Introduction

Burn injury occurs with high prevalence worldwide causing high morbidity and mortality despite great advances achieved in wound care.1 Wound healing occurs with restoration of skin integrity and function, leaving minimal scarring compared to the uninjured surrounding tissue.2,3 However, there are often obstacles to a successful healing process, including the crucial problem of insufficient skin tissue in the area of injury.1 For this reason, the healing process does not often regenerate a completely intact skin.1

Stem cell therapy has shown promise in the wound healing process.2,4 Despite our growing knowledge of regenerative medicine at the molecular level, stem cell-based therapy is not being practiced widely. This is due to several barriers to transferring harvested stem cells to the final target or loss of rejuvenation potential and differentiation in the recipient tissue. Recent studies have suggested new resources for harvesting stem cells.5-8 ADSCs have also been associated with dramatic outcomes in wound healing.9,10 Even so, there have been no demonstrations to show whether mechanically prepared adipose tissue concentration has a similar efficacy to ADSCs. Hence, we conducted the present study to measure the outcomes of wound healing using ADSCs by local delivery in a mouse model of a burn injury.

Methods and materials

Study design

This study was carried out in the animal laboratory
of Hazrat-e-Fatemeh Hospital in Tehran, Iran. All cellular and molecular procedures were performed in the Research Center of Royan Institute, Tehran, Iran, and in accordance with universal guidelines on the care and use of experimental animals.

Preparation of adipocyte solution and adipose tissue-derived mesenchymal stem cells

Ten healthy inbred male mice were sedated by a combination of intramuscular ketamine (30mg/kg) and lidocaine (5mg/kg). In a supine position, a longitudinal incision was made through the abdomen. Using a microsurgical set under sterile conditions, adipose tissue was dissected from the subcutaneous tissues of the belly, inner thigh, para-vertebral region, mesentery, and testes. The para-vertebral fat tissues were harvested through a peritoneal incision and the inner thigh fat was harvested through the lower part of the abdominal incision. Samples were transferred on a transfer media containing Streptomycin 1% and then washed 3 times to remove blood cells and other excess substances. Tissues were split into small pieces and finally divided into 2 separated groups.

In one group, collagenase type-I (0.07% solution) was added to the specimen and thereafter incubated for 1 hour at 37°C. Incubation was then extended for an additional 20 minutes after adding EDTA–trypsin. Finally, the trypsin was neutralized with 10% fetal bovine serum. Centrifugation at 300 rpm was done for 5 minutes to separate the ADSCs from the cell-free substances and non-adherent cells. Using a mesh filter, the solution was purified to form a homogenous liquid. Adherent fibroblast-like cells were removed by intermittent passages. The homogenized cell suspension was transferred into 6 tubes for characterization of the isolated ADSCs. Flocytometery was performed following introduction of the solution with anti mouse CD antibodies including CD44, CD90, CD105, and CD73. Statistical significance was obtained at p<0.05. Cellular viability was determined by dying with a 1:1 solution of trypan blue 0.4%. Using a neobar chamber, cells were attenuated by physiologic serum to form a suspension containing 1×10⁶ cells/ml.

The second group of adipose tissue derived specimens was rinsed 3 times with physiologic serum to remove excess cells and adherent substances. Following adequate passage, the sample was centrifuged at 300 rpm and finally a suspension of 1×10⁶ cells was obtained for transplantation.

Animal Models

Thirty healthy male Balb/c inbred mice, each weighing 40g, were selected from the experimental laboratory of our hospital. The mice were housed at a temperature of 25°C, a 12 hour light-dark cycle was set, and they were fed with chow and tap water. Following general anesthesia with the aforementioned protocol, a metal probe (1.5cm × 1.5cm), heated with water to 96°C, was placed on the back of mice for 8 seconds to create a standard 3° degree burn wound. All of the mice were randomly divided into the 3 groups: the ADSCs group, the adipocyte suspension group, and the control group.

Transplantation of adipocyte suspension and ADSCs

The burn wound of each mouse in the ADSCs group was injected with 1cc of the ADSCs sample. The adipocyte suspension group also had their wounds injected with 1cc of their sample. The control group received no treatment intervention.

Perioperative care and wound examination

All the wounds were dressed daily with silver sulfadiazine impregnated sterile gauze. Digital photographs were taken each week for macroscopic evaluation. To measure the wound surface and wound contracture, images were analyzed using “Image j144” software (www.imagej.com).

In the third week, the mice were scarified with ether over-infusion and biopsies were made to obtain full-thickness tissue. Specimens were prepared with Hematoxylin and Eosin stain and Masson’s trichrome. A pathologist blinded to the study groups investigated microscopic samples for inflammatory reactions, epithelialization, tissue granulation, fibroplasia, collagen synthesis and arrangements, and level of angiogenesis. Cells were counted by quantitative measures with cells/high power field.

Statistical Analysis

The data were analyzed using statistical package for social sciences (SPSS, version16, Chicago, Inc). Chi-square test, one-way ANOVA, and post-Hoc analysis were used, with values expressed as mean ± SD and n (%) where appropriate. A statistical significance was obtained at p<0.05.

Results

In both the adipose tissue suspension group and the control group, 1 mouse expired during the 1st week, while in the ADSCs group, 1 mouse died in the 3rd week. No infection or wound-related complication was otherwise reported in any of the groups.

Wound surface area

At the end of the 1st week, wound area measured 125.17±22 mm² in the ADSCs group, 121.76±21 mm² in the adipose tissue group, and 126.42±20 mm² in the control group. Table I summarizes the average weekly wound surface area measurements. Although there were lower values for the ADSCs group, followed by the adipose tissue and control groups, the repeated measures ANOVA did not reveal a statistically significant difference (p>0.05) (Fig. 1).
Eschar thickness
Thickness of eschar tissue was lower in the adipose tissue group ($2.05\pm1.8$ mm$^2$) compared with the ADSCs group ($2.33\pm1.4$ mm$^2$) and the control group ($3.4\pm3.1$ mm$^2$). However, the difference was not statistically significant ($p>0.05$) (Table I).

Inflammatory reactions
With regard to the inflammatory reactions, PMN and lymphocyte revealed smaller counts in the ADSCs group than in the two other groups. In contrast, macrophage counts were higher in this group compared with the adipose tissue and the control groups. However, none of these differences were statistically significant ($p>0.05$) (Table I).

Tissue regeneration
Epithelialization occurred in 5 mice from the ADSCs group and in 3 mice from each of the other two groups with no significant difference ($p>0.05$). Fibroplasia and collagen remodeling was also evident in 6 and 5 mice of the ADSCs and the adipose tissue groups respectively. However, this did differ significantly from the other groups ($p<0.05$). Moreover, fibroblast counts were higher in the ADSCs group followed by the control and adipose tissue group, although with no statistically significant difference ($p>0.05$) (Table I).

Angiogenesis
Neo-vascularization had the lowest degree in the adipose tissue group, and then in the ADSCs and control groups. However, the difference did not prove significant ($p>0.05$) (Table I).

Paired comparisons
Paired comparison of the 3 groups was performed to better illustrate each treatment approach. This comparison
revealed no significant differences in terms of wound healing, inflammation parameters, fibrosis, or angiogenesis (Tables II-IV).

**Discussion**

As regards wound healing, global attention is currently focused toward the regeneration of functional skin with similar integrity to the intact surrounding tissue rather than toward the mere covering of injured skin for cosmetic reasons. Stem cell therapies have shown promising results over the last decades. However, there is currently controversy regarding the ideal source of harvesting essential cells to be transplanted into the wound area. On the one hand, MSCs have been shown to promote wound healing by inducing immigration of inflammatory cells and subsequent triggering tissue granulation, fibroblast proliferation, and collagen synthesis. On the other hand, although not sufficiently investigated, adipose tissue-derived stem cells (ADSCs) are much more easily accessible. This is due to the availability of adipose tissue throughout the human body and the fact that there is no need to perform invasive bone-marrow aspiration biopsy, as well as the high concentration of stem cells in the obtained adipose tissue. However, it is also documented that implementing stem cells without providing a proper skeletal basis will not result in functional tissue because a biological matrix is highly required to integrate all cells and molecules to interact properly.

Our study demonstrated that ADSCs have the potential to accelerate the process of wound healing. Wound surface area and eschar thickness were smaller in the ADSCs group, and even more so from the 1st to the 3rd week (Fig. 1). However, no significant difference was observed between any pairs of these 3 groups. As per our study results, it is obvious that later inflammatory reaction occurs more remarkably in the adipose tissue and ADSCs groups. Moreover, collagen synthesis (Fig. 2) and remodeling (Fig. 3) are more favorable when using ADSCs and adipose tissue suspension. As Fig. 2 shows, blue staining, indicative of collagen synthesis, is more evident in the ADSCs group than in the other 2 groups. Furthermore, remodeling of collagen fibers is more organized in this group than in the adipose tissue and control groups, as the fibers have settled in a parallel direction together (Fig. 3). In this study, angiogenesis was less favorable in the ADSCs group than in the control group. This indicated that there are potentially other mechanisms by which stem cells play a role in wound healing.

Kim et al., in an experimental co-culturing of ADSCs obtained from human adipose tissue, showed that these cells promote healing by secreting growth factors and hence can be considered a suitable treatment for photoaging and wound healing. Another study by Maharlooei et al. interestingly demonstrated that mechanisms other than angiogenesis and accumulation of collagen fibers are proba-
bly involved in ADSCs induced wound healing.\textsuperscript{13} Using a mouse model, they applied ADSCs in diabetic wounds and concluded that, although stem cells accelerated the process of wound healing, angiogenesis and fibroblast migration are not significantly different between the study groups.\textsuperscript{13} A similar finding was also noted in our study as angiogenesis was not more favourable in the ADSCs group. However, in contrast to their study, fibroblast accumulation, collagen synthesis and remodeling were more desirable by application of ADSCs, which is consistent with the suggested mechanism in the literature for the role of stem cells in the acceleration of the healing process.\textsuperscript{3-5} This discrepancy may be due to the different methods used to evaluate vascular formation or deposition of collagen fibers. Inconsistencies in the findings may also be explained by the model used for studying wound healing: while our study involved ADSCs in burned mice, others have used diabetic or other types of burn models.

The transferring method has been considered a barrier to wide application of stem cell-based therapies in clinical practice. Recently, cell sheets and scaffolds have shown enhanced healing properties by successful transplantation of stem cells to an in-vivo setting.\textsuperscript{9,17} The effectiveness of the administration of ADSCs in association with biomaterial scaffolds vs. ordinary local delivery should be further investigated, as should the issue of whether this accounts for the lack of significant difference between our study groups. This could be achieved by evaluating engraftment of stem cells at histological examination. Levels of growth factor and their involvement in tissue restoration should also be noted when explaining the underlying mechanism by which stem cells improve wound healing. Sarojini et al. showed that MSCs have a chemotactic effect on fibroblasts.\textsuperscript{18} Another study revealed that the MSCs secrete factors facilitating wound closure by attracting fibroblasts and inducing keratinocyte migration.\textsuperscript{19} Although keratinocyte were not assessed in this study, chronic inflammatory cells such as lymphocyte and macrophage, as well as fibroblast, showed more cell counts in the ADSCs groups than in the controls. PMN cells, however, probably revealed a lower count due to the anti-inflammatory effect of stem cells.\textsuperscript{5,11}

There remain a few unanswered questions. It is not clear whether the culturing of ADSCs prior to transplantation is associated with better outcomes or whether the concentration/purification of fresh stem cells prove more effective. Additionally, it has been pointed out that application of cultured progenitor cells instead of freshly harvested ADSCs or cell-based derivatives in immunocompromised mice may be associated with tumor formation;\textsuperscript{7,20} a consequence which is poorly tolerated in this devastating condition.

Our study aimed to compare the efficacy of 2 differently prepared ADSCs - enzymatically purified stem cells and mechanically prepared adipose tissue - on the healing process of burn wounds in a mouse model. We used Mason’s trichrome staining to quantitatively measure collagen synthesis, deposition and remodeling. Moreover, via a scale optic lens, the thickness of the collagen fibers and fibroplasia were evaluated in comparison to the adjacent tissue area, showing that the ADSCs group produced thicker collagen fibers (Fig. 2). However, although in favor of the use of ADSCs, the differences were not statistically significant. For this reason, it is anticipated that future studies will overcome the limitations of this study.

Although it seems that enzymatically prepared ADSCs are potentially effective in accelerating the healing process in burn wounds, probably by attracting fibroblast and promoting regeneration of collagen fibers and remodeling, our results showed no differences among the 3 methods. Further studies are required to find the best method for using ADSCs. Due to easily performed preparation techniques, this could be considered as an alternative to cultured stem cells.

**Conclusion**

Our results showed that there is no difference in burn wound healing between mice treated with enzymatically prepared ADSCs, mechanically prepared adipose tissue or those in the control group.
RÉSUMÉ. Les cellules souches se sont révélées prometteuses en ce qui concerne le processus de cicatrisation des plaies de brûlures. Cependant, les sites donneurs de ces cellules sont encore sous enquête. L’objectif de cette étude est d’examiner l’efficacité des tissus adipeux de cellules souches dérivées pour accélérer la cicatrisation des plaies de brûlures du troisième degré dans un modèle de souris. À cette fin, quatre souris mâles consanguines sains de la race Balb/c ont été sélectionnées et mis en place comme un modèle expérimental. Elles ont été réparties au hasard en trois groupes de taille égale : le groupe de tissu adipeux de cellules souches dérivées, le groupe de tissu adipeux mécaniquement préparé, et le groupe de contrôle. Les plaies ont été examinées tous les jours jusqu’à ce que les souris aient été sacrifiées pour prélever des tissus dans la troisième semaine. Nos résultats ont montré que la zone de surface de la plaie et l’épaisseur de l’escarre étaient moins dans le groupe de tissu adipeux de cellules souches dérivées tout au long de la période de l’étude, mais il n’y avait pas de différence significative entre les groupes pour déterminer les valeurs des caractéristiques de la surface de la plaie. En termes de paramètres de la cicatrisation, de lymphocytes et de macrophages comptage de cellules sont plus grandes dans le groupe de tissu adipeux de cellules souches dérivées par rapport aux deux autres groupes. La fibroplasie, la synthèse du collagène et le remodelage étaient plus aberrant dans ce groupe. Cependant, il n’y avait pas de différence statistiquement significative dans les différences observées (p > 0,05). Bien que le tissu adipeux de cellules souches dérivées et cela préparé enzymatiquement semblent un traitement potentiel dans la cicatrisation des plaies, notre étude d’un modèle de souris n’a révélé aucune amélioration significative dans l’utilisation de cette option.

Mots-clés: cellules souches dérivées de tissu adipeux, plaie de brûlure, cicatrisation des plaies, modèle de souris

BIBLIOGRAPHY