Commentary Article

It Medical Team
https://www.itmedicalteam.pl/

Health System and Policy Research
2254-9137

2022
Vol.9 No. 10: 151

Comparison between the Irradiation Days and the Irradiation Daily Dose of Photobiomodulation Therapy (660nm) in Improvement of Spinal Cord Injury Complications

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Abstract

Objectives: Spinal cord injury (SCI) causes motor deficits, urinary incontinence and neuropathic pain. This study has been accomplished to provide a protocol for photobiomodulation therapy (PBMT) to gain a significant reduction in complications of the SCI. To date, it has not yet been determined whether a more prominent effect would be achieved by increasing the number of days of irradiation or daily dose of PBMT.

Methods: The study was performed in two steps. In the first step, comparison between the effects of PBMT (90sec), separately for two and four weeks on pain, and also improvement of movement (BBB score) was made. In the second step, the comparison to evaluate the effects of different durations of radiation (27, 45, 90 and 117 seconds) was done. Also, Oxidative stress and fibroblast invasion and time to gain spontaneous of urination were assessed in the both steps.

Results: Healing process in movement and pain stopped with discontinuation of radiation on week2 and fibroblast invasion resumed, on the other hand, no improvement was seen in the group receiving PBMT for 27 seconds compared to the ones receiving higher durations of laser radiation as the process of discernible improvement of movement and pain, so, that animals receiving 117s PBM have a higher BBB score even in the first 3days.

Conclusion: Number of radiation days is an important factor in improving the mobility; however, the daily dose of radiation is more essential in pain relief.

Keywords: Photobiomodulation therapy; Spinal cord injury; Motor function recovery; Hyperalgesia; GPX; SOD; MDA

Received: 03-October-2022, Manuscript No. Iphspr-22-12286; **Editor assigned:** 07-October-2022, PreQC No. Iphspr-22-12286; **Reviewed:** 18-October-2022, QC No.Iphspr-22-12286; **Revised:** 24-October-2022, Manuscript No. Iphspr- 22-12286 (R); **Published:** 31-October-2022, DOI: 10.36648/2254-9137.22.9.151

Introduction

Spinal cord injury (SCI) leads to paraplegia and chronic pain, also other side effects such as urinary incontinence, and depression which interfere with patient's quality of life [1]. Urinary incontinence is a common and painful problem after SCI that affects around half of the suffered population, beside to impaired mobility, difficulty to sex, and inability to perform personal task,

Citation: Riz AN, Ramezani F, Keihan, Kookli, Fazeli SM, et al. (2021) Comparison between the Irradiation Days and the Irradiation Daily Dose of Photobiomodulation Therapy (660nm) in Improvement of Spinal Cord Injury Complications. Health Sys Policy Res, Vol.9 No. 10: 151.

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lead to poor quality of life[2, 3].

Despite a great deal of research, no gold standard treatment for SCI has yet been established, and little overall success has been achieved in improving the functional recovery. Such as Ultra-early surgical interventions, including spinal cord decompression and spine fixation, which is routinely performed, until now[4].

It seems that SCI causes such severe damage to axons and blood vessels that the ability to recover is limited. The pathophysiology of SCI is considered to involve an initial injury (i.e. the primary phase) followed by a secondary phase in which oxidative stress and inflammation are critical components [5,6]. Also, consists of tissue swelling from hemorrhage and edema, inflammation, cytotoxic free radical and excitotoxicity substance generation, and excessive gliosis, which takes hours to months to develop [7]. One of the best validated secondary injury mechanisms in acute SCI concerns the post-traumatic generation of reactive oxygen species (ROS) and their resulting oxygen free radical-induced oxidative damage that result in axonal degeneration, glia scar and cavity formation [8].

All of this phenomenon occurs in a short time [9]. So in addition to selecting the appropriate type of treatment, the initiation time and duration of treatment is also very important.Variety of treatments have been proposed to use, yet none has been efficiently controlled neither the short-and long-term complications of the SCI [10, 12]. Among several other separate or adjuvant treatments, Photobiomodulation therapy (PBMT) has also been introduced which is the topic of discussion in this study which is a treatment that has been widely used in neurotrauma, neurodegenerative diseases and pain relive [13,14]. PBMT accelerates electron transport, ATP synthesis, oxygen consumption, membrane potential, and enhanced synthesis of NADH. It has been reported that PBMT is able to reduce neutrophil accumulation and edema after injury, through mechanisms that involve down-regulation of pro-inflammatory mediators, such as TNFα and IL-1β, as well as up-regulation of anti-inflammatory mediators, such as IL-10 and TGFβ. PBMT is able to stimulate the mitochondria, due to photon absorption by biological chromophores (e.g. cytochrome c oxidase) and nanostructured water. Mitochondria are involved in the production of ROS and in oxidative stress [15].

Therefore, the measurement of ROS and biomarkers of antioxidants and oxidative stress, such as glutathione peroxidase (Gpx), superoxide dismutase (SOD) and malondialdehyde (MDA), can be used to assess mechanisms and effects of SCI recovery.

The aim of the present study is to settle the dilemma whether the number of days of radiation or the duration dose of radiation would be the key element for improving the hyperalgesia or/ and the movement recovery. Also, CNS damage leads to scar formation. A glial scar is like a double-edged sword. On the one hand, it prevents the spread of lesions around, and on the other hand, it prevents the growth of axons. In fact, the site of injury can be classified into "glial" and "fibrotic". In animal studies, fibrotic scarring is more observed in penetrating lesions, which can rupture the dura and allow meningeal fibroblasts to invade the lesion site. The entry of fibroblasts into the lesion site follows infiltration of immune cells and inflammation. So, this study was designed in a way to comprehend not only the effect of each of these treatment protocols on antioxidant status, tissue repair, but also on fibroblast invasion.

Materials and Methods

Experimental design and animals

In this study, all experiments were performed on adult male Wistar rats (170-200 g). Efforts were made to minimize the number of animals in the experiment. The proposal with ethical code (IR.IUMS.REC 1397.31954) was approved by the IACUC of the Iran University of Medical Science, Tehran, Iran. Also, animal experiment was performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 or the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24 November 1986 (86/609/EEC). Rats were housed under controlled conditions (23 ± 2°C, 12 h light/dark cycle) with access to food and water ad *libitum*. The study was designed in two steps: In the initial step, assessment of effectiveness of 2- and 4-weeks of PBM radiation in improving movement and reducing pain. The best time was chosen, and in the second step, in which the effect of different PBM radiation doses (27, 45, 90 and 117 seconds) on pain and movement was evaluated. Finally, oxidative stress levels and tissue changes were also studied. 56 rats were randomly divided into 8 groups as follows: (1) Control group (n=6) intact animals; (2) SCI group (n=7) with spinal cord injury induced; (3) SCI+2 weeks of PBMT (n=6), starting 30 min after SCI induction, which underwent laser treatment 45 sec daily for two weeks.; (4) SCI+4 weeks of PBMT (n=6), starting half an hour after the SCI induction, which underwent laser treatment 45 sec, daily. (5) SCI+4 weeks of PBMT (n=6) for 27 sec (3 sec at each point), starting 30 min after SCI rats received PBMT daily (6) SCI+4 weeks PBMT (n=6), starting 30 minutes after SCI with 45 sec each day (5 sec at each point); (7) SCI+4 weeks of PBMT for 90sec (n=6) daily (10 sec at each point). (8) SCI+4 weeks of PBMT for 117sec (n=6) daily (13 sec at each point).

Spinal Cord Injury Induction

Animals were anesthetized by an intraperitoneal (IP) injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) and the spinal cord surgically exposed. The compression SCI model was performed at the level of T13-L1 of the spinal cord. To provide compression model of SCI, using an aneurysm clip (FST Company, Foster City, CA) power applied for 90 secs to deliver a force equal to 20 g/cm2. The muscles and skin were sutured separately. Post-surgery care included: Ringer's solution for prevention of dehydration (3 mL, IP, after surgery), penicillin G for 3 days after surgery (8 mg/100 g, IP), and bladder massage twice daily until animals can urinate spontaneously.

Photobiomodulation Therapy (PBMT)

In the present study, a diode CW laser was used with the parameters shown in (**Table 1**). Thirty-minutes after SCI induction (removal of the aneurysm clip), PBM irradiation was performed

Table 1: Laser Parameters.

Manufacturer	Heltschl Medizintechnik, Schlusslberg, Austria
Model identifier	Heltschl Medizintechnik, Schlusslberg, Austria
Year produced	2011
Number and type of emitters (laser or LED)	1 laser diode
Wavelength and bandwidth (nm)	660nm, 2 nm
Pulse mode (CW or Hz, duty cycle)	CW
Beam spot size at target (cm2)	0.197 cm^2
Total power (mW)	100 mW
Irradiance at target (mW/cm2)	500 mW/cm^2
Exposure duration (sec)	27, 45, 90, 117 sec
Radiant exposure (J/cm2)	13.7, 22.8, 45.6, 60 J/cm^2
Radiant energy (J)	2.7, 4.5, 9, 11.7 J
Number of points irradiated	9
Area irradiated (cm2)	1.5-2 cm^2
Application technique	at distance
Number and frequency of treatment sessions	28 sessions (daily for four weeks)
Total radiant energy over entire treatment course (J)	27, 126, 252, 327

on the lesion site, and at 8 points surrounding the injury site 5 mm away in each direction.

PBMT performed with two protocols: First protocol, the difference between the effects of two and four weeks of radiation was investigated and compared. The animals received 45 seconds of laser treatment daily.

Second protocol, the effect of different daily radiation times (3, 5, 10 and 13 seconds) (overall times 27, 45, 90, 117 seconds) for 4weeks was investigated and compared.

(Overall times 27, 45, 90, 117 seconds) for 4weeks was investigated and compared.

Behavioral Assessment

Functional Recovery assessment

Following surgery, the Basso Beattie and Brenham (BBB) test was assessed weekly to evaluate the motor function in a blinded manner for seven weeks. Each animal was allowed to freely move in an open field of 90 cm diameter, a wall height of 24 cm for 4 min. In groups that received different doses of laser daily, evaluation and scoring of movements began 24 hours after SCI induction and were repeated every day until the third day. The study was performed weekly until the end of the fifth week. Two independent observers scored separately based on the BBB scoring table scores 0 (complete paralysis) to 21 (normal walking) {Ferguson, 2004 #247}. The average score given by two observers was recorded as the main score.

Mechanical Hyperalgesia Evaluation (Pinch Test)

For hyperalgesia pain assessment, an Ugo Basile Analgesia Meter (Ugo Basile, Varese, Italy) apparatus was used. Mechanical stimulation was done with a weight connected to a lever. The increasing pressure produced by the pushing lever was determined using a ruler connected to the device. When the animal tried to respond to the applied pressure by removing its hind paw, the pressure increase would stop and the amount of pressure that led the animal to respond was recorded. This test was carried out twice on each leg with at least 5-min time intervals, and the mean of the results was recorded.

Urinate control spontaneously assessment

Time gained the ability of each animal to urinate spontaneously was recorded separately and reported.

Histological evaluation

H & E staining was performed for assessment of fibroblast invasion. After staining, digital images were captured (Olympus, magnification ×40).

Assays for MDA, Gpx and SOD measurement

Five weeks after the surgery, 3 animals from each group were randomly selected. After deep anesthesia as described above, the lesion sites were surgically re-exposed, and the injured sections were carefully removed.

The harvested tissues were quickly frozen and the samples were homogenized in Ripa buffer to prepare a supernatant. Glutathione peroxidase activity in the spinal cord tissues was determined using a Gpx ELISA kit (ZellBio GmbH, Ulm-Germany). The MDA Assay Kit (Zellbio Co, Germany) was used to measure malondialdehyde, a lipid peroxidation (LPO) marker. SOD assay kit (Zellbio Co, Germany) was used to measure SOD activity by converting superoxide anion to hydrogen peroxide and oxygen under enzymatic reaction conditions.

Statistical Analysis

Data were analyzed using Prism software version 6 and presented as mean and standard error. To compare the collected data from the behavioral assessments of different groups, two-way ANOVA analysis was used to Tukey follow-up test and one-way ANOVA analysis was used to evaluate the findings of ELISA test. In all analyses, $P < 0.05$ was considered significant.

Results

Motor function recovery

Motor function measured by BBB score was significantly reduced

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in all SCI groups compared to the control group rats (df: 7.319; F = 35; n=8; p < 0.001). In **Figure 1A**, in comparison between 2- and 4-weeks of PBMT, it was observed that the improvement of motor function recovery process depends on number of days of irradiation. In the third and fourth weeks, there is a significant difference between the two groups (P<0.01). As **Figure 1B** shows, during the first 3 days, the BBB score of SCI and 27sec PBMT group were almost the same as the SCI group without PMBT.

All groups had a noticeable difference compared to the control group (p<0.001). PBMT of group 90 sec and also 117 sec showed a higher BBB scores than PBMT of group 27 sec and 45 secs, on day 2, p<0.05 and day 3, p<0.001.

Figure 1C shows that during this 5-week study, no motor recovery was observed in animals receiving 4 weeks of 27 seconds of daily radiation compared to the SCI group. However, in the other groups, significant improvement of motor activity was observed. Altogether, a significant difference between the PBMT of 27sec group and the other groups was seen (p<0.001). Yet, there was no significant difference between motor activity of the PBMT of 45, 90, and 117sec groups.

Mechanical Hyperalgesia

Induction of spinal cord injury reduced the mechanical pain threshold compared to the control group (p<0.001) (**Figure 2A**). PBMT reduced the pain intensity for 2-wPBM and 4-wPBM groups, compared to the SCI group (p<0.001). However, the difference was observed compared to the control group until the end of the study (p<0.001). Pain intensity did not differ between 2 and 4-week PBM groups (**Figure 2B**). In groups, receiving 90 and 117 sectonds of PBM per day, pain decreased from week 3 and

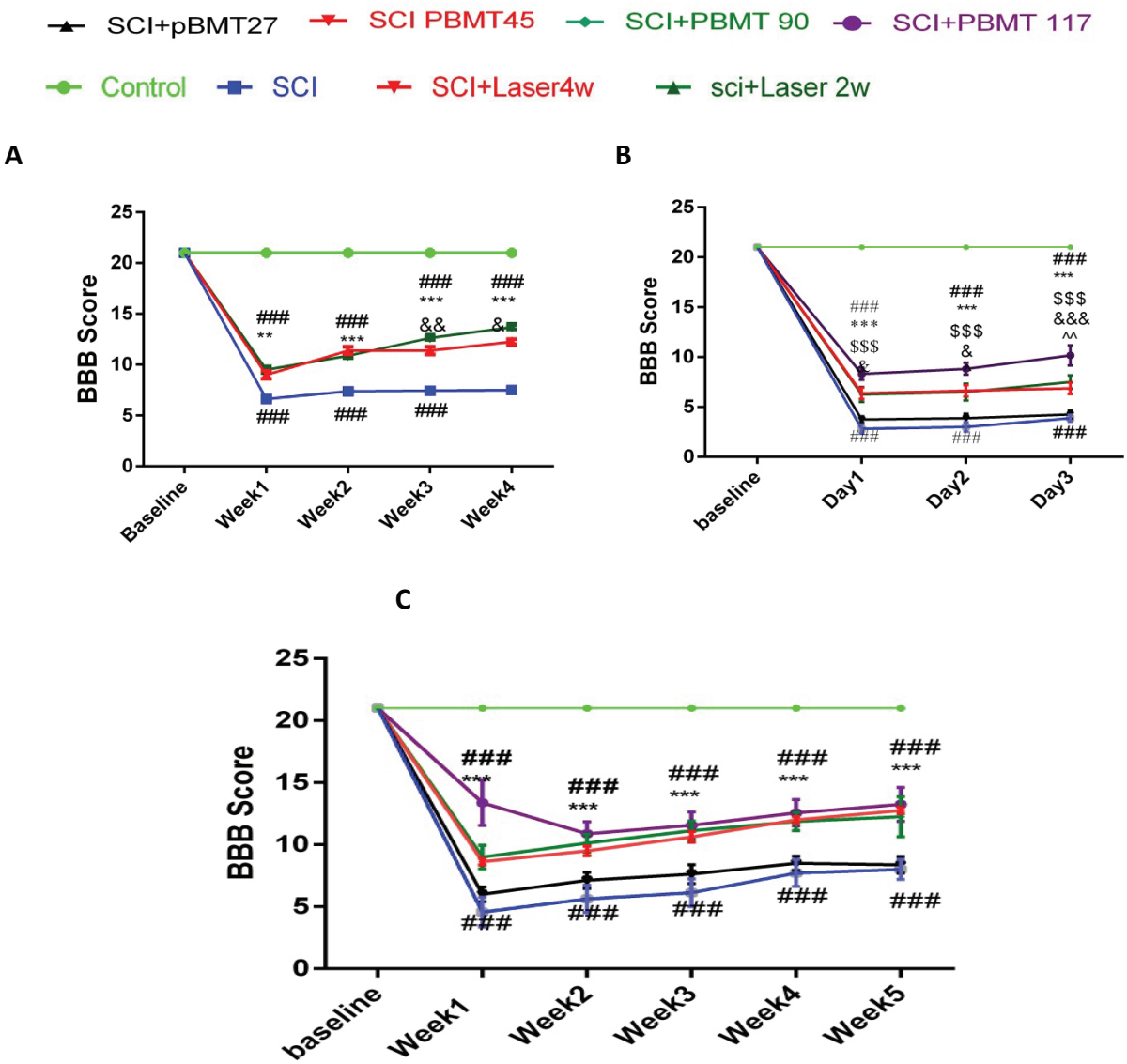

Figure 1 Effect of Photobiomodulation therapy (PBMT) on functional recovery (Basso Beatty Bresnahan (BBB) test). A) Compression 2weeks and 4weeks PBMT.B) Compression different irradiation times (27, 45, 90 and 117 sec) for 3 day) Compression different irradiation times (27, 45, 90 and 117 sec) weekly for 5 weeks. Data are expressed as the mean ± SEM.

showed the same result as the control group (**Figure 2C**).

Urination Control

Induction of spinal cord injury caused inability to urinate spontaneously (P<0.01). In rats, it sometimes takes 2 weeks or more to get the ability to urinate spontaneously. Urination control is regulated by a complex neural system in the brain and lumbosacral spinal cord. Following SCI, bladder is initially flaccid, but later, becomes hyper reflexive due to the injury of the reflex pathway by the SCI. Animal studies show that improved bladder function after SCI depends on part on the plasticity of the bladder afferent pathways. PBMT significantly reduced the number of days required for bladder massage compared to the SCI group (p<0.001). However, this improvement was observed significantly less in animals treated with PBM for 27 seconds per day. The difference with SCI was P <0.05 and with the control group was P <0.01.

Glutathione peroxidase activity

Glutathione peroxidase (GPX) levels decreased sharply in week 5 of SCI induction (df: 51; F = 12; n = 3; p <0.01). No difference in glutathione level was observed in comparison between groups receiving 2 and 4 weeks of PBMT. However, the level increased compared to the SCI in these groups (P<0.01) (**Figure 4A**). In PBM group 27sec, Gpx activity showed no significant increase at the end of the study compared to the control group (p <0.05). An increasing trend was observed among the other groups. The PBM 117 sec group showed a significant difference compared to the SCI and PBM 27 groups (**Figure 4B**) and the Gpx activity was as high as the control value (**Figure 4C**).

Superoxide dismutase activity

The results showed that SOD activity decreased significantly after SCI surgery compared to the control group (p<0.05). There was a difference in the 2-week PBM group compared to the control

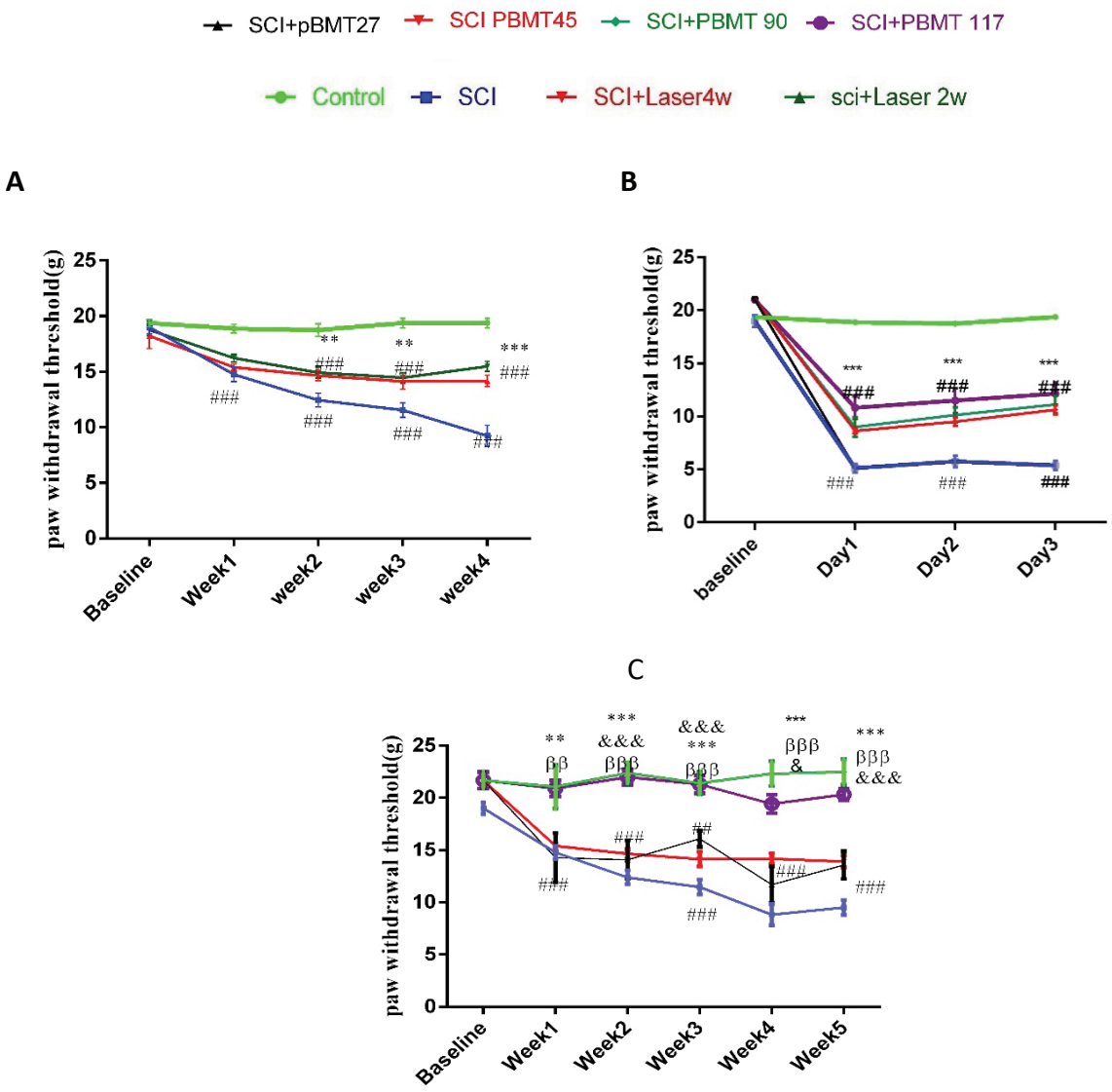

Figure 2 Induction of spinal cord injury, Pain intensity, PBM per day.

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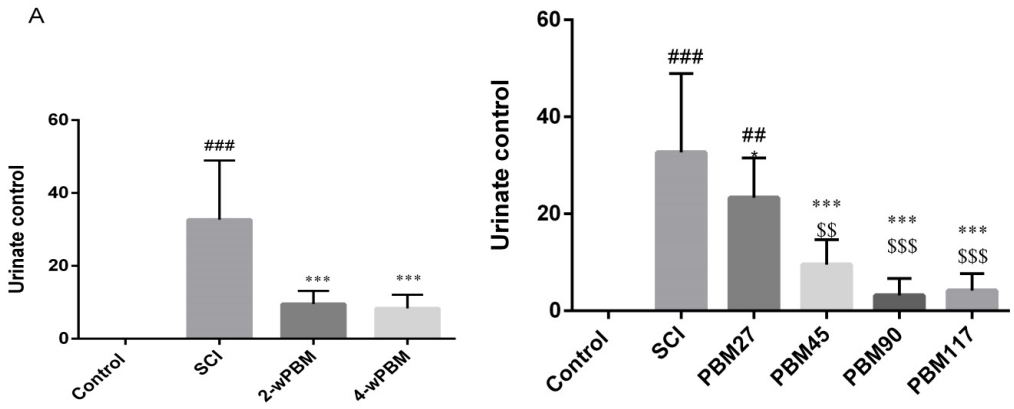

Figure 3 Evaluation and effect comparison of A) 2 and 4 weeks of photo-bio modulation therapy(PBMT) and B) durations of daily PBMT (27,45,90 and 117 seconds) radiation on obtaining the ability of urination control spontaneously. *** p < 0.001 versus SCI group; $$ p<0.01 $$$p<0.001 versus PBM27; ### p < 0.001 versus control group.

group) p<0.05), but in week 4 the difference was less observed (**Figure 4D**). In 27 sec group, SOD level was also significantly lower than the control group (p <0.05) (**Figure 3A**). SOD levels in 45, 90 and 117sec PBM groups were not significantly different from the control group (**Figure 3B**).

Malondialdehyde (MDA) activity

MDA used as an indicator of oxidative stress increased five weeks post SCI (p<0.05). The level of MDA at week 2 and 4 were not considerably different from the SCI group. Results in the 27sec PBM group showed MDA was higher compared to the control group (p<0.05) and was almost the same as the MDA level in SCI group. MDA activity levels in the PBM treated groups (45, 90, 117 sec) showed a decreasing trend (**Figure 4 E&F**), but values were not significantly different from the SCI group. However, these values were also not significantly different from the mean control value neither.

Fibroblast Count

The results showed that 2 weeks of PBM irradiation did not prevent the invasion of fibroblasts and a significant difference was observed with the control group (p<0.001). Also, 4 weeks of 27 seconds of PBM radiation per day, does not seem to be an adequate duration to reduce or prevent the invasion (p<0.001). Yet, 4 weeks of 45, 90 and 117 seconds per day of PBM irradiation prevented the invasion of fibroblasts. Differences were observed compared to the SCI and 27sec groups. (p<0.001) (**Figure 5A-C**).

Discussion

The results of this study showed that the compression model of SCI led to antioxidant activity (Gpx and SOD) reduction and to MDA level elevation (a well-known marker of oxidative stress). Simultaneously, the motor function in the animals was also greatly reduced. Mechanical hyperalgesia pain and urinary incontinence were also observed.

PBMT had a noticeable effect on functional recovery improvement. Both elements of concern as mentioned earlier i.e., days of radiation and the duration of daily radiation seem to be important in improving movement. On the grounds that after two weeks of PBMT, the healing process stopped with discontinuation of radiation, on the other hand, no improvement was seen in the group receiving PBMT for 27 seconds compared to the ones receiving higher durations of laser radiation as we already have demonstrated in Fig1B the process of discernible improvement of movement in the first 3 days, so that animals receiving 117s PBM have a higher BBB score.

It is important to mention that the type of radiation in that study was continuous 810-nm but their results similarly pointed that Photobiomodulation Promoted the Recovery of Motor Function, Reduced the Lesion Cavity Size, and Increased the Number of Surviving Neurons after Spinal Cord Injury.

The relationship between oxidative stress in the injured spinal cord and motor function has not yet been firmly established. There have been some studies that have used various antioxidant approaches such as tetrahydrocurcumin polydatin and coQ10 to improve motor dysfunction in SCI. The experimental data of this research reinforce the probability of association between the functional recovery and oxidative stress level after an SCI. In the SCI group, the motor function, pain threshold and urination control decreased as expected, also, antioxidant markers (Gpx and SOD) that have been shown functioning as neuroprotective substances decreased. Lack of urine control is a big problem that patients suffer from after SCI. Present study indicated, PBM radiation is also helpful to return, the ability of urine control. However, in case of urination control, the role of the dose (duration of radiation) received per day was more important. Daily Using PBM radiation of 90 and 117 seconds led to a faster period for rats to achieve intentional urination.

A study by Cevik et al. found that SCI causes oxidative stress damage in the bladder because the activity of oxidants such as MDA and nitric oxide increases, while levels of antioxidants such as Gpx and SOD decrease.

One way to counter the oxidative stress species, produced in SCI, is increment in Gpx which acts as an antioxidant and is able to

remove H2O2, one of the members of ROS system, by its reduction to water. In 2016, Janzadeh et al showed that GPX increased after PBMT. This rise possibly indicates the improvement function of PBMT on mitochondria.

In order to maintain normal cellular function, equilibrium between pro-oxidants and antioxidants is important. Therefore, a successful treatment for SCI should be able to maintain this balance as well as being able to improve motor function, pain relief and also, urinary control. The pathological processes occurring after SCI are complex and multifunctional. Considering the secondary phase of SCI, It has already been shown that the PBMT can exert pronounced anti-inflammatory effects after SCI. As well as after many other diseases and injuries.

It has been shown that anti-oxidative stress markers at levels below the normal range (GPX or/and SOD), are well-established to reflect inflammation in many physiological situations. These markers have been shown to be improved by PBMT delivered to animal models of wound healing muscle injury high intensity exercise and traumatic brain injury

Consistent with these studies, our results showed that PBM irradiation increases antioxidant levels of GPX. In case of the GPX, the daily dose of radiation was more important. The 2-week PBMT and PBMT of 27sec did not prevent the reduction of antioxidants. The same trend of increment was seen when SOD was evaluated,

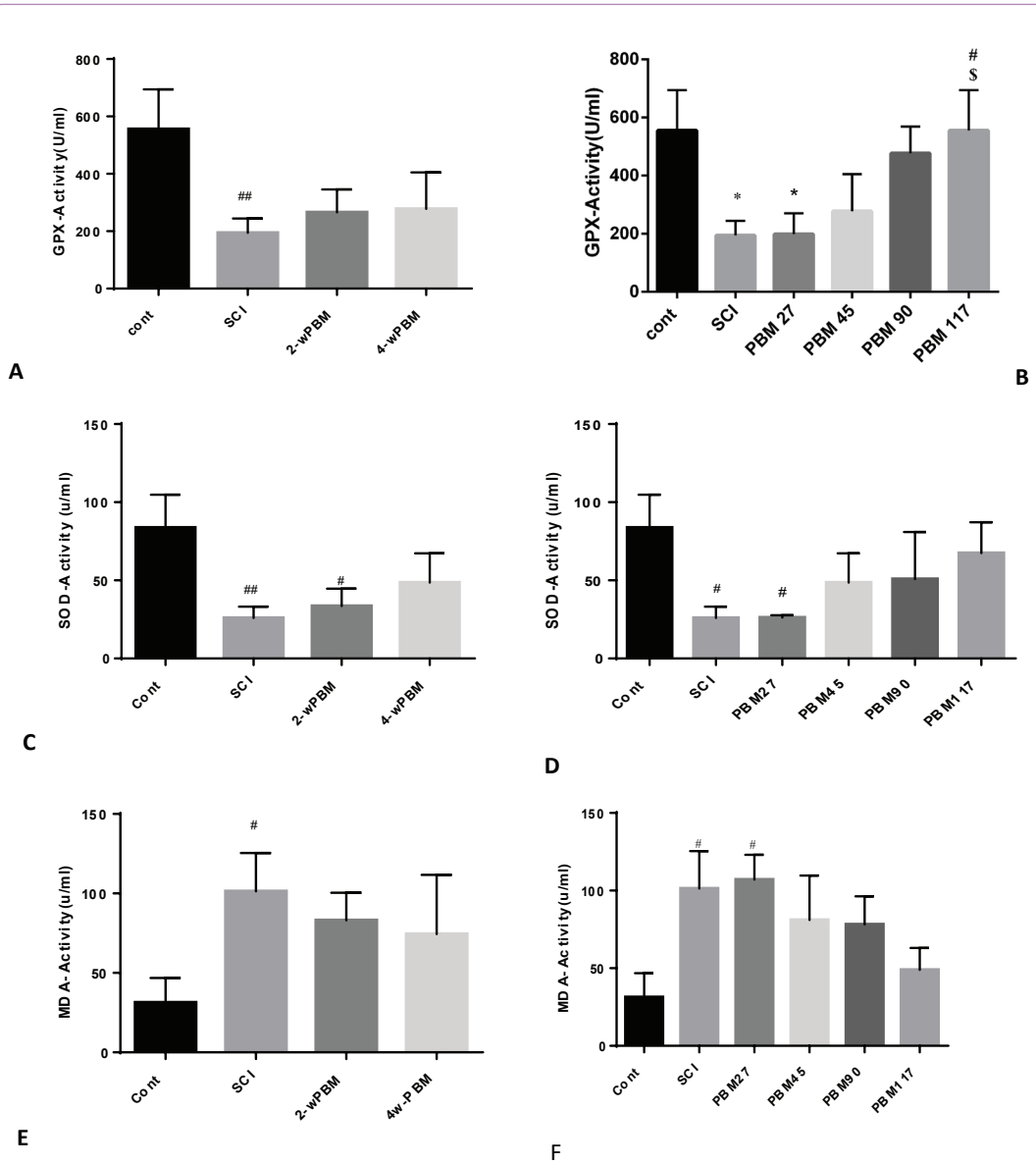

Figure 4 The effect of Photo Bio-modulation therapy(PBMT) on oxidative stress index in an animal model of spinal cord injury(SCI).A) Glutathione) GPX)2 and 4 weeks PBMT;B) Glutathione(GPX) at different times of PBMT(27,45,90 and 117 second);C) Ssuperoxide Dismutase) SOD)2 and 4 weeks PBMT; D) Superoxide Dismutase) SOD)at different times of PBMT(27,45,90 and 117 second);E) Malondialdehyde (MDA) 2 and 4 weeks PBMT;F) Malondialdehyde (MDA) at different times of PBMT(27,45,90 and 117 second). * p<0.05 versus SCI group; # <0.05p < 0.01 versus control group; $ versus PBM27.

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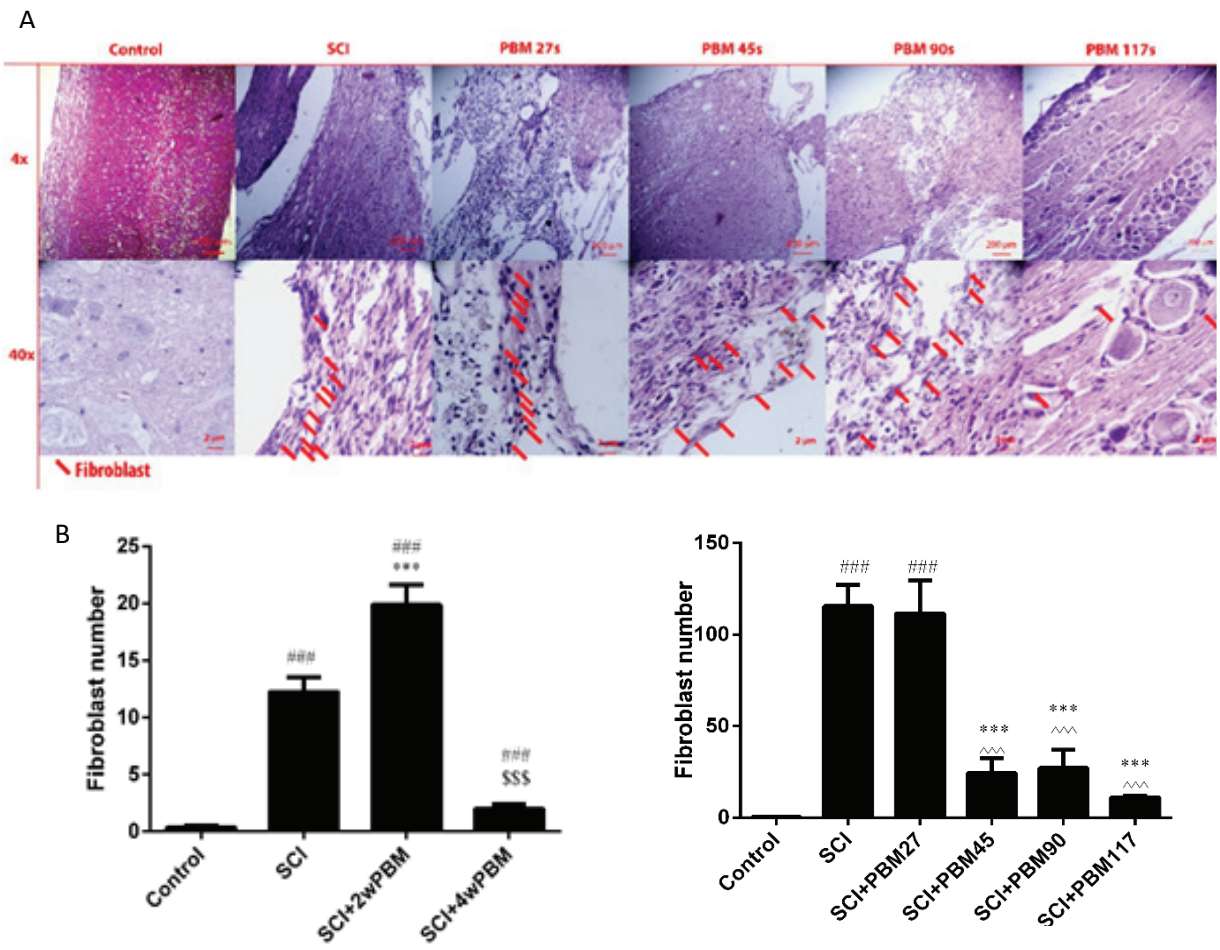

Figure 5 PBM irradiation an adequate duration to reduce or prevent the invasion.

however with a milder slope.

MDA is a well-known example of the lipid peroxidation. These reactive lipid by-products can damage cellular fluidity and permeability, alter the function of membrane-associated proteins, and bind to and inactivate other molecules including amino acids, proteins and nucleic acids. Glutamate excites toxicity. Over excitation of neurons in SCI and chronic pain attributes largely to the activation of glutamate receptors and pain. As we reported hyperalgesia and allodynia pain after SCI in this study.

Lipid peroxidation and oxidative stress has been proposed to play a role in the production of the glial scar, which is implicated in the failure of neural regeneration, pain, low BBB score.

The higher doses of PBMT started to become effective in a dose-dependent manner, specifically with the highest dose (117 sec or 11.7 J/day), causing not only the best improvement in motor function and pain, also a significant reduction in oxidative stress biomarkers. Also, with the effect of PBMT 117 on Gpx reaching statistical significance compared to SCI or PBMT of 27sec. Intermediate doses of PBMT showed a linear trend in improving oxidative stress markers, to the extent that they were no longer significantly different from control uninjured rats.

In assessment of MDA as an oxidative stress biomarker, although no significant reduction in 2 and 4 weeks of radiation and also different durations of PMBT was seen, yet a mild decreasing trend was observed as the duration of PMBT raised. Contradictory results of previous studies have shown that PBM can increase the rate of ROS production in some circumstances, while in other circumstances ROS and oxidative stress is reduced by PBM and antioxidants are increased.

SCI process is characterized by the production of a wide range of inhibitory factors that prevent axonal regeneration, as well as absence of other factors that promote regeneration. This imbalance has been termed “the microenvironment imbalance of SCI”.

As mentioned earlier, fibroblasts play an important role in scar formation causing prevention of axonal regeneration, and disability in movement. Therefore, reducing the entry of fibroblasts can be used as a strategy to help axonal renewal and repair. The PBMT is thought to assist preserving the dura intact by reducing inflammation and preventing fibroblasts from entering. Our study showed that number of days of radiation is as important as the duration of PBMT radiation each time. Scar-associated fibroblasts may be a source of stress-induced inflammatory exacerbations and pain. Inflammatory cytokine such as IL-1α–NF-κB–CCL2 signaling pathway, operating within

scar-associated fibroblasts, may be are involve for inflammation and chronic pain in fibrotic Scars.

In 2-week PBMT, high influx of fibroblasts and low threshold of pain and low BBB score were observed, probably due to fibrosis of the lesion site and inhibition of axon entry and repair after PBMT stooping. In other words, more days of radiation along with longer duration of radiation are both needed to prevent fibroblast entry.

In the PBMT of 27 sec group the BBB and urination control were similar to the untreated SCI group as were the oxidative stress markers. A low dose of PBMT (27 sec or 2.7 J/day) was clearly insufficient to produce any benefit to restoring motor function, pain reduction, urinary control, fibroblast invasion or to ameliorate the oxidative stress.

Conclusion

This research aimed to state that a proper adjuvant therapy with an appropriate protocol could help the routine decompression surgery and other emergent therapies to reach a higher level of recovery if used in medical practice. As 660 nm PBMT as a treatment was in line of our expectation in establishing an oxidative balance, controlling the urinary incontinence, reducing fibroblast entry, and improving movement and pain, practitioners should consider usage of the PBMT in medical and surgical emergencies. Based on this study we speculate that major complications of SCI can reduced by PMBT as the number of radiation days has been showed more important in improving the movement and the daily radiation dose in relieving the pain.

Conflict of Interest

MRH declares the following potential conflicts of interest. Scientific Advisory Boards: Transdermal Cap Inc, Cleveland, OH; BeWell Global Inc, Wan Chai, Hong Kong; Hologenix Inc. Santa Monica, CA; LumiThera Inc, Poulsbo, WA; Vielight, Toronto, Canada; Bright Photomedicine, Sao Paulo, Brazil; Quantum Dynamics LLC, Cambridge, MA; Global Photon Inc, Bee Cave, TX; Medical Coherence, Boston MA; NeuroThera, Newark DE; JOOVV Inc, Minneapolis-St. Paul MN; AIRx Medical, Pleasanton CA; FIR Industries, Inc. Ramsey, NJ; UVLRx Therapeutics, Oldsmar, FL; Ultralux UV Inc, Lansing MI; Illumiheal & Petthera, Shoreline, WA; MB Lasertherapy, Houston, TX; ARRC LED, San Clemente, CA; Varuna Biomedical Corp. Incline Village, NV; Niraxx Light Therapeutics, Inc, Boston, MA. Consulting; Lexington Int, Boca Raton, FL; USHIO Corp, Japan; Merck KGaA, Darmstadt, Germany; Philips Electronics Nederland B.V. Eindhoven, Netherlands; Johnson & Johnson Inc, Philadelphia, PA; Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany. Stockholdings: Global Photon Inc, Bee Cave, TX; Mitonix, Newark, DE. The other authors declare no conflicts of interest.

Acknowledgement

This study was supported by a scientific project grant (NO: 96-04-223-31954) financed by Iran University of Medical Science (IUMS). The authors would like to thank the ICPPM and Heltschl Company for the gift of the laser. MRH was supported by US NIH Grants R01AI050875 and R21AI121700.

Author contribution

Made a substantial contribution to the concept or design of the work; or acquisition, analysis or interpretation of data: Atousa janzadeh, Farinaz Nasirinezhad

1. Drafted the article or revised it critically for important intellectual content: Ali Neshasteh riz, Michael Hamblin, Fatemeh ramezani, Atousa Janzadeh
2. Surgery: Atousa janzadeh, Keihan Koukli,Alireza Fazeli
3. Bladder massage, behavioral test, Photobiomodulatyion Therapy: Keihan Koukli,Alireza Fazeli
4. Histology Staining: Ali Motamed, Alireza, Mohammadreza Asadi

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