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## Evaluation of wound healing activities of kefir products

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### ABSTRACT

Kefirs are natural probiotic compounds with antibacterial and anti-inflammatory properties which were tested in experimental burn injury. Kefir gels were prepared from an extract of continuously cultured kefir grains in MRS Broth medium for 24, 48 and 96 h. Similar burn injuries were made on dorsal skin surface of 56 rats. After 24 h the wounds were infected with *Pseudomonas aeruginosa*. The infected rats were divided in to 7 groups of 8 rats each. The base gel, silver sulfadiazine ointment, kefir 24 h gel, kefir 48 h gel, kefir 96 h gel and kefir grains 96 h gel were applied twice a day. Burn wound area was measured at baseline, one and two weeks. After two weeks the animals in all groups were sacrificed and whole skin wound areas were removed and percentage of epithelization, scar formation, inflammation and angiogenesis were evaluated. Results indicated that at the end of the 2nd week the percentage of wound size were lowest in order of kefir 96 h gel < kefir grains 96 h gel < kefir 48 h gel < kefir 24 h gel < silver sulfadiazine 1% < untreated and based gel groups. At the end of the 2nd week the percentage of inflammation was lower and percentage of epithelization and scar formation was higher in order of kefir 96 h gel, kefir grains 96 h gel, kefir 48 h gel, kefir 24 h gel, silver sulfadiazine 1%, base gel and untreated groups. In conclusion the kefir gel therapy was an effective therapeutic approach to improve outcomes after severe burn as compared to conventional silver sulfadiazine treatment.

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## 1. Introduction

Normal wound healing consists of a series of coordinated overlapping events or phases that involves acute and chronic inflammation, cell division, cell migration, chemotaxis and differentiation of numerous cell types [1,2]. These events are tightly regulated and results in wound healing and restoration of the structural and functional integrities of the damaged tissues [3,4]. Although in modern burn wound management,

topical antibiotics such as silver sulfadiazine dressing is mainly used [5,6], but due to its adverse effects, bacterial resistance and ineffective on healing process search for alternative compounds that speed the wound healing process is of an interest [7–9]. However the probiotic compounds may be of good choices. Probiotics are single strain or a mixture of different organisms and are claimed to strengthen the immune system, reduced inflammation and speed wound healing process following accumulation of lymphocytes, macrophages and poly morphonuclear in place of injury

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[10,11]. Kefir grains are a probiotic mixture of diverse spectrum of bacteria and yeasts [10]. The microorganisms present in the kefir grains produce lactic acid [12]. Such products due to antibacterial properties inhibits the proliferation of pathogenic microorganisms [13]. The anti-inflammatory properties of polysaccharide present in the kefir extract also influences wound healing process [14,15]. Kefir grains also stimulate innate immune responses in defense against pathogens [16,17]. However in the present study the effects of different kefir extracts were tested on wound healing on burn induced injury on rat skin (Fig. 1).

## 2. Materials and methods

### 2.1. Preparation of kefir extracts and kefir gels

Kefir grains (50 g) were continuously cultured in 100 g/l of MRS Broth medium for 24, 48 and 96 h. The supernatants of culture fermentation were centrifuged, filtered and named as kefir 24 h, kefir 48 h and kefir 96 h. Three types of kefir gel products were prepared from above three extracts named as kefir 24 h gel, kefir 48 h gel and kefir 96 h gel and one type of kefir gel were prepared from kefir grains 96 h named as kefir grains 96 h gel [18,19]. In brief the 100 g gel base was formulated by mixing 32 g ethyl cellulose 1%, 16 g glycerin and 52 g propylene glycol. The kefir gels were formulated by addition of 100 ml of different extracts or grain 100 g to 100 g gel base.

### 2.2. Antimicrobial determination of kefir in vitro

The minimum inhibitory concentration (MIC) parameters of kefir were determined in triplicates using 0.1 ml of bacterial suspensions ( $3 \times 10^8$  CFU/ml) in tubes containing 10 ml of MHB solution and the same amounts of kefir as described above. Tubes were mixed using a Vortex for 60 s and incubated at 37 °C for 24 h. MIC values were obtained based on the results for MIC values. Plates containing MHA medium were inoculated with 0.1 ml of the tubes showing no growth and incubated for 24 h at 37 °C [20].

### 2.3. Experimental protocol

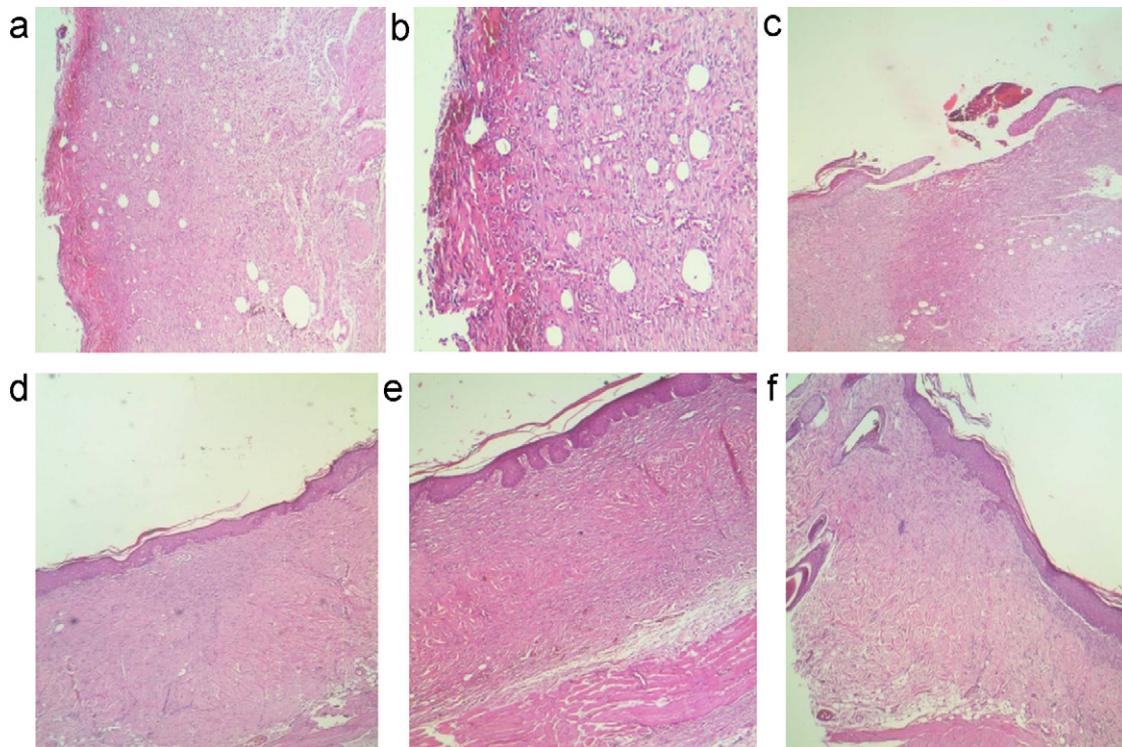
#### 2.3.1. Animals

Fifty-six male Wistar rats, aged six months old weighting  $200 \pm 10$  g were purchased from Pastor Institute Karaj city, I.R. Iran. The rats were caged under controlled conditions of light, room temperature and humidity for a week prior to study.

This study was approved by the ethical committee of Islamic Azad University, Tehran, Iran.

#### 2.3.2. Burn wounds induction

The 3rd degree burn wounds were induced on shaved area of dorsal skin of the rats under anesthesia (intraperitoneal injection of 100/5 mg/kg ketamin/xaylazin) using hot plate sized 3 cm  $\times$  1 cm at temperature of 156 °F or 69 °C for 3 s [21].



**Fig. 1 – Morphological changes of the rats skin lesions 14th days after burn wounds induction. Haematoxylin–eosin, 40 $\times$ . (a) Untreated rats; (b) rats treated with base gel; (c) rats treated with kefir 24 h gel; (d) rats treated with kefir 48 h gel; (e) rats treated with kefir 96 h gel; (f) rats treated with Kefir grains 96 h gel.**

The rats were placed in an isolated cage to inhibit transmission of infection. The wounds were examined after 24 h and in case of necrotic tissue, the same was removed. Debridement procedure under the standard way was done for all the animals.

2.3.3. *Microbial contamination of the burn wounds*

Twenty-four hours after burn wounds induction the burn wounds of all rats were inoculated at the same time with 0.1 ml of the *pseudomonas aeruginosa* (ATCC 27853) solution ( $3 \times 10^8$  CFU/ml) [22].

2.3.4. *Burn wounds treatment*

Twenty-four hours after the induction of infection the 56 infected rats were caged individually and divided into 7 groups of 8 rats each as follows:

1. Untreated group: the burn wounds received no medication.
2. Base gel group: the base gel was applied on burn wounds.
3. Silver sulfadiazine group: the silver sulfadiazine 1% was applied on burn wounds.
4. Kefir 24 h gel group: the kefir 24 h gel was applied on burn wounds.
5. Kefir 48 h gel group: the kefir 48 h gel was applied on burn wounds.
6. Kefir 96 h gel group: the kefir 96 h gel was applied on burn wounds.
7. Kefir grains 96 h gel group: the kefir grains 96 h gel was applied on burn wounds.

The gels and silver sulfadiazine thin layer were applied on burn wounds twice a day.

2.3.5. *Burn wounds infection assessment*

After one week any pus present at the site lesion in all rats were removed and cultured in blood agar medium and incubated at 37 °C. The cultures were checked after 24 h for the presence of *pseudomonas aeruginosa* using common laboratory tests [22].

2.3.6. *Burn wounds gross morphology assessment*

Wound area diameters were evaluated and measured by naked eyes on base line, one and two week's interval using planimetry procedure [23]. In brief wounds area were calculated by manually counting squares completely or half or more within the wound border using 1 mm<sup>2</sup> designed transparent graph paper. The initial wounds size using hot plate sized 3 cm × 1 cm were 300 mm<sup>2</sup> or 100%.

The percentage of wound size and recovery was calculated according to Eqs. (1) and (2)

Percentage wound area = 100

$$\times \frac{\text{wound area on day } x}{\text{wound area on base line (300 mm}^2\text{)}} \quad (1)$$

x is the day when the wound area is measured.

Percent of wound recovery = 100 – percent of wound area (2)

2.3.7. *Burn wounds histological assessment*

After 2 weeks the animals were sacrificed by spinal cord injury under anesthesia and 3.5 cm × 1.2 cm wound skin tissue in its full thickness were removed and paraffin embedded sections were prepared. The sections were cut with a microtome 2 μm thick, cutting perpendicular to the thickness of skin surface. The sections were stained with Haematoxylin–eosin. The percentage of epithelization, scar formation, inflammation and angiogenesis were evaluated in all specimens [24].

2.4. *Statistical analysis*

The data were analyzed by SPSS 10 software using ANOVA and Duncan mean comparison test. A value of  $p < 0.05$  was considered as statistically significant.

3. **Results**

3.1. *Antimicrobial activity of kefir in vitro*

MIC and MBC ratios of kefir extracts 24 h, 48 h and 96 h against the *pseudomonas aeruginosa* were ranged from 250 mg/ml (MIC) to 250 mg/ml (MBC) (Table 1).

3.2. *Microbial contamination of the burn wound*

The microbial contamination was observed after one week in all the wound tissue in the untreated and gel base groups. The microbial contaminations were observed in 4 and 2 rats in kefir 24 h gel and kefir 48 h gel groups, respectively.

No microbial contaminations were observed in the kefir grains 96 h gel, kefir 96 h gel and silver sulfadiazine groups. Microorganisms isolated from rats wounds were: *Staphylococcus aureus*, *Klebsiella*, *E. coli*.

3.3. *Gross morphology examination*

Results indicated that percentage of wound size were 3 cm on base line day for each rat in all the groups. At the end of the first week the percentage of wound size were significantly lower in kefir grains 96 h gel ( $p < 0.01$ ) and kefir 96 h gel ( $p < 0.01$ ) as compared to base gel and untreated groups as well as silver sulfadiazine treated group (Table 2). At the end of the 2nd week the percentage of wound size were significantly lower in kefir grains 96 h gel ( $p < 0.01$ ), kefir 96 h gel ( $p < 0.001$ ) and silver sulfadiazine 1% ( $p < 0.05$ ) as compared to base gel and untreated groups (Table 2).

**Table 1 – MIC and MBC ratios of kefir extracts 24 h, 48 h and 96 h against the *pseudomonas aeruginosa* (ATCC27853).**

	MIC	MBC
Kefir extract 24 h	250 mg/ml	250 mg/ml
Kefir extract 48 h	250 mg/ml	250 mg/ml
Kefir extract 96 h	250 mg/ml	250 mg/ml

**Table 2 – Percentage of wounds size after burn wounds induction at 1st, 7th and 14th days of treatment in 7 groups of 8 rats each (mean ± SD).**

Groups	Size of burn wound		
	First day	7 days	14 days
Kefir 24 h gel	95.0 ± 7.1	91.6 ± 2.3	85.1 ± 6.2
Kefir 48 h gel	95.0 ± 7.1	81.8 ± 4.6	74.6 ± 4.2
Kefir 96 h gel	95.0 ± 7.1	54.6 ± 2.8**	15.1 ± 2.1***
Kefir grains 96 h gel	95.0 ± 7.1	58.8 ± 6.2**	21.8 ± 3.0***
Untreated	95.0 ± 7.1	91.0 ± 6.2	89.6 ± 4.1
Silver sulfadiazine 1%	95.0 ± 7.1	92.5 ± 7.4	65.0 ± 5.2*
Base gel	95.0 ± 7.1	96.5 ± 3.5	92.5 ± 3.5

\*  $p < 0.05$ .  
\*\*  $p < 0.01$ .  
\*\*\*  $p < 0.001$ .

Percentage of wound size in all treated groups were compared to untreated and base gel groups. Percentage of wound size in kefir 96 h gel was significantly lowest at the end of 2nd week as compared to all other groups.

### 3.4. Histological examination

The percentage of inflammation, angiogenesis, epithelization and scar formation at the end of 2nd week in all groups is summarized in Table 2. Result showed that at the end of the 2nd weeks the percentage of epithelization and scar formation were significantly higher in kefir 24 h gel ( $p < 0.05$ ), kefir 48 h gel ( $p < 0.05$ ), kefir grains 96 h gel ( $p < 0.01$ ) and kefir 96 h gel ( $p < 0.001$ ), where as the percentage of inflammation were significantly lower in kefir 24 h gel ( $p < 0.01$ ), kefir 48 h gel ( $p < 0.01$ ), kefir grains 96 h gel ( $p < 0.001$ ) and kefir 96 h gel ( $p < 0.001$ ) as compared to silver sulfadiazine 1%, base gel and untreated groups. Angiogenesis were not significantly different between the groups. The data are summarized in (Table 3).

## 4. Discussion

Kefir extracts are typical probiotic mixture of several bacteria and yeasts with antimicrobial and inflammatory activity [25,26]. In present study the wound healing activity and antimicrobial effects of kefir gels were tested in experimental

burn wounds infected with *Pseudomonas aeruginosa* (ATCC 27853). Of three types of kefir gels tested the antimicrobial activity of kefir 96 h gel was similar to silver sulfadiazine ointment but wound healing time were lower in kefir 96 h gel as compared to silver sulfadiazine ointment. Furthermore the process of burn wound healing took place within 14 days for kefir gel 96 h in our study, but it was for 24 days for silver sulfadiazine reported in previous study [22]. These data indicated that, continuously cultured kefir grains in MRS Broth medium up to 96 h increases the wound healing properties of extract. The antimicrobial properties of kefir were reported on several microorganisms in laboratories as well as in human diarrheas disease and urinary tract infection [27-29]. Several mechanisms were reported for antimicrobial effects of kefir grains. Farnworth [12] reported that the antimicrobial effects of kefir grains are due to lactic acid and antibiotics produced by microorganisms. Kumthavee [30] proposed that, bacteriocin and lactic acids from *Lactobacillus rhamnosus* isolated from kefir grains are responsible for such antimicrobial effects. However several other mechanisms such as production of organic acids, ethanol, bacteriocines and hydrogenperoxyde, in fermented process were proposed for antimicrobial activity kefir extracts [30-32]. The anti-inflammatory property is also influence process of wound healing [17]. Medeiros et al. [33] reported that the positive effects of hyaluronic acid on burn injuries are due to its anti-inflammatory effects. The anti-inflammatory properties of polysaccharide present in kefir extract may also influence in process of wound healing [14,34]. However the lactic acid, acetic acid, polysaccharide and other chemicals present in kefir preparation are important factors for antimicrobial, anti-inflammatory and wound healing properties observe in present study. In support for this hypothesis the lactic acid and acetic acid concentration were higher in orders of kefir 96 h gel > kefir grains 96 h gel > kefir 48 h gel > kefir 24 h gel observed in another our unpublished articles. However in future studies we try to standardize kefir gel product by determination of lactic acid, acetic acid and polysaccharide concentration along with its burn wounds healing properties in animal studies. In conclusion the kefir gel therapy especially kefir 96 h gel with longer culture fermentation time strongly improves clinical outcomes after thermal injury as compared to conventional silver sulfadiazine treatment.

**Table 3 – Percentage of inflammation, angiogenesis, epithelization, and scar formation at 14th days of treatment in 7 groups of 8 rats each (mean ± SD).**

Treated groups	Scar formation	Epithelization	Angiogeneses	Inflammation
Kefir 24 h gel	12.5 ± 2.1	18.3 ± 2.4*	97.5 ± 3.5	16.1 ± 2.8**
Kefir 48 h gel	15.5 ± 3.0*	44.1 ± 1.4*	84.5 ± 4.3	16.0 ± 3.1**
Kefir 96 h gel	61.0 ± 5.6***	72.5 ± 6.5***	97.5 ± 3.5	11.4 ± 2.8***
Kefir grain 96 h gel	41.5 ± 5.2**	59.4 ± 4.2**	79.5 ± 2.1	15.2 ± 4.2**
Untreated	0 ± 0	2.5 ± 1.1	97.5 ± 3.5	97.5 ± 3.5
Silver sulfadiazine 1%	0 ± 0	5.0 ± 1.0	97.5 ± 3.5	95.0 ± 7.0
Base gel	0 ± 0	2.0 ± 0.1	95.0 ± 7.0	97.5 ± 3.5

\*  $p < 0.05$ .  
\*\*  $p < 0.01$ .  
\*\*\*  $p < 0.001$ .

Inflammation, epithelization and scar formation in kefir 24 h gel, kefir 48 h gel, kefir grains 96 h gel and kefir 96 h gel were significantly higher as compared to silver sulfadiazine 1%, base gel and control groups.

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## Conflict of interest statement

No conflict to disclose.

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