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The effects of Beeswax, Olive oil and Butter impregnated bandage on burn wound healing

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ABSTRACT

Background: Beeswax, Olive oil and Butter (BOB) are nutritive products that could support wound healing by adsorption to bandage. This study demonstrated the therapeutic effects of BOB on second degree burn.

Methods: Second degree burn model was created in rats. Experimental groups were assigned to Healthy, Burn, Silver Sulfadiazine (SS) and BOB. The effects of BOB were evaluated on skin regeneration, vesicles and bullae and fibroblast activity by histopathological analyses and wound contraction percent were determined. Transforming Growth Factor-Beta1 (TGF-β1) and Vascular Endothelial Growth Factor-alpha (VEGF-α) mRNA expressions were analyzed with Real Time-Polymerase Chain Reaction. All parameters analyzed at 3rd, 7th, 14th days.

Results: The BOB treatment increased TGF-β1 and VEGF-α expressions compared to Burn group. The histopathological analyses showed that epidermis and dermis layers injured due to burn. BOB treatment augmented the regeneration of these layers and increased fibroblast activity and keratinization which are play important role on the new blood vessels production. Also with the BOB treatment we showed wound contraction levels were higher than Burn and SS treatment.

Conclusion: This study demonstrated that beeswax-olive oil-butter mixture impregnated bandage treatment in a second-degree burn rat model improved burn wound healing and encouraged skin renewal via modulating tissue TGF-β1 and VEGF-α.

1. Introduction

Burn is very common trauma in all over the world that can treat with out-of-hospital treatments, can reach up to multiple organ failure which can lead to hemodynamic changes on skin, vascular and blood components. Treatment of severe burns requires long term hospitalization. According to American Burn Associations' data, only in a year in the United States, approximately 486,000 individuals are admitted for burn treatment to hospitals or emergency departments and 40,000 of these people get treatment in the hospital [1]. Nowadays, death rates due to burn injuries show decline

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because of the developments on the patient care and treatments [2].

Burn injury is directly related with the burn degree. Burns are classified first, second and third degree burns according to the affected skin layers. In the second degree burns, all of the epidermis layer and some part of the dermis layer was damaged [3]. Clinically, second degree burn wounds are characterized by edema, severe pain and blister formation. The rate of wound healing depends on the depth of skin damage and the occurrence of the infection [3].

Wound healing is a biological mechanism that involves cellular interactions of fibroblast, keratinocyte, immune and endothelial cells. It is also well known that wound healing involve some biochemical and cellular events like cell proliferation, inflammation, adhesion and neovascularization. TGF-β1 and VEGF-α play important roles on the formation of biomolecules during the wound healing process. It is believed that VEGF is the most effective in all growth factors due to trigger angiogenesis in wound [4]. VEGF also trigger the collagen production and epithelization [5]. TGF-β is more effective than others growth factors on the polypeptide production, cell growth and differentiation during wound healing process [6].

Today some topical agents which have anti-microbial, anti-inflammatory and anti-oxidant effects are being used on burn healing [7–9]. Unsalted butter and olive oil are rich in mineral, vitamin and unsaturated oil content and these contents both have anti-inflammatory effect and provide nutrition for the skin during treatment [10,11]. Olive oil compounds have a lot of benefit effects in healing burned skin [12] and using olive oil and beeswax mix on burn healing treatment showed therapeutic effect [13]. Topical application of olive oil and beeswax have protective effects on skin defects [14,15]. Beeswax is commonly used especially in folk medicine. Beeswax has antioxidant and antimicrobial effects and it was shown that beeswax increases cytokines in skin cells [14,16]. Beeswax, Olive oil and Butter mixture impregnated bandage can provide positive results to burned skin like a new skin dress.

Recent studies show the protective effects of some new methods like skin dressing that preserves the wound integrity and humidity [17]. Especially protecting the humidity of the wound help the healing process in order that humidity keeps the wound area alive. But there is not an exact treatment method on this problem yet, so more researches on burn and wound healing process are necessary. So this study aimed to investigate BOB treatments as a new and complex protocol for burn induced wound injury and provide information to future clinical approaches.

2. Materials and methods

2.1. Animals

60 male rats (Albino Wistar-220-250 g) were received from the Medicinal and Experimental Application and Research Centre of Ataturk University (ATADEM). The animal care and experimental protocols were approved by the Experimental Animal Ethics Committee of Ataturk University, under protocol number 36643897-102. The rats were housed in standard conditions. Tap water and standard rat food were given ad libitum.

2.2. Experimental design

Four groups were created;

Group 1: Healthy group (n=6)

Group 2: Burn control group (n=18)

Group 3: Silver Sulfadiazine (SS) group (n=18)

Group 4: Beeswax+Olive oil+Butter (BOB) treatment group (n=18)

2.3. Bandage preparation

Beeswax (5 g), Olive oil (10 ml) and Butter (10 g) mixed in a beaker and were heated. Liquid mixture was prepared with bain–marie method. This mixture was impregnated to bandage and after impregnation these bandages were sterilized. The bandages were sterilized by gamma irradiation at 25 kGy by Gamma-Pak sterilization Ind. & Trd. Inc. (Istanbul, Turkey). Sterile bandages were cut as large as enough to cover the wound area.

2.4. Burn model

All rats were anesthetized by thiopental (intraperitoneal) and sevofluran (%5-inhaled) combination. Left backs of rats was shaved and to induce second degree burn model, hot cylinder that filled with boiled water applied to the skin of rats (95 °C for 15 s) [18]. This heat exposure caused a uniform second-degree burn on the left back side of the skin. The animals were prevented from dehydration by 5 ml of normal saline injection.

The animals were divided in four separate groups after 24 h. Nothing was applied to Group 1 (the healthy group), nothing was applied to Group 2 (burned control group), Group 3 was SS, (silver sulfadiazine), (treated group single dose per day), Group 4 was BOB, (treated group once per day).

24 h after induction of burn model, we started treatment protocols. For evaluation of wound healing rates, wound images were photographed and the lesion sizes was determined at 3rd, 7th, 14th days. After determination of macroscopic wound healing examination, the tissues were kept at −80 °C for molecular analyses. The rest of the tissues were transferred immediately into 4% formaldehyde for histopathological analyses.

2.5. Contraction measurement

We investigated percent contraction levels on burned skin tissue of rats by investigator programme. The size of the wound areas on the first day was taken notice as 100% and wound areas on subsequent days were compared with the first day. Following formula was used for calculation of lesion results [19];

Percentage of wound contraction=(Initial wound size − Specific day wound size)/Initial wound size × 100

2.6. Total RNA extraction and cDNA synthesis

Tissues (20 mg) were stabilized in RNA Stabilization Reagent (RNAlater, Qiagen), and then shred using the Tissue LyserII (Qiagen). Total RNA was purified using RNeasy Mini Kit

Qiagen according to the instructions in Qiaqube (Qiagen). The RNA samples were reverse-transcribed into complementary DNA by High Capacity cDNA Reverse Transcription Kit. Reverse transcription was carried out at by Veriti 96 Well Thermal Cycler (Applied Biosystem). The cDNA concentration and quality was assessed and quantified by using the Epoch Spectrophotometer System and Take3 Plate (Biotek) [9].

2.7. Real-time quantitative PCR analyses

Skin tissue relative TGF-β1 and VEGF-α expression levels determined with StepOne Plus Real Time PCR System technology (Applied Biosystem). TaqMan Gene Expression Assays Rn00572010_m1 for rat TGF-β1, Rn01511601_m1 for rat VEGF-α and Rn00667869 for rat β-actin (Applied Biosystems) were used for PCR amplification. β-actin was used as standard control protein in each tissue. For each tissue, determinations for all target genes (TGF-β1, VEGF-α and β-actin) were performed as described prefiously [9,20]. All data are expressed as fold-change in expression compared to the expression in other animal grups, using the $2^{(-\Delta\Delta Ct)}$ method.

2.8. Histopathologic analyses

Samples were taken for histopathological studies with a small excision containing part of the wound area from skins. Tissue samples were fixed in 10% neutral formalin. Tissues were embedded to Paraffin wax and sections were cut 4-5 μm thickness and stained with haematoxylin and eosin. Histopathologic assesment were done under light microscopy (Olympus BX 51, Japan) and scored including hypereosinophilic appearance, coagulation necrosis, inflammatory cell, odema, vesicles/bullae, fibroblast activity, epithelial regeneration, keratinization levels.

2.9. Statistical analysis

For the statistical analysis, we used SPSS 20.0 software. Results are presented as means ± standard deviation (SD). Significant differences were detected between all groups, compared to Healthy group (*$p<0.05$, **$p<0.01$, ***$p<0.001$) and compared to Burn group (#$p<0.05$, ##$p<0.01$, ###$p<0.001$) by one-way ANOVA followed by Tukey test.

3. Results

3.1. TGF-β1 and VEGF-α expressions

As shown in Fig. 1, the TGF-β1 gene expression was increased in the all burn groups (Burn 3d, Burn 7d and Burn 14d) by 46.11-fold, 27.31-fold and 40.2-fold, respectively, compared to the Healthy groups ($p<0.05$). In the SS treatment groups, TGF-β1 gene expression was increased, (SS 3d, SS 7d and SS 14d) by 28.79-fold, 53.02-fold and 42.31-fold, respectively, when compared to Healthy groups ($p<0.05$). BOB treatment increaesed TGF-β1 mRNA expression in all BOB groups (BOB 3d, BOB 7d and BOB 14d) by 61.85-fold, 74.67-fold and 58.51-fold, respectively, compared to Healthy groups as shown in Fig. 1 ($p<0.05$). BOB treatment significantly augmented TGF-β1 expressions especially at 7th day compared to SS and Burn groups ($p<0.05$).

Similarly, VEGF-α mRNA gene expression increased (Fig. 2) in the Burn 3d, 7d and 14d group rats compared to the Healthy group (1.30-fold, 1.27-fold and 1.24-fold, respectively). In the SS treatment groups, VEGF-α gene expression was significantly increased, (SS 3d, SS 7d and SS 14d) by 1.47-fold, 1.65-fold and 1.45-fold, respectively, when compared to Healthy group ($p<0.05$). BOB treatment significantly increased the VEGF-α expressions by 1.6-fold, 1.93-fold and 1.62-fold respectively for BOB 3d, BOB 7d and BOB 14d groups compared to Healthy group

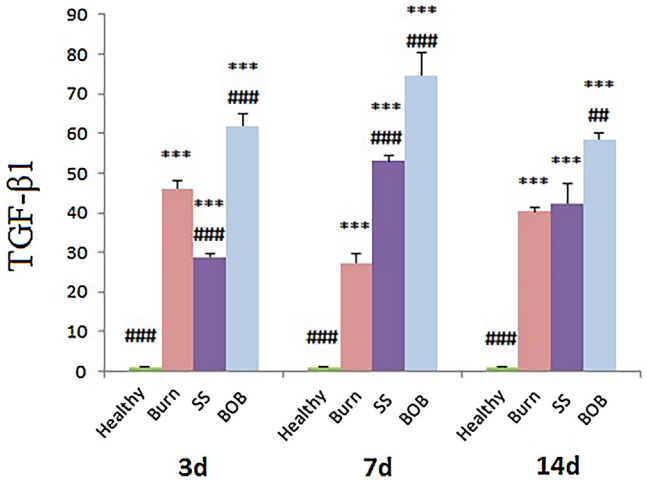

Fig. 1 – Relative mRNA expression Levels of TGF-β1 in skin of experimental rat groups. SS: Silver Sulfadiazine, BOB: Butter, Olive oil, Butter mixture impregnated bandage. The expression of mRNAs was detected using quantitative real time PCR analysis. β-actin was used as the reference gene. Results are expressed as relative-fold change compared with Healthy animals. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Each bar expressed as mean value ± SD. Significant differences were detected between all groups, compared to Healthy group (*$p<0.001$) and compared to Burn group (##$p<0.01$, ###$p<0.001$) by one-way ANOVA followed by Tukey test.**

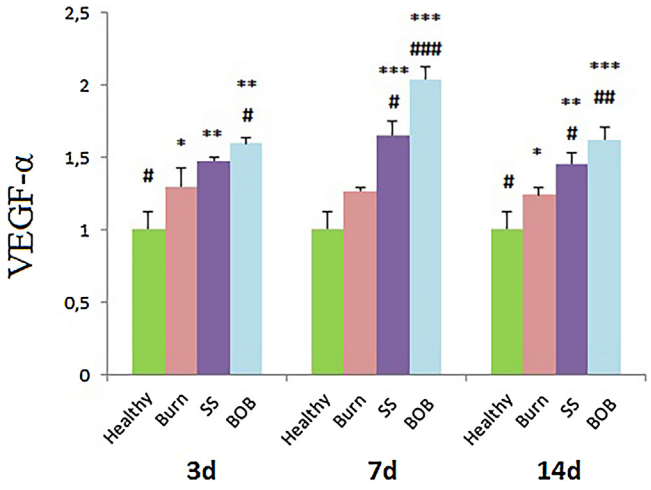

Fig. 2 – Relative mRNA expression Levels VEGF-α in skin of experimental rat groups. SS: Silver Sulfadiazine, BOB: Butter, Olive oil, Butter mixture impregnated bandage. The expression of mRNAs was detected using quantitative real time PCR analysis. β-actin was used as the reference gene. Results are expressed as relative-fold change compared with Healthy animals. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Each bar expressed as mean value ± SD. Significant differences were detected between all groups, compared to Healthy group (*p < 0.05, **p < 0.01, ***p < 0.001) and compared to Burn group (#p < 0.05, ##p < 0.01, ###p < 0.001) by one-way ANOVA followed by Tukey test.

($p < 0.05$). There was no significantly difference between the BOB and SS treatment groups as shown in Fig. 2 ($p < 0.05$).

3.2. *Histopathologic results*

The parameters of histopathological lesions and assessment of healing were described in Table 1. The healthy animal's skin tissues showed normal structure (Fig. 3a). The epidermis and part of the dermis are injured severely in all groups on day three (Fig. 3b–d). The epidermis and dermis were seen severe hypereosinophilic. Coagulative necrosis with acute inflammatory reaction of the epidermis and dermis were seen in all groups at day 3 (Fig. 3b–d). Histopathological assessments on days, seven, and fourteen showed the burn healing to be better in the BOB and SS groups with respect to the healthy group. Regenerative and reparative attempts in the epidermal layer were also observed (Fig. 4d–f). In BOB treated group regenaration was seen on seventh day. Inflammatory cell, specifically neutrophils infiltration without an epithelial layer was noted in upper (Fig. 4d,e) and lower epidermis (Fig. 4f).

The eschar had fallen off in all groups by the seventh and fourteenth day of the trial and epidermis was observed to have developed in BOB and SS groups. But, still inflammatory reactions and edema with osinophilic apperance were seen (Fig. 4d–f). However, the epithelial layer in the BOB group had a better histology when compared to other groups (Fig. 4f).

3.3. *Contraction results*

We investigated percent of contraction levels in burned skin tissue of rat by İmage J. program, as shown in Fig. 5. Contraction levels were significantly higher in BOB treatment groups compared to SS and burn control groups. The contraction analysis showed that BOB treatment group at

Table 1 – Histopathological scores in burn and treatment groups.

Histopathologic parameters	Burn (untreated group)			SS			BOB		
	Days			Days			Days		
	3rd	7th	14th	3rd	7th	14th	3rd	7th	14th
Hypereosinophilic appearance	+++++	++++	++++	++++	+++	++	++++	+++	++
Coagulation necrosis	++++++	++++	+++	++++	+++	++	++++	++	+
Inflammatory cell	++	++	+++	+++	+++	++++	++	+++	+++
Edema	+++++	++++	+++	++++	++	+	++	+	+
Vesicles/bullae	+++++	++++	++++	++++	++	+	++++	+	–
Fibroblast activity	++	+	++	++	+++	+++	+++	+++	+++
Epithelial regeneration	–	–	–	–	–	++	–	+	++
Keratinization	–	–	–	–	–	+	–	–	+

– negative, + very mild, ++ mild, +++ mild to moderate, ++++ moderate, +++++ severe.

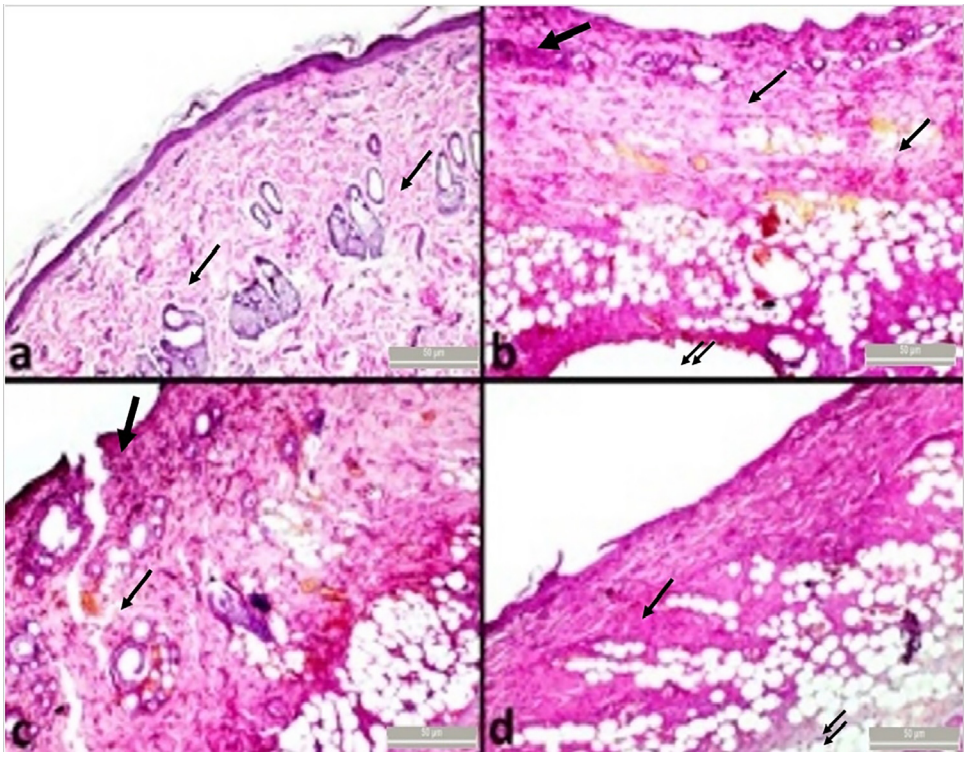

Fig. 3 – a: Skin of healthy animal. Haematoxylin and eosin. Magnification ×100. b-d: Burn on third day, all group animal skins showed hypereosinophilic appearance was the dominant histological finding (Thick arrow). Upper layer of dermis displayed interstitial inflammatory cell infiltration (Thin arrow). Vesicles and bullae form in the dermis were in the deep layer of dermis (Double arrows). Haematoxylin and eosin. Magnification ×40.

3d, 7d and 14d showed a significantly increased contraction levels compared to the Burn control group ($p < 0.05$). Despite of the SS treatment group that is positive control group, BOB treatment was more effective than Silver sulfadiazine at percent contraction increasing.

4. Discussion

Burn wound treatments aim to recreate the vascularization, re-epithelization and collagen production of the burned skin. Still now researchers aimed to show therapeutic effects of directly using of herbal cures especially plant mixtures and extracts. Recently researchers developed more useful and technological methods like biomaterials, bio-gels and effector material impregnated bandages to protect the integrity and humidity of wound. In this study we showed the therapeutic effects of butter, olive oil and beeswax mix impregnated bandage on second degree burn model in rats. We showed the therapeutic effects of BOB through the histopathological assesment, the expression of tissue inflammatory cytokines and the wound contraction levels.

In the literature there are a lot of studies about the burn and the burn wound healing that shows the lesion failure and epidermis and dermis layer injury [9,21,22]. Histopathological analyses is the most important parameter on the skin defects like burn injury because skin is firstly effected from these defects. Also in the treatment, skin layer regeneration is one of the most important indicator of the burn wound healing [22]. According to our histopathological assesment BOB treatment showed a regenerative effects in the dermis and the epidermis layers. Especially fibroblast activity and epithelial regeneration were important to show healing as a result of our study. Near all the histopathological parameters in the burn treatment skin humidity must be protected and so wound healing can be provided [17]. Olive oil and butter that are nutritive products can use on the treatment for burned skin [13,23]. Although these nutritive products can regenerate skin layers, protecting the skin humidity and long time application of these agents by bandage are more helpful on wound healing. During the wound healing process there is another mechanism that provides the histopathological regeneration: growth factors.

Growth factors have roles on proliferation, angiogenesis and granulation tissue formation, and are important during the wound healing process [24–26]. In the present study we analysed Transforming Growth Factor-beta1 (TGF-β1) and Vascular Endothelial Growth Factor-alpha (VEGF-α). VEGF-α play a lot of roles on the angiogenesis that is important on the skin regeneration process [27]. Li et al. showed decreased VEGF-α mRNA expressions in the burned skin [28]. Similar to this study we showed decreased VEGF-α levels in the burned skin while BOB treatment increased VEGF-α expressions. BOB treatment provided the new blood vessels formation by increasing VEGF-α levels and also this result were supported with our histopathological assesments. Like the VEGF-α, TGF-

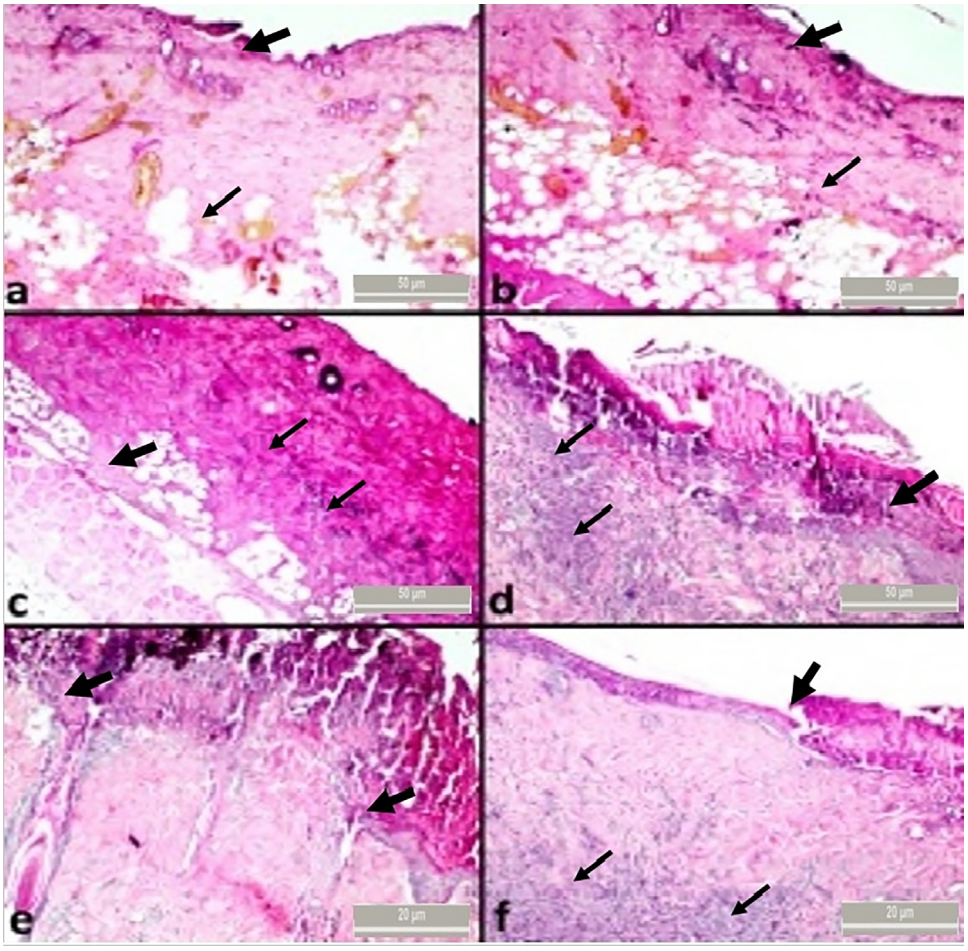

Fig. 4 – a: Skin of seventh day of Burn group. Severe hypereosinophilic and necrotic layers are seen (Thick arrow). Edema and bullaes are seen (Thin arrow). Haematoxylin and eosin. Magnification ×100. b: Skin of fourteenth day of Burn group. Severe hypereosinophilic and necrotic layers are seen (Thick arrow). Edema and bullaes are seen clearly (Thin arrow). Haematoxylin and eosin. Magnification ×100. c: Skin of SS treated group at seventh day. Edema and bullaes are seen in deep layer of epidermis through to muscular layer (Thick arrow). Inflammatory cells are seen between bullaes and upper layer of epidermis (Thin arrow). Haematoxylin and eosin. Magnification ×100. d: Skin of SS treated group at fourteenth day. Bullaes and edema is decreased. Inflammatory reaction is seen all layer of skin (Thin arrow). Epithelial regeneration has begun (Thick arrow). Haematoxylin and eosin. Magnification ×100. e: Skin of BOB treated group at seventh day. Epithelial regeneration is get started (Thick arrow). Haematoxylin and eosin. Magnification ×100. f: Skin of BOB treated group at fourteenth day. Epithelial regeneration is get started and keratinization is observed on newly regenerated epithelial layer (Thick arrow). Inflammatory cells are seen in epithelial layer and deep layer (Thin arrow). Haematoxylin and eosin. Magnification ×100.

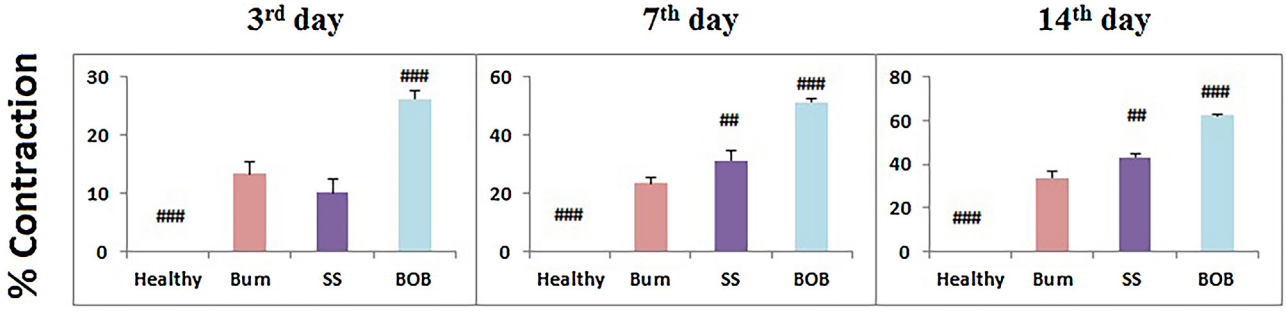

Fig. 5 – Wound contraction percent analyses of the rat skin. SS: Silver Sulfadiazine, BOB: Butter-Olive Oil-Butter mixture impregnated bandage. Results are means ± SD. Significant differences were detected between all groups compared to Burn group (##p < 0.01, ###p < 0.001) by one-way ANOVA followed by Tukey test.

β1 also play important roles on wound healing central [26,29,30].

Today it is well known that TGF-β stimulates the myofibroblast differentiation, a hallmark of fibrotic diseases [31]. Also TGF-β induces keratinocytes, fibroblasts, endothelial cells, and monocyte cells which are the important cell types involved in wound repair [32]. In a study Schultze et al. showed less fibrosis and prominent collagen types I–IV fibres production by inhibition of the TGF-β activity [33]. Thus TGF-β might be the driving force behind the new connective tissue formation. Ghahary et al. announced that healthy skin formation have more TGF-β mRNA levels than burned skin [34]. Parallel to these results we showed less TGF-β1 mRNA expression than normal skin. Also BOB treatment induce fibroblast activity, epithelial regeneration and keratinization by increasing the TGF-β1 levels. Also in a study there is an opposite result that researchers showed TGF-β isoforms especially TGF-β1 delay re-epithelization and inhibition of the TGF-β1 promote wound healing [35]. But in the same study it was shown that TGF-β inhibition reduces the fibrosis and myofibroblast differentiation during wound healing process [35]. This contradictory results may be due to searching on the different tissue wound because they studied on corneal wound healing where as we studied on the skin wound.

In the present study we demonstrated that growth factors help the new tissue formation by increasing the fibroblast activity, epithelial regeneration and keratinization. These results helped us to show regeneration of the skin layer. But we needed visual wound area analyses to say BOB completely help the skin regeneration. So we analysed the wound contraction percent. Wound contraction need myofibroblasts that are transformed from fibroblasts by TGF-β signals [31]. Myofibroblast differentiation is induced by the keratinocytes and fibroblasts and requires TGF-β activity [36]. According to our TGF-β1 level results there was a parallesim with the contraction levels. We showed a higher contraction with the BOB treatment. With these results we demonstrated that wound surface area was decreased and BOB treatment help the healing.

5. Conclusion

In the present study we demonstrated that the BOB treatment helped the skin regeneration by inducing fibroblast activity and keratinization via modulating tissue TGF-β1 and VEGF-α. To use the Beeswax, Olive oil and Butter mixture impregnated bandage showed significant therapeutic effects on second degree burn wound. Our results showed that BOB treatment positive effects are not only in skin regeneration but also in wound healing process especially in visual healing. So we are suggesting that BOB treatment is helpful and cost efficient protocol that would be used in clinic for the burned patient's treatment. However future studies are required to confirm the BOB utility in different wound dressing substrates.

Conflict of interest

All authors declare no conflict of interest.

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