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Research Article

Anti-inflammatory Effects of Low Level Laser Therapy and *Streptococcus thermophilus* on Wound Assessed Histomorphologically: A Comparative Study in Diabetic Rat's Model

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Received: March 16, 2020; **Published:** June 30, 2020

Abstract

Background and Objectives: Worldwide, diabetic wound is a major concern. In diabetic wounds, medical professionals require excellent therapy choice with less devastating consequences. Laser therapy and *Streptococcus thermophilus* are potent therapeutic agents. The topical effects of laser and *Streptococcus thermophilus* on the inflammatory phase of diabetic injury are compared in this research.

Material and Methods: The total 18 rats were divided randomly into three groups and six rats were placed into each group. Diabetes mellitus was induced in rats by administration of Alloxan intraperitoneally. After induction of diabetes, wounds were created in all rats. Control group received normal saline, group B irradiated with 10 J/cm^2 for 30 sec with Low Level Laser Therapy, group C was given 1 ml *Streptococcus thermophilus* application containing 10^{10} to 10^{11} CFU/ml organisms topically. Neutrophils, lymphocytes and macrophages were counted on the 3rd and 7th day of therapy to determine the inflammatory status of diabetic wound.

Results: In comparison to *Streptococcus thermophilus* and normal saline with p values < 0.02 the rats were handled with Low level laser therapy showed a significant reduction in the number of neutrophil counts with increased number of macrophages and lymphocytes.

Conclusion: In diabetic wounds, both low level laser therapy and *Streptococcus thermophilus* exerted their anti-inflammatory effects; however, low level laser therapy showed greater preceding anti-inflammatory impacts as compared with *Streptococcus thermophilus*.

Keywords: *Diabetes Mellitus; Low Level Laser Therapy (LLLT); Streptococcus thermophilus; Neutrophils; Lymphocytes; Macrophages*

Introduction

With an ever increasing occurrence of Diabetes Mellitus (DM) with estimates of over 370 million individuals worldwide, it is becoming more and more prevalent in every part of the world. Individuals who suffer from DM are usually at a danger of post-operative complications linked with non-union of operational wound as a result of systemic diseases mainly due to chronic diabetes [1]. Diabetes causes impaired healing of wounds because of inflammatory response imbalances, modified cytokine release, and modified synthesis of collagen, decreased formation of new vessels with decreased tensile strengths [2]. Delayed wound healing, causing reduction in strength of wound, impaired wound contracture, increases chances of wound infection which prolongs hospital stay as well as increases mortality rates [3]. WHO estimations showed by 2030, in 191 countries there will be 366 million more people with diabetes [4]. The anti-inflammatory effects of low-level laser therapy has been in clinical practice for alleviating pain and also used to speed up healing progression in patients having chronic ulcers, muscular injury, surgical wounds and burns [5-7]. Laser therapy biomodulatory effects are based on the theory that cell's photoreceptors like hemoglobin, oxy-hemoglobin, and melanin as well as cytochrome c absorb photon energy. Photoreceptor absorbs electrical energy and converts it into chemical energy [8]. Cytochrome c absorbs photon which generates a redox state change in mitochondria and/or ion pumps through ATP synthesis across the inner mitochondrial membrane. Intracellular calcium (Ca^{+2}) is also enhanced, stimulating RNA as well as DNA synthesis, activating an intracellular signal cascade [9]. Anti-inflammatory effects of probiotics proposed by following factors including duplication of DNA, increases synthesis of protein, regulating oxidative stress

as well as modulate cytokine secretion [10]. The above actions contribute to bio-modulating of numerous cells that are responsible for regeneration of tissue, which includes; increasing percentage of fibroblast, mitosis activity, increased angiogenesis, change in cytokine synthesis, with assistive conversion of fibroblasts to myo-fibroblasts that have an influence in healing process [11]. The effects have been shown in both pre-clinical as well as clinical research trails. Nevertheless, due to lack of normalization of dosimeter as well as light delivery procedures and inadequate knowledge of association between cells with molecular action mechanisms causes limitation for usage of the above mentioned therapy technique [12]. Significance of the dose vs the moment of irradiation is under researched [13]. Isolated form of *Streptococcus thermophilus* applied in the form of liquid for a week as a topical therapeutic agent in management of wound repair, shows well established recovery of wound. An increase in tensile strength was observed on topical application of *Streptococcus thermophilus* that can easily be liquefied in the stratum corneum significantly, that decreases the loss of water, includes enhance the immune response against antigens and bacteria, as well as establishing barrier function in the epidermis [14]. Probiotics supposedly increased the cell line of human keratinocytes cultivated with *Streptococcus thermophilus* tonic cells. Concentrations in treated keratinocytes were stated to raise 50 - 60-fold after 18h with regards to the basal value [15]. *Streptococcus thermophilus* contained therapeutics as used in patients with atopic dermatitis evidence an improvement in symptoms such as erythema, scaling and pruritus after 2 weeks of application [16].

Material and Methods

Eighteen male adult Wistar albino rats (having 100 - 150 grams of weight) were kept in different plastic cages. They were divided into 3 groups of 6 rats, with 3 rats in each cage with access to water and normal diet. The animals were monitored daily. This d ethical principles for animal experimentation in Pakistan. Experimental study was experimental study was conducted after approval from the ethical committee of Al-Tibri Medical College and Hospital Karachi campus, according to the guidelines of animal care anperformed at Al-Tibri Medical College and Hospital. Karachi. Pakistan

Chemical induction of diabetes

Post fasting for 24h with free access to water, all rats were induced with diabetes with a one-time intra-peritoneal (IP) injection of alloxan monohydrate (Sigma, Germany) diluted in chilled normal saline at a dose of 120 mg/kg of body weight of rats. Following 72 hours of administration of alloxan injection, blood sugar was measured in rats using a glucometer to determine confirmation of diabetes. The blood glucose level was measured every third day for 15 days for diabetic status of rats. Blood samples were taken from the tail of rats by needle pricking. The criterion for diabetes was taken as fasting glucose level of higher than 200 mg/dl for three consecutive days. Animals with fasting blood glucose greater than 200 mg/dl for three consecutive days were selected for the experiment.

Experimental design

Sample size was calculated by the "E" formula, E = (Total numbers of animals- total number of groups) then E= (7 × 3) - 3. E = 21 - 3, E = 18. From the Total 21 rats 18 were selected and divided into three groups, 6 rats in each group. Group A was tagged as a control group, Group B was treated with Low level Laser therapy and Group C was were treated with topical *Streptococcus thermophilus*. Control group received normal saline topically once daily. Group B received 10 Joules (J)/cm^2 for 30 sec of low laser therapy topically once daily. (THOR DDII laser therapy device with multi-diode cluster probe) was used. This therapy was performed by using the wavelength of 660 nm and power of 100 mW with doses of 10 J/cm^2 for 30 seconds that was taken from the Isra Institute of Rehabilitation Sciences, Karachi Pakistan. Group C were received application of *Streptococcus thermophilus* topically of 1 ml containing 10^{10} to 10^{11} CFU/ml bacteria once daily. The strains of *Streptococcus thermophilus* were isolated from yogurt. The *Streptococcus thermophilus* strains were identified and authenticated by the Microbiology Department of Pakistan Council of Scientific and Industrial Research Karachi, Pakistan (PCSIR).

Wound formation

1.9% of ethanol was used to induce anesthesia in a closed plastic container. Cotton soaked with ethanol was placed in wide plastic container; rats were placed inside and monitored for the state of unconsciousness. Induction took 5 - 10 minutes and then rats were taken out from the plastic container. Fur was removed from dorsal surface of rats each animal with an electric shaver. The skin was cleaned with a 0.12% chlorhexidine solution. After induction of anesthesia, incisional round wound of about 2.5 × 2.5 cm was produced on the central portion of the dorsum of rats by using of Scalpel blade of 8 mm, the size of wound was measured with the help of ruler. After wound formation the rats were kept in their labeled cages. All three groups were treated topically once daily according to the treatment plan after 24 hours of wound formation.

Histological examination

At 3rd and 7th day of experiment the granulation tissue and scab was formed at wound site and its samples (complete thickness of edges and its surrounding skin). Tissue was removed, by using the scalpel and fixed in 10% buffered formalin (pH 7.4), were cleaned in xylene and embedded in paraffin, followed by dehydration through alcohol in ascending order. The rotary microtome was used to cut the tissues in 5-µm section from each animal sample and stained with hematoxylin and eosin for morphological analysis, placed on albumenized glass slides. An experienced pathologist performed the allocation of the samples to the different groups and performed the analysis, searched the complete extension of each sample with a light microscope and recorded inflammatory cells count by using reticule.

Microscopic parameter

1. Neutrophil count
2. Number of macrophages
3. Number of lymphocytes.

Statistical analysis

Data was analyzed by using the software statistical package for social sciences (SPSS) version 23.0. ANOVA (Analysis of Variance) was used for comparison between the groups which was followed by Post-hoc Tuckey's test for multiple comparisons. All values were expressed as mean ± S.D. Statistical significance was set at $p \leq 0.05$.

Results

In all rats, intra peritoneal alloxan monohydrate injection was administered. The RBS was monitored in group A, B, and C after three days of injection. RBS was subsequently checked for evaluation of diabetic status. After confirmation of diabetic status, RBS was checked on the sampling day as shown in the table 1. After topical therapy, the slides were prepared on day three and seven to observe the neutrophils, lymphocytes and macrophages under a light microscope.

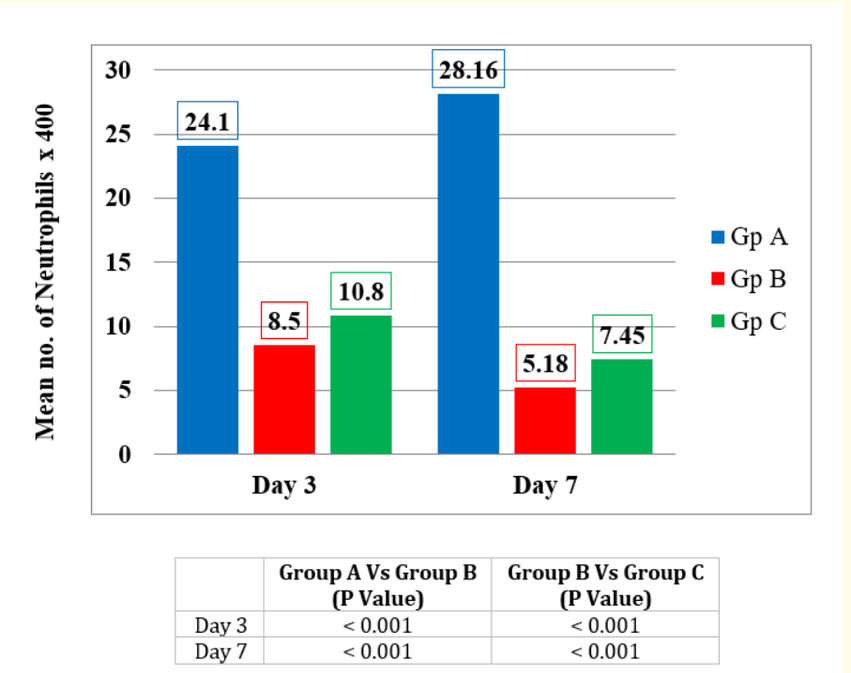
No: of Days	Group (A)	Group (B)	Group (C)
Day 3	297 ± 16.690	300 ± 15.690	280 ± 19.690
Day 7	298.75 ± 18.07	310.75 ± 19.07	278.75 ± 11.07
Day 14	312 ± 19.08	313 ± 14.08	318 ± 10.08
Day 17	315 ± 33.0	324 ± 13.89	330 ± 17.99
Day 20	299 ± 12.9	290 ± 14.9	334 ± 30.9

Table 1: *Showing mean value of random blood sugar.*
This table is showing mean value of RBS after I.P injection of Alloxan monohydrate on day 3rd, 7th and 14th.
After that 3rd and 7th day of tissue sampling that is day 17 and 20 in Group A, B and C

The mean neutrophil count: There was a significant reduction in number of neutrophil count among the animals of laser treated group compared with control and *Streptococcus thermophilus* on day 3rd and 7th as shown in figure 1A and 2A-2C.

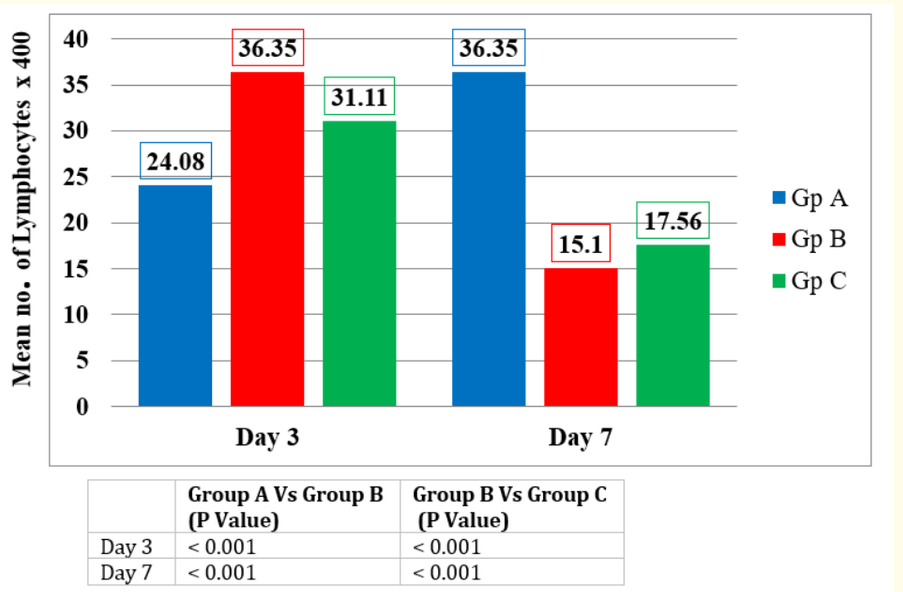
The mean lymphocyte count: The mean Lymphocyte count in laser therapy group increased significantly compared to control and *Streptococcus thermophilus* on third day. There was a significant reduction in lymphocytes in the laser treated group on day 7th compared with control and *Streptococcus thermophilus* group as shown in figure 1B and 2A-2C.

The mean macrophage count: There was a significant increase in macrophage count in laser treated group compared with control and *Streptococcus thermophilus* 3rd and 7th day of experiment as shown in figure 1C and 2A-2C.



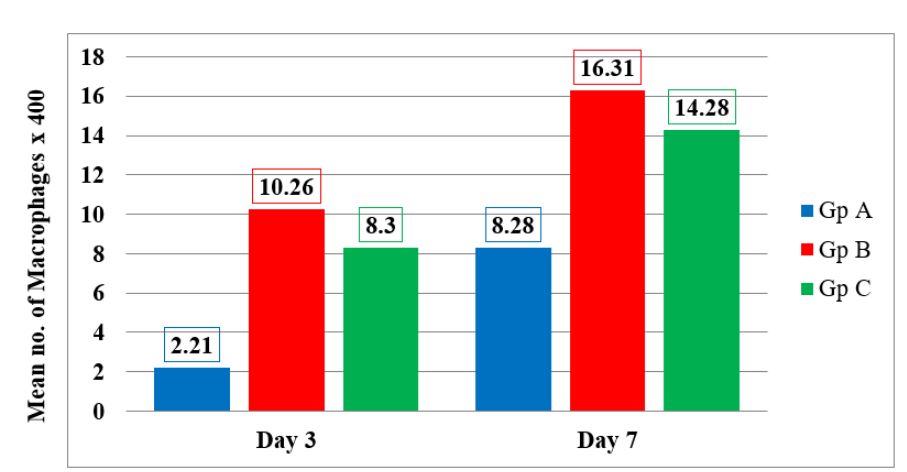
	Group A Vs Group B (P Value)	Group B Vs Group C (P Value)
Day 3	< 0.001	< 0.001
Day 7	< 0.001	< 0.001

Figure 1A: *Bar chart showing comparison of number of neutrophils (0.81 cm2 areas x400). There was significant reduction in number of neutrophils in group B (laser therapy) on day three and seven compared with Group A (normal saline) and C (Streptococcus thermophilus). P value < 0.05* (significant).*



	Group A Vs Group B (P Value)	Group B Vs Group C (P Value)
Day 3	< 0.001	< 0.001
Day 7	< 0.001	< 0.001

Figure 1B: *Bar chart showing comparison of number of lymphocytes (0.81 cm2 area x 400). There was significant increase in number of lymphocytes in group B (laser therapy) on day three and decrease on day seven compared with Group A (normal saline) and C (Streptococcus thermophilus). P value < 0.05* (significant).*



	Group A Vs Group B (P Value)	Group B Vs Group C (P Value)
Day 3	< 0.001	< 0.001
Day 7	< 0.001	< 0.001

Figure 1C: *Bar chart showing comparison of number of macrophages (0.81 cm2 area x400). There was significant increase in number of Macrophages in group B (laser therapy) on day three and seven compared with Group A (normal saline) and C (Streptococcus thermophilus). P value < 0.05* *(significant).*

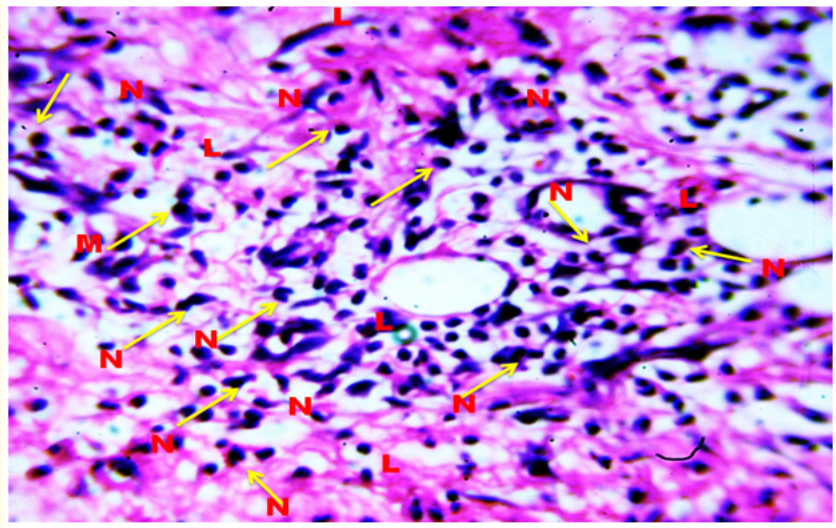


Figure 2A: *(H&E staining) showing neutrophils (N), lymphocytes (L) and macrophages in group A (Control).*

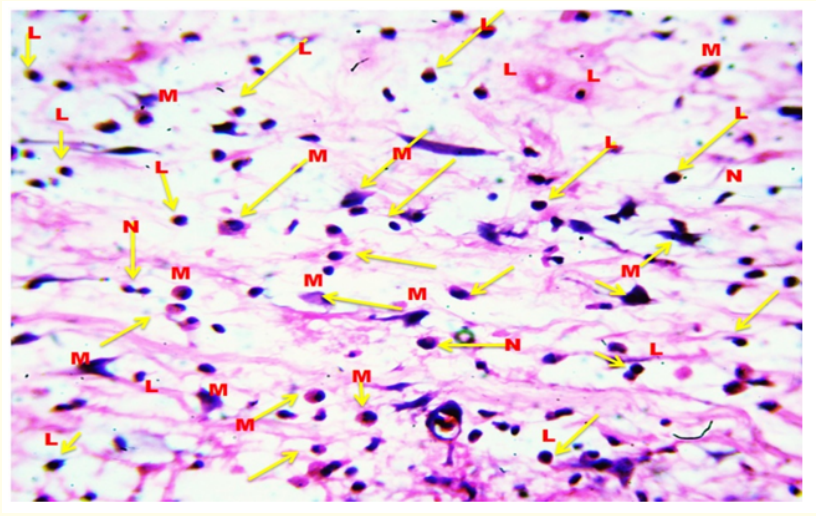


Figure 2B: *(H&E staining) Showing Neutrophils (N), lymphocytes (L) and macrophages in group B (laser therapy).*

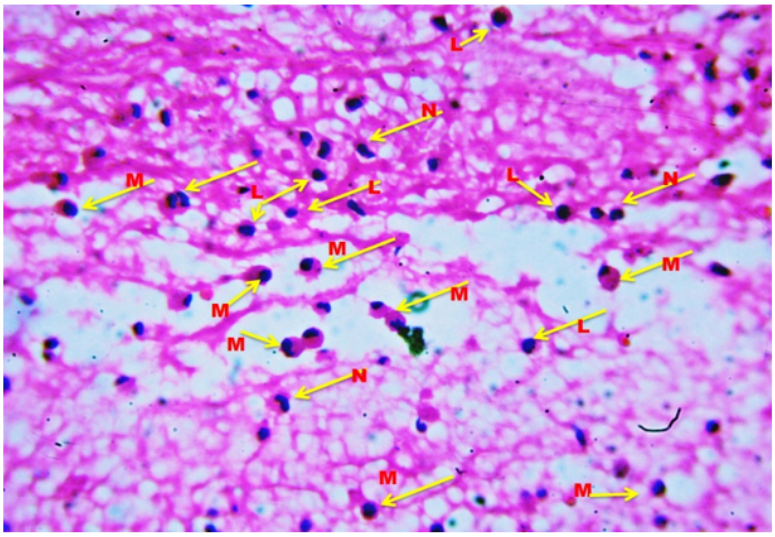


Figure 2C: *(H&E staining) Showing neutrophils (N), lymphocytes (L) and macrophages in group C (Streptococcus thermophilus).*

Discussion

In medical cases, low-level laser therapy has been frequently utilized in variety of procedures. It offers positive impact in numerous ways like wound healing, pain associated with musculoskeletal-system of the body; it also helps to reduce the inflammation in number of

cases [17]. Owing with enhanced aggregated neutrophils and reduction of macrophages at injury site causes prolongation of inflammatory stage in diabetic wounds. The protein glycation results in alterations of basement membrane of capillary endothelium. The presence of high glucose levels in blood and thickness of basement membrane create a ground for alterations in its permeability level. This leads to chronic inflammation and vulnerable infective wound. On each occasion of wound appearance; it instigates with first phase of the inflammatory process in which several chemical mediators are released such as histamine, nitric oxide, interleukins-IL-1 and IL-6. It results in the migration of numerous cells like neutrophils, lymphocytes, monocytes/macrophages [18]. The pro-inflammatory mediators for example TNF-α, IL-1β and IL-6 while IL-10, TGF-β are included in the category of inflammatory mediators that supposed to perform an imperative part of inflammatory process. The drastic change in these chemical mediators was observed when treated by He-Ne laser therapy that result in reduction of inflammation [19]. According to Albertini., *et al*. LLLT have great impact in reduction of certain chemotactic agents like TNFα, IL-1 β and IL-6. According to Albertini LLLT extensively make changes in the mRNA that encodes the information of these mediators [20]. In this research, it was noticed that LLLT treated group showed significant reduction in neutrophil and lymphocyte count at wound site with increased availability of macrophages that eventually regulate the inflammatory stage in diabetic rats compared to other groups.

Study conducted by Alves., *et al.* 2013 disclosed that LLLT provides a great share in reduction of inflammation by decline in number of neutrophil count. These results are in consistent with current research where the count of neutrophils in the laser-treated group was considerably reduced [21]. *Streptococcus thermophilus* established evidence regarding its valuable anti-inflammatory effects and its potential use for the treatment of inflammatory bowel disease. One of the research on patients of acute colitis treated by *Streptococcus thermophilus* in which IL-10 concentration was observed to increase with decrease in pro-inflammatory mediators. In turn, it offers better anti-inflammatory activity [22]. In current research, the group treated with *Streptococcus thermophilus* showed neutrophil and lymphocyte count to be decreased with an increase in macrophage count at the site of wound. *Streptococcus thermophilus* strains were treated in different types of *in vitro* cellular models that were cultured with *Streptococcus thermophilus* and *in vivo* mouse models with colitis. It comprised of antioxidant, anti-inflammatory characteristics that delivers beneficial effects by restricting the damage to large intestine [23]. One of the latest research revealed that LLLT boost up the supply of neutrophils during the initial three days of wound healing, results in removal of debris with abolishment of bacterial growth and other microorganisms. Eventually organization of the wound bed for new cellular growth starts. The neutrophil count begins to decrease after three days and the acute inflammatory infiltrate is replaced, which is followed by more myo-fibroblasts along with fibrous tissue in wound area in LLLT treated group [24]. In the current research, it was observed that LLLT and *Streptococcus thermophilus* both decreases the duration of the inflammatory phase by decreasing the count of neutrophils and increase the supply of macrophages compared to the normal saline group. But in case of laser group the results showed decrease neutrophil count with earlier supply of more macrophages than *Streptococcus thermophilus* and saline.

Conclusion

In diabetic wounds, both low level laser therapy and *Streptococcus thermophilus* exerted their anti-inflammatory effects; however, low level laser therapy showed greater preceding anti-inflammatory impacts as compared with *Streptococcus thermophilus*.

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