



# Intravenous Administration of Granulocyte-Colony Stimulating Factor for Stem Cells Mobilization and Third Degree Burn Wound Healing in Rats

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## Abstract

**Introduction:** Burns are one the most common skin damages which require medical intervention to be fully-recovered. In this light, tissue engineering field presents a wide variety of strategies including both scaffold-based and cell-based approaches to recover the damaged site.

**Materials and Methods:** In this study, the effects of granulocyte-colony stimulating factor (G-CSF) administration on mobilization of the bone marrow mesenchymal stem cells (MSCs) into defect area and treatment of the skin burn wound was examined *in vivo*. The G-CSF was injected intravenously into rats subjected to third degree burn wound. At days 3, 5, 7, 15 and 30 post-injections, the defect site was removed and investigated by H&E and Malory's trichrome staining. The number of MSCs in blood samples was also determined by flow cytometry assay.

**Results:** According to the results, intravenously administration of G-CSF significantly increased collagenesis and number of fibroblast cells infiltrated into the burned site, while decreased the severity of acute inflammatory response and amount of inflammatory cells comparing to control. The number of MSCs in bloodstream, representing the rate of MSCs migration, showed a 4-fold increase in the experimental group compared to control.

**Conclusions:** The current study suggests the potential of intravenously administration of G-CSF as an effective strategy for treatment of severe burn injuries.

**Keywords:** Burn, Granulocyte-Colony Stimulating Factor (G-CSF), Mobilization, Wound Healing, Mesenchymal Stem Cell

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## Introduction

Skin burn injury is considered as a critical clinical issue that profoundly affects the life quality of patients.<sup>1-3</sup> It has been reported that about 2 million people are burned annually in the United States, among which around 80 000 are hospitalized and 6500 of them die.<sup>4</sup> The healing process of skin in burn injuries depending on the degree and severity of the damage, ranges from 3 (in superficial partial-thickness burns) to

several weeks (in deep partial-thickness and full-thickness burns) (4-8). Burn wound healing is a complicated process that involves many cellular and molecular mechanisms and is orchestrated by cytokines and growth factors secreted from cells (such as macrophage and neutrophils) into the injured area. These factors affect the proliferation and migration of keratinocytes and fibroblasts.<sup>9-11</sup>

Epidermal growth factor (EGF), vascular endothelial

growth factor (VEGF), transforming growth factor beta (TGF- $\beta$ ) and granulocyte-colony stimulating factor (G-CSF) are major cytokines and growth factors involved in this process.<sup>12</sup> Among those, G-CSF is a cytokine which specifically stimulates differentiation of bone marrow progenitor cells to granulocytes and macrophages.<sup>13</sup> The G-CSF also, stimulates proliferation of keratinocytes in an autocrine manner which results in regeneration of damaged skin.<sup>14</sup> *In vitro* studies have shown that G-CSF enhances re-epithelialization through increasing the proliferation of keratinocytes.<sup>12</sup> In addition, it has been demonstrated that G-CSF augments the migration and proliferation of endothelial and bone stromal cells from bone marrow into blood stream.<sup>15</sup> Taken together, G-CSF plays a crucial role in burn wound healing as a cytokine.<sup>9,15</sup> To fully treat third degree burn wounds, early dermabrasion and escharectomy followed by covering with skin grafts are recommended.<sup>8,16</sup> Stem cell therapy has been established to overcome the obstacles associated with the application of skin substitutes such as difficulty and expensiveness.<sup>17</sup>

Stem cells, especially bone marrow-derived ones, have been found to be a promising cell source for treating burn injuries in recent decades. Easy accessibility and differentiation capabilities to a variety of cell types such as blood vessel cells, perifollicular cells, perisebaceous gland cells, keratinocytes and fibroblasts, which all play vital roles in the healing process, have made stem cells a potential source for cell therapy.<sup>18</sup> It has been shown that efficiency of burn healing with mesenchymal stem cells (MSCs) transplantation is higher than other types like, fetal fibroblast cells. Nevertheless, lack of telomerase activity, low maintenance and proliferation rate of these cells *in vitro* have limited the applications of such cells.<sup>19</sup> Regarding the fact that the G-CSF may affect the mobilization of MSCs from bone marrow, in the current study, this cytokine has been administrated intravenously in the third-degree burn wound model of rats and then its effects were evaluated on MSCs infiltration and wound healing *in vivo*.

## Materials and Methods

### *In Vivo* Study

The current study was performed on 40 adult male Sprague-Dawley rats (weighing 180–220 g) purchased from Pasteur Institute of Iran and acclimatized for one week under standard conditions prior to the experimental study. The rats were anesthetized by an IP injection (5 mg/kg body weight) of a 4:1 solution of ketamine hydrochloride and xylazine (Sigma, USA). After a general anesthesia induction, the back skins of animals were shaved by an electric clipper and then, exposed directly to boiling water (98°C) for 30 seconds. The leather cover was used in order to avoid burning of uninvolved areas.<sup>20</sup> The rats were randomly divided into 2 experimental groups: in the first group, the burned animals were treated with G-CSF and in the second one, normal saline solution was applied (each group containing 5 rats). After burning, the animals were intravenously administrated with G-CSF and normal saline for 9 consecutive days.<sup>21</sup> The study was performed according to the guiding principles in the care and use of animals (Ethical code: IR.TUMS.REC 1390.926).<sup>22</sup>

### Flow Cytometric Analysis

The flow cytometric analysis was performed to determine the mobilization rate of the MSCs from bone marrow into the blood stream. Half an hour after the last dose of G-CSF and normal saline injection, a 5-mL blood sample was taken from each animal. The mononuclear cells were isolated from the blood samples using ficoll (Sigma, USA). The isolated cells were then incubated with monoclonal anti-CD90 (FITC, Santa Cruz) and monoclonal anti-CD106 (PE, Santa Cruz) for 30 minutes. Anti IgG1-PE (Santa Cruz) and IgG1-FITC (Santa Cruz) served as negative controls. The population of CD90 and CD106 positive cells, representing the MSCs, were measured by flow cytometry (Facs caliber, Becton Dickinson).<sup>23</sup>

### Histological Analysis

Histological observations were carried out 3, 5, 7 and 30 days after drug administration. To this end, five rats were randomly selected from each group. The histological specimens were collected from different areas of the burned site using a biopsy punch. The samples were fixed in 10% formalin, dehydrated through a series of ethanol concentrations (70%, 80%, 90%, 100% in distilled water), followed by embedding in xylene and paraffin. The samples were then sectioned serially (8  $\mu$ m in thickness) by a microtome (Rotary microtome, MICRO DS 4055). The sections were stained with Harris Hematoxylin (EM Science) and eosin Y (Sigma, USA), and Malory's trichrome for histological analysis.<sup>24,25</sup> The rate of collagen synthesis, the presence of inflammatory cells and number of fibroblast in the defected area were the major criteria evaluated in this study.

### Statistical Analysis

The independent sample *t* test was used for statistical data analysis. A *P* value of  $\leq 0.05$  was defined as the level of significance.

## Results

### Flow Cytometric Analysis

According to the obtained results from the flow cytometry assay, the number of CD160<sup>+</sup>/CD90<sup>+</sup> cells in peripheral blood significantly increased (approximately 4-fold) in the G-CSF group compared to the control group (Figure 1). The average number of MSCs in the experimental and control groups was 624 cells/mL and 155 cells/mL, respectively.

### Histological Analysis

The histological observations revealed that the burn wound healing in the experimental group was remarkably faster than those in the control group (Figures 2 and 3).

The microscopic observations showed that the G-CSF administration significantly affected the number of fibroblast cells in the burned area of the experimental group in comparison to the control group. As a result, the inflammatory cells such as lymphocytes had a significant decrease in the G-CSF group compared to the control group by day 15 post-administration (Figure 2). The average number of fibroblast cells in the G-CSF group showed a 4.5-fold increase in

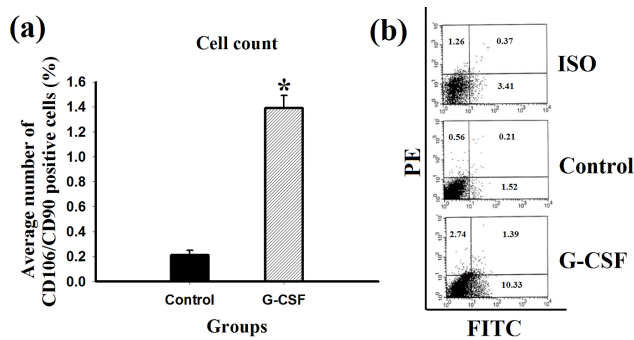
comparison to the control group after day 15 (28.8 and 6.4 cells in experimental and control, respectively) ( $P \leq 0.01$ ) (Figure 4).

Figure 3 shows the active fibroblast cells (black arrow) and collagen fibers in the control and collagen fibers and fibrocyte cells in the experimental samples, respectively. It is observable that, the G-CSF administration positively increased the

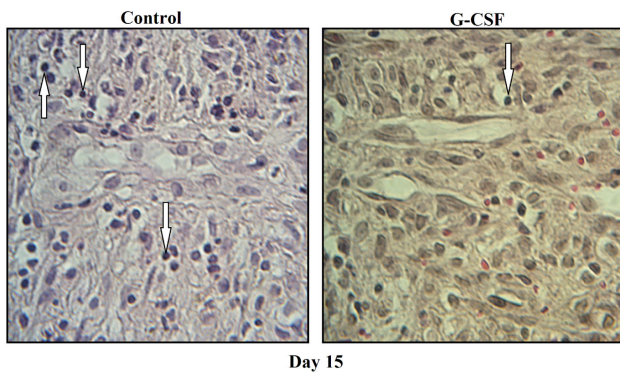
fibroblast infiltration and collagenesis in the defect area. The Malory's Trichrome stained samples showed a remarkable collagenesis increase in the experimental group compared to the control group after days 15 and 30 (Figure 5). As it is shown in Figure 5, the collagenesis in G-CSF administrated group was clearly observed by day 15.

**Discussion**

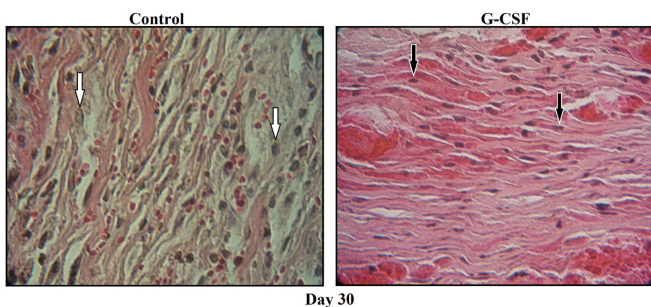
The efficiency and therapeutic applications of bone marrow-derived stromal cells in soft tissue engineering, especially skin, have been reported by several studies.<sup>26-28</sup> In this line of research, Mansilla et al have found that the number of these cells will increase in peripheral blood after burn injuries.<sup>27</sup> Additionally, several studies have demonstrated that both migrations of stem cells and regeneration of damaged tissues are mediated by some growth factors such as G-MCSF. In



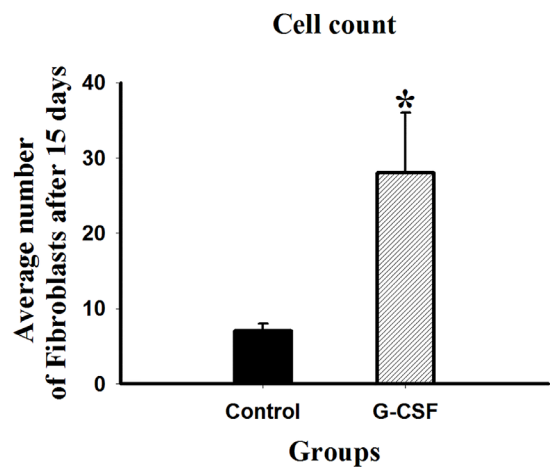
**Figure 1.** There was a 4-fold increase in number of MSCs in experimental group (average 1.39 %) compared to control (average 0.21 %) (a). The flow cytometry results indicate the number of CD106 (PE) and CD90 (FITC) positive cells among the mononuclear cells isolated from the blood samples of rats (b). Asterisk indicates a significant difference with control ( $P$  value  $\leq 0.001$ ).



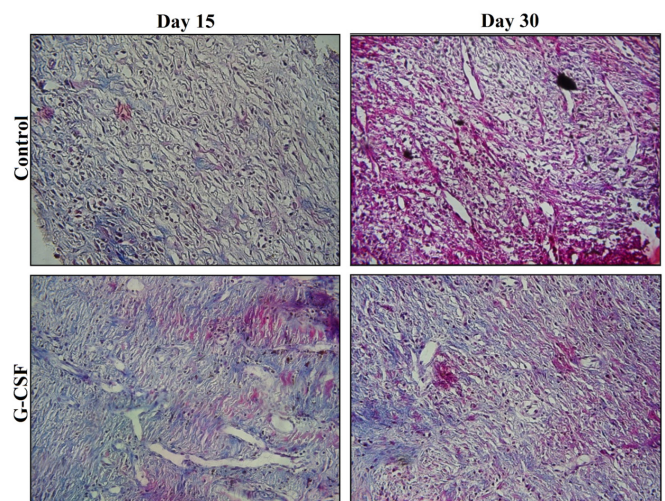
**Figure 2.** The number of inflammatory cells (lymphocytes) was compared between H&E-stained specimens of the control and experimental group on day 15. Number of these cells decreased remarkably in the G-CSF group in comparison with control. Arrows show inflammatory cells.  $\times 10$  magnification.



**Figure 3.** H&E Stained Samples of the G-CSF and Control Groups on Day 30. A large numbers of active fibroblast cells (white arrow) and a few numbers of collagen fibers (red fibers) were observed in the control samples. In the G-CSF sample, the fibrocyte cells (black arrow) and collagen fibers (red fibers) were observed numerously compared to control.  $\times 10$  magnification.



**Figure 4.** The Number of Fibroblast Cells in Defect Area on Day 15. The fibroblasts showed a 4.5-fold increase in the G-CSF compared to control group. Asterisk indicates a significant difference with control ( $P$  value  $\leq 0.001$ ).



**Figure 5.** The Comparison of the Collagenesis Between the Malory's Trichrome Stained Samples of the G-CSF and Control Groups After Days 15 and 30. As it is clearly observable, the collagenesis remarkably increased in the G-CSF group compared to control in both days 15 and 30.  $\times 40$  magnification.

the current study, the role of G-CSF administration has been represented on the mobilization of bone marrow-derived stromal stem cells into the peripheral blood and its healing potential of third-degree burn injury created in rats.

The results obtained from this study revealed that the administration of the G-CSF significantly increases the number of MSCs in peripheral blood 4-fold ( $P \leq 0.05$ ). These results were consistent with other studies conducted in this area.<sup>19,21,29,30</sup> The mechanisms by which the G-CSF affects the migration of MSCs are not fully understood, but seems to be associated with SDF-1/CXCR4 ligand/receptor pair. The SDF-1, as a chemokine, and its receptor CXCR4 are found to have an essential role in stem cells homing.<sup>31</sup> It has been reported that G-CSF reduces the concentration of SDF-1 in bone marrow and leads to release the progenitor cells into the peripheral blood.<sup>32</sup> The histological observations showed a decrease in the number of inflammatory cells in the experimental samples compared to the control samples 15 days post-injection. According to other studies, these findings could be due to the anti-inflammatory role of mesenchymal stem cells. These results were in line with those obtained from Shen et al's<sup>33</sup> study in which locally administration of G-CSF in the rat full-thickness wounds significantly decreased the inflammatory cells within the wound bed.

The mesenchymal cells can eventually differentiate to various cell types such as collagen producing fibroblasts.<sup>34,25</sup> The present study showed a significant difference in the number of fibroblasts among experimental and control samples ( $P \leq 0.01$ ) in day 15. The MSCs are involved in the formation of extracellular matrix (collagen type I, IV and fibronectin) through secretion of numerous cytokines and growth factors and thereby provide a microenvironment to support cell growth and maturation, especially in damaged tissues. Chen et al showed the potential of MSCs in secretion of some paracrine factors that are associated with the repair of damaged tissues.<sup>36</sup> In the present study, the collagen fibers were clearly observed in biopsy specimens of the test group by day 15 post injection. The higher collagenesis rate in the experimental group was quite evident in comparison to the control. Additionally, the G-CSF group showed a decreased cellular infiltration and increased number of fibroblast cells when compared with control samples. The therapeutic role of the G-CSF in this study may not only be due to the increase of bone marrow-derived cells infiltration, but also results from its effects on the migration of endothelial precursors and hematopoietic stem cells into defect area. All of these cells are responsible for angiogenesis and wound healing through secretion of angiogenic factors such as VEGF, bFGF and Ang-1.<sup>37</sup> On the other hand, the role of G-CSF in induction of endothelial cell proliferation has been previously reported.<sup>38</sup> Furthermore, the G-CSF administration on cardiac defects found to increase the expression of the adherent molecules such as ICAM-1 at the site of damaged blood vessels as well as the infiltration of MSCs.<sup>39</sup> Taken together, the data of this study strongly demonstrates the positive effects of G-CSF on migration of bone marrow-derived cells and eventually, healing the skin lesions, that is concordant with other studies

that showed the role of MSCs in skin injuries healing without using the G-CSF.<sup>18,27,28,36</sup>

## Conclusions

The increasing importance of cell therapy in burn wound healing and the necessity of developing a safe and effective therapeutic method, has attracted the attention of researchers for designing an alternative strategy to minimize the challenges associated with stem cell therapy such as rejection and low proliferation rate. Recently, researchers have focused on the application of growth factors involved in tissue regeneration rather than stem cells as an alternative strategy in cell therapy, in damaged tissue repair. In this line of research, the efficacy of G-CSF administration in MSCs migration and burn injury healing in rats was examined in this study. All the collected data clearly indicated the potential role of the G-CSF in MSCs infiltration to the defect site and third-degree burn wound healing. Therefore, this study can serve as a pilot to further investigate the therapeutic abilities of the G-CSF in soft tissue engineering.

## Authors' Contributions

MG, AS, NN and MJS designed the concept of the study. MG wrote the manuscript. NP, AS and NN equally performed the experiments. BF revised the manuscript. AS and NN contributed equally to this research.

## Conflict of Interest Disclosures

The authors declare no conflict of interest neither in terms of financial issues nor in regards to the publication of this study.

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