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Full Length Research Paper

The effect of *Lactobacillus brevis* isolated from Iranian traditional cheese on cutaneous wound healing in rats

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Today, there is a great interest in healing cutaneous wounds. Probiotics are defined as different microorganisms that may have positive effects on preventing or treating special pathologic conditions. It is also possible to use probiotics in wound healing processes. Since much research has not been done on probiotics, this study is designed to investigate the effects of *Lactobacillus brevis* on cutaneous wound healing. Twenty two strains of *lactobacillus* isolated from traditional dairy products of Iran are investigated for exopolysaccharide (EPS) production applying the phenol-sulfuric acid method. *L. brevis* selected for its high exopolysaccharide (EPS) and mucoid coloniese production. A full-thickness wound (1.5 × 1.5 cm) was made on the back of each rat (45 rats in 3 groups). Two groups of holding control and experimental ones treated by eucerin and eucerin containing *L. brevis*. Additionally, negative control groups did not receive anything. On days 1, 3, 7, 14 and 21 rats were killed and wound samples were collected for histological and statistical studies. On day 21, The percentage of wound healing (99.53%) and inflammation in the experimental groups compared with the control (90.55%) and negative control groups (91.14%) were significant ($p<0.001$). In contrast to the control and negative control groups, the number of neutrophils and macrophages in the experimental groups showed a faster reduction ($p<0.01$). The current study presented a significant decrease in inflammation and an acceleration of wound healing in *L. brevis* treated rats as compared to the control and negative control groups. Further studies are required to develop a detailed mechanism of *L. brevis* during cutaneous wound healing.

Key words: Cutaneous wound, exopolysaccharide, healing, *Lactobacillus brevis*, probiotic.

INTRODUCTION

The primary function of the skin is to serve as a protective barrier from the environment. Loss of the integrity of large portions of the skin as a result of injury or illness may lead to a major disability or even death (Cordoso et al., 2010; Johnston, 1990).

Wound healing is the restoration of physical integrity to internal and external structures. The process involves intricating interactions between the cells and other numerous factors (Savunen and Viljanto, 1992; Schwartz, 1984). Appropriate treatment and care are essential to accelerate the healing process and to prevent infection and chronicity of the wound. Different means and approaches have thus far been used for this end.

The primary goals of the treatment of wounds are rapid wound closures and functional and aesthetically satisfactory scars. Recent advances in cellular and molecular biology have greatly expanded our understanding of the biological processes involved in wound repair and tissue regeneration (Jeffrey et al., 2010) and have ultimately led to improvements in wound care.

Different chemical agents have been used for wound healing but each agent has negative side effects (Weinstein-Oppenheimer et al., 2010; Sasidharan et al.,

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have a beneficial effect on both the prevention and treatment of specific pathological conditions (Salminen et al., 2010; Settanni and Moschetti, 2010). Also, these bacteria have been known as activator elements shape of pro-inflammatory cytokine and chemokine (Laws et al., 2001; Yamaguchi et al., 2009). In the early twentieth century, Metchinkoff was the first person who proposed that the ingestion of bacteria has beneficial effects on natural micro intestine (Metchnikoff, 1907). The only study on the use of probiotics in cutaneous wound healing was done by Kamila et al. (2004). By using kefir, the study found the bacteria both to decrease inflammation and to restore the wounded area (Rodrigues et al., 2004).

Also, probiotic bacteria produce exopolysaccharides (EPS) that can be connected to a cell's surface or that can discharge into the environment (Burd and Huang, 2008; Low et al., 1998). 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.

Current studies have presented the promotion of wound healing in some diseases and chronic disorders with the aid of herbal extracts are more challenging than ever before. EPS have many roles like immune-stimulatory (Shivanada et al., 2010) and anti tumor activity (Arul et al., 2007; Vander Wal et al., 2009). Also, phosphate groups in EPS are highly regarded in activating macrophages and lymphocytes (Berger et al., 2005; Foligné et al., 2010).

The function of lactic acid bacteria depends on special strains and species. Whereas, the treatment effect belongs to different species, large experiments on these bacteria are performed in different bases. The goal of this experiment is to study the effect of *Lactobacillus brevis* on cutaneous wound healing.

MATERIALS AND METHODS

Animals

Male Wistar rats weighting 250 to 280 g were housed under normal conditions of light, room temperature and humidity. This study was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC). The animals were separated into three groups (n = 25), negative control, control and experimental, for the days 1, 3, 7, 14 and 21. Twenty-five rats were kept as negative controls and their wounds left untreated. The second group of 25 rats was used as controls whose wounds treated with the eucerin alone. In the third set of 25 rats, the experimental group, the wound was treated with eucerin containing *L. brevis*.

Induction of wounds and drug administration

First, the rats were anaesthetized with ketamine and xylazine. After shaving the dorsal hair, one open excision full-thickness wound that was approximately 1.5 × 1.5 cm long wounds were created on the back of each mouse by using shablon.After the wounding process, each mouse was housed in a sterilized cage and given autoclaved food and redistilled water in order to prevent bacterial infection. After 24 h passed following the wounding process, the wounds in the control and experimental groups were treated topically once daily.

The mice were killed at 1, 3, 7, 14, and 21 days after wounding. Paraffin embedded sections were prepared from skin samples, including samples from the wound, and 4 mm of the surrounding skin were harvested by means of a surgical blade. The sections were 6 mm thick and the cut was perpendicular to the skin surface and included the whole thickness of the skin. Serial sections were stained with haematoxylin–eosin. These 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.

Lactobacillus brevis

In this study, some strains of *Lactobacillus* were isolated from Iran's traditional dairy products. The Lactobacilli strain was identified by Ebrahimi et al. (2011a) 16S rRNA gene sequencing and showed 98% similarities to *L. brevis* (GQ423768) (Ebrahimi, et al. 2011a). The *Lactobacillus* was then cultured in an MRS agar medium and incubated for 48 h at 37°C. Following this, the bacteria on the surface of the culture were collected with a sterilized kolle handle.

In order to prepare the ointment, 10^{10} to 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 preserver. The culture and eucerin were mixed thoroughly until a uniform income was produced and immediately applied on the wounds in the experimental group. For the control group, however, we used 5 ml of eucerin for each of 5 mice.

Wound contraction determination

The maximum length and width of each wound were measured with calipers in tertian. Digital photographs of the wounds were taken on days 1, 3, 7, 14 and 21. 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 contraction was determined from the difference between the initial and final areas of the wounds. Mean values were then calculated. The results were expressed in cm^2 and the percentage of wound closure was calculated as the 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.

Statistical analysis

All values were expressed as the mean ± S.D. Data from diffusion experiments were evaluated using the least squares method and adjusted to the data. Animal group comparisons used one-way ANOVA.

RESULTS

Cicatrizing activity

The cicatrizing activity of *L. brevis* in the rat model is

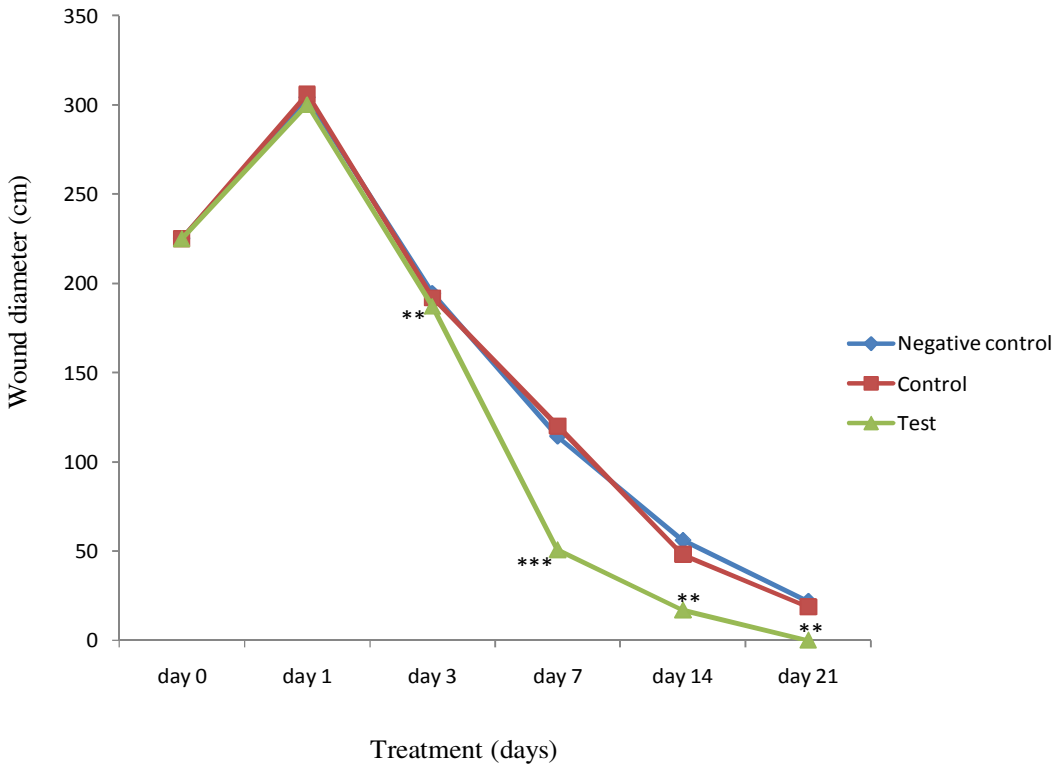

Figure 1. Cicatrizing activity in skin lesions. Data represent untreated animals (Negative control), animals treated with eucerin (Control), and animals treated with *Lactobacillus brevis* (Test). ** p< 0.01. *** p < 0.001 as compared with corresponding control and negative control using one-way ANOVA.

presented in Figure 1. The wound's diameter in the experimental group, which was treated with *L. brevis*, experienced a faster reduction than both the negative control and the control. At the 7_{th} day of the experiment, the wounds treated with *L. brevis* were smaller than the other groups' wounds (Figure 1).

The rates of contraction in the control and experimental wounds are depicted in Figure 2. The *L. brevis*-treated wounds were found to contract much faster. The epithelialization period of the treated wounds with *l. brevis* presented a significant decrease (P < 0.001).

Histological examination

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

Values are expressed as mean ± SE for 75 animals. The significant level of experimental group compared with control group $(P<0.01)^{**}$, $(P<0.001)^{***}$ and with negative control group $(p<0.01)^{\#\#}$, $(P<0.001)^{\#\#\#}$ using one-way ANOVA.

Also, the reduction in the number of neutrophils in the experimental group on the third (P<0.01), seventh, fourteenth and twenty first days of study was statistically vital as contrasted to the two other two groups (P<0.001). The number of macrophages on the third day (P<0.01) of study illustrated 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 group than control and negative-control groups (Table 1).

DISCUSSION

The clinical treatment of skin loss continues to be a major problem in surgical procedures. A therapeutic agent selected for the treatment of wounds should ideally improve one or more phases of healing without producing deleterious side effects.

The condensation rate of the wound was declined by moving skin to the center of the wound since the rate was dependent on the size of the open wound. This process induced the contractile properties of active fibroblasts, specifically myofibroblasts, in the bud tissue of wounds (Darby and Hewitson, 2007; Valander et al., 2009). Tests that measured the wound and calculated the percentage

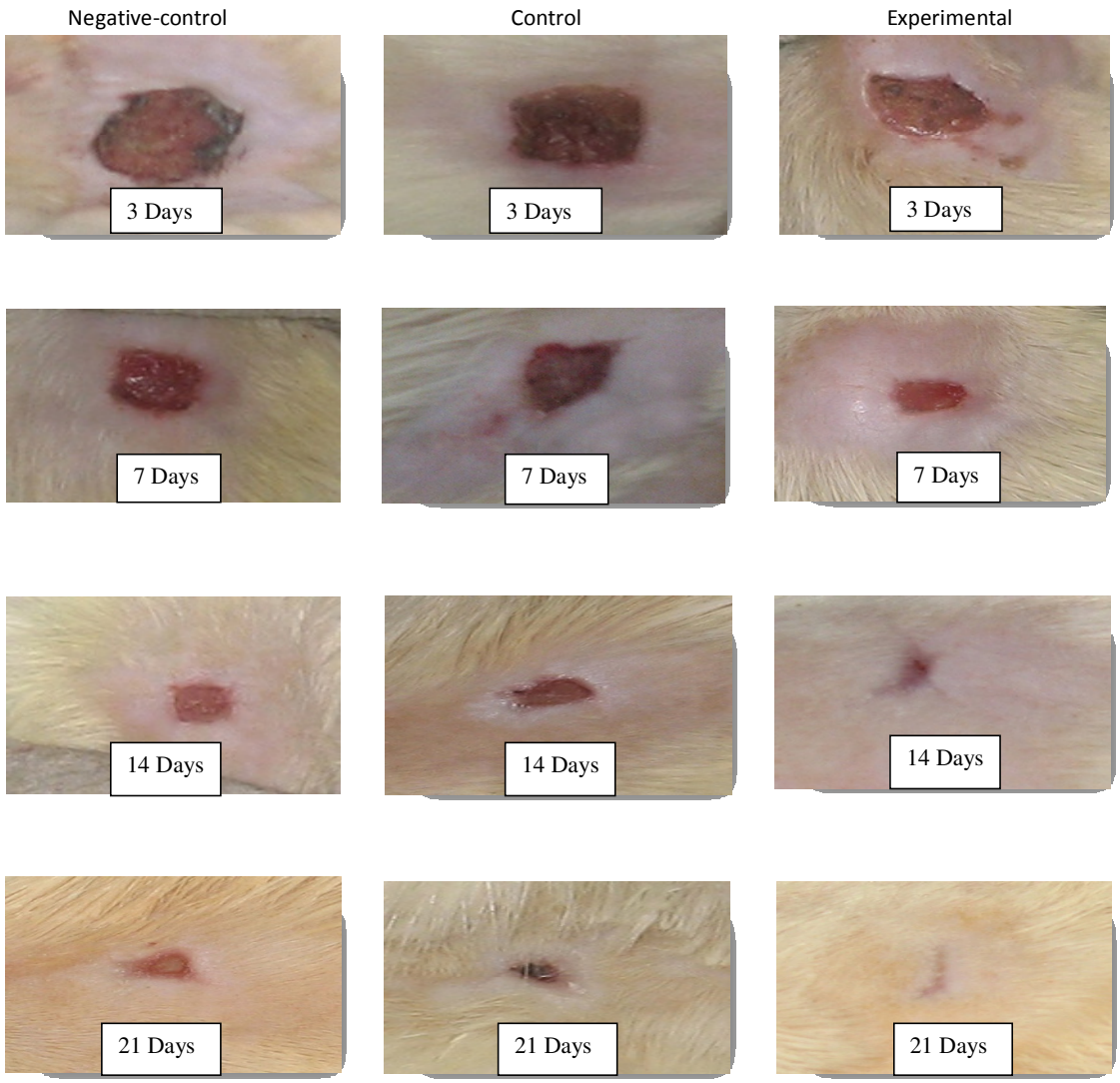

Figure 2. Photographic representation of contraction rate on different days. (a) Negative-control rats untreated; (b) control rats treated with eucerin; (c) experimental rats treated with *Lactobacillus brevis.*

of the healed wound showed that the treatment in the experimental group was significantly different, specifically in regards to the healed percentage than with the control and negative control groups.

In this study, although there is an increase in the wound area within 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 in the early days of the study because of skin and muscle tension. Blood clots form in two instances: when there are sores in the skin's epidermis and dermis layers and also when vascular peripheral blood cells are damaged and thrown into the area covered by the wound. After the blood plasma from this process comes in contact with collagen, the clotting reaction in which platelets accumulate and become granulated is initiated (Arul et al., 2007). This phenomenon causes hemostasis, which attracts inflamed cells to the wound site. These platelets-born are the production of tissue growth factor and platelet-derived growth factor which they role as a chemical absorbent for neutrophils and monocytes, that they are used in the subsequent phases (Boirivant and Strober, 2007).

In terms of coordination, there was no difference between the histometric results and the hypothesized theory. The reduction of the wound size was an effect of a reduction in the wound's inflammation. The wound's progress and results were presented in part. Conclusively, we found that using *L. brevis* resulted in the largest percentage of the wound healing in the shortest amount of time.

Tissue pathology studies show a reduction in neutrophile cells in the wound area. Since neutrophile cells are central in the wound's inflammation, an issue associated

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

Day	Groups	Variables		
		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	** 2.23 ± 0.337	** 11.46 ± 0.446	** 5.34 ± 0.209
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	*** 1.53 ± 0.430	** 9.23 ± 0.921	*** 11.25 ± 0.303
14	Negative control	3.02 ± 0.182	9.40 ± 0.910	11.82 ± 0.362
	Control	2.78 ± 0.184 ##	8.55 ± 0.745 ##	12.02 ± 0.520 ###
	Experimental	** 1.47 ± 0.210	** 3.550 ± 0.630	*** 4.13 ± 0.474
21	Negative control	2.02 ± 0.146	7.91 ± 0.765	7.20 ± 0.367
	Control	2.40 ± 0.221 ###	8.55 ± 0.715 ##	7.45 ± 0.342 ###
	Experimental	*** 1.25 ± 0.478	** 1.91 ± 0.228	*** 1.13 ± 0.183

Values are expressed as mean±SE for 75 animals. The significant level of experimental group compared with control group ($P<0.01$)**, ($P<0.001$)*** and with negative control group ($p<0.01$)$^{\#\#}$, ($P<0.001$)$^{\#\#\#}$ using one-way ANOVA.

with an increase in the area's macrophages, the decrease could be the evidence of the positive impact of the product in improving treatment of inflammations (Heidari et al., 2004; Ebrahimi et al, 2009b). Our review is focused on the third day which is the inflammation stage of wound healing. We found that the total number of neutrophils in the experimental group was significantly less than both the control group and the negative control group. On day 7, the wound was treated with a topical ointment containing cultured bacteria. 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. In cases of granulated tissue formation, fibronectin can perform a major role for cell growth and cell migration which facilitates wound contraction. Moreover, fibronectin is considered a suitable anchor for a regeneration stage (Qiu et al., 2006). Since wound contraction and cell regeneration are compatible with each other, we suggest that *L. brevis* has caused the inflammation stage of the wound to pass by quickly to the end. Consequently, the healing stage begins earlier. The foregoing results provide that *L. brevis* has improved the 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 diminishing the time required for full recovery. Throughout lessening inflammation – or, in other words, by adjusting the phase in which inflammation occurs – the inflammation stage is accelerated and thus the wound healing process is respectively hastened (Rea et al., 2009).

On days 14 and 21, total fibroblast declining institution in experimental group 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 prevent creating white ugly closure (Price et al., 2008).

The present investigation describes some unique features of the therapeutic effect of probiotics, specifically in regards to the wound healing process of the dermal layer of rats. In contrast to untreated wounds, wounds treated with *L. brevis* showed a larger number of infilitrating cells such as: macrophages, neutrophils, and fibroblasts. During granulation tissue formation, as contraction proceeds and resistance increases, fibroblasts differentiate into myofibroblasts. The presence of myofibroblasts is considered to be characteristic of tissue undergoing contraction (Qiu et al., 2007). The greater number of myofibroblasts in lactobacillus brevis-treated wounds may be partially responsible for the faster

wound contraction.

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