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Full Length Research Paper

Comparison of the effects of *Lactobacillus brevis* and *Lactobacillus plantarum* on cutaneous wound healing in rats

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The benefits of probiotic microorganisms have been tested in several studies and they show many positive effects on human health like reduction of serum cholesterol, stimulation of immune system and prevention or treatment of human infections. This study has shown the activity of *Lactobacillus brevis* and *Lactobacillus plantarum* isolated from Iranian traditional cheese on cutaneous wound and describes the difference in healing activity between these two *Lactobacilli*. Some strains of *Lactobacillus* isolated from traditional dairy products of Iran were investigated for exopolysaccharide (EPS) production using the phenol-sulfuric acid method. *L. brevis* and *L. plantarum* were selected because they have high exopolysaccharide (EPS) production. A full-thickness wound (1.5 × 1.5 cm) was made on the back of each rat (45 rats in 4 groups). Two groups, experimental 1 and experimental 2, were treated by *L. brevis*, and *L. plantarum* that were added to eucerin. A control group was treated with eucerin and a negative-control group, additionally, did not receive anything. On days 1, 3, 7, 14 and 21, the rats were killed and wound tissue samples were collected for histological and statistical studies. The percentage of wound healing and inflammation in the experimental groups on day 21, when compared with the control and negative control groups, were significant ($p \leq 0.05$). In contrast to the control and negative-control groups, the number of neutrophils in the experimental groups was reduced in the later phase of wound healing. The current study showed a significant reduction in inflammation and an acceleration of wound healing in wounds treated with *Lactobacilli* as compared to the control and negative control groups. Further studies are required to develop a mechanism of *L. brevis* and *L. plantarum* during wound healing.

Key words: Cutaneous wound, exopolysaccharide, *Lactobacillus brevis, Lactobacillus plantarum*.

INTRODUCTION

Skin as the first immune barrier plays effective roles in maintaining human health. Loss of the integrity of large portions of the skin as a result of injury or illness begins a chain of processes for healing (Cordoso et al., 2010; Johnston, 1990). In general, wound healing proceeds in three interrelated dynamic and overlapping phases namely, inflammation, granulation, and remodeling (Janis et al., 2010; Savunen and Viljanto, 1992; Schwartz, 1984).

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Different chemical agents have been used for wound healing but each agent has negative side effects (Weinstein-Oppenheimer et al., 2010; Sasidharan et al., 2010; Shivanada et al., 2010; Tramontinal et al., 2002). Appropriate treatment and care is essential to accelerate the healing process and to prevent infection and chronicity of the wound. A therapeutic agent selected for the treatment of wounds should ideally improve one or more phases of healing. Probiotics and its exopolysaccharide, were both tested for antimicrobial and healing activities in chronic ulcers but though, they have many strains and species the only study on the use of probiotics on cutaneous wound healing was done by

Rodrigues et al. (2004).

These bacteria have been known as beneficial factors that have many effects on human health like reduction of serum cholesterol, stimulation of immune system and prevention or treatment of human infections (Salminen et al., 2010; Settanni and Moschetti, 2010). Also, probiotic bacteria produce exopolysaccharides (EPS) that can be connected to a cell's surface or that can discharge into the environment. Among the wide variety of EPS-producing microorganisms, lactic acid bacteria (LAB) have gained much attention because of their GRAS (generally recognized as safe) status (Laws et al., 2001; Vinderola et al., 2006). EPS have many roles like immunostimulatory (Shivanada et al., 2010), anti tumor activity (Arul et al., 2007). Also, phosphate groups in EPS play an important role in activating macrophages and lymphocytes (Foligné et al., 2010).

Despite different strains and species, there are few researches on the effect of these bacteria on cutaneous wound healing have been done. The goal of this experiment was to study the effect of *L. brevis* and *L. plantarum* on cutaneous wound healing and compared their differential effect on wound healing.

MATERIALS AND METHODS

Animals

Male Wistar rats weighting 250–280 g were housed under controlled conditions of light, room temperature and humidity. The animals were separated into four groups (n=25), negative control, control, experimental 1 and experimental 2, and the rats of each groups were killed at 1, 3, 7, 14, and 21 days after wounding. Twenty-five rats were kept as negative controls, where the wounds were left untreated. A second group of 25 rats were used as controls, where the wounds were treated with eucerin alone. In the third set of 25 rats, the experimental 1 group, the wounds were treated with eucerin contain *L. brevis* and the last set of 25 rats, the experimental 2 group, wound of each rat was treated with eucerin which contain *L. plantarum*. This study was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC).

Induction of wounds and drug administration

Briefly, the rats were anaesthetized with ketamine (15 mg/kg) / xylazine (20 mg/kg), the skin of rats were shaved and a 1.5 cm^2 full-thickness open excision wound was made in the back of each rat using shablon and the skin was removed with scalpel.

After the wounding process, each rat was housed in a sterilized cage and given autoclaved food and redistilled water in order to prevent bacterial infection. Twenty four hours after the wounding, the wounds in the control and experimental groups were treated topically once daily.

Lactobacillus strains

In this study, EPS production in some strains of *Lactobacillus* isolated from Iranian traditional dairy products were investigated by Tajabady et al. (2011) using the phenol sulfuric method (Dubios et al., 1956). The *Lactobacilli* strains were identified by 16S rRNA gene sequencing and showed 98% similarities to *L. plantarum* (GQ423760) and *L. brevis* (GQ423768) and were selected because they had more EPS production (Tajabady et al., 2011a). These two selected strains of *Lactobacillus* were then cultured in an MRS agar medium and incubated for 48 h at 37°C. Following, the bacteria on the surface of the culture and EPS secreted by them to aid in the treatment of wounds were collected with a sterilized kolle handle (Vander Wal et al., 2009).

In order to prepare the ointment, 10^{10}-10^{11} CFU/ml bacteria that had been collected every day after a 48-h culture were added to 4 ml of eucerin for each of 5 mice as a preservative. The culture and eucerin were mixed thoroughly until a uniform income produced and immediately applied on the wounds in the two experimental groups. However, for the control group, we used 5 ml of eucerin for each of 5 mice.

Wound contraction determination

Digital photographs of the wounds were taken on days 1, 3, 7, 14 and 21. The maximum length and width of each wound was measured with calipers in tertian. Subsequently, the area of the wound was calculated from these measurements as a function of time that had passed during the treatment. The degree of con-traction was determined from the difference between the initial and final areas of the wounds. Mean values were then calculated for each group of 5 animals (treated with *L. brevis* ointment, *L. plantarum* ointment, control and negative control). The results were expressed in cm^2.

The percentage of wound closure was calculated as follows:

[(Area of original wound - Area of actual wound)/Area of original wound] × 100.

The inside edge of the calipers exactly matched the edge of the wound.

Histopathology

The rats were sacrificed at 1, 3, 7, 14, and 21 days after wounding with ether and the tissues from the wound site including the whole thickness of the skin and the surrounding skin of the individual animal was removed. These samples were then separately fixed in 10% formalin, dehydrated through graded alcohol series, cleared in xylene, and embedded in paraffin wax.

Serial sections of 5-7 mm were cut and stained with hematoxylin and eosin (Tang et al., 2007). The sections were used for counting inflammation cells such as neutrophils and macrophages in 100 fields of view in different sections from wound area under a light microscope and numbers were expressed in percentage.

Statistical analysis

All results were expressed as mean ± S.D and two-way analysis of variance were applied to test statistically significant differences among groups. A probability value of ≤ 0.05 was considered significant. All statistical analyses were performed using SPSS statistical version 16.0 software package (SPSSs Inc., Chicago, IL).

RESULTS

The rates of contraction of negative control, control, experimental 1 and experimental 2 wounds are depicted

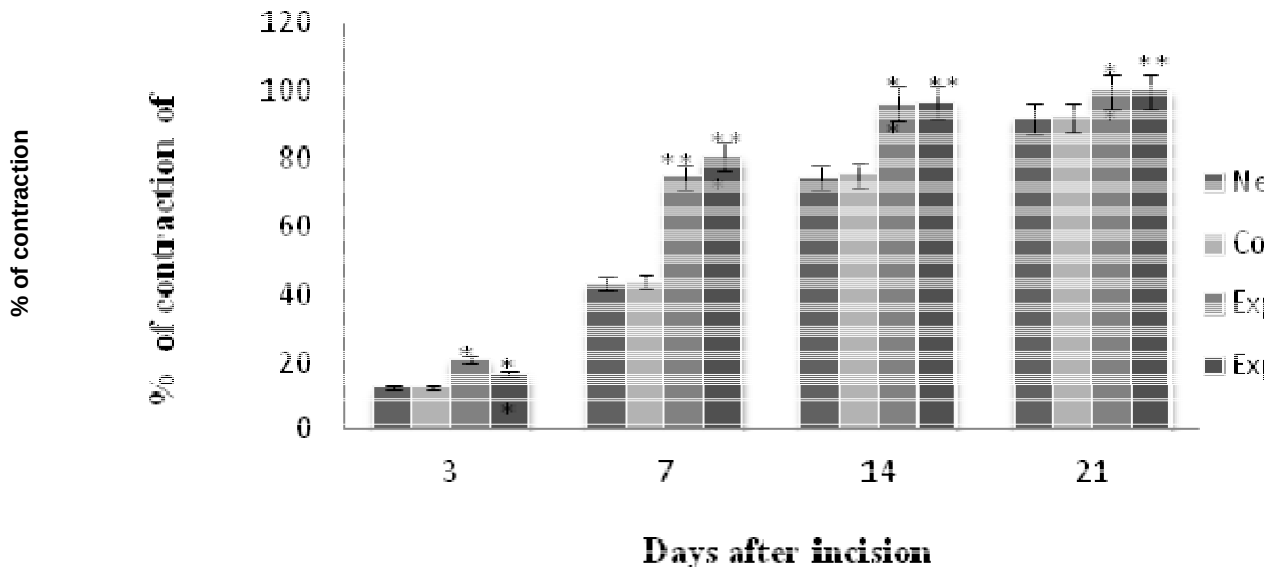

Figure 1. The rate of contraction in negative control, control, *Lactobacillus brevis* treated (experimental 1) and *Lactobacillus plantarum* treated (experimental 2) at different days. Values are expressed in mean ± SE. ** p < 0.01; *** p < 0.001 as compared with corresponding control and negative control using two-way ANOVA.

in Figure 1. In addition, according to the results of measurement of the longitudinal or the perpendicular length of wounds, the wound sizes of negative control and control groups were significantly larger than that of experimental groups at all days after the incision.

Both the wound treated with *L. brevis* (experimental 1) and *L. plantarum* (experimental 2) resulted in a faster reduction of the wound diameter than the 2 other groups. However, the *L. brevis*-treated wound were found to start healing much faster than the other groups but, at day 7 of the experiment, the wound treated with *L. plantarum* were smaller than the *L. brevis*-treated wound (P<0.001). In all groups, wounds were covered by a dehydrated wound scab at day 1 after incision. By 21 days after the incision, the wounds fully healed in all experimental groups rats based on the macroscopic closure of the incision interface and restoration of an epithelial cover (Figure 2).

The wound area increased in the early days of the study, the results are justified since the increase is in compliance with the inflammation phase. Additionally, the wound area increased because of skin and muscle tension (Figure 3).

The number of fibroblasts in the experimental 1 and 2 groups at day 3 (P<0.01) and day 7 (P<0.001) of study showed a statistically significant increase than that of both the negative-control and the control group. Reduction in the number of fibroblasts in the experimental 1 and 2 groups on the fourteenth (P<0.001) and twenty first days of study (P<0.01) was statistically significant than the other two groups (Table 1).

Also, the reduction in the number of neutrophils in the experimental 1 group on the third, seventh days (P<0.001), fourteenth and twenty first days (P<0.01) of study was statistically significant when contrasted to the negative control and control groups. The only difference between experimental 1 and experimental 2 groups was seen on the third day of study, increase in the number of neutrophils was observed in experimental 2 group (P<0.001) (Figure 4).

On the third day of study, the number of macrophages in the experimental 1 group and in the experimental 2 group (P<0.001) showed a significant increase in contrast to the control and negative control groups. Cell counting results also confirmed a significant reduction in the number of macrophages on the seventh, fourteenth and twenty-first days (P<0.001) in the experimental 1 and 2 groups than the control and negative-control groups (Figure 4).

DISCUSSION

Human was always looking for a solution to treat wounds to accelerate healing process and to prevent infection and becoming a chronic wound. Finding an effective therapeutic agent can be a new window to this science and help to heal wounds require extra attention like minor surgery and burns (Jeffry et al., 2010; Berger et al., 2005). The benefits of probiotic microorganisms have been tested in several studies and they show many positive effects on human health like reduction of serum cholesterol, stimulation of immune system and prevention or treatment of human infections. Cicatrizing properties of the probiotics themselves and their derived products have not been previously described. This study has shown the activity of two strains of *Lactobacillus* isolated

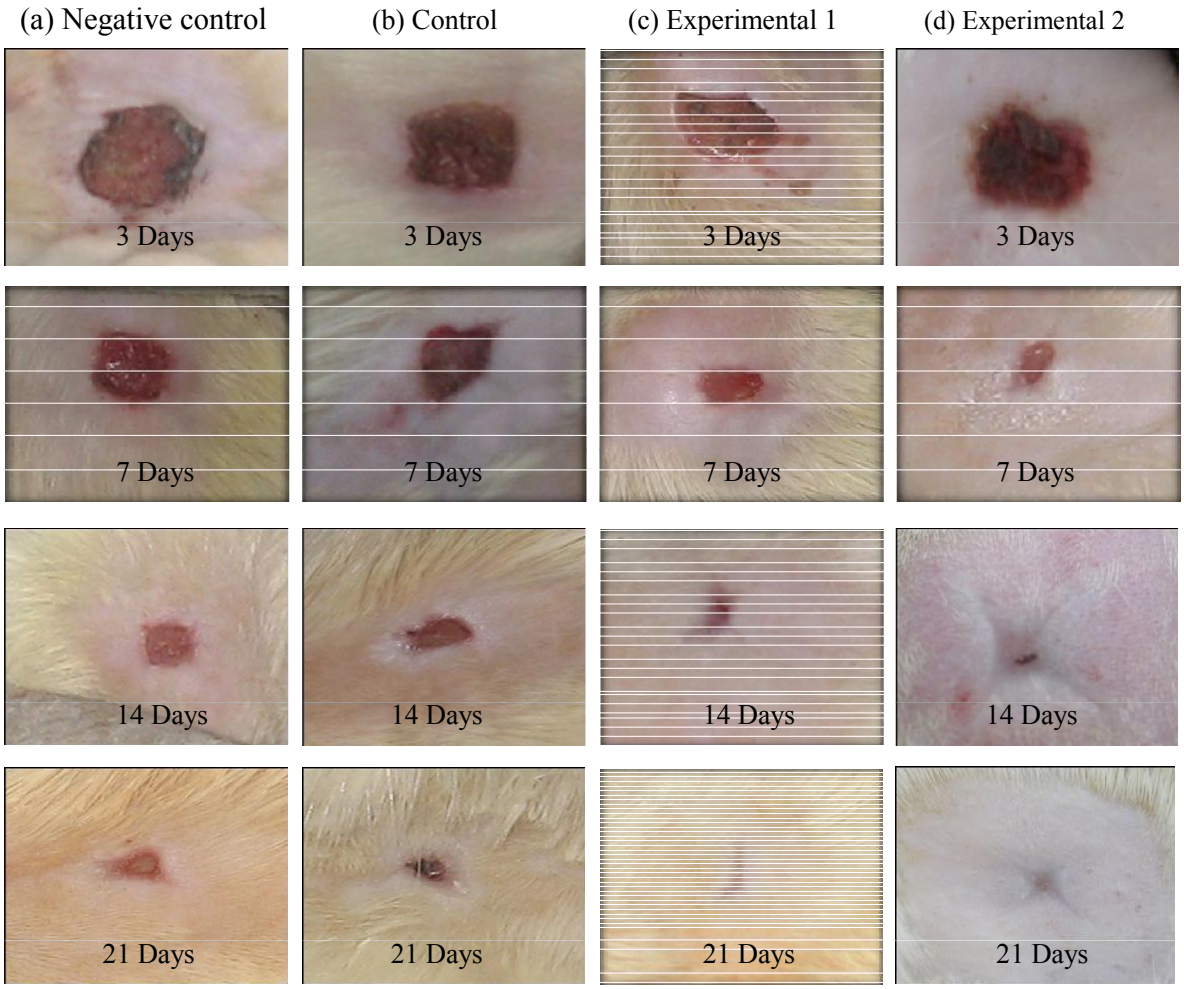

Figure 2. Photographic representation of contraction rate on different days. (a) Negative control, (b) Control, (c) *Lactobacillus brevis*-treated (experimental 1) and (d) *Lactobacillus plantarum*-treated (experimental 2) (Magnification × 10).

Iranian traditional cheese on cutaneous wound and describes the difference in healing activity between these two *Lactobacilli*.

It has been demonstrated by live probiotic strains induce the production of protective cytokines that enhance epithelial cell regeneration and inhibit epithelial cell apoptosis (Eswara et al., 2010). In a result of a study cytokine- induced apoptosis was prevented in intestinal epithelial cells in the presence of *Lactobacillus rhamnosus* GG (Yan and Polk, 2002). Probiotic bacteria in a culture of mouse or human colon cells activated anti apoptotic Akt/protein kinase B and inhibited activation of the proapoptotic p38/mitogen activated protein kinase by tumor necrosis factor-α (TNFα), IL-1α, or interferonγ (IFNγ) (Neish et al., 2000). Inhibitions of apoptosis enhance survival of intestinal cells and promote proliferation during recovery from epithelial injury (Hausmann, 2010). Also probiotic metabolites have been reported to induce angiogenesis, proteoglycans deposition and heal wounds (Halper et al., 2003; Resta-Lenert and Barrett, 2003; Valdez et al., 2005).

In this study, *L. brevis* and *L. plantarum* was tested for cicatrizing activity in rats with dorsal injuries. Animals treated with these two *Lactobacilli* showed better wound healing compared with those groups that receive eucerin (control) and negative control group that receive nothing to compare with control group. The control and negative control groups did not show any significant difference. Natural process of wound healing was observed in these two groups so that until the twenty-first day of study, wounds could be seen on the skin of rats while only cicatrix was left on the skin of the experimental groups.

Also, no infection was found in the experimental groups because probiotics prevent infection in wounds by an antimicrobial mechanism that involves secrete antimicrobial peptides, inhibition of bacterial invasion and inhibit pathogenic bacteria adhesion to epithelial cells (Boirivant and Strober, 2007).

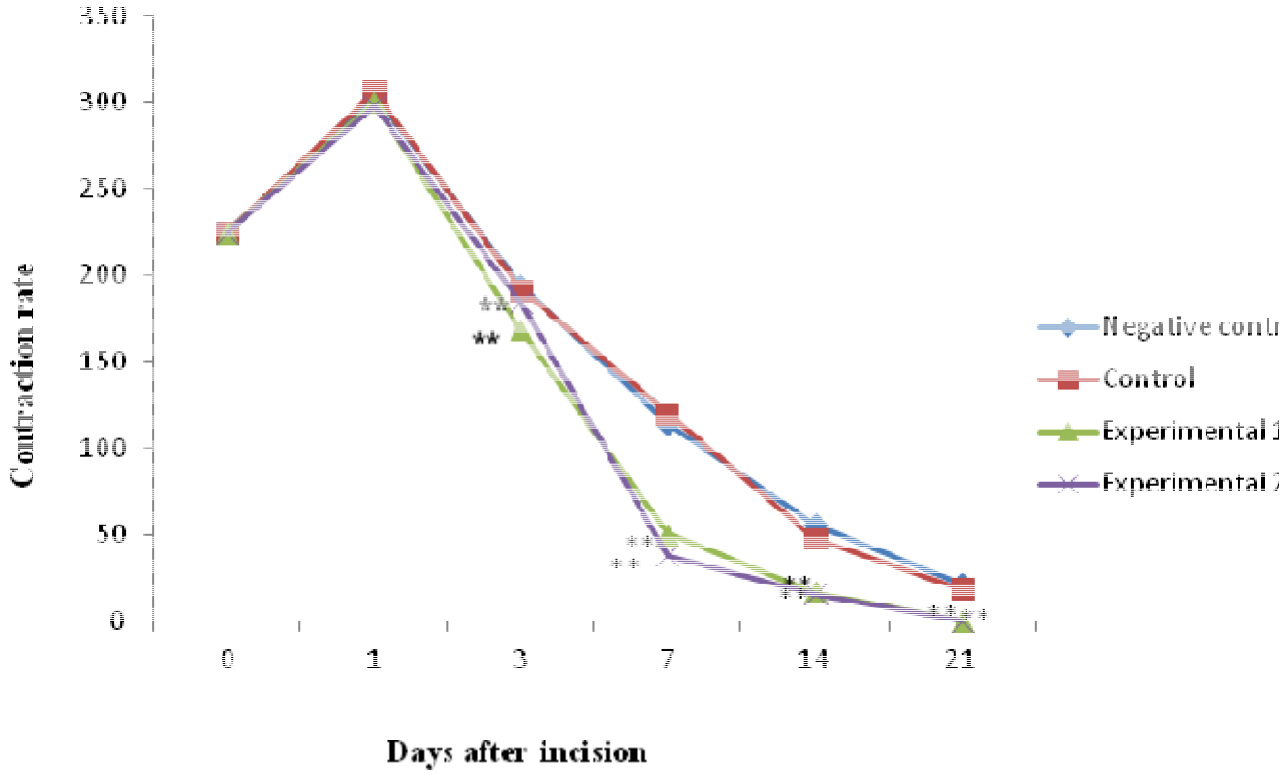

Figure 3. Cicatrizing activity in skin lesions. Data represent untreated animals (Negative control), animals treated with eucerin (Control), animals treated with *Lactobacillus brevis* (Experimental 1) and animals treated with *Lactobacillus plantarum* (Experimental 2). *** $p < 0.001$ as compared with corresponding control and negative control using two-way ANOVA.

Table 1. Histological indices of wound healing in negative-control, control, experimental 1 and experimental 2 groups in days 1, 3, 7, 14 and 21.

Day	Variables / Groups	Neutrophile	Macrophage	Fibroblast
3	Negative control	11.05 ± 0.351	5.12 ± 0.557	1.40 ± 0.532
	Control	10.77 ± 0.274	4.92 ± 0.4545	2.11 ± 0.277
	Experimental 1	*** 2.23 ± 0.337	*** 11.46 ± 0.446	** 5.34 ± 0.209
	Experimental 2	*** 7.49 ± 0.013	*** 11.86 ± 0.517	** 4.770 ± 0.121
7	Negative control	5.266 ± 0.283	16.73 ± 0.568	5.98 ± 0.45
	Control	5.86 ± 0.285	16.46 ± 0.522	5.47 ± 0.33
	Experimental 1	*** 1.53 ± 0.430	*** 9.23 ± 0.921	*** 11.25 ± 0.303
	Experimental 2	*** 1.22 ± 0.251	*** 7.472 ± 0.487	*** 14.59 ± 0.018
14	Negative control	3.04 ± 0.182	9.40 ± 0.910	11.82 ± 0.362
	Control	2.78 ± 0.184	8.55 ± 0.745	12.02 ± 0.520
	Experimental 1	** 1.47 ± 0.210	*** 3.550 ± 0.630	*** 4.13 ± 0.474
	Experimental 2	** 1.15 ± 0.908	*** 2.910 ± 0.039	*** 4.22 ± 0.311
21	Negative control	2.02 ± 0.146	5.91 ± 0.765	7.20 ± 0.367
	Control	2.40 ± 0.221	5.55 ±0.715	7.45 ± 0.342
	Experimental 1	** 1.25 ± 0.478	*** 1.91 ± 0.228	** 1.13 ± 0.183
	Experimental 2	** 1.03 ± 0.029	*** 1.84 ± 0.112	** 2.57 ± 0.309

Values are expressed as mean ± SE for 100 animals. The significant level of experimental 1 and experimental 2 groups compared with negative control and control groups (P<0.01) **, (P<0.001)*** using two-way ANOVA.

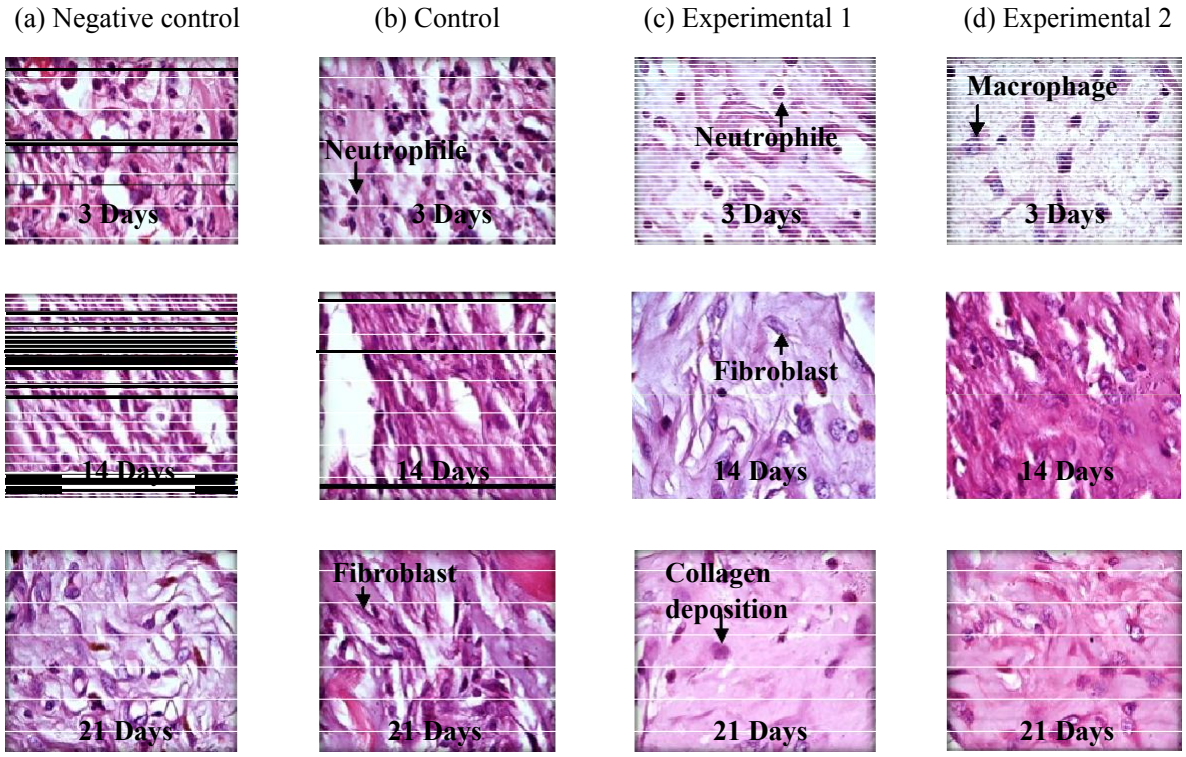

Figure 4. Photographic representation of inflammation cells on 3, 14 and 21 days. (a) Negative control, (b) Control, (c) *Lactobacillus brevis*-treated (experimental 1) and (d) *Lactobacillus plantarum*-treated (experimental 2) (Magnification × 100).

On the first day of this study, no difference was seen between the groups. Hyperemia, edema and inflammation were the dominant phenomena observed on the first day of study.

Our review is focused on the third day, which is the inflammation phase of wound healing. Because of anti inflammation and healing activities of probiotics, we expected to reduce inflammation in two groups treated with *Lactobacillus* than the other groups (Heidari et al., 2004; Tajabady et al., 2009b). We found that the total number of neutrophils was significantly less than the negative control group, the control group and experimental 2 groups. On the third day of this study, inflammation phase was finished in the *L. brevis*-treated group and proliferative phase had started when the other groups are beginning inflammation phase. However, in the experimental 2 group, the *L. plantarum* attracted neutrophils to wound sites but, there was a delay compared with *L. brevis*-treated group. *L. plantarum* Increased IL-10 synthesis specifically that results in increased secretion of macrophages (Hegazy and El-Bedewy, 2010; Pathmakanthan et al., 2004). For this reason, we noticed an increased in number of macrophages on the third day of study rather than the seventh day. In contrast to the control and negative control groups, the topical cream caused a significant increase in the number of fibroblasts and a reduction in neutrophils.

Tamawski et al. (2005) suggested that Probiotics were able to stimulate some growth factors like FGF, EGF, PDGF, TGFβ and cytokines that cause fibroblasts to migrate and proliferate in wound tissue (Tarnawski, 2005). A growth factor is a naturally occurring substance capable of stimulating cellular growth, proliferation and cellular differentiation. The results of this study showed that in the groups treated with *L. brevis* and *L. plantarum* the total number of fibroblasts was significantly more than the negative control group and the control group therefore, leads to improved wound healing process from the third day onward by reducing the wound's area, by increasing the percentage of the wound that heals over time, and ultimately by reducing the time required for full recovery.

On days 14 and 21, total fibroblast declining institution in experimental groups compared with the negative control and control groups that is confirmed the restructuring phase and on the other hand renewed earlier phase of collagen synthesis occurred at this stage and collagen bundles with a more in diameter and varies transverse connection between the collagen molecules. Collagen fibers make similarities between the wound after the initial repair tissue to before surgery and prevents creation white ugly closure (Valander et al., 2009; Price et al., 2008; Darby and Hewitson, 2007). In

relation to this issue, rats of control and negative control groups also exhibit bleeding near the wound site for an extended period of time, even after the surface wound has closed but wounds of the experimental group had a higher strength.

The current study provides firm evidence supporting the fact that probiotics have positive effect on cutaneous wound healing too. Also, the results of this study showed that the *L.brevis*-treated group reduced inflammation faster than *L. plnatarum*-treated group and there is a delay in attraction of neutrophile and starting the first stage of wound healing in this group than *L. brevis* group. We demonstrate that the *L. brevis* started earlier than *L. plantarum* in wound healing phases but in total, both groups showed a faster reduction in wound area than control and negative control groups. Further studies are required to develop a detailed mechanism of *L. brevis* and *L. plantarum* during wound healing in human.

Conclusion

The current study focused on different effects of *Lactobacilli* on cutaneous wound healing. The results of this study showed a significant reduction in inflammation and an acceleration of wound healing in wounds treated with *Lactobacilli* as compared to the control and negative control groups. With a delay in starting healing process in experimental 2 group but, at day 7 of the experiment, the wound treated with *L. plantarum* were smaller than the *L. brevis*-treated wound. In contrast to fully treated wound in both experimental groups at day 21 of the experiment, in most *Lactobacilli*-untreated rats, the scab remained until 21 days, or there was a still gapping and red wound field with a scaly surface lacking an epidermal covering.

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