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Effect of phototherapy with low intensity laser on local and systemic immunomodulation following focal brain damage in rat

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ABSTRACT

Brain injury is responsible for significant morbidity and mortality in trauma patients, but controversy still exists over therapeutic management for these patients. The objective of this study was to analyze the effect of phototherapy with low intensity lasers on local and systemic immunomodulation following cryogenic brain injury. Laser phototherapy was applied (or not-controls) immediately after cryogenic brain injury performed in 51 adult male Wistar rats. The animals were irradiated twice (3 h interval), with continuous diode laser (gallium–aluminum–arsenide (GaAlAs), 780 nm, or indium–gallium–aluminum–phosphide (InGaAlP), 660 nm) in two points and contact mode, 40 mW, spot size 0.042 cm^2, 3 J/cm^2 and 5 J/cm^2 (3 s and 5 s, respectively). The experimental groups were: Control (non-irradiated), RL3 (visible red laser/ 3 J/cm^2), RL5 (visible red laser/5 J/cm^2), IRL3 (infrared laser/ 3 J/cm^2), IRL5 (infrared laser/5 J/cm^2). The production of interleukin-1IL-1β (IL-1β), interleukin6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α) was analyzed by enzyme immunoassay technique (ELISA) test in brain and blood samples. The IL-1β concentration in brain of the control group was significantly reduced in 24 h ($p < 0.01$). This reduction was also observed in the RL5 and IRL3 groups. The TNF-α and IL-6 concentrations increased significantly ($p < 0.01$ and $p < 0.05$, respectively) in the blood of all groups, except by the IRL3 group. The IL-6 levels in RL3 group were significantly smaller than in control group in both experimental times. IL-10 concentration was maintained stable in all groups in brain and blood. Under the conditions of this study, it is possible to conclude that the laser phototherapy can affect TNF-α, IL-1β and IL-6 levels in the brain and in circulation in the first 24 h following cryogenic brain injury.

1. Introduction

Injury to the central nervous system (CNS) leads to a well-regulated inflammatory response, which is important for tissue regeneration [1–3]. This inflammatory response involves activation of microglia, brain macrophages, and astrocytes; processes likely mediated by the release of inflammatory cytokines. Numerous cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-10 (IL-10) are induced rapidly after acute CNS insults [4,5]. They are expressed in a temporal and special pattern consistent with their involvement in subsequent neuronal death.

IL-1β and TNF-α are known pro-inflammatory cytokines which have been detected in brain tissue of experimentally brain-injured rats [6,7] and in the cerebrospinal fluid of patients with severe brain injuries [8,9]. IL-10, on the other hand, has been found to inhibit expression of IL-1β and TNF-α and therefore has anti-inflammatory effect [10,11]. IL-6 and TNF-α can either enhance or inhibit neuronal injury, probably depending on the time course and extent of expression. These mediators play a vital role not only propagating the neuroinflammatory response, but also serving as useful indicators of its presence [12].

Phototherapy with low intensity laser has shown favorable results on the inflammatory response modulation [13–17]. The laser phototherapy modulates various biological processes by increasing mitochondrial respiration and ATP synthesis, facilitating wound healing and promoting the angiogenesis process in injured ischemic organs [18–20]. The laser phototherapy has been presented as a new modality with beneficial effects in treatment of long-term incomplete peripheral nerve injury [21 and reviewed in 22]. Moreover, it was demonstrated that the transcranial laser phototherapy applied after embolic stroke in rats [23] caused significant improvements of neurological scores. More recently, studies have

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demonstrated that laser phototherapy in brain traumatized by a weight-drop device caused a significant long-term functional neurological benefit to mice [24]. On the other hand, laser phototherapy in brain after cryogenic injury caused an increase in the cerebral vascular permeability [25] which could be deleterious for the brain regeneration. Then, although the laser phototherapy emerges as an alternative or auxiliary therapy for traumatic injuries on peripheral as well as central nervous system tissues, its effects are still controversial. Thus, the objective of this study was to analyze the effect of phototherapy with low intensity lasers after cryogenic brain injury on the production of pro- and anti-inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6 and IL-10).

2. Material and methods

2.1. Sampling

Fifty-one adult male Wistar rats (*Rattus norvegicus albinus*) weighting an average of 250–350 g were used. All animals were kept under constant temperature and with free access to food and water. All experimental procedures were approved by the Ethical Committee of the School of Medicine, University of São Paulo.

2.2. Cryolesion and phototherapy with low intensity laser procedures

The brain cryogenic trauma was carried out as previously described [26] with modifications. Fig. 1 illustrates the brain cryogenic trauma and laser phototherapy experimental procedures. Briefly, the animals were injured under anesthesia with a mixture of Ketamine (0.15 mg/kg, i.p.) and Diazepam (5 mg/kg, i.p.). The anesthetized animals were fixed on the stereotaxic frame (Fig. 1). The injury site coordinates corresponded to the C17-motor region according to Paxinos and Watson [27]. The skull above the left frontoparietal cortex was exposed (Fig. 1A and B) and a focal cryoinjury on the surface of the brain was produced with liquid nitrogen at −80 °C. The cryogenic injury was done in standardized conditions, as follows: a cylindrical copper probe (7.06 mm diameter) was immersed into liquid nitrogen for 90 s and then applied on the top of the duramater during 40 s (Fig. 1C and D).

The laser phototherapy was applied on two points at the injured site immediately after lesion according to the experimental groups. The laser irradiation was done in contact. For controlling the aseptic conditions prior to the irradiations the laser handpiece was cleaned with 70% alcohol solution and was coated with a physical barrier (thin translucent plastic film) (Fig. 1E). Non-irradiated lesions were used as controls. After irradiation, the injured sites (irradiated and non-irradiated) were coated with bony wax (Fig. 1F) and the skin incisions were sutured. The animals were kept at 20 °C and then returned to their cages. Three hours later, under gentle restriction, without anesthesia, the animals received a second irradiation. This irradiation was done in contact to the rat skin immediately over the lesion site. The rationale to perform two irradiations resides on the efficacy improvement of the phototherapy with low intensity laser when applied in a fractioned approach [28]. Control animals were submitted to the same environmental conditions of irradiated animals; however, the laser equipment was kept off.

Laser irradiation was delivered with semiconductor diode laser equipment (MM Optics Ltda, São Carlos, SP, Brazil) using the parameters described at Table 1. The LaserCheck power meter (Coherent, Inc., Santa Clara, CA) was used to verify the output power of the laser equipment.

The rats were sacrificed at two time points: 6 and 24 h postlesion. Immediately after sacrifice samples of blood were collected from the heart of the animals by using a syringe. The blood was centrifuged (300*g* for 5 min at 4 °C), and then the supernatant was collected. The ipsilateral brain cortex containing the injured site (Fig. 1G) was dissected and both, blood supernatant and brain tissue were frozen in liquid nitrogen and stored at −80 °C for later cytokines production analyses using the Enzyme-Linked Immunosorbent Assay (ELISA).

For illustrative purposes the brain of one rat was fixed in 10% formalin for obtaining light microscopic images of the injured site.

2.3. Experimental groups

After brain cryogenic trauma the animals were randomly divided into five groups (n = 10 animals per group):

- Control: no laser irradiation.
- RL3: 660 nm laser irradiation, 3 J/cm^2 per point.
- RL5: 660 nm laser irradiation, 5 J/cm^2 per point.
- IRL3: 780 nm laser irradiation, 3 J/cm^2 per point.
- IRL5: 780 nm laser irradiation, 5 J/cm^2 per point.

2.4. Elisa

The rat injured brain tissue was assayed twice using a quantitative sandwich enzyme immunoassay technique. The Human IL1-β (DY501), TNF-α (DY510), IL-6 (DY506) and IL-10 (DY522) ELISA kits (DuoSet from R&D System, Minneapolis, USA) were used following the manufacturer's instructions. The absorbance was read in a micro plate reader (Genious Reader, TECAN Salzburg – Austria) using a 450 nm filter.

2.5. Data analysis and statistics

The experiments were done in five replicates for all treatments. The concentrations of all cytokines in pg/ml are presented as mean ± standard error of the mean (SEM). The data were compared by two-way ANOVA complemented by the Tukeýs test or using the Kruskal–Wallis complemented by the Dunn's test. The level of significance was 5% ($p \leqslant 0.05$).

3. Results

The diagram of Fig. 2A illustrates the brain cryogenic trauma site. Histologically, the cryogenic trauma caused a focal lesion at the ipsilateral brain cortex represented by an apparent pale core region surrounded by a homogeneous eosinophilic stained tissue (Fig. 2B). There was a marked edema with tissue vacuolization at the core lesion associated to minor bleeding and congestion of cortical blood vessels (Fig. 2C). Neutrophil polymorph nuclear leukocytes were present at the site of the cryogenic lesion 6 h after injury (Fig. 2C).

TNF-α, IL-1β, IL-6 and IL-10 were detected in the brain and in the blood of all animals. The mean concentrations (pg/ml) of all cytokines studied (e.g. TNF-α, IL-1β, IL-6 and IL-10) in the brain and in the blood are presented in Tables 2 and 3, respectively.

The amounts of all cytokines in the brain samples of irradiated groups (RL2, RL5, IRL3 and IRL5) were similar to those detected in the rat cryo-injured brain with no further treatment (control group). The only difference in cytokines amounts between control and irradiated groups was observed in the blood. The concentrations of IL-6 in the RL3 group were significantly smaller than those of control samples at both experimental times ($p < 0.05$). The blood of the remaining irradiated groups presented concentrations of all cytokines similar to those of control samples.