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Original Research

Hypoxia and laser enhance expression of SDF-1 in muscles cells

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Abstract: Targeted homing of transplanted mesenchymal stem cells (MSCs) is a decades old discussion in regenerative medicine. It has been proved that stromal cell-derived factor-1 (SDF-1α) is a potent chemoattractant of MSCs. Therefore, different strategies have been used to increase secretion of SDF-1α in damaged tissues to elevate targeted homing of MSCs. Previous studies have revealed that increased SDF-1α expression in hypoxic necrotic tissues and also low-level laser exposure enhanced angiogenesis in injured tissues. Herein, human skeletal and cardiac muscle cells (HSKM and HCM) were treated with hypoxia and low level laser to see their effects on expression of SDF-1α and on MSCs migration towards these treated cells. The optimal treatment conditions were determined by investigating the cellular viability after treatment. Real-Time PCR and Western blot analysis were done to study the expression of SDF-1α in treated cells. Migration potential of MSCs toward hypoxic and laser treated cells was investigated via migration assay. MTT assay revealed that laser and hypoxia treatment had no effect on the viability of HCM, HSKM compared with Glioblastoma cells. Real-Time PCR showed 16- and 90-fold elevation in mRNA of *SDF-1α* in HSKM and HCM cells, respectively, in laser treated with 12 J/cm^2 intensity. In these two groups, selected as optimal conditions, *HIF-1α* expression showed maximum fold changes that might be partly because of response to treatments help to SDF-1α expression. It can be concluded that hypoxia and laser treatments may recruit MSCs and applied as a useful strategy for the further targeted stem cell homing.

Key words: Targeted Homing, Hypoxia, Laser, SDF-1α, Muscle cells, Mesenchymal stem cells.

Introduction

Stem cell therapy has been considered as a promising tool for degenerative diseases and disorders as their self-renewal property has the potential to return an impaired organ to its proper functioning. Targeted stem cell therapy is a decades long discussion in stem cell transplantation to overcome the challenges like escape of cells etc. Finding techniques to enhance chemotaxis and retention of the implanted mesenchymal stem cells (MSCs) for maximum effectiveness in MSC based stem cell therapy is an evolving step in regenerative medicine. MSCs are multipotent adult stem cells having self-renewal and multi-lineage differentiation potential. These cells have been preferred for stem cell therapy because of their regenerative potential, secreted growth factors and immunomodulatory role preventing immune cell activation (1,2). Various studies have proven the role of chemokine stromal cell-derived factor-1 (SDF-1α) in stem/progenitor cell chemotaxis and organ-specific homing of MSCs in ischemic tissue because of its cognate receptor CXC chemokine receptor 4 (CXCR4) located on the surface of MSCs (3–6). SDF-1α (stromal derived factor-1) is a small molecule, whose N-terminus binds and activates chemokine receptors especially CXCR4. The CXCR4 receptor is expressed on several cell types, including blood cells (i.e. lymphocytes, monocytes, etc.), haematopoietic stem cells (HSCs), and embryonic stem (ES) cells (7–9). In a number of studies, CXCR4 upregulation was observed in MSCs when they were exposed to hypoxia for a short duration of time (6,10,11). These pre-conditioned MSCs have been shown attractants toward SDF-1α expression. All ischemic tissues expressed a significant amount of SDF-1α which attract MSCs for their regeneration mechanism. Search for non-invasive strategies to enhance SDF-1α expression and their validity for a number of tissues and cells may help to reduce challenges in stem cell therapy. In addition to SDF-1-CXCR4 axis, HIF-1α as a transcription factor responsible to regulate the *SDF-1α* expression, plays significant role in cellular migration. Studies have described the Hypoxia as stabilizer of HIF-1α protein in later inflammatory phases because ofHIF-1 binding sites in SDF-1α promoter. Thus, stabilized HIF-1 enhances SDF-1α expression in hypoxic/injured tissues initiating chemoattraction of CXCR4$^+$ stem cells for the purpose of tissue repairing and regeneration (12).It has been proposed here that physical treatments like laser treatment and hypoxia may enhance SDF-1α expression which is an attractant of implanted MSCs and can be an alternative to chemicals for targeted homing of implanted stem cells. It has been demonstrated in this study that cardiac and skeletal muscle cells showed enhanced expression of SDF-1α when treated with hypoxia and laser concluding that hypoxic treatment or laser treatment can be a clinical strategy to made MSCs transplantation more

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Table 1. Different time period intervals for hypoxia and laser exposure.

	Hypoxia Treatment Protocol		Laser Treatment Protocol	
Cell Type	**Hypoxia (1% O2) treatment**	**Normoxia incubation time after hypoxia treatment**	**Laser Treatment (time of exposure)**	**Normal incubation time after laser treatment**
HSKM	4 h 8 h 24 h	0 h, 24 h &72 h	0 J/Cm^2 (Sham Control) 6 J/Cm^2 (113 sec) 12 J/Cm^2 (226 sec)	24 h
HCM	4 h 8 h 24 h	0 h, 24 h &72h	0 J/Cm^2 (Sham Control) 6 J/Cm^2 (113 sec) 12 J/Cm^2 (226 sec)	24 h
Glioblastomas	4 h 8 h 24 h	0 h, 24 h &72h	0 J/Cm^2 (Sham Control) 6 J/Cm^2 (113 sec) 12 J/Cm^2 (226 sec)	24 h

effective in regenerative medicine

Materials and Methods

In this study, human skeletal muscles cells (HSKMs), human cardiac myocytes (HCMs) and Glioblastoma Cells were cultured, treated by hypoxia or laser exposure and analysed to investigate the effect of physical parameters on the targeted homing of these cells after transplantation. All experiments were performed in triplicate.

Cell culture

Primary HSKM and HCM cells and also Glioblastoma Cell line were purchased from Pasteur Institute (Tehran, Iran). HSKM and HCM were cultured in DMEM Low Glucose/Ham's F-12 (Gibco, USA) 50% mix supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 5ug/ml Insulin (Sigma, Germany) and 50ng/ml bFGF (Royan, Iran) without penicillin-streptomycin. Glioblastoma cells were cultured in RPMI1640 (Biowest, USA) supplemented with 10% FBS without penicillin-streptomycin. Cells were expanded for two passages and treated after the 2nd passage. In the migration experiment, previously characterized adipose-derived MSCs were used; the isolation and expansion of MSCs was done as previously published (13).

Treatment of Cells

Hypoxia Treatment

Cells were incubated in a pre-adjusted 37°C incubator specified for hypoxic conditions (95% N_2, 5% CO_2, and 1% O_2) at different time intervals as given in table 1. Based on different studies regarding the effect of hypoxia on rheumatoid synovial fibroblasts and endothelial cells (14), our primary cells and glioblastoma cell line were undergone in different incubation times for both hypoxia and post-treatment normal conditions. These time intervals for hypoxia treatment were 4, 8 and 24 hours. Post-conditioning normoxia incubation was a 37°C incubator with 5% CO_2. We took the samples from cells: A) immediately (0hour) after hypoxia treatment (4, 8 and 24 hours); B) after 24 h- and C) after 72 h- post-incubation in normal conditions. Control group was not incubated in hypoxia conditions.

Laser Treatment

Cells were exposed by two irradiation doses of 6 J/Cm^2 and 12 J/Cm^{2}using He/Ne laser with 6328A° (632.8 nm) wavelength with the 2mW power as a source of low level laser. Cells at the time of laser treatment were plated in 96 well culture plates in a very specific designed pattern to avoid the effect of scattering laser light on surrounding cells as shown in figure 1. The culture media was supplemented with 0.5% FBS to minimize the absorption capacity of FBS and to maximize the laser activity. Incubation time and laser treatment durations were based on the laser treatment protocol as given in table 1. Sham control group was used as negative control. Post-conditioning incubation was a 37°C incubator with 5% $CO_2$for 24 hours. In all exposed cells and sham groups, we gave 24 h in normal cell culture conditions to let them enough period of time for showing expression effects. We took the samples from cells after 24 h post-exposure incubation.

MTT assay

Cell viability for hypoxia and laser treated cells, was determined by using MTT assay. Upon Treatment of cells by laser and hypoxic conditions, 100 µL of culture medium and 10 µL of 12 mM MTT (Sigma, Germany) stock solution were added to each well. Plates were incubated at 37°C for 20 minutes and then 150 µL of 100% DMSO solution (Sigma, Germany) was added. The absorbance was determined at 490 nm using ELIZA reader (BioTek, Germany).

Reverse transcription-qPCR

Total RNAs were extracted using tripure solution (Roch, Germany) and treated with DNase I (Fermentase, USA). 1µg of total RNA was used for cDNA synthesis using cDNA synthesis kit (Fermentas, USA) according to the manufacturer protocol and qPCR was performed

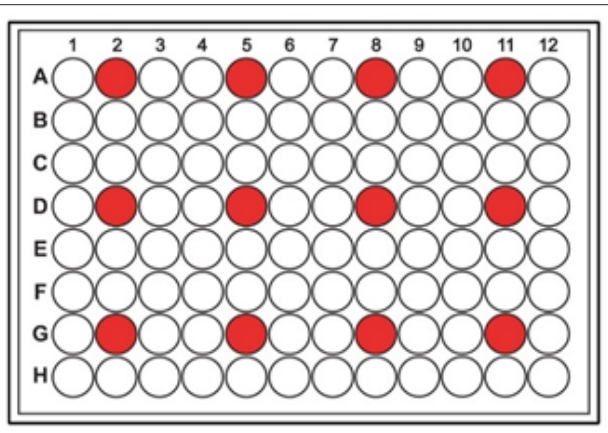

Figure 1. Specific designed cellular pattern in 96 well culturing plates for laser treatment.

Table 2. Real-time PCR primer sequences.

Gene	Sequences (5'-3')	PCR product size (bp)
β-actin	F: GCGGGAAATCGTGCGTGACATT R: GATGGAGTTGAAGGTAGTTTCGTG	232
SDF-1α	F: TGTTTGTGCTGTGGTGTGTCC R: ATCTAAGGTTGGGGGAGGTG	165
HIF1	F: TCACCACAGGACAGTACAGGATGC R: CCAGCAAAGTTAAAGCATCAGGTTCC	303

using human-specific primers (summerized in table 2) and SYBR Green qPCR Master Mix (Parstoos, Iran) by BioRadCFX96 thermocycling machine.

For each primer pair, the annealing temperature was optimized by gradient RT-PCR. The expression (E) of each target mRNA relative to human β-actin in hypoxia and laser-treated cells (HCM and HSKM) was calculated based on the cycle threshold (Ct) as $E=2-\Delta(\Delta Ct)$, in which $\Delta Ct=Ct\ target-Ct\ \beta\text{-actin}$ and $\Delta(\Delta Ct)=\Delta Ct\ untreated-\Delta Ct\ treated$ (28).

Western Blotting

In each time point of harvesting for sampling, HSKM, HCM and Glioblastoma cells were washed with ice-cold PBS and cells were lysed using tripure solution (Roch, Germany) for protein extraction. After vigorous pipetting, the suspension transferred to a canonical tube and was centrifuged for 15 min at 12000g and 2-8°C. After centrifugation, the upper phase containing RNA was collected to study genetic expression at transcriptional level and from the remaining two phases containing DNA and Protein, the proteins were aspirated carefully. To purify proteins, DNAs were precipitated out using 100% ethanol. Acetone and 2% glycerol was added to the obtained phenol ethanol supernatant enriched with protein, and protein was separated as precipitate. This precipitated protein was washed three times with 0.3 M guanidine hydrochloride dissolved in 95% ethanol and 2% glycerol. The washed proteins were dissolved in 1% SDS and were incubated at 60°C for 10 min with periodic vortexing to have maximum dissolution. Finally, protein samples were labelled and stored in -80°C freezer for western blot analysis.

Protein samples were thawed and dissolved in 5X sample Buffer. These samples were loaded and separated on sodium dodecyl sulphate polyacrylamide gel (SDS–PAGE). The proteins were electrophoretically transferred onto a polyvinylidenedi fluoride (PVDF) membrane (Bio-Rad, USA) and then blocked with TBS plus Tween 20 (TBST) containing 5% non-fat dry milk for 3 h at room temperature. The membrane was incubated for 2 h with 1:300 diluted SDF-1α antibody (Santa-Cruz Biotechnology, USA) and 1:300 diluted β-actin antibody (Santa-Cruz Biotechnology, USA) as housekeeping antibody, appropriately diluted in TBST containing 3% non-fat dry milk. The immune complexes were visualized with 1:2000 diluted appropriate horseradish peroxidase-conjugated secondary antibodies (Santa-Cruz Biotechnology, USA) and enhanced chemiluminescence (Bio-Rad, USA).

Cell Migration Assay

A conventional Boyden chamber assay using a Transwell cell culture chamber (Corning Costar Italia, Italy) was performed to find out the effects of SDF-1α chemokine on MSCs motility and migration. 50000 adipose derived MSCs were seeded for each well of chamber (the upper side of a polyvinylpirrolidone-free polycarbonate filter having 6.5-mm diameter and 8-µm pore size) and cultured in 50 µL of serum-free medium. The lower compartment was filled with the condition medium of HCM cells, either treated with Hypoxia 4 h followed by 72 h normoxiaor exposed to 12 J/Cm^2 Laser. Plates were left in the incubator for 24h, at 37◦C, 5% CO2. At the end of the incubation, cells were mechanically removed from the upper surface of the filter by wiping them with a cotton bud. Cells that had migrated to the lower surface were fixed with methanol for 15 min, washed with PBS. Then, trans-wells were stained with DAPI (Sigma, Germany). Invasive capability was measured according to a method modified from Albini et al. (12).

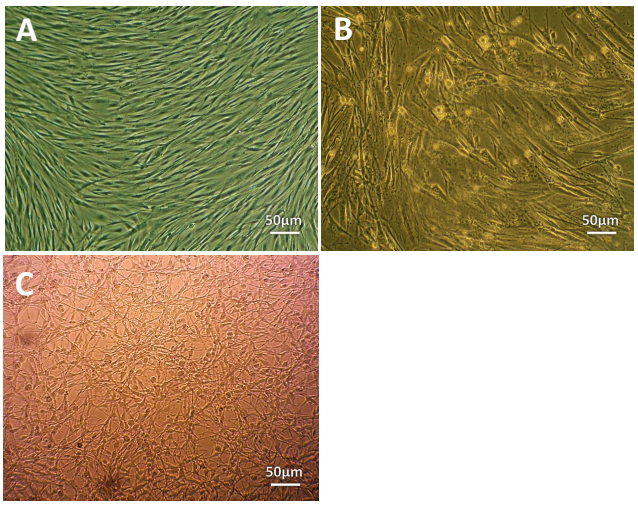

Figure 2. Morphological characterization of *in vitro* expanded cells at passage 2. (A)HSKM, (B) HCM and (C) Glioblastoma cells.

Statistical Analysis

All data were subject to statistical analysis using Graph Pad Prism v 6.0 for the analysis of qPCR, MTT and Migration assay results. The significance of difference between the experimental groups and controls was assessed by one-way ANOVA dunnett's test. The results are presented as the means ± standard deviation (SD).

Results

Cell culture and MTT assay

HSKM, HCM and Glioblastoma cells were expanded (Figure 2) and after passage 2, MTT assay was performed to determine the viability of cells.

Comparing cell viability between different subgroups underwent hypoxia has revealed that four hours hypoxia followed by 72 hours normoxia (4H/72N) has

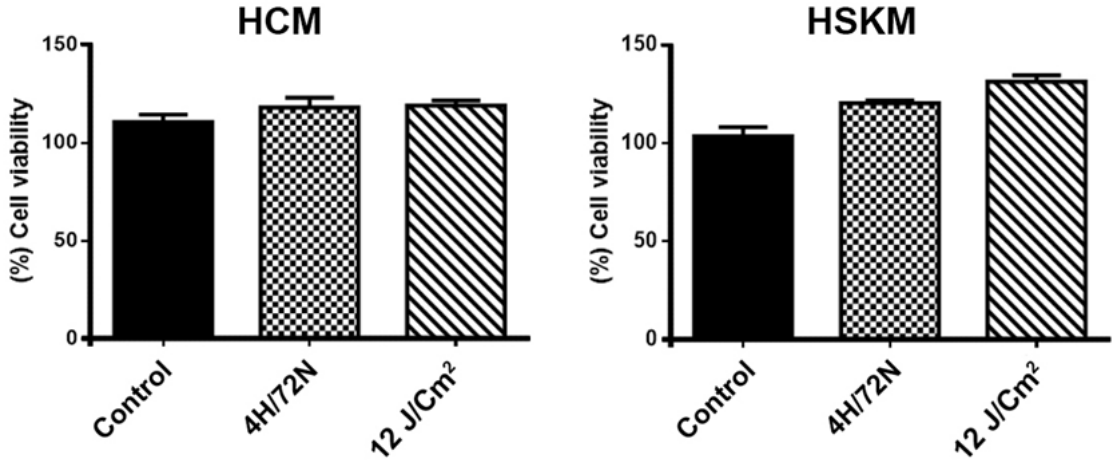

Figure 3. Cell viability after hypoxia and laser treatment. Hypoxia and laser have no effect on cell viability of (A) HCM and (B) HSKM compared with Glioblastoma (control). In hypoxia group, four hours hypoxia followed by 72 hours normoxia (4H/72N) and in laser group, 12 joules laser per square centimetre (12 J/Cm^2) were selected as preferred conditions based on previous MTT experiments in each group.

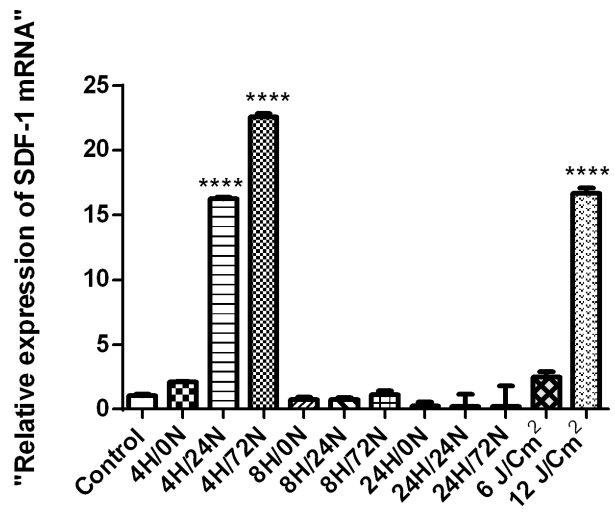

Figure 4. Real time PCR results of *SDF-1α* mRNA expression in HSKM. Changes in the expression of *SDF-1α* after different times of hypoxia treatments are shown in relation to untreated HSKM, displaying a substantial upregulation of this gene after 4h of hypoxia treatment followed by 24h and 72h normoxic incubation, and 12 J/Cm^2 laser treatment. Results were statistically evaluated by using one-way ANOVA with post hoc test **, P < 0.001 was considered significant as compared with control. Abbreviation: Number units are in hours; H: Hypoxia conditions; N: Normal incubation condition.

the best effect on cell viability and in laser subgroups, the highest cell viability was belong to cells underwent 12 J/ cm^2 laser followed by 24 h normal conditions (Data was not shown).

To see any probable effect of hypoxia and laser treatment on cell viability, it was compared with Glioblastoma. The results revealed that hypoxia and laser didn't change the viability of cells significantly compared to Glioblastoma as control (Figure 3).

SDF-1α expression Analysis

Expression at transcriptional level

SDF-1α expression in HSKMs increased significantly in the groups of 4H/24N and 4H/72N hypoxia treatment and laser 12 J/Cm^2 (16 fold, 22 fold and 16 fold respectively). In HCM, *SDF-1α* expression level was increased significantly, 45-fold, 68-fold, 59-fold and 90-fold for 4H/24N,4H/72N (Hypoxia treatment group), laser 6 J/Cm^2 and 12 J/Cm^2 respectively. Comparative expression of *SDF-1α* has been given in the figures 4 and 5. From this data, we choose cells showing dramatic increase of *SDF-1α* expression in cells treated in 4h hypoxic conditions and then re-oxygenation for 72h and 12 J/Cm^2 of laser treatment for analysing the *SDF-1α* expression at transplantation level in coming sections.

Expression at translational level

Results observed by Real-Time PCR regarding SDF-1α expression gene in HCMs after 4 hours of hypoxia and laser treatment were confirmed by western blot analysis as shown in figure 6. Highest expression of the SDF-1α was observed when cells were treated by 12 J/Cm^2 laser dose. ImageJ 2.0 software was used to analyse the intensity of bands obtained in western blot figure 7.

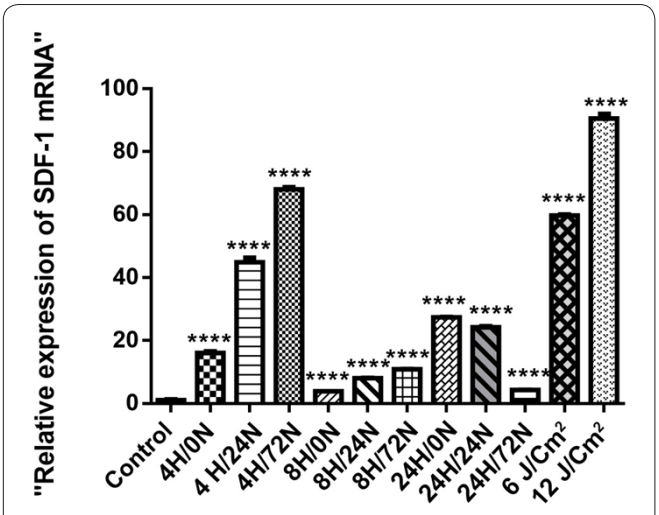

Figure 5. Real time PCR results of SDF-1α mRNA expression in HCM. Changes in the expression of SDF-1α after different time intervals of hypoxia treatments are shown in relation to untreated HCM, displaying a substantial upregulation of this gene after all treatments. Results were statistically evaluated by using one-way ANOVA with post hoc test **, P < 0.001 was considered significant as compared with control. Abbreviation: Number units arein hours; H: Hypoxia conditions; N: Normal incubation condition.

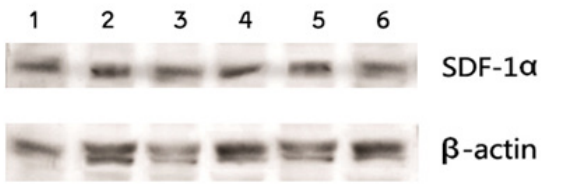

Figure 6. Protein expression analysed in cells under hypoxia and laser treatment in control and hypo 4/0, hypo 4/24, hypo 4/72, laser 6J and laser 12J groups by western blot and numbered as 1, 2, 3, 4, 5, 6 respectively (a) Protein bands observed in X-ray film by western blot. (b) Image based analysed data showing fold expression of after hypoxia and laser treatment.

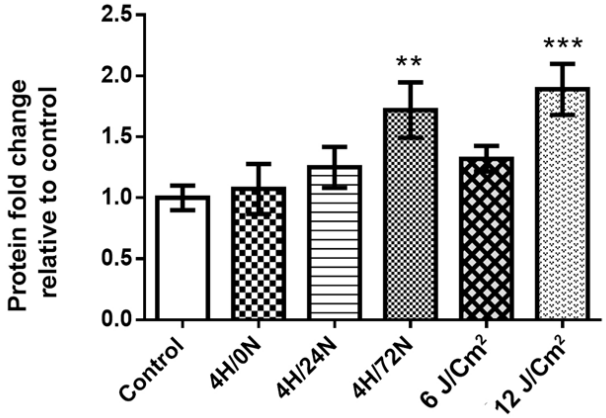

Figure 7. Quantitative expression of SDF-1α at translational level in HCM cells when treated with Hypoxia and Laser. Abbreviation: Number units are hours; H: Hypoxia conditions; N: Normal incubation condition.

Determination of *HIF-1α* gene expression by real-time PCR

Real-time PCR results showed increased expression of *HIF-1α* in HCM cells as well as HSKM cells when they were treated for 4 hours in hypoxic condition plus 6 J/Cm^2 and 12 J/Cm^2 laser treatments as shown in figure 8. *β-actin*, as an internal control of each sample was used for normalization.

Cell Migration Boyden Chamber Assays

Migration is an essential step of stem cell homing events. In this study, we used a conventional Boyden chamber assay (corning) to study the cellular migration capabilities (Figure 9(a)). For this purpose, MSCs were subjected to migration for 24 h from the upper chamber through the insert toward the condition medium collected from HCM cells, treated with Hypoxia 4 h and 72 h incubation or Laser 12j in receiver wells. Data reported in Figure 10 is showing a significant increase of the migration ability of MSCs as compared to control containing serum-free medium.

Discussion

SDF-1α is a chemokine that plays an important role in the trafficking and migration of blood cells as well as stem cells (15,16). Expression of SDF-1α increases in the heart about one hour after the induction of hypoxia (17). it was conjectured that this process plays a role at the beginning of tissue repair and angiogenesis (18). Based on these findings, it can be concluded that

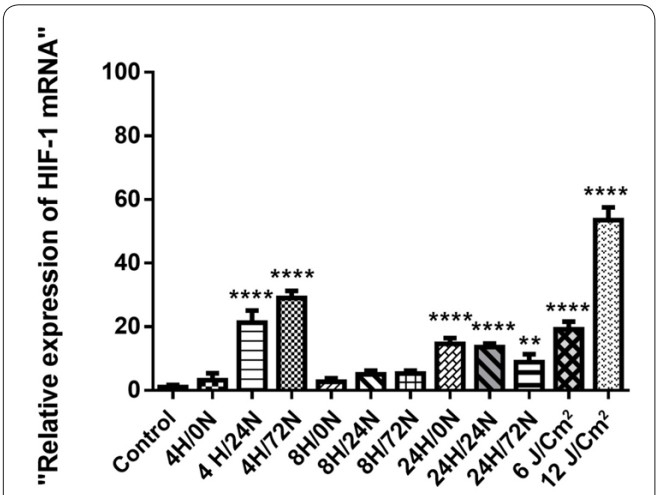

Figure 8. Real time PCR results of HIF-1α mRNA expression in HCM. Changes in the expression of HIF-1α after different time intervals of hypoxia treatments are shown in relation to untreated HCM. Results were statistically evaluated by using one-way ANOVA with post hoc test **, P < 0.001 was considered significant as compared with control. Abbreviation: Number units are hours; H: Hypoxia conditions; N: Normal incubation condition.

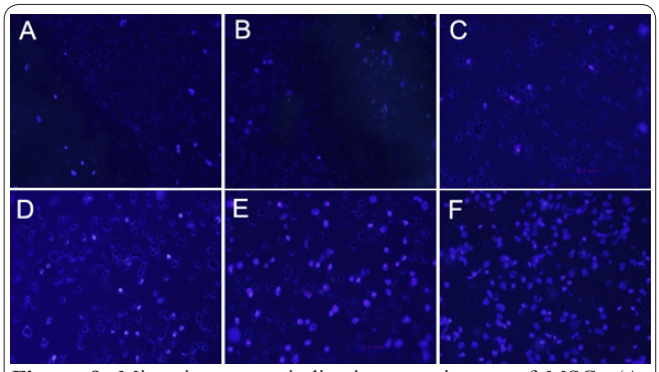

Figure 9. Migration assay indicating recruitment of MSCs (A: Untreated Cells showing no recruitment of MSCs, B: Control negative showing no recruitment, C: Condition medium containing AMD3100 showing no recruitment of MSCs, D: Hypoxic treatment showing recruitment of MSCs, E: Laser treatment showing recruitment of MSCs, F: Control Positive (FBS enriched medium) showing recruitment of MSCs towards FBS).

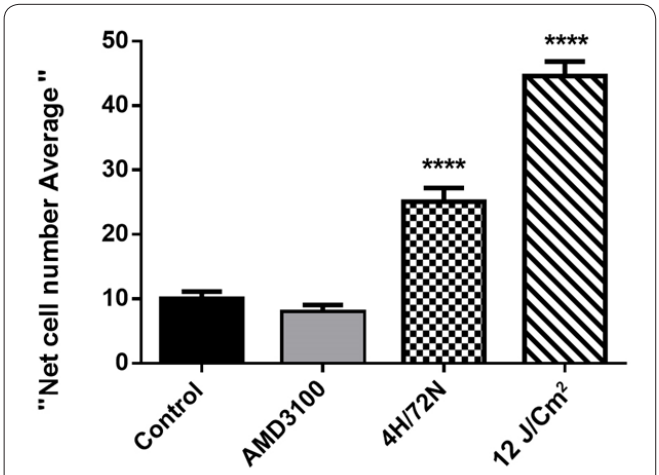

Figure 10. Average number of Ad-MSCs migrated toward condition medium collected from HCM cells, treated with Hypoxia 4 h and 72h normaxic incubation or Laser of 12 J/Cm^2 **, P < 0.001 was considered significant as compared with control (HCM untreated and HCM condition medium containing AMD3 100). Abbreviation: Number units are inhours; H: Hypoxia conditions; N: Normal incubation condition.

SDF-1α expression induced by hypoxia is an effective way for triggering cellular migration and angiogenesis in damaged tissues (19). Several studies have demonstrated that induction of SDF-1α alone is sufficient to increase cell migration and angiogenesis and improve the damaged tissue recovery. It was proved that SDF-1α regulates the migration and angiogenesis of bone marrow cells expressing CXCR4 in vivo (20). SDF-1α and its receptor CXCR4 play an important role in the migration of stem cells to ischemic heart. It is also involved in migration of early generating cells, angiogenesis and deployment of endothelial cells to ischemic tissue (21). Previous studies have shown that the expression of SDF-1α leads to systemic migration of the stem cells. Transient expression of SDF-1α after myocardial infarction have significant implications for potential clinical strategies to regenerate and restore heart functions after myocardial infarction following stem cells transplantation and their natural migration to the damaged heart muscle (20). In animal models such as rats and mice, expression of SDF-1α increased when hypoxic condition were created in heart, therefore hypoxia can act as a prominent factor to attract stem cells toward the damaged tissues (22). In another study, expression of SDF-1α in endothelial cells has been observed when cells were exposed with hypoxic and radiation exposure. It was also observed that activity of SDF-1α and HIF-1α has been increased when cells exposed to radiation. It was concluded that hypoxia and radiation together significantly affect the expression of SDF-1α instead of being alone (23). Low level laser also has been applied on damaged liver, arteries and bone marrows and increased expression of SDF1-α was observed in damaged tissues (24–26).

SDF1-α has been known as an important player of cell migration and considering the importance of hypoxia and laser radiation effect on the expression of SDF-1α, this study was aimed to evaluate the effects of these two factors on expression of SDF-1α in muscle cells for the improved and targeted migration and implantation of stem cells in ischemic heart tissue. It was concluded that, increasing SDF-1α expression as a ligand of CXCR-4 receptor in damaged tissues, may be a successful approach in order to obtain enhanced stem cell recruitment and implantation (1,7,9,23).

In this regard, HCMs and HSKM cells were exposed to hypoxic condition and different doses of laser energy to find out the optimum treatment time. Every group of cells under hypoxic treatment was incubated in normal oxygen (normoxic) condition for 0, 24 and 72 hours time intervals. Based on the results, it was observed that Cells (HCM and HSKMs) showed increased expression of SDF-1α when were treated in hypoxic condition for 4 hours and then re-oxygenated for 72 hours, as well as 12 J/Cm^2 laser treatment. But in other preconditioning treated cells, especially in hypoxia treated groups, very low expression of SDF-1α was observed at transcriptional and translational level when compare with 4H/72N group. These results could possibly be due to excessive exposure of cells under the hypoxic conditions. Gradual increased expression of SDF-1α was observed with the passage of time in all hypoxic preconditioning groups when they were incubated in normoxic conditions except the 24 hours hypoxia group only which was might be due to the lethal dose of hypoxia condition killing cells (figure 5 and 7). Similar to these results have also been reported by Stempien-Otero et al. on endothelial cells (27).

Nowadays, in most studies, repeated injections of cells to ischemic/damaged tissues, is the main strategy to enter the inductive factors in the body (3–5). In this study, hypoxia and low-level laser were selected as inductive factors providing hypoxia condition, especially in the muscles can be achieved easily with an exercise program and low level laser irradiation is also an easy approach to implement. Therefore, by a clinical perspective and practical look at this issue, in addition to the possible effects of these two factors on the improvement of stem cell homing, this strategy will relive patients from frequent and painful injections. The results of this study can offer new modalities for improvement of cell therapy in the case of muscular diseases.

Acknowledgement

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