Over-Expression of CXCR4, a Stemness Enhancer, in Human Blastocysts by Low Level Laser Irradiation

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Abstract

Background: The key role of chemokine receptor CXCR4 in the maintenance of stemness property of stem cells has been shown recently. The low level laser irradiation (LLLI) is being used currently in a wide variety of clinical cases as a therapeutic tool for wound healing, relieving pain and destroying tumor cells. The aim of this study was to evaluate the effect of LLLI mimicking low level laser therapy (LLLT) on the expression level of CXCR4 gene a few hours after irradiation on human blastocysts.

Materials and Methods: After the development of human embryos to the first grade blastocyst stage, they were irradiated with a low power Ga-Al-As laser at a continuous wavelength of 650 nm and a power output of 30 mW. Total RNA of the irradiated blastocysts and control groups was isolated in groups of $1x2$ J/cm^2, $2x2$ J/cm^2, $1x4$ J/cm^2 and $2x4$ J/cm^2 LLLI. Specific real-time PCR primers were designed to amplify all the two CXCR4 isoforms yet identified. RNA amplifications were done for all groups.

Results: We showed for the first time that LLLI makes the human blastocysts to increase the expression level of CXCR4 a few hours after irradiation. Moreover, it was shown that two irradiation doses with one day interval can cause a significant increase in CXCR4 expression level in human blastocysts.

Conclusion: This study revealed that LLLI could be a proliferation motivator for embryonic cell divisions through enhanced over-expression of CXCR4 level.

Keywords: CXCR4; Human blastocysts; Low-level laser irradiation (LLLI); Proliferation; Stemness

Introduction

Low level laser therapy (LLLT) refers to the use of red-beam or near-infrared lasers with a wavelength of 600-1100 nm and an output power of 1-500 mW. This type of radiation is a continuous wave or pulsed light that consists of a constant beam of relatively low energy density (0.04-50 J/cm^2), and the laser is directed at the target tissue or monolayer of cells using powers measured in milliwatt (mW). Low level low-levels of red/near-infrared light illumination (1-2). At low doses (2 J/cm^2), the LLLI stimulates proliferation, while at high doses (16 J/cm^2) LLLI is suppressive, pointing to the dose dependence of biological responses after light exposure (3). However, stimulation in cell proliferationhas been shown to occur outside these ranges (4). A number of different laser light sources, including helium-neon, ruby and gallium- aluminum-arsenide, have been used to deliver LLLI in different treatments and on

different schedules. LLLI transmits energy at low levels and therefore does not emit heat, sound or vibrations. Its reactions are non-thermal because there is no immediate increase in the temperature of the laser-irradiated tissue. Experiments following LLLI exposure have shown that the immediate increase in heat of the target tissue is negligible (± 1 °C) (5). It has been confirmed by many investigators that the temperature remained unchanged in fibroblast suspensions during LLLI irradiation (6). As well as reported announced that a temperature increase of less than 0.065°C, with laser irradiation of 40 mW/cm^2, using a microthermo probe in a monolayer of cells. In contrast, high-energy lasers (e.g., carbon dioxide lasers and neodymium-YAG lasers) are able to raise the tissue temperature high enough to cut and vaporize them (7).

Applications of LLLI in regenerative medicine
The LLLT has been used for wound healing since a few decades ago. It is also widely applied in different branches of regenerative medicine (e.g., tissue regeneration) (8) and dentistry, where it is used to enhance the healing process (9). Earlier, the LLLI was shown to have beneficial effects on variety of pathological and clinical conditions including pain relief and inflammatory condition (10). Recently, it has been reported that LLLI could also enhance the proliferation of mesenchymal and cardiac stem cells (11), and differentiation of human embryonic stem cells (12).

Cell signaling after LLLI
Cell proliferation is a very important physiological effect of LLLI. The LLLI is used in basic experimental cell culture procedures and clinical practice (13). Nonetheless it promotes the proliferation of multiple cells, mainly through the activation of mitochondrial respiratory chain and the initiation of cellular signaling. The alterations in photoacceptor function are the primary reactions, and the subsequent alterations in cellular signaling and cellular functions are secondary reactions (14). The primary reactions after light absorption are singlet-oxygen hypothesis, redox properties alteration hypothesis, nitric oxide hypothesis, transient local heating hypothesis and superoxide anion hypothesis (15). The secondary reactions after light absorption are cellular signaling pathways, including the mitochondrial retrograde signaling (16).

CXCR4 as an involved gene in stemness
A superfamily of chemoattracting molecules called chemokines are cytokine-like proteins that bind to and activate a family of chemokine receptors. More than 50 chemokines have been identified, and they

are divided into 4 families on the basis of the positions of 4 conserved cysteine residues: CXC, CX3C, CC and C (17). Chemokine receptors are seven-transmemberane receptors coupled to G-proteins, with their C-terminus in the cytoplasm, three extracellular and three intracellular loops as well as an N-terminus outside the cell surface. One of the intracellular loops of the chemokine receptors couples with heterotrimeric G-proteins and that mediate ligand binding to the receptor which initiates a cascade of signal transduction events (18).

There is a high degree of redundancy in the chemokine family as multiple chemokines bind to the same receptor (19).

Yet chemokines and their receptors have been identified to have important roles in inflammation, infection, tissue injury, allergy, cardiovascular diseases and malignant tumors (20). Regulating metastasis is one of the most important roles of chemokines and the chemokine receptors. Chemokine receptors may potentially facilitate tumor dissemination at each of the key steps of metastasis, including adherence of tumor cells to endothelium, extravasation from blood vessels, metastatic colonization, angiogenesis, proliferation and protection from the host response via activation of key survival pathways such as ERK/MAPK, PI-3K/Akt/mTOR or Jak/STAT (21). Of the chemokine receptors the CXCR4 is a highly conserved G-protein which couples seven-span transmembrane receptors (GPCR) that binds the ligands such as CXCL12α and CXCR4. It has received considerable attention since it serves as a co-receptor for entry of T-tropic (X4) HIV viruses that target CD4$^+$ T cells (22). The CXCR4 is expressed in different cell types (e.g. central nervous systems and immune systems) during the development. It can also mediate migration of resting leukocytes and hematopoietic progenitors in response to CXCL12 functioning in a number of physiological processes (23). In the marrow and regulating stem cell trafficking, differential expression of CXCR4 in CD34$^+$ progenitor cells may be involved in maintaining hematopoietic progenitor cells (24). For example, the deficiency of CXCL12/CXCR4 axis leads to circulatory, CNS, immune and hematopoietic defects. This axis plays an important role in the embryonic development (25). CXCR4 is over-expressed in more than 23 human cancers, including breast, ovarian, melanoma and prostate cancers (26). Although CXCR4 is expressed in a broad array of tissues, its expression is low or absent in many normal tissues, including breast and ovary (27). Vascular endothlial growth factor (VEGF) is a known inducer of CXCR4 expression and it has been shown that Hypoxy Inhibiting Factor-1 (HIF-1) acts as an upstream to induce VEGF and the heterodimeric

transcription factor responsive to oxygen concentrations in tissues. It can also up-regulate CXCR4 expression. CXCR4 is functionally crucial for maintenance of stemness in drug-resistant non-small cell lung cancer cells (28).

Materials and Methods

Human embryos and ethical approval

Human embryos used for the present study were donated (the consent was given) from couples who underwent *in vitro* fertilization (IVF) program for infertility treatment at reproduction medicine unit, Department of Obstetrics and Gynecology, Shariati Hospital, Tehran University of Medical Sciences (TUMS) in accordance with medical research ethical regulations of the Ministry of Health and Medical Education of Iran.

Culture of human embryos

Fresh normal and frozen-thawed normal fertilization at pronuclear (PN)- stage embryos were transferred to droplets of global® medium (Life Global) supplemented with 10% Serum Substitute Supplement (SSS™) (Irvine Scientific), covered with light oil (Life Global), and cultured at 37 °C, 5% O_2 60% CO_2, 89% NO_2. The resulted blastocysts were then prepared for 72 h for low level laser treatment.

Irradiation on human blastocysts via laser

The blastocysts were irradiated with a low power Ga-Al-As laser (Metron Inc., Australia) at a continuous wavelength of 650 nm and a power output of 30 mW based on grading system reported by Gardner (93). Blastocysts were irradiated at a distance of 15mm from the laser probe tip. The beam diameter of the laser was 6 mm.

The provided blastocysts were divided into two groups. The first and the second groups were irradiated for 30 s (2 J/cm^2) and 30 s (4 J/cm^2), respectively. Furthermore, each group was divided into two subgroups, one with no irradiation and the other treated with irradiation for the second time, a day after the first irradiation treatment and with the identical laser wavelength (2x2 J/cm^2 and 2x4 J/cm^2). Each group consisted of five wells. Blastocysts in 96-well plates were seeded in wells far from each other to avoid overlapping or scattered irradiation. The cover of the plate was off during the irradiation period and treatment was done at room temperature. Control blastocysts were subjected to the same procedure as the irradiated cells but the laser was not turned on. In order to avoid influence of second-order variables, the blastocysts of two experimental groups, including the control group (non-irradiated), were exposed to the same environmental and stress conditions such as temperature, humidity and light.

Regarding the light, during the experiment, all dishes were covered by black box to prevent light exposure. Identical samples from each group were taken for random cell counting 2, 4 and 6 days after the first irradiation. Then we compared the effect of laser with different power outputs on cell proliferation.

Culture of human blastocysts

To culture the isolated blastocysts, the culture medium was changed to human embryonic stem cell culture medium, consisting of knockout™ DMEM (Invitrogen) supplemented with 20% knock out serum replacement, 1% glutamax™, 0.5 mM β-mercaptoethanol, 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 1% nonessential amino acids and 8 ng/ml bFGF (Invitrogen). Blastocysts were cultured 7 days until the inner cell mass (ICM) outgrowths were formed.

Proliferation assay

Blastocysts proliferation of all eight cell groups (irradiated and controls), was evaluated on the 4th day after the first irradiation, by 3- (4, 5-Dimethylthiazol-2-yl) -2, 5-diphenyltetrazoliummbromide (MTT) assay. In order to determine cell numbers, the culture medium was removed and 100 μl of fresh culture medium containing 50 μl of MTT (5 mg/ml, Sigma) was added to each well. The cells were then incubated at 37 °C for 4 h. The color was extracted with 100 μl DMSO at 37 °C for 6 m. OD_{490}, which was directly related to the viable cell numbers, was determined at room temperature and cell growth curves were plotted.

RNA isolation and cDNA synthesis

Total RNA was isolated from all eight cell groups (groups irradiated at 1x2 J/cm^2, 2x2 J/cm^2, 1x4 J/cm^2, 2x4 J/cm^2 LLLI, and their controls) using Trizol Reagent (Invitrogen) according to the manufacturer's instructions, and dissolved in RNase-free water. The quality and quantity of the extracted RNAs were measured by gel electrophoresis and spectrophotometry, respectively. To remove the whole DNA contamination, equal amounts of RNA were treated with RNase-free DNase (Takara, Japan) and then the first strand of cDNA synthesized by reverse transcriptase (Takara, Japan), using both oligo dT and random hexamer primers (Takara, Japan) according to the manufacturer's instructions. For each sample, a no-reverse transcription control was used in parallel to the DNase-treated RNA to detect non-specific amplification of genomic DNA.

Reverse transcription quantitative real-time PCR

Specific real-time PCR primers (MWG Biotech, Germany) were designed using GenRunner software

to amplify all two CXCR4 isoforms ((isoform a (accession No, NM_001008540.1) and isoform b (accession No, NM_003467.2)). We used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (accession No, NM_002046.4) as a reference gene or internal control in our measurement. PCR reaction was performed using 3 µl of cDNA or no-reverse transcription sample with 10 µl SYBR Premix Ex Taq II master mix (TaKaRa) U of Taq and 0.5 µM of each primer in a 25 µl PCR reaction. Real-time PCR reaction supplemented with ROX reference Dye II was used for all amplification reactions. The amplification carried out using the following cycling conditions: initiation at 95 °C for 5 m, amplification for 40 cycles with denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s and extending at 72 °C for 15 s and final extension at 72 °C for 10 m. All real-time PCR reactions were carried out by the ABI 7500 Real-Time PCR systems (Applied Biosystems, FosterCity, CA).

Table 1. Primers designed for CXCR4 and GAPDH

Gene name	F/R	Primer	Product (bp)
CXCR4	F	GGTCCATGGTTACCAGAAGA	364
	R	GTCATCTGCCTCACTGACGTTG	
GAPDH	F	GCCACATCGCTCAGACAC	115
	R	GGCAACAATATCCACTTTACCAG	

Forcompensatig variations in the amount of input RNA and the efficacy of reverse transcriptase, GAPDH mRNA was also quantified as internal control, and the expression data were normalized to GAPDH expression value in each sample. PCR products were separated on a 1% agarose gel, stained with ethidium bromide and visualized under the UV light. Direct DNA sequencing (MWG, Germany) confirmed the PCR products.

Statistical analysis
Data are presented with mean±SD of three independent experiments (n=3 for each individual group and assay). The data were compared by ANOVA test followed by the Tukey test. The level of significance was 5% ($p < 0.05$).

Results
Effect of LLLI on the proliferation of human blastocysts
Proliferation of human blastocysts was detected in all eight cell groups (groups in which human blastocysts were irradiated at 1x2 J/cm², 2x2 J/cm², 1x4 J/cm²

and 2x4 J/cm² LLLI. The number of viable human blastocysts, expressed as OD_{490}, was detected by the MTT assay on the 3rd day of the first irradiation by related medium. The OD_{490} in groups irradiated at 2x2 J/cm² and 2x4 J/cm² was significantly higher than that in the non-irradiated groups ($p<0.01$). Also OD_{490} in groups irradiated at 2x4 J/cm² and 2x2 J/cm² was significantly higher than that one irradiated at 1x4 J/cm² and 1x2 J/cm², respectively ($p<0.01$).

The effect of LLLI on human blastocysts after irradiation
RNA was extracted using Trizol Reagent (Invitrogen), according to the manufacturer's instructions, from two biological replicates 12 h after irradiation. CXCR4 expression levels were determined from RNA isolated at 0 and 12 h after the treatment and quantified relative to GAPDH expression level. The control time point (t=0) expression level was set to 100% and treated samples were shown as a percentage of the control (Fig. 1)
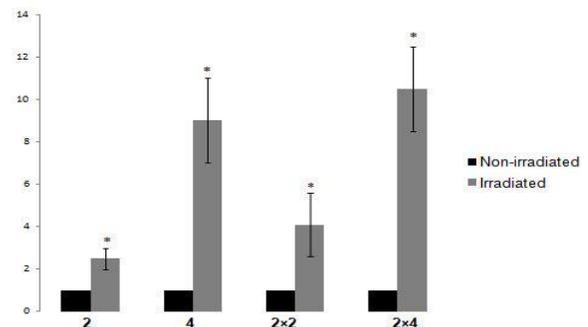
Figure 1. The effect of different doses of low level laser irradiation on human blastocysts, as the expression level of CXCR4 transcript was analyzed by real-time quantitative RT–PCR in human blastocysts after 2 J/cm² and 2x2 J/cm² (two times irradiation each time with 2 J/cm²), 4 J/cm² and 2x4 J/cm² (two times irradiation each time with 4 J/cm²) LLLI in comparison with their paired non-irradiated (* P-value < 0.05)

Discussion
We showed for the first time that low power laser irradiation makes the human blastocysts to increase the expression level of CXCR4 a few hours after irradiation. Moreover, we demonstrated that two irradiations with one day interval cause a significant increase in CXCR4 expression level in human blastocysts.
This finding is important in regenerative medicine and reproductive technologies. Self-renewal, which is considered as the ability of stem cells to be divided and reproduced, discriminates these cells from the other cells. As we mentioned earlier, the key role of the chemokine receptor CXCR4 in the maintenance of stemness property of stem cells has been observed

and published recently (20). Blastocysts are pleuripotent stem cells (12). Some genes are common stemness regulators, such as SOX2, OCT4, Nanog, KLF4; however, the role of CXCR4 has not been shown clearly. This finding is the first report of CXCR4 in human blastosyst irradiated with laser. Thus, LLLI could be a candidate tool for non-dividing freeze-thawed *in vitro* fertilization (IVF) embryos through over-expression of CXCR4. In regard to possible molecular mechanism, the possibility of binding of CXCR4 to more than one ligand, as occurs for many chemokine receptors, may lead to identification of novel ligands or characterization of different expression levels of its common cognate receptors such as CXCL12 in blastocysts. LLLI may trigger an intracellular cascade signaling pathway through which a conformational change in molecules involved in cell proliferation and self-renewal of blastocysts may occur or may activate or inactivate enzymes that catalyze cell proliferation, which requires further studies to be cleared.

Conclusions
LLLI may be a candidate tool for motivation of non-dividing freeze-thawed *in vitro* fertilization (IVF) embryos through over-expression of CXCR4 as we showed in this study for the first time.

Acknowledgements

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