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Protective properties of kefir on burn wounds of mice that were infected with *S. aureus*, *P. auroginasa* and *E. coli*

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Abstract: Burns and burn wounds are very sensitive to infections and cause a large amount of death worldwide. Although burn wound is sterile at the beginning, because of the risk factors such as prolonged hospital stay, immune suppression and burn affecting large surface area, colonisation with *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* occur. For the burn therapy, one of the most important ways is to control bacterial infections. A probiotic fermented milk product kefir has antioxidant, antimicrobial, antiinflammatory, anticancer and various health promoting features. This study aims to examine possible protective properties of kefir which was used on the burn wounds that were infected with *S. aureus, P. auroginasa and E. coli*. *Swiss albino / Balb-c mice* were seperated into four groups: (1) used as control group, (2) second-degree burn model+ burn wounds were infected with *P.aeruginosa* + *S.aureus* + *E.coli*, (3) second-burn wounds were treated with sterile pads dressed with kefir and (4) second-degree burn+burn wounds were infected with *P. aeruginosa* + *S.aureus* +*E.coli* before being treated with sterile pads dressed with kefir. The serum biochemical results verified the histopathological results and our findings showed that kefir is an effective product with cell-protecting properties.

Key words: Second-degree burn; Kefir; Wound healing; Infections; *S.aureus*; *P.aeruginosa*; *E.coli*; Mice.

Introduction

Burns are one of the health issues in modern life due to their serious damages to the patients and to the family relationships (1). Burn area infection is a prominent problem in burn treatment and is the most common factor of fatality after burns (2,3). The microorganisms causing burn infection may be infected from the environment during the treatment of patients in burn units or from another patient treated in the same unit (4,5). Burn wound infections by *Pseudomonas aeruginosa*, *Escherchia coli* and *Staphylococcus aureus* are more common causes of mortality (2,6). *S.aureus* that is gram-positive is the a major reason of burn wound infections (3) and commonly end up with septicaemia (7,8). In the gram-positive bacteri infected burns hyperthermia, leukocytosis, behavioral disorders, mental confusion are usually seen and around of the wound cellulitis and exuded maceration occur. Hypothermia and leukopenia are common in burn infections caused by gram negative bacteria. And the patients may be confused (4).

Alternative medicines with natural products are cheaper options and becoming increasingly common (9,10). Probiotic products are reported to strengthen the immune system, reduce wound healing process and inflammation following lymphocyte accumulations in wound area (11,12). Probiotics are being used for allergic diseases, bacterial vaginosis, urinary and gastrointestinal system infections (13). Probiotic microorganisms can inhibit the adverse effect of pathogens, strengthen the immune response and intestinal barrier (14,15). Potential health research has focused on fermented milk from cows, ewes and goats milk such as kefir which is well known as a major source of probiotic (16-18). Kefir is a famous fermented product with its potential health benefit properties that arise from the microbial species that kefir grains have which are also associated with kefir fermentation (19). Kefir grains include a rich microbial community formed by bacterial and yeast microflora responsible for the kefir fermentation (20,21). Kefir contains rich health-benefiting properties, exhibits antioxidative, antimicrobial, anticarcinogenic and different health supporting activity (22,23). Kefir addition has diverse health benefits such as gastro-intestinal cell proliferation, antibacterial, anti-mutagenic, anti-inflammatory, antiallergic, hypocholesterolemic, anticarcinogenic, antidiabetic, β-galactosidase, scavenging activity, lactic acid content, lipid and blood pressure level effect, protection against apoptosis, bacterial colonization and immune system support properties (22). Furthermore, kefir benefits on wound healing process with anti-inflammatory activity of polysaccharide present in kefir product (24,25).

In the this experimental study, we aimed to examine the wound healing process and antimicrobial activities of kefir in second-degree burn injuries infected with *S.aureus, P.aeruginosa* and *E.coli* in mice.

Materials and Methods

Kefir Fermentation

In this study, freeze-dried kefir culture was commercially purchased from Faculty of Agriculture, Dairy Product Technology, Ege University, Turkey to produce kefir.This lightly natural aromatickefir grains with1 lt pasteurized cow milk were preferred for kefir fermentation. The method used for kefir fermentation was reported by Marshall et al. fermented kefir was incubated for 24h at the room temperature (24-26 °C) (26). At the end of the fermentation period, kefir was cooled to about +4 °C and kept at this temperature for usage. The kefir products after fermentation were centrifuged. Supernatant portions were taken and used in the study. Kefir was matured at 4°Cfor at least one day before use and fermentation was renewed daily and used fresh.

Preparation of Bacterial Cultures and contamination of the burn wounds

Escherichia coli (ATCC23276), *Staphylococcus aureus* (ATCC26542) and *Pseudomonas aeruginosa* (ATCC 25338) control strains were commercially purchased from SACEM Hayat Teknolojileri A.S., Turkeyto use in the study.Blood Agar Base was used for *Staphyloccocus aureus* (ATCC26542). The Selective Agar Base was used for *Pseudomonas aeruginosa* (ATCC 25338). Eosin Methylene Blue agar was used for *Escherichia coli* (ATCC23276). The three bacteria were all diluted to $1.5x10^5$ CFU.mL^{-1} with 0.9% sodium chloride solution and the experiment was repeated three times to determine the minimum inhibitory concentration (MIC).

Antimicrobial determination of kefir in vitro

In order to test the MIC parameters of the kefir, kefir which were kept for 24 h were added to the tubes containing 10 ml Müller Hinton Broth (MHB).Then samples were prepared containing 0.1 ml of bacterial suspension ($3x10^8$ CFU / ml).The mixture was stirred using vortex for 60 seconds, then, incubated at 37 °C for 24 h. MIC levels were obtained. Then, MHA medium was sown, and no growth samples were included in the study (27). MIC values of kefir for *S.aureus* were 2.42 mg·mL^{-1}, *P.aeruginosa* 7.9 mg·$mL^{-1}$and *E.coli* were 4.55 mg·mL^{-1}.

Animals and burn model preparation

Swiss albino / Balb-c mice were provided from Kobay Experimental Animals Lab. San. Tic. A.S. The experimental protocol of this study was approved by the ESOGU Experimental Animals Ethic Committee (Protocol no: 618-2 / 30.11.2017) prior to the experiment. 28 male, 3 months, 25-30 g *Swiss albino / Balb-c* mice were used for this study. Seven (7) mice were used for each group. Before the experiment, the animals were kept for at least 1 week in 22 ± 2 °C temperature, 60±5% humidity, 12 h light/dark cycle and fed with pellet diet and tap water. The mice were randomly seperated into 4 groups (each group include 7 mice): *(1)* used as control group, (2) second-degree burn model+burn wounds were infected with *P.aeruginosa+S.aureus+E. coli,* (3) second-degree burn wounds were treated with sterile pads dressed with kefir and (4) second-degree burn + burn wounds were infected with *P.aeruginosa+ S.aureus+E.coli* ($3x10^8$ CFU/ml) before being treated with sterile pads dressed with kefir. The mice were intramuscularly anesthetized with ketamine/xylazine (80/12 mg/kg) combination and their dorsal hair was shaved with a sterilized clipper. Then the shaved skin was disinfected with 70% (v/v) ethanol. Burn wounds were formed on dorsal area of shaved mice using a metal rod (1.5 cm diameter) heated over the boiling water for 30 s and pressed to the target area surface for 20 s according to Zhang et al. (28). After that, the animals were individually kept in order to inhibit other mice disturbance at the wound healing process. Two animals from 2ndgroup died due to unknown reasons.

Kefir was matured at 4 °C for at least one day before use. Daily amounts of kefir were brought to the experiment medium with in tubes for usage. After mice were infected to prevented from contamination they were left in separate cages during the experiment. Kefir fermentation was renewed daily and used fresh. Since the mice were separated by individual cages during the experiment, the contamination of sterile wounds with bacteria around the mouse was prevented. After the burn wounds were formed, 2nd and 3rd groups burn wound was inoculated with 0.1 ml of *S.aureus, P.aeroginasa* and *E.coli* solution ($3x10^8$ CFU/ml). And after we had infected the mice with bacteria we applied kefir for 7 days.

Measurement of wound surface area

Daily photographs of all mice that included in the study were taken. Wound area was measured in millimeters square by area calculation method.

Histological study

Burn tissue biopsies were 0.5x0.5x0.5 to 1x1x1 cm and 100-500 mg. Biopsy samples were taken together with tissues both containing and not containing burn area. A collagen fiber, inflammatory cell, blood vessel and granulation tissues were examined under a microscope. This tissues were stained with hematoxylen-eosin, Brown Hopp gram stain and periodic acid-schiff (PAS) (4).

Taking serum from mice

On the last day of experiment, intracardiac blood was taken under appropriate anesthesia. The blood was used for eveluate leucocytes, thrombocytes, neutrophil, monocyte and lymphocyte. The collected bloods were centrifuged at 2000 x rpm for 10 mins to separate the serum. All serum were kept at -20 °C until the working day.

Statistical analysis

The findings were showed as means ± S.E.M. Statistical analysis was applied by using One Way Analysis of Variance and Kruskal-Wallis One Way Analysis of Variance on Ranks Test. Value of $p<0.05$ accepted as considering statisticallyimportant. Each experiment was repeated at least three times.

Results and Discussion

Burns are suitable areas for bacterial increase and infection, when patients remain in the hospital for a long

time (29). Burn injuries are so traumatic and physically debilitating that these can adversely affect almost all the organs (30). Because of epidermisis lost after burn injury, the epidermis becomes susceptible to infections and this causes significant morbidity and mortality in burn trauma cases. As a matter of fact; from our findings in activity, exhaustion, trembling and weakness were seen in the burn + bacteria infected 2nd group when we compared to theother groups. Burn wounds can be treated with different methods depending on the severity of the burns. Researches (31,32) demonstrated that topical antibiotics treatment is mostly used but due to its adverse effects such as bacterial resistance and insufficient on wound healing process, search for alternative natural products isa growing interest. It was observed from our findings that mice in kefir treated burn group are seen more mobile and healthy when compared to burn + infection group.

Tissue inflammation occurs after exposure to thermal heat in the tissues of the burnt stasis region and tissue edema is seen. During this inflammation, cytotoxic cytokines and reactive oxygen species (ROS) are released from the neutrophils collected in the medium. Due to prolonged inflammation, burned cytokines and ROS are not able to compensate antioxidant mechanisms, resulting in damage to vital structures such as lipid, protein and nucleic acid (33). Ma et al.(34)reported that neutropenia is one of the common complication of burn. Our result showed thatleucocytes, thrombocytes, neutrophil, monocyte and lymphocyte numbers were increased in the 2nd group (Table 1). This is a sign of severe tissue damage and inflammation in the burn area. Therefore, it was important to search more efficient agents with fewer adverse effects for healing of burns. Lopitz et al. (35) reported that kefir is found to act against pathogenic bacteria. Also it was demonstrated that kefir can stimulate innate immune responses in defense against pathogens (36,37). According to our results, the number of neutrophil, leucocytes, thrombocytes, monocyte and lymphocyte is lower in the kefirgiven 3rd group than all the other groups despite of the burn injury (Table 1). Therefore, we may infer that topical application of kefir can concurrently protect burn wounds from infection and accelerate the healing process.

Kefir can be used as topical treatment for burn injury for its anti-microbial activity. Our findings showed that kefir dressing healed the burn wound and reduced the burn wound area increasingly (Fig. 1). We may infer from this; for burn treatment, the important way is to control the bacterial infection. Parallel to our studyHuseiniet al. (25) observed that the lactic acid, acetic acid, polysaccharides and other chemicals present in kefir are important factors for wound healing properties and woundsareimportantlylower in kefir compared to control, base gel and silver sulfadiazine dressing groups.

Our histological results also showed the wound heal-

Figure 1. The appearance of the2nd, 3rd and 4th Groups'burn wounds with escharon last days of study before dissection.

Table 1. The number of leucocytes, thrombocytes, neutrophil, monocyte and lymphocyte. Kruskal Wallis One Way Analysis of Variance on Ranks. (Median (25%-75%)).

	Groups	Mean±Std. Deviation	Median (25%-75%)	P	Multiple Comparisons
Leucocytes 10^6 mm^3	Group 1 (Control)	11,36±1,91	10,60 (9,95-12,99)	0,001	1-2
	Group 2	19,67±2,34	20,00 (17,50-22,00)		
	Group 3	14,20±2,06	14,10 (12,65-16,20)		
	Group 4	16,37±1,99	16,60 (14,15-18,20)		
Thrombocytes 10^6 mm^3	Group 1 (Control)	275,33±34,79	277,00 (250,00-303,50)	0,004	1-2, 1-3, 1-4
	Group 2	403,33±40,69	399,00 (367,50-428,00)		
	Group 3	390,67±49,78	396,00 (342,50-425,50)		
	Group 4	401,00±58,06	385,00 (363,00-431,00)		
Neutrophils 10^6 mm^3	Group 1 (Control)	26,67±3,27	27,00 (23,50-30,00)	0,012	1-2, 1-4
	Group 2	38,97±6,11	39,40 (32,60-44,65)		
	Group 3	31,60±15,65	37,50 (25,80-40,05)		
	Group 4	38,37±1,42	38,50 (37,50-39,30)		
Monocytes 10^6 mm^3	Group 1 (Control)	2,07±0,33	2,10 (1,75-2,40)	0,001	1-2
	Group 2	3,80±0,42	3,90 (3,60-4,05)		
	Group 3	3,07±0,35	3,10 (2,75-3,30)		
	Group 4	3,20±0,40	3,15 (2,80-3,58)		
Lymphocytes 10^6 mm^3	Group 1 (Control)	70,67±6,02	69,00 (65,50-78,00)	0,076	Non-significant
	Group 2	81,67±8,04	85,00 (74,00-88,00)		
	Group 3	79,00±4,86	80,00 (76,00-82,50)		
	Group 4	80,33±9,16	83,00 (73,00-88,00)		

Table 2. In the histopathologic evaluation; epithelial, vascular and fibroblastic proliferation, collegenization, inflammatory cells infiltratesis evelauted.Method for scoring histopathologic parameters: 0-no findings. 1-low level findings. 2-middle level findings. 3-significant level findings.

	Epithelial Proliferation	Vascular Proliferation	Fibroblastic Proliferation	Collegenization	Inflammatory cells infiltrates
Group1 (Control)	0	0	0	0	0
Group 2	2.7	3.1	3	2.9	2.9
Group 3	0,8	1	1	1.2	1
Group 4	2.1	2	1.9	2.3	2

ing and antimicrobial effect of the kefir at the cell level. As a matter of fact, in the 3rd group it was seen that kefir could protect tissue cells in spite of the infected burn wound (Fig. 2).

At the end of the experiment, wound tissues were removed from the mice for histopathologic examination. The results confirmed that skin samples with burn wounds were exposed to more severe infiltration of inflammatory cells and necrosis of the epidermis and hypodermis compared to normal skin tissues.The tissues taken from kefir treated 3rd group were seen as a thick and well developed epidermal layer similar to normal skin.Additionally, inflammatory cells infiltrates was less than control.Histological findings showed that kefir may support epidermal regeneration and accelerate wound healing (Fig. 2, Table 2). Epithelization, collegenization and eschar reduction were found to happen in much shorter time in the kefir administered 3rd group to compared with burn+infection 2nd group and control (Table 2). Also, based on Table 2 findings, we can indicate that kefir dressingimportantly accelerates angiogenesis in burn tissue. Likewise, it is observed that kefir had a positive effect on fibroblastic proliferation and collegenization during burn wound healing (Table 2). Huseini et al. (25) reported that epithelization and scar formation were markedly higher while inflammation were markedly less in kefir treated group compared to silver sulfadiazine and control (1st) groups.

Although inflammation is an significant situation in the wound healing, it can also inhibit the healing process (38). Shupp et al. (33) explored the prominent inflammatory cell infiltration of burn wound progression and proposed that after burn injury, the burn wound experiences a prolonged inflammatory response in which neutrophils release cytotoxic cytokines and ROS. Moreover, persistent neutrophil aggregation in postcapillary venules contributes to vascular occlusion and edema. As a matter of fact, in present study, inflammatory cell infiltration increased in the 2nd group compared to the the other groups.

Based on our findings, we can say that kefir dressing accelerates angiogenesis and vascular proliferation in mice burn skin (Table 2). Similar to our study, Zhang et al.showed that SOP dressing on burn wound decreases wound contraction and epithelization process and supports collagenization compared to control (28).

From the findings of this study we may infer that kefir could accelerate the healing of second-degree burns with its potent antibacterial property. Similarly Huseiniet al.showed that kefir has better wound-healing activity than conventional silver sulfadiazine treatment (25). A study byKamila et al. showed that kefir has a better wound healing activity comparingto the clostebol-neomycin treatment(39). Also, Rodrigues et al. reported

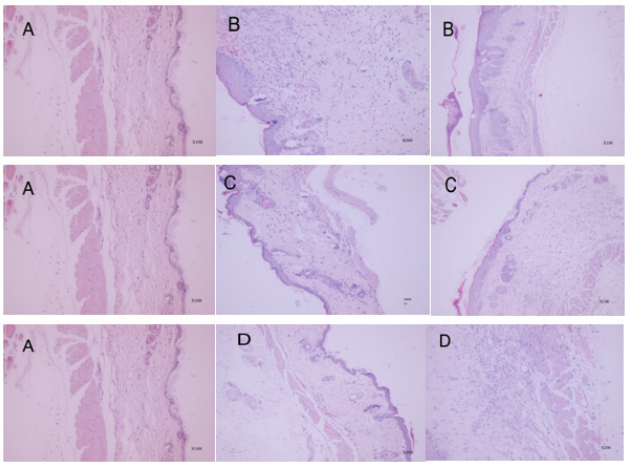

Figure 2. Haematoxylin and eosin (H&E) staining of skin tissue. **A-** Microscopic images of control group, **B-**Microscopic images of burn + infected 2nd group, **C-**Microscopic images of burn + kefir treated 3rd , **D-**Microscopic images of burn + infection + kefir treated 4th group

Table 3:. The body weight of mice after kefir treatment at the end of experiment. Data are expressed as mean ± S.D.

Groups	Body weight before treatment (gr)	Body weight after treatment (gr)
Group 1	26.42	31.25
Group 2	28.15	23.42
Group 3	27.24	29.38
Group 4	28.76	25.61

that rats treated with kefir, showed a faster healing activity comparing to clostebol-neomycin treatment on infected-wound (40).

From the findings on Table 3 it was seen that no important body weight changes were observed in all the groups at the end of the experiment. However, it was observed that the body weight decreased in the 2nd and 4th groups, especially in the 2nd group. In the 3rd group mice gained weight, this can be a result of kefir (Table 3).

There covery signs in the results of biochemical findings were verified the histopathological results. Our results indicate that kefir is an effective burn wound healing product with cell and tissue protecting activities. Kefir which is a promising treatment for the second-degree burns is an important antimicrobial. According to the results, it could be concluded from our findings that kefir accelerates the healing of second degree burn wound and prevents infection influentially. We may infer that kefir is a good candidate for a therapeutic product for burn treatment and has an effective antibacterial activity against burns but in-depth more studies will be needed to evaluate its clinical application on humans. Furthermore, considering the lack of information on the

effect of kefir on the development of burn wound, these results can provide a preliminary platform for future researches.

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Author contributions
Concept – *S.C.Y.*; Design – *S.C.Y.*; Supervision – *S.C.Y.,* C.D., Resource – S.C.Y.; Materials – *S.C.Y., C.D., M.C., A.A;* Data Collection and/or Processing – *S.C.Y., C.D.;* Analysis and/or Interpretation - *S.C.Y., C.D., M.C., A.A;* Literature Search – *S.C.Y., C.D.;* Writing – *S.C.Y.* Critical Reviews – *S.C.Y.*

Conflicts of interest
There are no known conflicts of interest associated with this publication.We confirm that the manuscript has been read and approved by all named author.

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