Journal of Photochemistry & Photobiology, B: Biology 159 (2016) 179–185

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Low level laser therapy accelerates bone healing in spinal cord injured rats

Carla Christina Medalha, Ana Lúcia Yaeko Silva Santos, Suellen de Oliveira Veronez, Kelly Rossetti Fernandes, Angela Maria Paiva Magri, Ana Claudia Muniz Renno *

Department of Biosciences, Federal University of São Paulo, Av. Ana Costa, 95, Vila Mathias, Santos 11050-240, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 22 July 2015
Received in revised form 23 March 2016
Accepted 24 March 2016
Available online 29 March 2016

Keywords:
Low level laser therapy
Spinal cord injury
Bone
Rats

ABSTRACT

Bone loss occurs rapidly and consistently after the occurrence of a spinal cord injury (SCI), leading to a decrease in bone mineral density (BMD) and a higher risk of fractures. In this context, the stimulatory effects of low level laser therapy (LLLT) also known as photobiomodulation (PBM) have been highlighted, mainly due to its osteogenic potential. The aim of the present study was to evaluate the effects of LLLT on bone healing using an experimental model of tibial bone defect in SCI rats. Twenty-four female Wistar rats were randomly divided into 3 groups: Sham group (SG), SCI control group (SC) and SCI laser treated group (SL). Two weeks after the induction of the SCI, animals were submitted to surgery to induce a tibial bone defect. Treatment was performed 3 days a week, for 2 weeks, at a single point over the area of the injury, using an 808 nm laser (30 mW, 100 J/cm^2; 0.028 cm^2, 1.7 W/cm^2, 2.8 J). The results of the histological and morphometric evaluation demonstrated that the SL group showed a larger amount of newly formed bone compared to the SC group. Moreover, a significant immunoexpression of runt-related transcription factor 2 (RUNX2) was observed in the SL group. There was no statistical difference in the biomechanical evaluation. In conclusion, the results suggest that LLLT accelerated the process of bone repair in rats with complete SCI.

1. Introduction

Spinal cord injury (SCI) is characterized by a complete or partial loss of autonomic, sensory and motor functions and is caused by interruption of neural signal conduction along the axonal tracts [1]. SCI results in partial or complete paraplegia or tetraplegia, and is responsible for severe complications such as the loss of mobility, dysfunctions of many systems and accentuated decrease in bone mass [2]. Bone metabolism is widely affected by SCI, marked by a significant decrease in the rate of bone formation and an increase in bone resorption (with a higher osteoclast activity), which is responsible for rapid bone loss and lower bone mineral density (DMO) [2] in humans [3–5] and in rats [4,6]. Bone loss significantly increases the risk of fracture, causing severe clinical, psychological and financial complications [7].

In this context, there is a critical need to develop technologies capable of stimulating bone tissue and accelerating fracture consolidation in patients with SCI, improving their health status [8]. Recently, a significant body of evidence has been accumulated demonstrating that low level laser therapy (LLLT) and photobiomodulation (PBM) is effective in reducing post-injury inflammatory processes, accelerating soft tissue healing and stimulating the formation of new blood vessels [9–13]. The exact mechanisms underlying the therapeutic effects of PBM are not yet well established but there is evidence that laser light is absorbed by chromophores in the cells, especially those known as cytochrome *c* oxidase (CCO) [14]. CCO stimulation induces photochemical reactions and the production of energy in the cell [15]. This increased energy, in the form of higher adenosine triphosphate (ATP) production, is used to perform various cellular tasks. This is accompanied by the upregulation of transcription factor activation and the modulation of reactive oxygen species (ROS) [16,17]. Moreover, it seems that LLLT may cause photodissociation of nitric oxide (NO) from CCO, also improving cell respiration [14]. Thus, the application of light directly affects cell proliferation and migration, modulation in the levels of cytokines, growth factors and inflammatory mediators, and increases tissue oxygenation [18–20].

Based on these effects, recent studies demonstrated that LLLT was capable of stimulating healing in damaged bone tissue, including in SCI rats [14,21]. A previous study demonstrated that the AsGaAl laser irradiation (830 nm, 30 mW/cm, 250 J/cm^2) was capable of improving histomorphometrical properties of tibias of rats in an experimental model of SCI [6].

Although the stimulatory action of LLLT on bone cell proliferation and *in vivo* bone formation has already been demonstrated, data about the interaction between this therapy and fracture healing in SCI rats are fairly limited in literature. In view of the aforementioned on bone cell proliferation and *in vivo* bone formation, it was hypothesized that LLLT may accelerate bone tissue metabolism in SCI rats, culminating in the improvement of bone healing. Consequently, the present study

* Corresponding author.

http://dx.doi.org/10.1016/j.jphotobiol.2016.03.041
1011-1344/

aimed to evaluate the *in vivo* tissue response to the LLLT using a standard tibial bone defect model in SCI rats [6,22]. Histology, immunohistochemistry and biomechanical analysis were used to evaluate bone tissue healing.

2. Materials and Methods

In this study 24 female Wistar rats (8 weeks old, weighing between 180 and 200 g) were used. This study was approved by the Animal Care Committee guidelines of the Federal University of São Paulo (450326). The animals were divided into 3 groups (n = 8 per group). Sham group (SG): healthy animals submitted to the tibial bone defect model; SCI control group (SC): SCI animals submitted to the tibial bone defect model without treatment; SCI laser treated group (SL): SCI animals submitted to the tibial bone defect model and treated with LLLT.

2.1. Spinal Cord Injury Surgery

Except for the SG, all animals were anesthetized with ketamine (90 mg/kg) and xylazine (13 mg/kg) and placed over a heating pad to maintain body temperature. After trichotomy, a posterior incision in the thoracic spine (at the T9–T10 level) was performed and the spinous process was removed to expose the spinal cord. Using an ophthalmic surgical scissor, a complete transection of the spinal cord was made. Finally, the skin was closed using staples (Agraven®, Instruvet BV, The Netherlands). To minimize post-operative discomfort, buprenophine (Temgesic; Reckitt Benckiser Health Care Limited, Schering-Plough, Hoddesdon, UK) was administered intraperitoneally (0.02 mg/kg) directly after the operation, and subcutaneously for 5 days after surgery. Manual emptying of the bladder was done twice per day for 7 days after SCI [6,23].

2.2. Bone Defect Surgery

Two weeks after the induction of the SCI, in all experimental animals, bilateral, non-critical size bone defects were surgically created in the upper third of the tibia (10 mm distal of the knee joint). Surgery was performed under sterile conditions and general anesthesia, induced by intraperitoneal injection of ketamine/xylazine (Dopalen/Anasedan, Sespo Indústria e Comércio Ltda., Jacareí, SP, Brazil) (80/10 mg/kg). The medial compartment of the tibia was exposed through a longitudinal incision in the shaved skin. A standardized 1.5 mm diameter bone defect was created by using a motorized drill under copious irrigation with saline solution. Holes were compressed with gauze for 5 min and the cutaneous flap was replaced and sutured with absorbable Vicryl® 5-0 (Johnson & Johnson, St. Stevens-Woluwe, Belgium) polyglactin [9,24].

2.3. LLLT Treatment

LLLT treatment was started 24 h after the induction of the bone defect and was repeated every 48 h for 2 weeks. An AsGaAl laser (Photon Lase III, DMC Equipment, São Carlos, SP, Brazil) was used. LLLT parameters are showed in Table 1. The irradiation was performed at a single point on the skin, over area of the defect, by the punctual contact technique. Animals were euthanized 24 h after the last application of LLLT, by CO_2 suffocation, and both tibias were harvested for analysis. These parameters were chosen based on our group's previous work [23,9].

Table 1
Laser parameters

Parameters	
Wavelength	808 nm (infrared)
Laser frequency	Continuous output
Optical output	30 mW
Spot size	0.028 cm²
Power density	1.07 W/cm²
Energy density	100 J/cm²
Total energy	2.8 J
Time per point	94 s
Application mode	Stationary in skin contact mode

2.4. Locomotor Function Evaluation

Two weeks after the SCI surgery, motor functional behavior was evaluated using the Basso, Beattie, Bresnahan (BBB) scale [25]. The BBB rating scale is a 21-point system based on operationally defined behavioral features to follow up recovery progression the lesion. BBB is used to predict anatomical and behavioral outcomes and provides an evaluation a view after SCI. For this analysis, animals were individually placed on a circular plastic floor where its behavioral recovery was observed and recorded for 5 min. The analysis was performed by two observers (ALYSS and SOVS), in a blinded manner.

3. Analysis

3.1. Histological Procedures

After harvesting, left tibias were fixed in 10% buffered formalin for 24 h, washed in running water for 24 h and subjected to decalcification in 10% solution of ethylene diamine tetra-acetic acid (EDTA) (Merck KGaA, Darmstadt, Germany) for approximately 40 days. After decalcification, specimens were dehydrated in a graded series of ethanol and embedded in paraffin.

For each paraffin block, thin sections (5 μm) were prepared perpendicular to the medial-lateral drilling axis of the implants, using a microtome (Leica Microsystems, SP1600, Nussloch, Germany). At least, three sections of each specimen were stained with hematoxylin and eosin (H.E. stain, Merck KGaA, Darmstadt, Germany) for qualitative and quantitative histological analysis.

3.2. Qualitative Histological Analysis

Sections of each specimen were examined using light microscopy (Leica Microsystems AG, Wetzlar, Germany). Two experienced observers (ALYSS and SOVS) performed the evaluation in a blinded manner. The tibial bone defect area was qualitatively analyzed for the presence of granulation tissue, inflammatory process, newly formed bone and tissue organization.

3.3. Morphometry

All histological sections were quantitatively scored using computer-based image analysis techniques (Axioplan 2, Carl Zeiss, Jena, Germany). The analysis was performed by two observers (SOVS and ALYSS), in a blinded manner. From digitalized images of the defect (×10), the amount of newly formed bone was determined within two regions of interest (0.9 mm^2), i.e.: ROI1 (upper half) and ROI2 (lower half). This method of analysis was established in a previous study conducted by our team (11). The amount of newly formed bone was determined in square micrometers in each ROI and the total newly formed bone was represented as ROI1 + ROI2 (Fig. 1).

3.4. Immunohistochemistry

The expression of RUNX2 and receptor activator of nuclear factor κβ ligand (RANKL) was used to evaluate bone metabolism. Paraffin was removed with xylene from serial sections of 5 μm and the sections were rehydrated in graded ethanol. They were then pretreated in a microwave with 0.01 M citric acid buffer (pH 6) for 2 × 5 min cycles each at 850 W, for antigen retrieval. The material was preincubated with 0.3% hydrogen