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The effects of combined low level laser therapy and mesenchymal stem cells on bone regeneration in rabbit calvarial defects ☆

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ABSTRACT

Objective: This study evaluated the effect of Low Level Laser Therapy (LLLT) and Mesenchymal Stem Cells (MSCs) on bone regeneration.
Background data: Although several studies evaluated the effects of MSCs and LLLT, there is little information available regarding *in vivo* application of LLLT in conjunction with MSCs.
Methods: Forty-eight circular bone defects (6 mm in diameter) were prepared in the calvaria of 12 New-Zealand white rabbits. The defects of each animal were randomly assigned to 4 groups: (C) no treatment; (L) applying LLLT; (SC) filled with MSCs; (SCL) application of both MSCs and LLLT. LLL was applied on alternate days at wavelength of 810 nm, power density of 0.2 W/cm^2 and a fluency of 4 J/cm^2 using a Gallium–Aluminum–Arsenide (GaAlAs) diode laser. The animals were sacrificed after 3 weeks and then histological samples were evaluated to determine the amount of new bone formation and the remaining scaffold and inflammation.
Results: The histological evaluation showed a statistically significant increase in new bone formation of LLLT group relative to the control and the other two experimental groups ($p < 0.05$). There was no significant difference in bone formation of the control group compared to experimental groups filled with MSCs. Laser irradiation had no significant effect on resorption of the scaffold material. In addition, inflammation was significantly reduced in LLLT group compared to the control defects and the other two experimental groups.
Conclusion: Low level laser therapy could be effective in bone regeneration but there is no evidence of a synergistic effect when applied in conjunction with MSCs.

1. Introduction

Bone defects are not always straightforward to repair [1]. A variety of methods have been developed to enhance bone repair including autogenous bone grafts or synthetic materials used to fill up the defects [1,2]. Up until recently, the use of autologous grafts has been considered as the gold standard of treatment for bone regeneration [1]. However, its implementation has been restricted due to difficulties in the procedure of obtaining autogenous bone, including inadequate bone supply, donor site pain, infection, remaining scar and risk of nerve injury [1,2]. Other types of bone materials including demineralized bone matrix, Hydroxyapatite, Tricalcium phosphate, etc., are not as effective as the autogenous bone due to lower osteo-inductive capacity [3,4]. Efforts to overcome the problem led to the development of tissue engineered bone regeneration [5].

Bone tissue engineering benefits from Mesenchymal Stem Cells (MSC) along with bone material (natural or synthetic) instead of

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using autografts [5,6]. The bone material is actually a resorbable scaffold which carries the stem cells into the defect and helps to preserve the bone volume [4,5]. The MSCs, on the other hand, are the vital components which are responsible for new bone formation [5]. These cells are known as a group of multi-potent, self-renewing progenitor cells which are able to differentiate into all types of cells with the mesenchymal origin, including osteoblasts, chondroblasts and myoblasts [5,6]. They are easily obtainable from adult bone marrow and some other tissues such as fat tissue and periosteum [7]. Although their high proliferation and differentiation potential could be effective in regeneration of bone defects, several studies demonstrated just a moderate improvement of new bone formation by using MSCs [7,8]. In these studies, a variety of osteo-inductive techniques have been used to increase the rate, quantity and quality of bone repair, including biochemical compounds (growth factors, bone morphogenic proteins) and physical stimulus (low level laser, ultrasonic waves) [5,9].

In the past decade, several studies have evaluated the effects of Low Level Laser Therapy (LLLT) on bone regeneration [9–11]. It has been showed that *in vitro* application of LLLT could lead to higher activity of alkaline phosphatase enzyme, increased intra-cellular calcium concentration and increased activity of osteoblasts leading to a higher rate of new bone formation [12–14]. In addition, several *in vivo* studies have revealed that LLLT promotes the rate and amount of new bone formation in standardized bone defects and also improves bone healing in artificial fractures [11,15,16]. However, there is little information about the *in vivo* effects of using LLLT in conjunction with MSCs on bone healing. The aim of this study was to evaluate the effect of LLLT alone and in conjunction with autologous MSCs on bone regeneration of calvarial defects in rabbits.

2. Materials and methods

2.1. Animals

Fifteen male New Zealand white rabbits aged between 8 and 12 months and with a mean weight of 3320 ± 370 g were used in this study. The bone marrow sampling procedure commenced 1 week after adaptation. All animals were housed separately in large, well-lit standard cages in an animal laboratory controlled for temperature (21 °C) and maintained with a daily photoperiod of 12 h of light. Each animal had *ad libitum* access to food and water. All experiments followed the guidelines of the Iran Animal Care Committee and were approved by the AJA University of Medical Science animal care committee, Ethical Approval No. 88/12/12.

2.2. Bone marrow sampling

Intramuscular injection of 50 mg/kg ketamine hydrochloride (100 mg/mL, Alfasan, Woerden-Holland) and 10 mg/kg xylazine hydrochloride (20 mg/mL, Alfasan, Woerden-Holland) were used for anesthetizing the rabbits. After shaving and disinfecting the region, almost 3 mL of bone marrow were aspirated from the humerus of the animals using the Jamshidi aspiration needle and a 10 mL syringe containing 3000 U of heparin. All procedures were carefully performed under sterile conditions to avoid bacterial infection of the samples.

2.3. Mesenchymal stem cells preparation

The bone marrow aspirates were suspended in 5 mL Dulbecco's modified Eagle's medium (DMEM, Gibco, Germany) supplemented with 100 IU/mL penicillin (Sigma, USA), 100 IU/mL streptomycin (Sigma, USA) and 10% Fetal Bovine Serum (FBS). The cells were plated in 75 cm^2 culture flasks at a density of 10^5 cells/mL in an atmosphere of 5% CO_2 and 37 °C [17]. After 1 week, cells attached to the bottom of the flasks were washed by phosphate buffered saline (PBS – Gibco, USA) and culture medium was replaced with fresh DMEM [17]. The flasks were incubated and medium replacement took place twice per week until the cultures became confluent. At 60–70% confluence, second passage cultures of MSCs were washed with PBS and trypsinized with trypsin/EDTA (0.2%). MSCs labeled with vital fluorescent dye (PKH26) 24 h before the surgical procedure to track cells proliferation. The cells were then suspended in collagen type I (Koken, Japan) at a density of 10^5 cells/mL and loaded onto Bio-Oss scaffolds in preparation for surgery.

2.4. Bio-Oss resorbable scaffold

Bio-Oss was used in several studies as the scaffold for MSCs [17–19]. Bio-Oss is a resorbable natural bone substitute usually used for bone regeneration in the oral cavity [16]. It is originally deproteinized bovine bone in particulate form with high interconnecting porosity which makes it suitable for angiogenesis and also cell proliferation and migration [17–19]. It is integrated into the natural modeling and remodeling procedures and the new bone deposited directly on the Bio-Oss particles. These features make Bio-Oss a reliable scaffold in tissue engineered bone regeneration.

2.5. Surgical procedure

Animals were intramuscularly anesthetized as described above. After shaving and disinfecting the animals' head, a full thickness incision was made over the midline to expose the underlying parietal bone. Four symmetric circular full thickness bone defects were made through the calvaria using a trephine bur with an outer diameter of 6 mm as described in previous studies [20] (Fig. 1). These bone defects in each animal served as four different experimental groups: control (C), laser (L), stem cells (SC) and combination of laser and stem cells (SCL). Scaffolds and MSCs were inserted into the SC and SCL defects and the other two defects (C and L) remained empty. The L and SCL defects received one treatment of LLLT before closing the incision. Finally the periosteum and the skin were tightly sutured in two different layers with an absorbable 4-0 suture material (Vicryl, Ethicon, USA) to seal the area and keep the scaffolds in their places. All the animals received subcutaneous injection of enrofloxacin (5 mg/kg, Baytril, Bayer Corp, USA) and tramadol hydrochloride (4 mg/kg, Tehran Chemie Pharmaceutical Co, Tehran, Iran) for 5 days after the surgery. The animals were sacrificed after 3 weeks by an overdose of sodium pentobarbital.

2.6. Low level laser therapy

The laser therapy for L and SCL defects was initiated at the time of surgery and then continued every other day for 3 weeks. A continuous emission mode Gallium–Aluminum–Arsenide (GaAlAs) diode laser (THOR Photomedicine Ltd. UK) with a wavelength of 810 nm, power output of 200 mW, power density of 0.2 W/cm^2, spot size of 1 cm^2, distance of 0.5 cm, period of 20 s and fluency of 4 J/cm^2 per session was utilized as the source of LLLT.

2.7. Histologic and histomorphometric evaluation

After 3 weeks, all the animals were sacrificed and the calvariae were removed and fixed in 10% buffered formalin solution for 2 weeks. The specimens were soaked in 14% EDTA solution for decalcification while their softening or calcification was controlled frequently as they were to be cut by microtome device. After