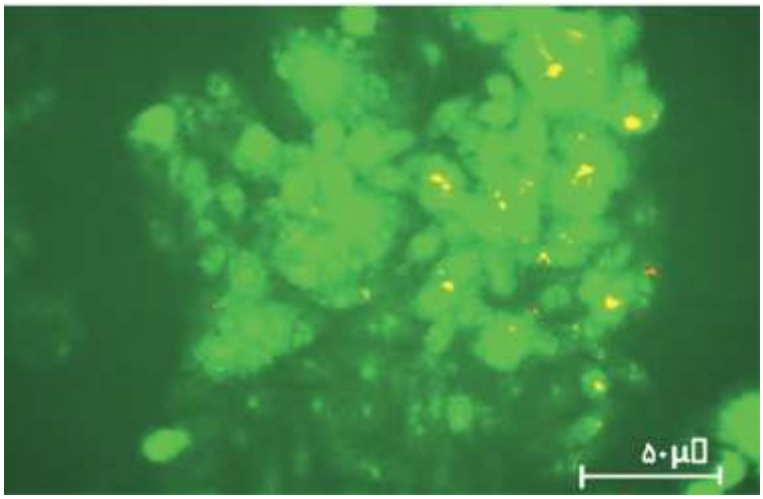

Figure 6B

Figure 6: Cell viability measured by MTT assay method in untreated fibroblast cells and fibroblast cells cultured on chitosan. Optic Density (OD) indicated cell viability (Figure 6A). PI/AO live/dead assay in fibroblast cells cultured on chitosan (red color shows dead cells (11%) and green color shows live cells (89%)) (Figure 6B)

Macroscopic observation and wound area measurement

Macroscopic observation (Figure 7) showed accelerated wound healing in treatment groups compared to control group on days 7, 14 and 21 post-treatments. Animals treated with chitosan + fibroblast cells 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 no significant difference in wound area between the group treated with chitosan + fibroblast cells and groups treated with chitosan or fibroblast cells on days 7 and 21 post-treatment (Figure 8).

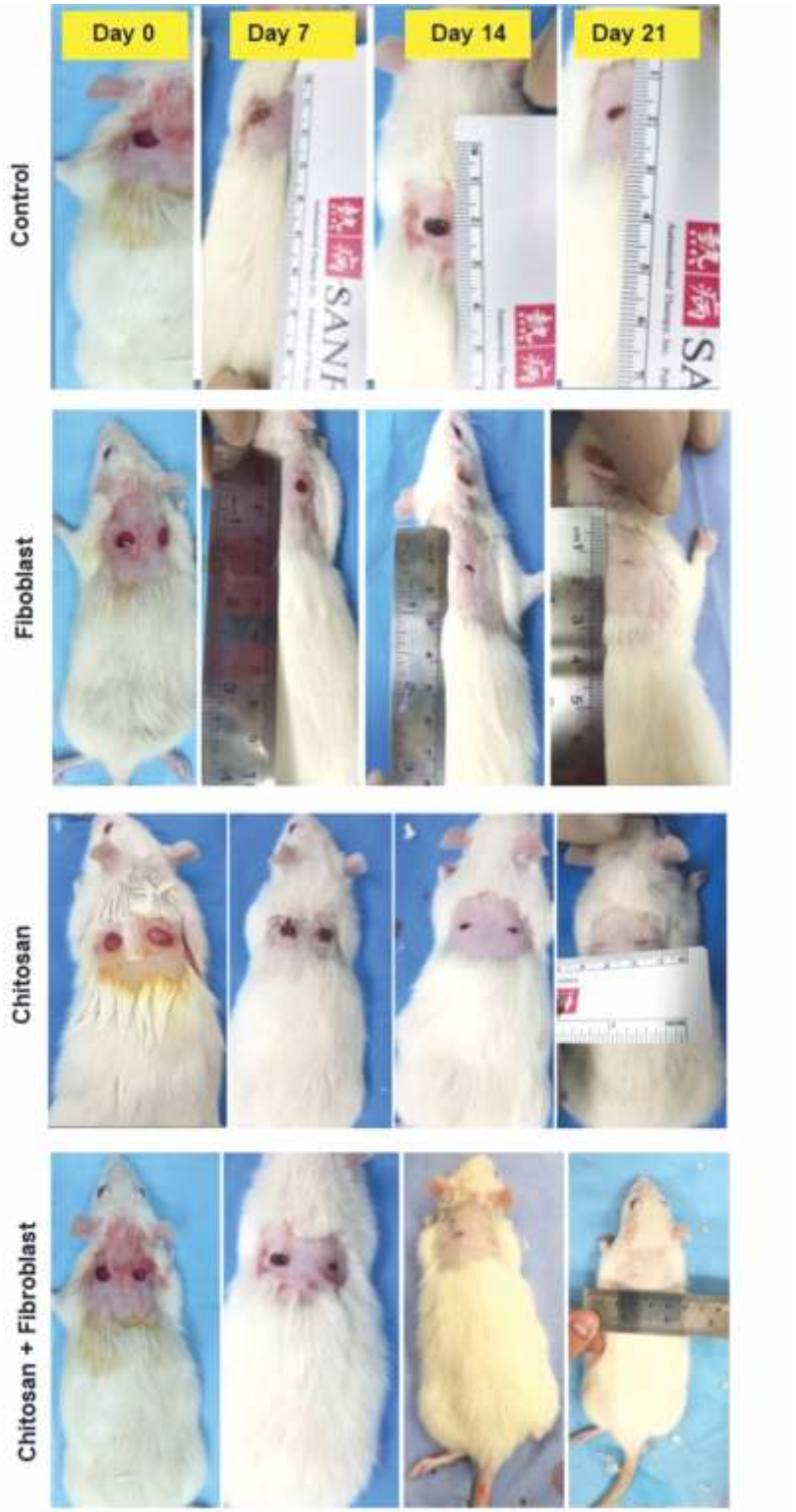

Figure 7: Wound closure on days 0, 7, 14 and 21 in control and experimental rats (n=24)

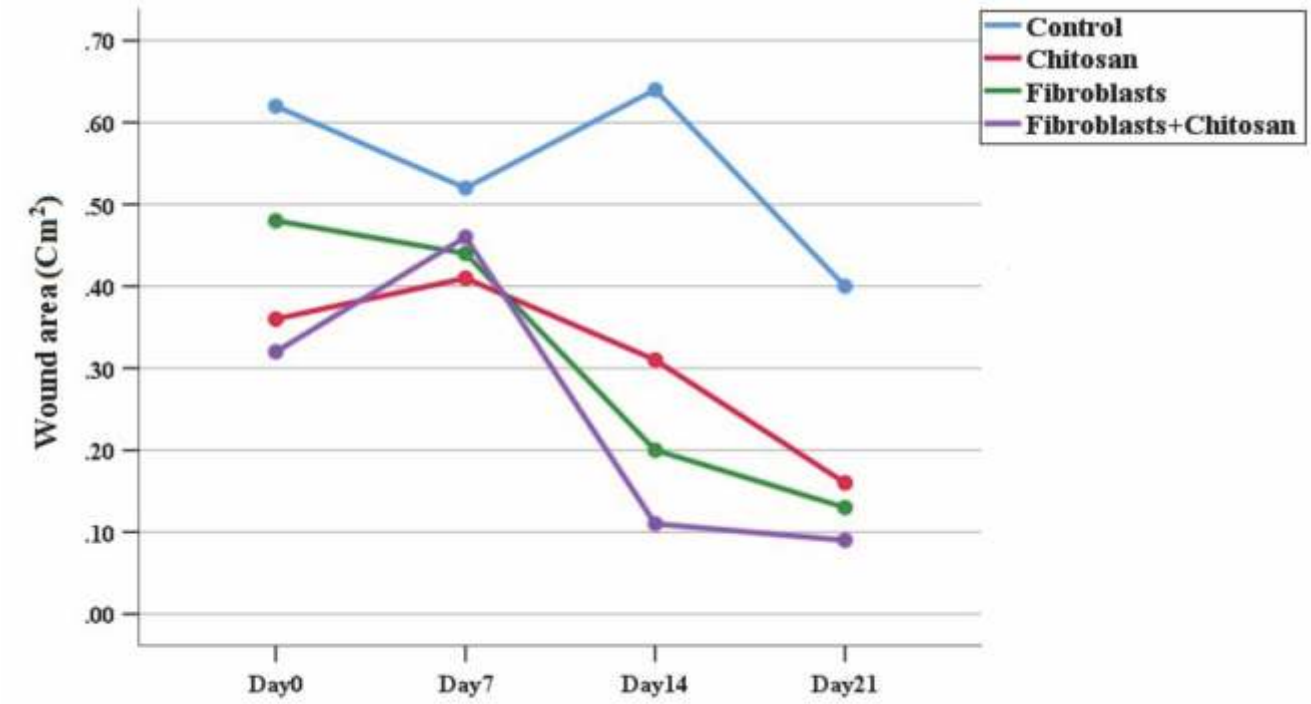

Figure 8: Wound area on days 0, 7, 14 and 21 in control and experimental rats (n=24)

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 had stimulated the 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 chitosan+ fibroblast than other groups. Epithelial tissue started to form during first week post-treatment and continued to expand during weeks 2 and 3 after treatment. The epithelial formation was higher in group treated with chitosan + fibroblast than other groups on days 7, 14 and 21 post-treatments (Figure 9).

Biometric analysis

The thickness of the skin (epidermis + dermis) was significantly more in group treated with chitosan + fibroblast than control group and also in the groups treated with chitosan, and fibroblast on days 7, 14 and 21 after treatment (Figure 10).

The skin (epidermis + dermis) density was significantly more in group treated with chitosan + fibroblast than control group and the groups treated with chitosan, and fibroblast on days 14 and 21 after treatment (Figure 11). On day 7, skin density was significantly more in rats treated with chitosan + fibroblast than control group (and not other groups) (Figure 11).

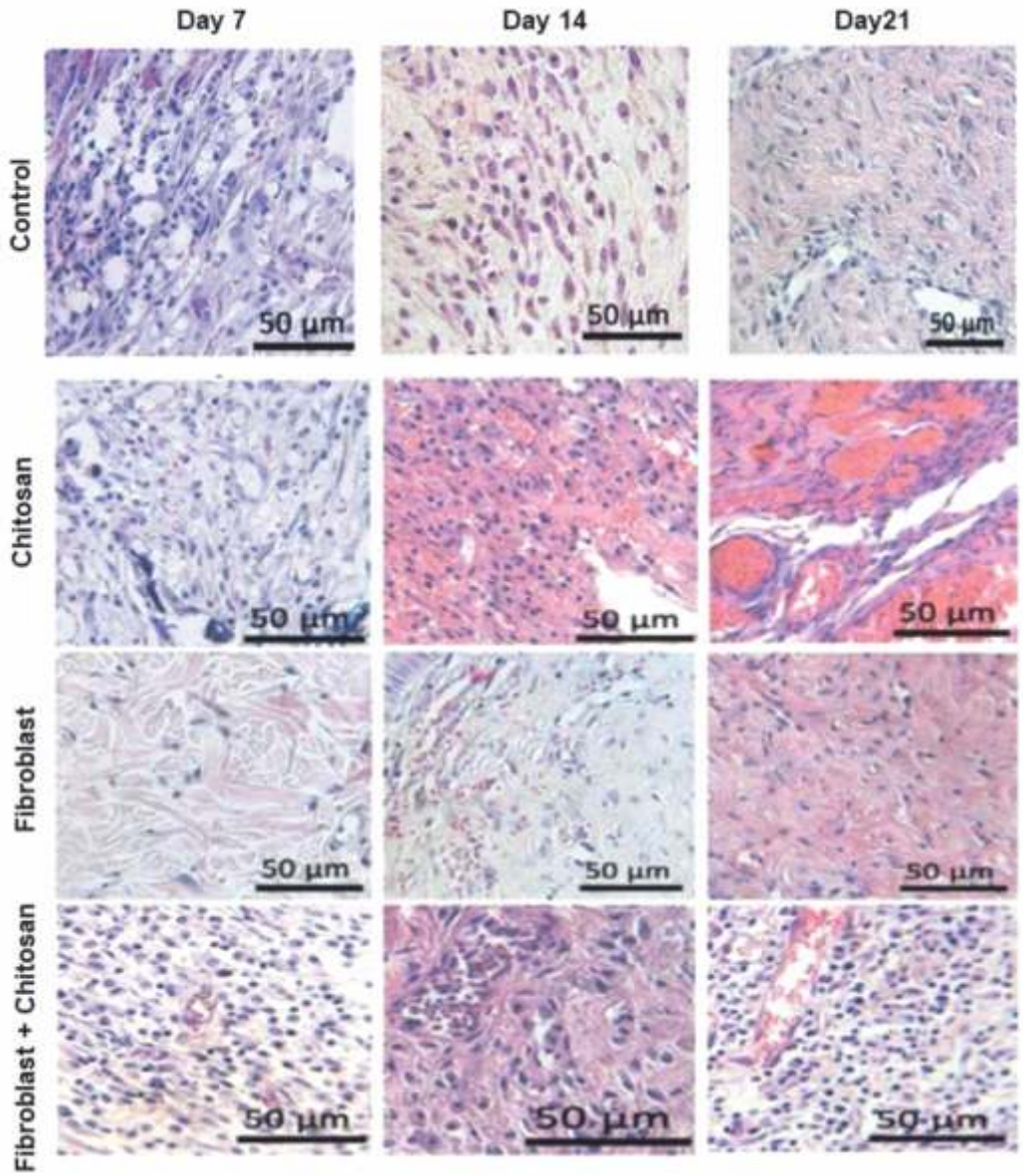

Figure 9: Histological sections of harvested wound area specimens on days 7, 14 and 21 in control and experimental groups

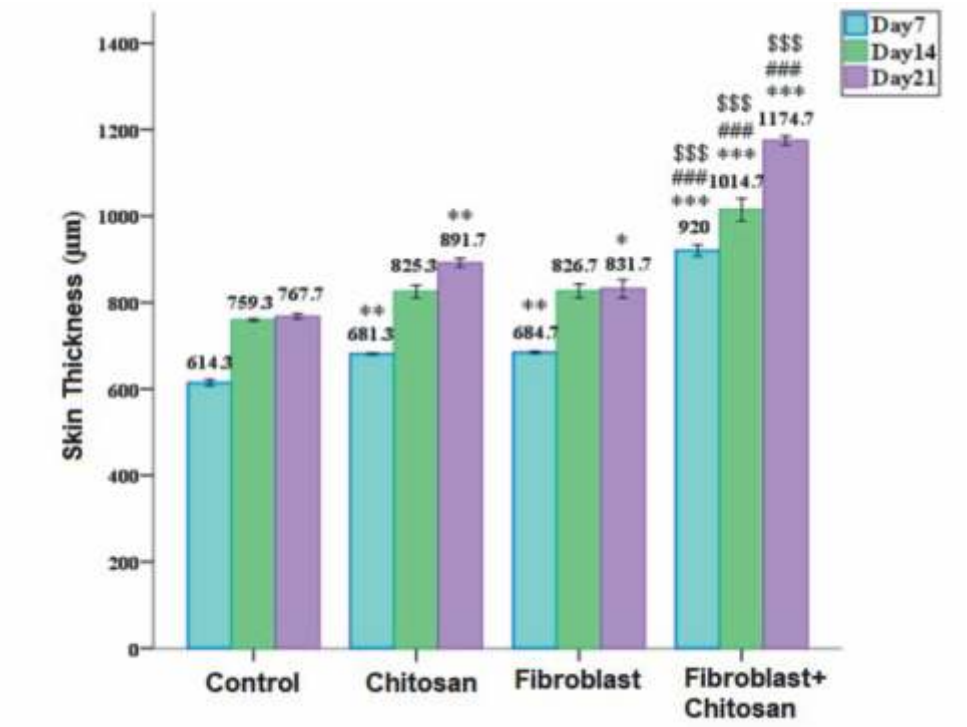

Figure 10: Skin thickness in control group (n=6) and groups treated with chitosan (n=6), fibroblast (n=6), and "chitosan + fibroblast (n=6)". *, # and $ represent a statistically significant difference compared to control group, and groups treated with chitosan, and fibroblast, respectively (*: p <0.001, **: p <0.01, *: p <0.05, ###: p <0.001, $$$: p <0.001)**

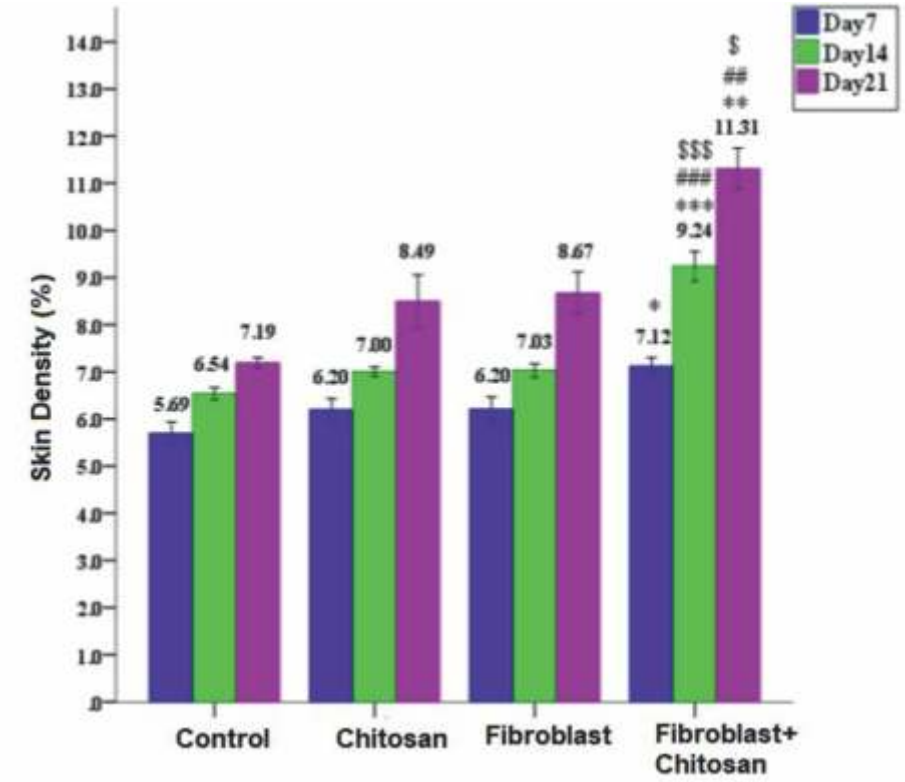

Figure 11: Skin density in control group (n=6) and groups treated with chitosan (n=6), fibroblast (n=6), and "chitosan + fibroblast (n=6)". *, # and $ represent a statistically significant difference compared to control group, and groups treated with chitosan, and fibroblast, respectively (*: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ###: $p < 0.001$, ##: $p < 0.01$, $$$: $p < 0.001$, $: $p < 0.05$).**

Skin gross elasticity (R2) and net elasticity (R5) were higher in group treated with "chitosan + fibroblast" on days 14 and 21 after treatment than control group and groups treated with fibroblast, and chitosan. On day 7 after treatment, R2 did not show significant change in group treated with "chitosan + fibroblast" compared with other groups; however, R5 was higher compared to control group and group treated with chitosan. In group treated with "chitosan + fibroblast", 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 (Figures 12, 13 and 14).

Obtained results from ultra-sound imaging showed that skin thickness and density was higher on day 21 after treatment in diabetic animals with burns treated with "chitosan + fibroblast" than other groups (Figure 15).

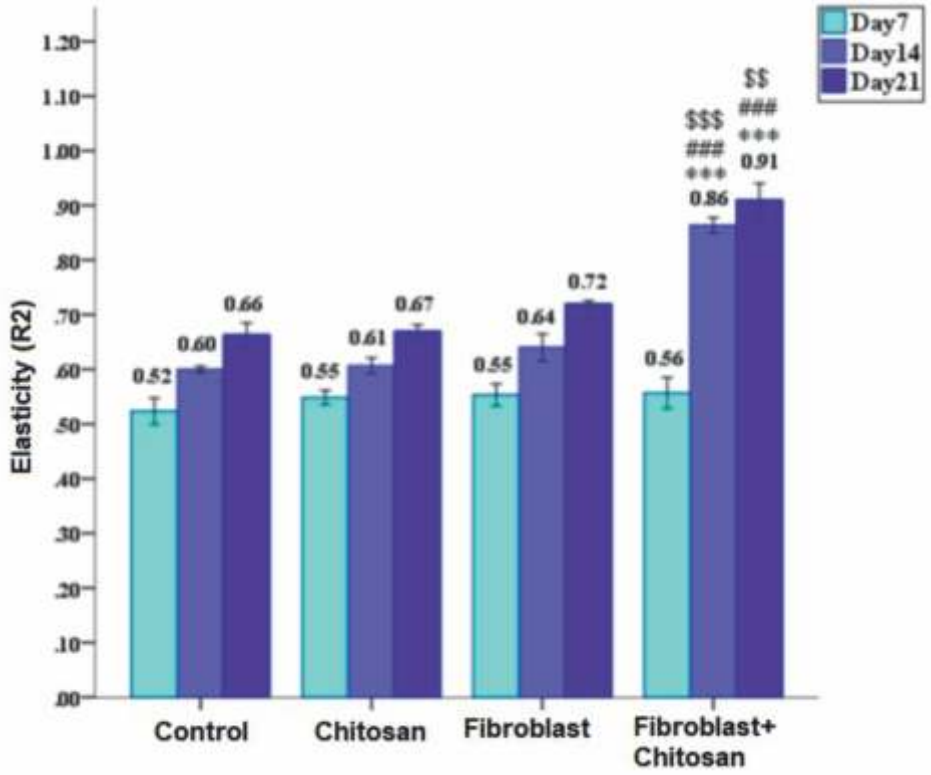

Figure 12: Skin elasticity (R2: gross elasticity) in control group (n=6) and groups treated with chitosan (n=6), fibroblast (n=6), and "chitosan + fibroblast (n=6)". *, # and $ represent a statistically significant difference compared to control group, and groups treated with chitosan, and fibroblast, respectively (*: p <0.001, ###: p <0.001, $$$: p <0.001, $$: p <0.01).**

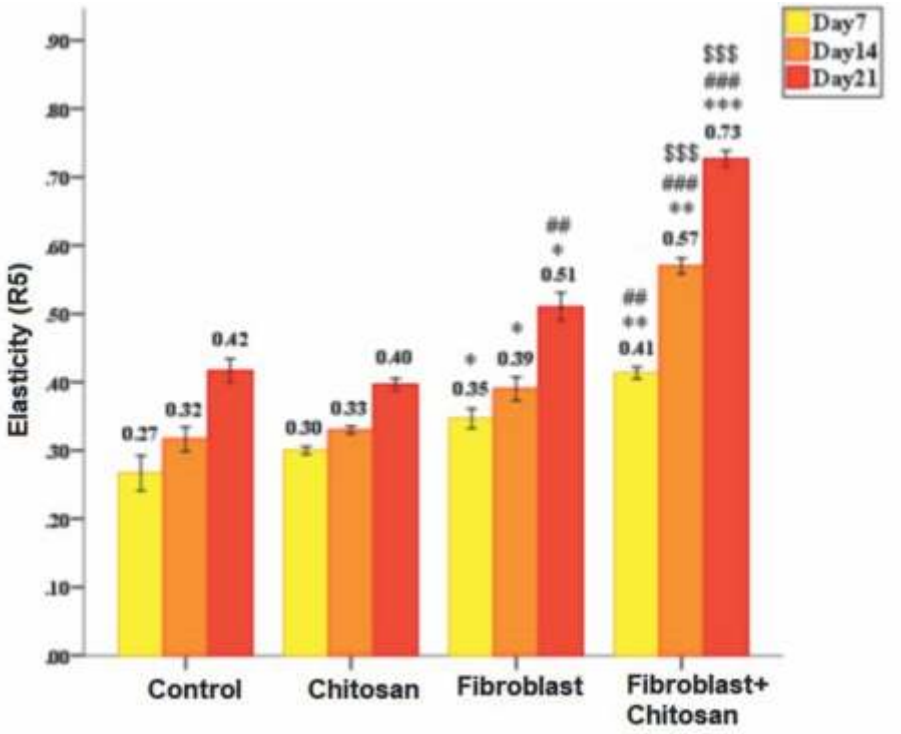

Figure 13: Skin elasticity (R5: net elasticity) in control group (n=6) and groups treated with chitosan (n=6), fibroblast (n=6), and "chitosan + fibroblast (n=6)". *, # and $ represent a statistically significant difference compared to control group, and groups treated with chitosan, and fibroblast, respectively (*: p <0.001, **: p <0.01, *: p <0.05, ###: p <0.001, ##: p <0.01, $$$: p <0.001)**

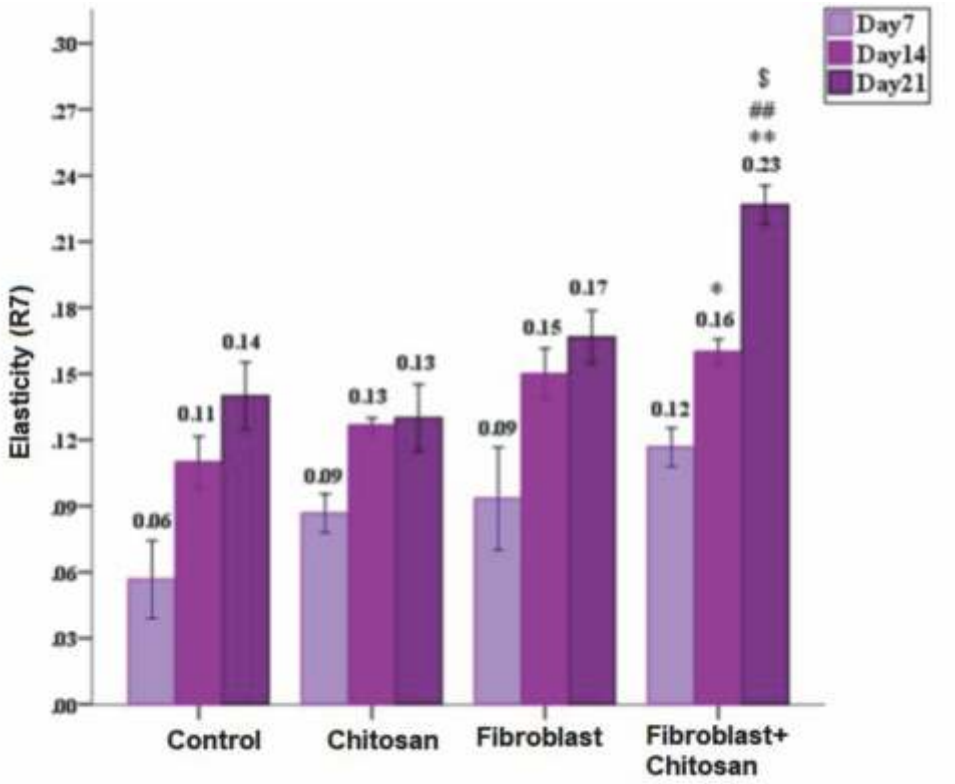

Figure 14: Skin elasticity (R7: recovery after deformation) in control group (n=6) and groups treated with chitosan (n=6), fibroblast (n=6), and "chitosan + fibroblast (n=6)". *, # and $ represent a statistically significant difference compared to control group, and groups treated with chitosan, and fibroblast, respectively (: p <0.01, *: p <0.05, ##: p <0.01, $: p <0.05).**

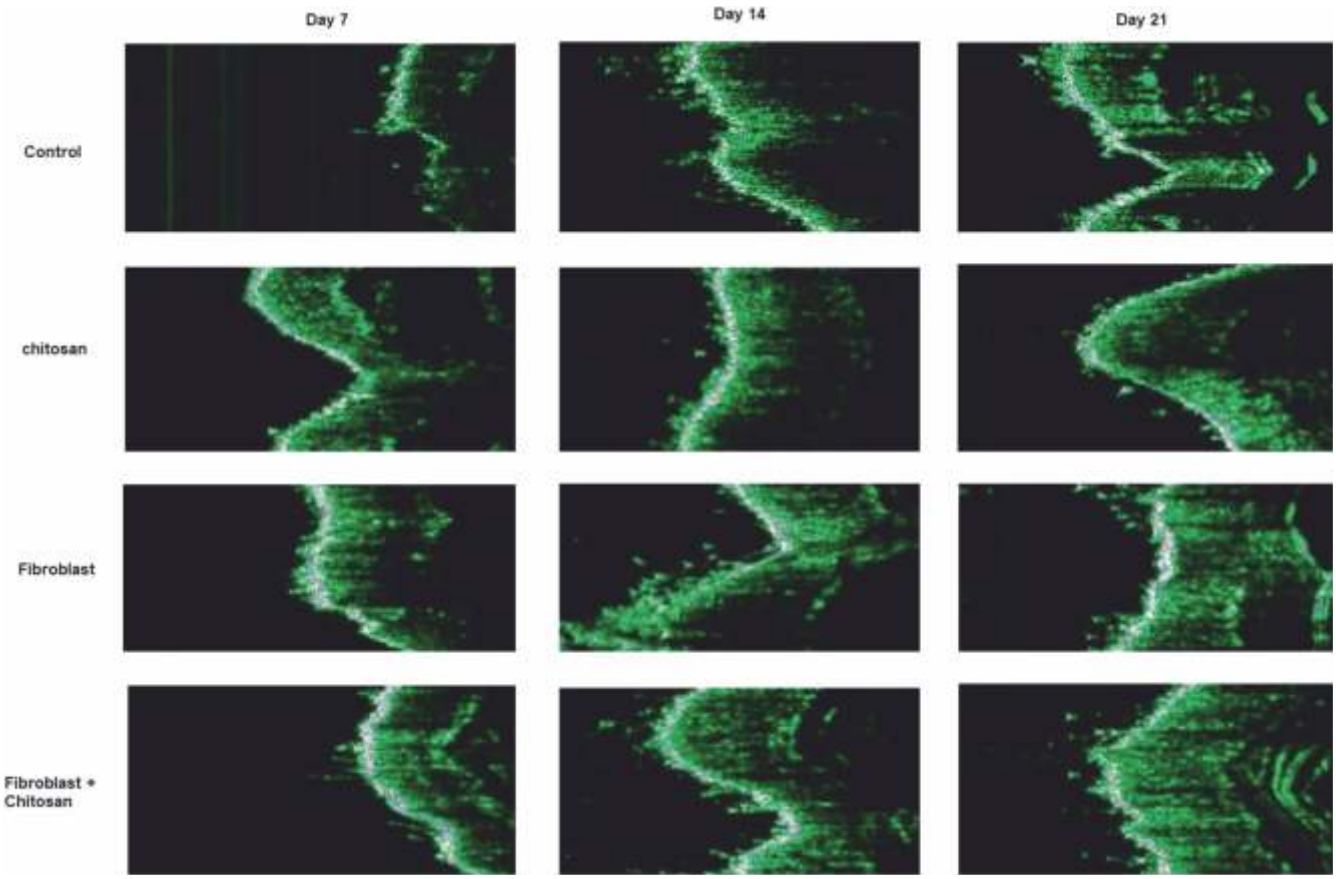

Figure 15: Ultra-sound imaging for measurement of density and thickness of wound area skin using a 75 MHz ultrasound probe on days 7, 14 and 21 after treatment.

Discussion

Many studies have evaluated the efficacy of fibroblast cells and bioactive materials like chitosan hydrogel or scaffold on wound regeneration [17], however, due to challenging results obtained from previous and recent research on efficacy of fibroblast cells on wound healing, there is still a need for more research assessing the effects of fibroblast cells on wound healing process using biomaterials including chitosan hydrogel.

We applied neonatal foreskin derived fibroblast cells for burn wound healing in diabetic rats. The studies have shown that the production of IL-6, IL-8, and CXCL1 by neonatal fibroblasts is reduced during prenatal and neonatal course, which causes reduced inflammation in wound healing [18]. In comparison to adults, clinical evidence also indicated that regeneration is quicker and almost scar less at an early newborn age partly caused by fibroblast cells function [19]. It has been shown in our study that neonatal fibroblast cells had low apoptosis offering a great potential for cutaneous wound healing. Chitosan hydrogel also did not show significant cytotoxic effects on neonatal foreskin derived fibroblast cells indicating that the chitosan hydrogel is a safe biomaterial for wound dressing when the fibroblast cells are used for

wound healing. Suitable attachment and migration of fibroblast cells cultured in chitosan hydrogel and also well penetration of these cells into cross-linked chitosan polymer were observed in our study, which may be due to chitosan porosity and density of cultured cells. [20].

Macroscopic and microscopic observation of wound site:

Animals treated with chitosan + fibroblast showed the highest accelerated wound healing process and completed formation of epidermis in the wound site 21 days after wound-establishment. In consistent with these results, study by Bai *et al.*, showed that diabetic wounds treated with Bone-marrow Mesenchymal Stem Cells (BM-MSCs) cultured in chitosan hydrogel, stimulated complete wound healing 15 days after wound excision in contrast to the control group. Bai *et al.*, also showed that chitosan hydrogel + BM-MSC and chitosan hydrogel groups represented meaningfully faster wound healing progress in comparison to the control in rat models [2]. According to an experimental research by Shen *et al.*, wounds in diabetic mice, in the group treated with sulfated chitosan-doped Collagen type I (Col I/SCS group) had higher wound regeneration speed compared to collagen type 1 received and control groups. This group also gained complete wound healing on day 18 post-treatment while wounds in control and collagen type 1 (Col I group) groups weren't completely healed but, there was no significant difference in wound healing rate among three mentioned groups [21].

Microscopic examination of harvested specimens represented lowest numbers of macrophages on days 7, 14 and 21 in the wound site in chitosan + fibroblasts group compared with other groups. This might be due to lower expression of genes that are involved in inflammatory responses and macrophage attraction to the wound site by neonatal fibroblasts [22], which causes a shorter inflammatory phase and subsequently faster progression of wound healing; this might be also due to oxygen permeability of chitosan which promotes wound regeneration [13] In a similar study, lower expression of genes involved in inflammation phase of wound healing and more efficient wound regeneration was significantly observed in the group treated with neonatal fibroblasts compared with the group treated with adult fibroblasts [19]. Seventh, 14th, and 21st days after treatment, the chitosan + fibroblasts group consistently had a higher percentage of epithelial cell formation and also represented complete epidermis formation. This could be attributed to the ability of chitosan in improving re-epithelialization [23] and also production of Fibroblast Growth Factors (FGFs) by neonatal fibroblast cells which aids in migration, differentiation, morphogenesis and plasminogen activity of human keratinocytes and also proliferation of human epithelial cells [11, 24].

Our findings indicated that wound healing progress speed, re-epithelialization and epidermis formation were remarkably higher in animals treated with chitosan + firoblasts compared with the group treated with fibroblast cells, which can be attributed to the hypothesis that chitosan causes better function of fibroblast cells by amplifying cell proliferation, stabilizing and improving production of fibroblast cells growth factors [13, 15]. It has also been shown that chitosan can induce fibroblast cell proliferation [25], however, it has an anti-angiogenesis property [26].

Biometric analysis

Thickness of wound region skin in rats treated with chitosan + fibroblasts was significantly higher than other groups. On the 7th and 21st day after treatment, the skin tissue thickness in wound area was significantly more in chitosan group than control group while it didn't differ significantly from the group treated with fibroblast cells. In consistent with these findings, Naim Kittana *et al.*, showed that wounds treated with chitosan and different concentrations of chitosan–single-wall carbon nanotubes and chitosan–multi-wall carbon nanotubes had remarkably higher epidermis thickness compared with the control group[27].

Skin elasticity and recovery after deformation parameters were higher in animals treated with chitosan + fibroblasts than in all other groups on day 21. It might be related to the notion that fibroblast cells produce collagen and elastin, which improve skin elasticity and density, respectively [28]. Solubilized elastin, like tropoelastin, can communicate with the elastin–laminin receptor. The actions of solubilized elastin mediated by this receptor include elastin production, chemotaxis, and proliferation of fibroblasts, keratinocytes, and smooth muscle cells [29], which improve wound healing process and may justify higher re-epithelialization, thickness and elasticity in groups treated with fibroblast cells, and with chitosan + fibroblast cells compared to the control group.

It has also been reported that collagen synthesis is improved by chitosan [26], which can be the major reason of better function of chitosan + fibroblasts group in increasing the elasticity and density of wound region skin compared to fibroblast cells group. In a study by Mu Hu *et al.*, it was proved that cartilage tissues treated with chitosan/hyaluronic acid hydrogels had significantly higher elastin content; however, in contrast to the beneficial properties of chitosan, biomechanical properties of chitosan are weak and it has also been shown that chitosan fiber adheres poorly to the wounds and absorbs low exudate [30] which may limit its wide-spread clinical application as a wound dressing.

Current study had some limitations in measuring growth factors in wound site tissue and also expression of regenerative genes involved in skin repair in dermal and epidermal cells which are great subjects that can be addressed in future research.

Conclusion

The results of the present study show that dressing the burn wound in diabetic rats with neonatal fibroblast cells seeded into chitosan scaffold significantly accelerates wound healing process through accelerated epithelial formation and increased skin tissue density and elasticity.

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