35th Annual International Conference of the IEEE EMBS
Osaka, Japan, 3 - 7 July, 2013

Effects of Low Level Light Irradiation on the Migration of Mesenchymal Stem Cells Derived from Rat Bone Marrow*

Wen-Tyng Li, Chih-Wei Chen, and Po-Ya Huang

Abstract—**Low level light irradiation (LLLI) was found to exert positive effects on various cells *in vitro*. The aim of this study was to investigate the effect of LLLI on the migration of rat bone marrow mesenchymal stem cells (rbMSCs). Light irradiation was applied at the energy density of 4 J/cm^2 using red (630 nm) and near infrared (NIR, 850 nm) light emitting diodes (LEDs). Wound healing assay showed both red and NIR light irradiation increased cell mobility. Red and NIR light enhanced transmembrane migration of rbMSCs up to 292.9% and 263.6% accordingly. This agreed with enzymatic activities of MMP-2 and MMP-9 enhanced by irradiation. F-actin accumulation and distribution correlated to increased migration in light-irradiated MSCs. Reactive oxygen species production as well as the expression of pFAK and pNF-κB were elevated after red and NIR LLLI. The study demonstrated that red and NIR LLLI increased rbMSCs migration and identified the phosphorylation of FAK and NF-κB as critical steps for the elevated cell migration upon LLLI.**

I. Introduction

Mesenchymal stem cells (MSCs) play an important role in tissue regeneration which involves the processes of mobilization of stem cells from the bone marrow, homing of these cells to the site of injury, and differentiation of the stem cell into a functional cell of the injured. Some important mechanisms for MSCs to migration to injured tissues, including chemoattractants, paracrine factors, membrane receptors, and intracellular signaling molecules. Extracellular matrix and biophysical factors also play an important role in guiding migration of MSCs [1]. One key feature of MSCs-based therapy is that MSCs must find their way to disease target after cell transplantation. Enhancing the migratory capability of MSCs to increase the number of MSCs reaching relevant disease target may improve the overall therapeutic efficacy of MSC transplantation.

Low level light irradiation (LLLI) is considered part of light therapy and part of physiotherapy, which has been studied and used clinically for over 30 years. The light is typically of narrow spectral width in the red or near infrared (NIR) spectrum (600-1000 nm), with an irradiance between 1 mW/cm^2 to 5 W/cm^2. The light source is usually a low power laser or light emitting diode (LED) in the range of 1 mW to 500 mW [2-4]. Previously, we studied the influence of red light (630 nm, 15 mW/cm^2) and near-infrared (NIR, 850 nm, 10 mW/cm^2) LED irradiation on rat MSCs and found that the parameters for the maximal increase of MSCs proliferation were multiple dose of 630 nm and 850 nm LLLI at 4 J/cm^2. Under these parameters, the osteogenesis of MSCs was promoted [5-7]. The use of LLLI at 632.5 nm has been demonstrated to promote endothelial cell proliferation, migration and NO secretion via the activation of PI3K/Akt pathway [8]. Red (660 nm) and infrared (958 nm) laser irradiation increased motility of hematopoietic stem cells up to 126 and 151% accordingly [9]. LLLI at 804 nm to autologous bone marrow helps to recruit MSCs to the infarcted area and reduce scarring and ventricular dilatation following myocardial infarction [10, 11].

The effect of LLLI using noncoherent LED for modulating MSCs mobilization has not yet been investigated. The aim of this study was to investigate the effect of red and NIR LED used in LLLI at 4 J/cm^2 on migration of rat bone marrow mesenchymal stem cells (rbMSCs).

II. Materials and Methods

A. Animals

Female Wistar rats (150-250 g) were used for MSC isolation. The rats were obtained from National Laboratory Animal Center (Taipei, Taiwan). All the procedures were approved by the Institutional Animal Care and Utilization Committee of Chung-Yuan Christian University before the experiments.

B. Isolation of Mesenchymal Stem Cells and Culturing

Rat MSCs were isolated as described previously [7]. Bone marrow was collected from the femurs and tibias by flushing with serum-free alpha minimum essential medium (α-MEM) (Invitrogen, GIBCO/BRL, CA, USA) containing 2 mM L-glutamine, 100 U/ml antibiotic-antimycotic and 0.1 mM Nonessential amino acids. Cells were filtered through a 100-μm nylon mesh (BD Bioscience, CA, USA) and subjected to 40% percoll density gradient centrifugation. The mononuclear cell layer was collected from the interface and suspended in α-MEM supplemented with 10% fetal bovine serum (FBS) (Hyclone, UT, USA). After 24 h incubation at 37^{o}C and 5% CO_2, nonadherent cells were discarded and the adherent MSCs (passage 0) were grown to 90% confluence in fresh α-MEM containing 10% FBS prior to subculturing.

*Research supported in part by the National Science Council, Taiwan, R.O.C. under Grants NSC 100-2221-E-033-026-MY3, NSC 101-2627-E-033-001, and NSC 101-2632-M-033-001-MY2.

W.–T. Li is with the Department of Biomedical Engineering and Center for Nanotechnology, Chung-Yuan Christian University, Chung-Li, 32023 Taiwan, R.O.C. (corresponding author to provide phone:+886-3-2654543; fax: +886-3-2654599; e-mail: wtli@cycu.edu.tw).

C.-W. Chen was with the Department of Biomedical Engineering, Chung-Yuan Christian University, Chung-Li, 32023 Taiwan, R.O.C. (e-mail: kok029@hotmail.com).

P.-Y. Huang is with the Master Program in Nanotechnology, Chung-Yuan Christian University, Chung-Li, 32023 Taiwan, R.O.C. (e-mail: huangpoya@hotmail.com).

MSCs between passages 2 and 4 were used in the following experiments.

C. Light Irradiation

The light source used was a home-made LED array (22 x 31 lamps) designed to fit over a standard 96-well microplate (12.5 x 8.5 cm) for cell culture. LED lamps were purchased from Lenoo Electronic Co. Ltd. (New Taipei City, Taiwan). The material used for Red LED was AlInGaP and had an emission wavelength peaked at 630 nm and a bandwidth of 34 nm. The material used for NIR LED was GaAlAs and had an emission wavelength peaked at 850 nm and a bandwidth of 84 nm. The distance from the top of the LED array to the surface of cell monolayer was 1.2 cm. The irradiance at the surface of the cell monolayer was measured by a power meter (Orion, Ophir Optronics Ltd., UT, USA) and could be adjusted by direct current from a standard power supply. The radiant exposure could be regulated by the time period of LED irradiation, which was calculated as follows: radiant exposure (J/cm^2) = time (sec) x power (W) / surface (cm^2). To obtain the energy dose of 4 J/cm^2, exposure time for red light LED array was 4.27 min under power density of 15 mW/cm^2, and that for NIR LED array was 6.40 min under 10 mW/cm^2. During the irradiation experiments, all culture plates were maintained under semi-dark conditions at room temperature and atmosphere for the length of the irradiation. Rat MSCs without LED exposure placed under the same condition were used as control.

D. Wound Healing Assay

Wound healing assay is a classic method of evaluation *in vitro* cell migration. Rat MSCs ($2x10^5$ cells) were seeded on a 6-cm culture dish. After 24 h of cell culture, a sterile 1 ml pipette tip was use to make a straight scratch on the cell monolayer. The LLLI protocol was undertaken using radiant exposure of 4 J/cm^2. Forty-eight hours after irradiation, the cells were stained with Liu's stain according to the manufacture's protocol (Tonyar Biotech Inc., Tauyuan, Taiwan). To assess the migration of cells back into the wound region, images were acquired with an inverted microscope equipped with a digital CCD camera (Leica DMIL, Leica Microsystems, Wetzlar, Germany). The distance of migration was analysed using ImageJ software (NIH, Bethesda, MD, USA).

E. Transwell Migration Assay

The capacity of rbMSCs to migrate through a cell permeable membrane was assessed using 12 mm-diameter transwell chamber (Millipore, County Cork, Ireland) with polycarbonate membrane inserts (8 μm pore size). Each chamber was placed in a 3.5-cm culture dish containing α-MEM supplemented with 10% FBS. One thousand cells were seeded onto the upper compartment of the chamber and incubated for 24 h. Prior to LLLI, the medium in the upper compartment was replaced with α-MEM supplemented with 2% FBS. Forty-eight hours after light exposure of 4 J/cm^2, the cells that had not migrated were removed from the upper surface of the polycarbonate membrane using cotton swabs, and those that migrated to the lower surface of the polycarbonate membrane were fixed in 1.5% glutaraldehyde and stained with hematoxylin-eosin stain. Migration was determined by counting the cell number with a microscope. Five visual fields were chosen randomly for each assay. The average number of the migrating cells in five fields was taken as the cell migration number of the group.

F. Detection of Reactive Oxygen Species

The reactive oxygen species (ROS) assay was performed in a 6-well microplate seeded with $3x10^5$ MSCs per well. The cells were incubated in α-MEM containing 2% FBS and 20 μM 2,7-dichlorodihydrofluorescein diacetate (Sigma-Aldrich, MO, USA) for 1 h. Cells were lysed with 0.5% Triton X-100 in PBS buffer after LLLI. The fluorescence of dihydrofluorescein diacetate (DCF) was analyzed immediately by a fluorescence microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific, Inc., MA, USA) using a 488 nm excitation wavelength and a 538 nm emission wavelength. The protein amount in each well was determined by Bicinchoninic Acid Protein Assay Kit.

G. Gelatin Zymography

Gelatin zymography was performed using supernatant from MSCs ($3x10^4$) received LLLI. The samples were stored at -20°C until use. The gelatinolytic activity of matrix metalloprotease (MMP) from the supernatant was assayed under non-reducing conditions. Equal volumes of sample and 2X non-reduced Tris-Glycerol SDS sample buffer were mixed and resolve by a SDS polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin. The gel was then incubated overnight with Zymogram developing buffer at 37°C and stained with a Coomassie blue solution containing 40% methanol, 10% glacial acetic acid and 0.05% Coomassie blue (Sigma-Aldrich, MO, USA). A clear band against a blue background represents enzyme activity.

H. Western Blot Analysis

Cells ($2x10^5$) were harvested at 3 and 36 hr after LLLI, and sonicated 3 times in 200 μl of Homo buffer (10 mM Tris at pH 7.4, 1 mM EDTA, 1mM EGTA, 50 mM NaCl, 50 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1% Triton X-100, 1 mM PMSF, 1 mM Benzamidine and 0.5 μg/ml Leupeptin) on ice. After centrifuging at 15000 x g for 20 min at 4°C, the resulting supernatant was used as the cell extract. The cell extract (~40 μg of protein) was resolved by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with buffer containing 5% skim milk in 0.5% Tween 20, 15 mM NaCl and 20 mM Tris at pH 7.4 for 1 h at room temperature, and then incubated with primary antibodies (phospho-focal adhesion kinase (pFAK) at 1:800, and phospho-nuclear factor kappa B (pNF-κB) at 1:1000) (Epitomics, Inc., CA, USA) overnight at 4°C. The membrane was incubated with secondary antibody conjugated with horse radish peroxidase (1:2000) for 2 h at room temperature. The proteins in the membrane were visualized by using Western Blotto Luminol Reagent (Origene Technologies, Inc., MD, USA). The protein content of each sample was normalized by re-probing the same membrane with GADPH antibody. Briefly, the blotted membrane was incubated with 200 ml of stripping buffer (62.5 mM Tris.HCl at pH 6.7, 100 mM β-mercaptoethanol and 2% w/v SDS) at 50°C for 30 min with occasional agitation to strip

off the bound antibodies. After washing six times in TBST buffer (20 mM Tris.HCl at pH 7.4, 0.5 M NaCl and 0.05% Tween 20), the stripped membrane was immunoblotted with GADPH antibody (1:1000). Quantities of protein were analyzed with ImageJ software.

I. Immunofluorescence Microscopy

Rat MSCs were seeded in 24-well plates at a density of $1x10^4$ cells per well. Five minutes after LLLI, cells were washed with PBS buffer and fixed with 3.7% glutaraldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS buffer for 5 min prior to staining. Cells were incubated in the solution containing 5 μg/mL Hoechst 33258 and 300 U/ml Rhodamine-phalloidin for 30 min. After further washes, fluorescence images were collected on a fluorescence microscope.

I. Statistical Analysis

Quantitative data were expressed as means ± standard deviation (SD) from at least three independent experiments and assessed using Wilcoxon Signed Rank Test. Significance was accepted if $*P<0.05$.

III. Results and Discussion

A. LLLI Promoted MSC Migration

The analysis of the monolayer of MSCs after irradiation by wound healing assay showed red and NIR light irradiation increased cell mobility up to 183% and 161% respectively (Figure 1A, 1C). Red and NIR light enhanced transmembrane migration of rbMSCs up to 292.9% and 263.6% accordingly (Figure 2B, 1D). The results of both analyses show that MSCs received red light irradiation have higher mobility than those with NIR treatment.

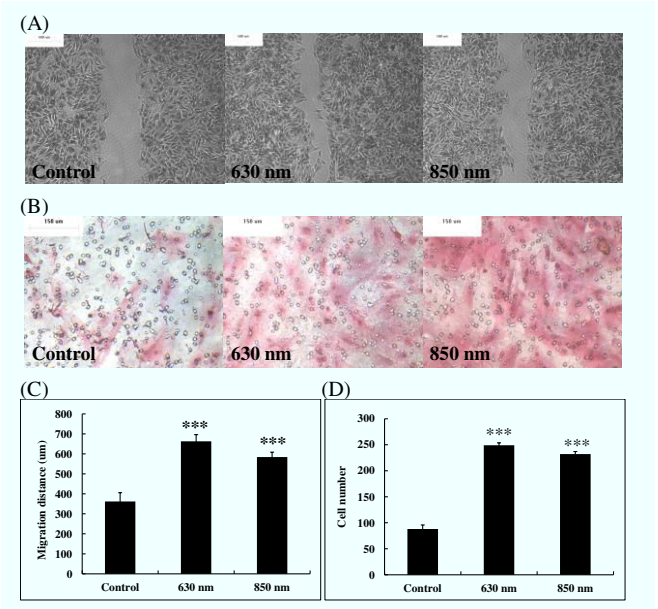

Figure 1. Influence of red and NIR light irradiation on rat MSC migration. The migration of rbMSCs were analyzed by wound healing (A, C) and transwell migration (B, D) assays. Forty-eight hours after red and NIR light exposure of 4 J/cm^2, the distance of wound closure (C) was quantitated by ImageJ software and the transmembrane migrated cells (D)were counted. The cells without irradiation were taken as control group. Significant increase in migration of irradiated cells compared to control cells is indicated by *** ($P<0.001$). Error bars show the SD from three independent experiments.

B. LLLI Enhanced Intracellular ROS Level of MSCs

The DCF fluorescence of MSCs was evaluated immediately after LLLI. As shown in Figure 2, red and NIR irradiated cells resulted in significant increase of DCF fluorescence up to 30% and 21% respectively. The result indicates that ROS may play a role in MSC migration subjected to LLLI.

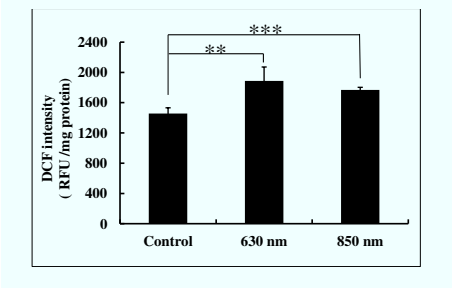

Figure 2. Influence of red and NIR light irradiation on intracellular ROS level of rat MSCs. The ROS level of rbMSCs were analyzed immediately after red and NIR light exposure of 4 J/cm^2. The cells without irradiation were taken as control group. Data is expressed as the mean fluorescence/mg protein. Error bars show the SD from three independent experiments. Significant increase in migration of irradiated cells compared to control cells is indicated by ** ($P<0.01$) and *** ($P<0.001$).

C. LLLI Increased MMP-2 and MMP-9 Enzymatic Activities

As analyzed by gelatin zymography, the enzymatic activities of MMP-2 and MMP-9 were detected at 3 hr after red and NIR light irradiation (Figure 3). The result suggests that the release of MMP-2 and MMP-9 from MSCs was facilitated by LLLI, thus led to increased cell migration.

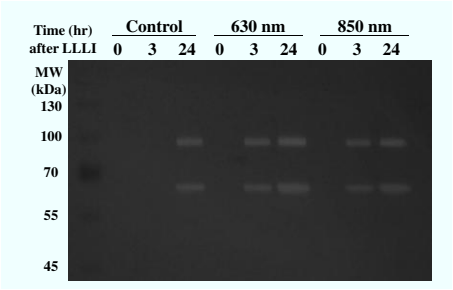

Figure 3. Influence of red and NIR light irradiation on gelatinase MMP-2 and MMP-9 activity of rat MSCs. The MMP-2 and MMP-9 enzymatic activities of rbMSCs were analyzed by gelatin zymography at 0, 3 and 24 hr after red and NIR light exposure of 4 J/cm^2. The cells without irradiation were taken as control group. A clear band at approximately 92 kDa corresponding to pro-MMP-9 activity and another band of the activated MMP-2 at approximately 62 kDa are visible.

D. LLLI Increased p-FAK and p-NF-κB Expression

As demonstrated by western blot analysis, phosphorylation of FAK and NF-κB was elevated in red and NIR light irradiated MSCs after 36 and 3 hr (Figure 4A). The expression levels of pFAK in Red and NIR groups were 1.4 and 1.7 fold of control group (Figure 4B). The expression levels of pNF-κB in Red and NIR groups were 11.2 and 12.8

fold of control group (Figure 4C). The study identified the phosphorylation of FAK and NF-κB as a critical step for the elevated cell migration upon LLLI.

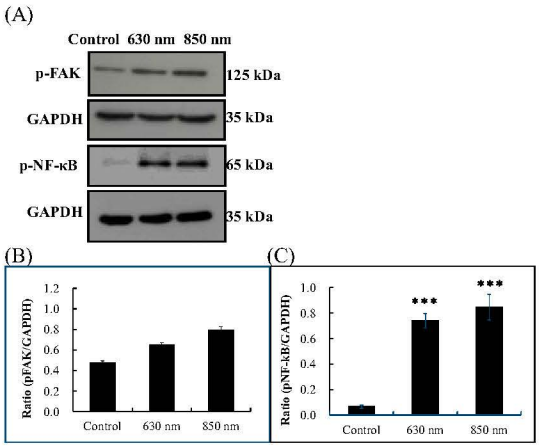

Figure 4. Influence of red and NIR light irradiation on pFAK and pNF-κB expression of rat MSCs. The expression of pNF-κB and pFAK of rbMSCs were analyzed by immunoblotting at 3 and 36 hr after red and NIR light exposure, respectivley (A). The cells without irradiation were taken as control group. The densities of p-FAK (B) and p-NF-κB (C) bands were normalized relative to those of GADPH by ImageJ software. Semi-quantitive analysis of protein expression shown here is expressed as the mean ± SD from three independent experiments (**P<0.01; *** P<0.001).

E. Immunofluorescenc Microscopy

Immunofluorescence staining revealed that cytoskeletal f-actin pattern of rat MSCs changed after low level red and NIR light irradiation (Figure 5). LLLI stimulates accumulation of F-actin at the leading edge of MSCs.

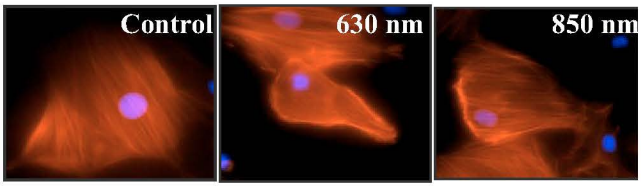

Figure 5. Influence of red and NIR light irradiation on cytoskeletal F-actin pattern of rat MSCs. The nuclei were stained by Hoechst 33258 (blue) and F-actin microfilaments were stained with rhodamine-phalloidin (orange) at 5 min after red and NIR light exposure of 4 J/cm^2. The cells without irradiation were taken as control group.

In conclusion, the current study demonstrated that red and NIR LLLI could increase rbMSCs migration. We provided the evidence that LLLI exerts its photostimulating effect via phosphorylation of FAK and NF-κB, thus leading to increased MMP-2 and MMP-9 enzymatic activities. ROS may play an important role as a messenger in the stimulatory effect of LLLI.

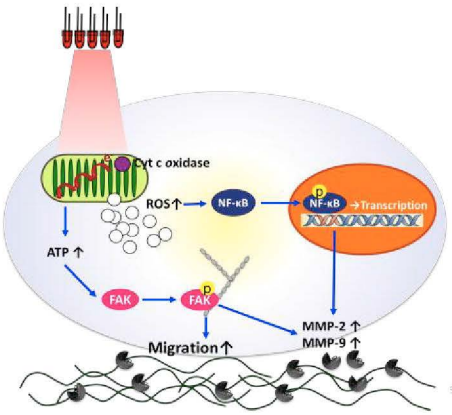

Figure 6. A model of the signaling pathways of LLLI enhancing MSC migration.

REFERENCES

[1] L. Li, Z. Wang, J. Jiang, and M. Zhao, "Signaling pathways involved in migration of mesenchymal stem cells," *Trends Bio/Pharmaceutical Industry,* vol. 6, pp. 29-33, 2010.

[2] H. T. Whelan, R. L. Smits, Jr., E. V. Buchman, N. T. Whelan, S. G. Turner, D. A. Margolis, *et al.*, "Effect of NASA light-emitting diode irradiation on wound healing," *J Clin Laser Med Surg,* vol. 19, pp. 305-14, Dec 2001.

[3] T. I. Karu, *Low power laser therapy* vol. 48. Boca Raton, FL, USA: CRC Press, 2003.

[4] W. Posten, D. A. Wrone, J. S. Dover, K. A. Arndt, S. Silapunt, and M. Alam, "Low-level laser therapy for wound healing: mechanism and efficacy," *Dermatol Surg,* vol. 31, pp. 334-40, Mar 2005.

[5] T.-Y. Hsu and W.-T. Li, "The influence of low level near-infrared irradiation on rat bone marrow mesenchymal stem cells," in *2008 ICBME Proceedings*, Singapore, 2008, pp. 2072–2075.

[6] W. T. Li, H. L. Chen, and C. T. Wang, "Effect of light emitting diode irradiation on proliferation of human bone marrow mesenchymal stem cells," *J Med Biol Eng,* vol. 26, pp. 35-42, 2006.

[7] W. T. Li, Y. C. Leu, and J. L. Wu, "Red-light light-emitting diode irradiation increases the proliferation and osteogenic differentiation of rat bone marrow mesenchymal stem cells," *Photomed Laser Surg,* vol. 28 Suppl 1, pp. S157-65, Aug 2010.

[8] C. H. Chen, H. S. Hung, and S. H. Hsu, "Low-energy laser irradiation increases endothelial cell proliferation, migration, and eNOS gene expression possibly via PI3K signal pathway," *Lasers Surg Med,* vol. 40, pp. 46-54, Jan 2008.

[9] L. V. Gasparyan, G. Brill, and A. M. Makela, "Influence of low level laser radiation on migration of stem cells," in *Proc. of SPIE. Laser Florence 2004*, 2005, pp. 1-5.

[10] H. Tuby, L. Maltz, and U. Oron, "Induction of autologous mesenchymal stem cells in the bone marrow by low-level laser therapy has profound beneficial effects on the infarcted rat heart," *Lasers Surg Med,* vol. 43, pp. 401-9, Jul 2011.

[11] H. Tuby, L. Maltz, and U. Oron, "Implantation of low-level laser irradiated mesenchymal stem cells into the infarcted rat heart is associated with reduction in infarct size and enhanced angiogenesis," *Photomed Laser Surg,* vol. 27, pp. 227-33, Apr 2009.