THE EFFECTS OF CHRYSIN ON BURN HEALING

Muhammet Gokhan Turtay[1], Osman Ciftci[2], Asli Cetin[3], Sukru Gurbuz[1], Hakan Oguzturk[1], Nese Basak[4], Cemal Firat[5], Taner Guven[6], Kasim Turgut[7]

[1]Department of Emergency Medicine, Faculty of Medicine, Inonu University, Malatya, Turkey - [2]Department of Medical Pharmacology, Faculty of Medicine, Inonu University, Malatya, Turkey - [3]Department of Histology and Embryology, Faculty of Medicine, Inonu University, Malatya, Turkey - [4]Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Inonu University, Malatya, Turkey - [5]Department of Plastic Surgery, Faculty of Medicine, Inonu University, Malatya, Turkey - [6]Department of Emergency Medicine, State Hospital of Malatya, Malatya, Turkey - [7]Department of Emergency Medicine, State Hospital of Adiyaman, Adiyaman, Turkey

ABSTRACT

Introduction*: This study aims to investigate the effects of chrysin on burn healing in burned model rats.*

Materials and methods*: Rats were burned and randomly divided into four groups. Group 1; the group whose burn wound was left to secondary healing. Group 2; the group to which bacitracin neomycin sulfate pomade was topically administered. Group 3; the group to which chrysin was administered topically and with gastric gavage. Group 4; the group was assigned as the group to which chrysin and bacitracin neomycin sulfate was administered.*

Results*: On days 3, 7 and 14, blood samples and skin biopsies were taken. Necrosis, congestion, hemorrhage and IL-1β were found to have the lowest levels on all days when bacitracin and chrysin were administered together. This group was also found to have the lowest level of TNF-α serum levels on days 7 and 14.*

Conclusion*: We conclude that chrysin is effective in the treatment of burn wounds when used separately, but when combined with topical bacitracin pomade application, it is more effective for healing.*

Keywords: *Chrysin, Bacitracin, Burn, Rat.*

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Introduction

Burn traumas are an important part of hospital admissions. Despite the advances in the treatment of burns in recent years, they can cause serious consequences if they are not treated quickly and effectively.

The importance of the stasis zone, also known as a salvageable zone, in burn treatment is known[(1)]. In the treatment of burn wounds, the recovery of stasis zone has been focused. Also, the studies have focused on the recovery of this zone. After the burn, alterations such as neutrophil activation which leads to local tissue damage by triggering vascular thrombosis, local tissue edema, and vascular permeability leading to increasing of inflammation cause transformation of the stasis zone into the ischemic and necrotic state[(1)]. The inflammatory response in burn cases is associated with local and systemic tissue damage and lipid peroxidation[(2, 3)].

After the burn, macrophage activity, production of pro-inflammatory mediators, interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), transforming growth factor beta (TGF-β), leukotriene B4 (LTB4) and lipoxygenase metabolites are shown to increase[(4-6)]. Pro-inflammatory cascade plays a role in some post-burn complications[(7)]. For

these reasons, montelukast and β-glucan which have anti-oxidant and anti-inflammatory effects have been shown to be effective in the treatment of burn[1, 8].

Also, the infection that may occur in burn cases is one of the most important factors that disrupt wound healing. Topical antimicrobial agents such as bacitracin, neomycin, silver sulfadiazine and mafenide are used for infection in local burn wounds[9].

Studies have shown that flavonoids have antioxidant, anti-inflammatory, radical scavenging, antiallergic, antiviral and anticarcinogenic effects[10, 11]. The chrysin from flavonoid components is present in parsley, thyme, celery, honey, and propolis is determined to have effects of anti-inflammatory and free radical level reducing[12-14]. Due to these reasons, chrysin may be useful in the treatment of burn traumas both topically and systemically.

This study aimed to investigate the effects of flavonoid chrysin on burn healing in burned rats.

Materials and methods

This study was approved by the Inonu University Ethics Committee of Experimental Animals with 2013 / A-10 protocol number. In the study, a total of 28 male Sprague-Dawley rats which reached sexual maturity (weights 250-300 g) were used. The rats were fed ad libitum (Food and drinking water). Rats were kept at room temperature and in 12 h light-dark cycle. The burn was formed by a heated brass comb in rats[15]. The brass comb consisted of four prongs (1 × 2 cm) divided by three 5 mm notches. All the rats were anesthetized intraperitoneally with a mixture of 80 mg/kg ketamine and 5 mg/kg xylazine. The backs of the rats were shaved, the brass comb was kept in a boiling water bath for 5 min, and then the brass comb was contacted to the shaved area for 30 s with little pressure. The same procedure was performed again on the other side. The interspace showed the zones of stasis, and the burnt areas showed the coagulation zones.

Rats were randomly divided into four groups and there were seven rats in each group.

Group 1 (Control group); the group whose burn wound was left to secondary healing after the burn was formed.

Group 2 (Bacitracin group); the group to which bacitracin neomycin sulfate pomade (Thiocilline®) w*as topically administered twice daily.*

Group 3 (Chrysin group); the group to which chrysin was administered topically and by gastric gavage.

Group 4 (Chrysin + bacitracin group); the group was assigned as the group to which chrysin and bacitracin neomycin sulfate was administered as described above. After the burning process, 10 ml/kg of an isotonic saline solution were given subcutaneously to the rats in all groups. Also, in order to provide standardization, 2 cc of isotonic saline solution with no chrysin was administered by gastric gavage to Group 1 and 2. On days 3, 7 and 14, blood samples and elliptical skin biopsies including stasis and coagulation zones of 0.5 × 1 cm in size were taken. At the end of the 14th day, rats were sacrificed under anesthesia. TNF-α and IL-1β from blood samples and histopathological studies from tissue biopsies were examined.

Histopathological analysis

For light microscopic evaluation, skin samples were fixed in 10% formalin and embedded in paraffin blocks. Paraffin-embedded liver tissue was cut into 5 μm thick sections, mounted on slides and stained with Hematoxylin-Eosin (HE). Tissue samples were examined using a Leica DFC280 light microscope and a Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Histopathological damage score was calculated by histopathological changes. Scores were given as 0, none; 1, mild; 2, moderate; 3, severe for each criterion. Statistical analyzes were performed with SPSS 13 and MedCalc program. The mean values of all groups were compared with nonparametric Kruskal-Wallis and Connover tests. Mann-Whitney U test was used to assess the significance of the differences between the groups by using binary comparisons. All results were expressed as the mean ± standard error (SE) and $p < 0.0001$ values were considered statistically significant.

Determination of cytokine levels

Cytokines were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits according to the manufacturer's instructions. TNF-α and IL-1β levels were determined by using anti-rat ELISA kits by Krishgen Biosystems International (CA. 90603 USA). Plates were read at 450 nm using the CA-2000 ELISA microplate reader and washer (CIOM Medical Co., Ltd. in China). The amounts of cytokines in the samples were calculated from the standard curves of recombinant cytokines by using the linear regression method. All values were presented as mean ± SE and significant differences ($p < 0.05$) were given in the tables. For statistical analysis, the computer program SPSS 18.0 was used.

Results

In Group 1; on the 3rd day (Figure 1B) and 7th day (Figure 1C), apparent necrosis regions were detected on epidermis and dermis. In addition, on the 3rd day, very dense mononuclear cell infiltration, on the 7th day several mononuclear cell infiltration (Figure 1C), intense hemorrhage and vascular congestion (Figure 1D) were observed. On the 14th day, a small amount of necrosis area, mononuclear cell infiltration, and dramatically reduced vascular congestion were detected (Figures 1E, 1F).

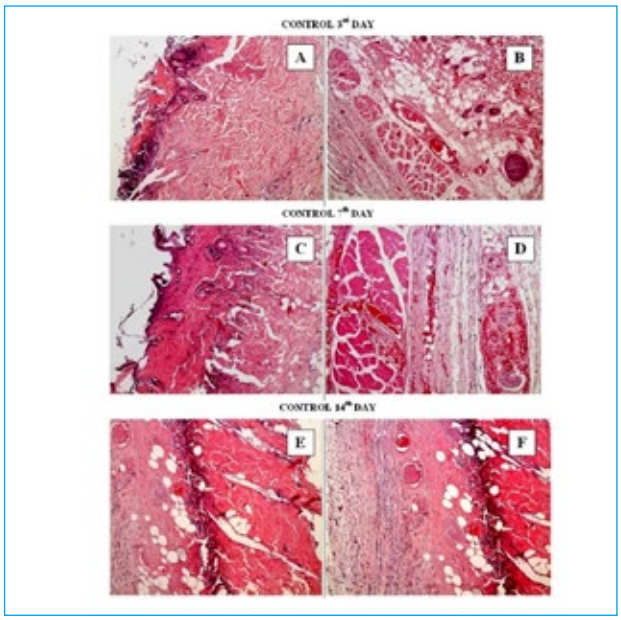

Figure 1: Histopathological images of Group 1, on days 3 (A, B), 7 (C, D) and 14 (E, F) (HE; X 10).

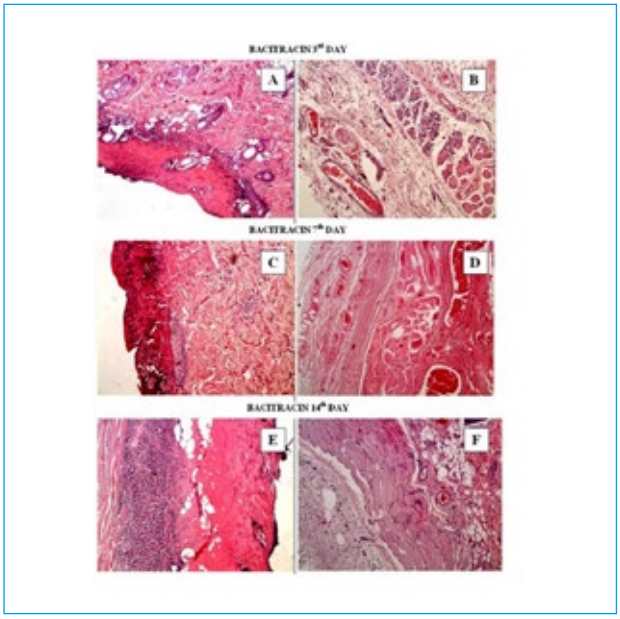

Figure 2: Histopathological images of Group 2, on days 3 (A, B), 7 (C, D) and 14 (E, F) (HE; X 10)

In Group 2; on the 3rd day (Figure 2B) and 14th day (Figure 2E), dense necrosis areas were detected in the epidermis and dermis. On 7th day, necrosis in the epidermis and dermis decreased (Figure 2C). On 3rd and 7th days, a small amount of mononuclear cell infiltration (Figure 2A, 2C), on the 14th day, dense mononuclear cell infiltration were detected (Figure 2E). On 3rd day, several vascular congestions (Figure 2B), on 7th day, intense vascular congestion and hemorrhage were observed (Figure 2D).

In Group 3; on the 3rd, 7th and 14th days, mild necrosis areas were detected in the epidermis and dermis. Group 3 had less mononuclear cell infiltration compared to other experimental groups on days 3, 7, and 14 (Figures 3A, 3C, 3E). Marked vascular congestion on day 14 (Figure 3F) and a relatively small amount of vascular congestion on day 7 was observed (Figure 3D).

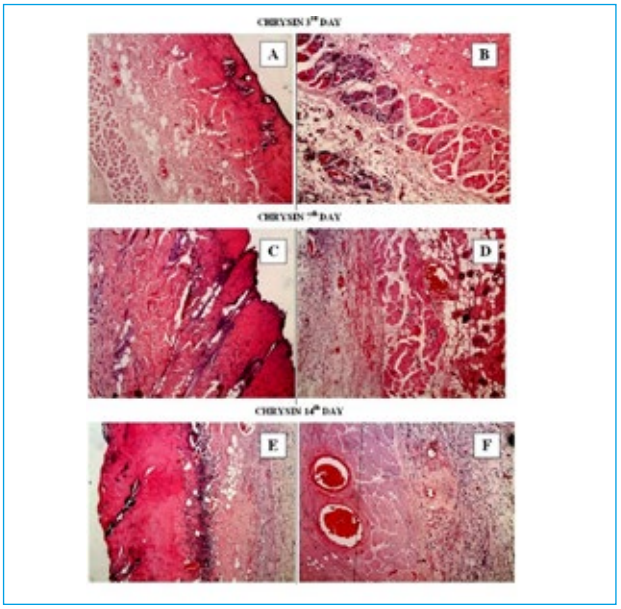

Figure 3: Histopathological images of Group 3, on days 3 (A, B), 7 (C, D) and 14 (E, F) (HE; X 10).

In Group 4; on day 3 (Figure 4A, 4B) and day 7 (Figure 4C, 4D) necrosis in epidermis and dermis layer was detected. On day 14 (Figure 4E, 4F) significantly decreased necrosis in epidermis and dermis was detected. Mononuclear cell infiltration was intense on day 3, and mononuclear cell infiltration was significantly reduced on days 7 and 14 (Figures 4B, 4D, 4F). On day 7, a very small amount of vascular congestion was observed (Figure 4D). Vascular congestion and hemorrhage were not observed on day 14 (Figure 4F).

Regarding necrosis; on the 3rd and 7th days, the groups in which bacitracin and chrysin were adminis-

tered separately, less necrosis was observed compared to the control group. Although chrysin had lower necrosis levels than that of bacitracin in all days, the difference was not statistically significant. However, when bacitracin and chrysin were administered together, there was a significant decrease in the level necrosis on all days compared to the groups that were administered separately and to the control group. When bacitracin and chrysin were administered together, the cell infiltration decreased by the time, when compared to the other groups, and it reached the lowest level on day 14th. Bacitracin and chrysin co-administration also lead to the lowest levels of congestion and hemorrhage during the experiments as compared to the groups administered separately and the control group (Table 1).

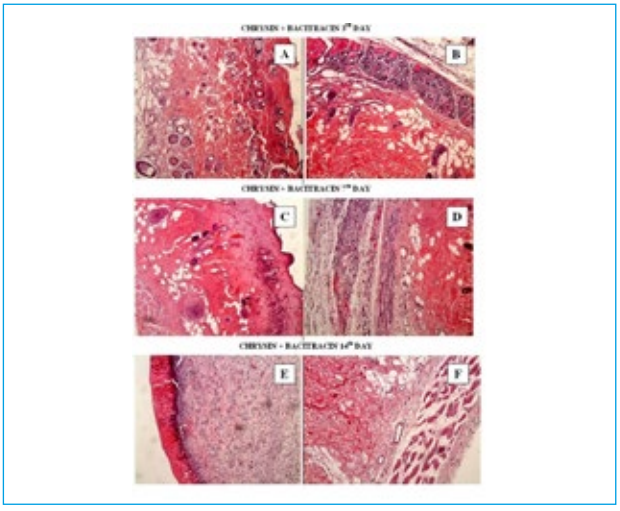

Figure 4: Histopathological images of Group 4, on days 3 (A, B), 7 (C, D) and 14 (E, F) (HE; X 10).

Regarding serum IL-1β values; when bacitracin and chrysin were administered together, the serum IL-1β level decreased significantly by time. When chrysin was administered alone, it had significantly lower levels of IL-1β levels at all time the points when compared to the group to which bacitracin was administrated alone and control group. However, co-administration of bacitracin and chrysin resulted in the lowest levels of IL-1β serum levels compared to the groups to which drugs were administered separately or the control group (Table 2).

Regarding serum levels of TNF-α; when bacitracin and chrysin were administered together, the serum TNF-α level decreased significantly by time. When chrysin was administered alone, the serum levels of TNF-α significantly decreased on all days compared to the control group or to which bacitracin was administered alone. However, when bacitracin and chrysin were administered together, it was found that it caused the lowest levels of TNF-α on the 7th and 14th days compared to groups to which they were administered separately and to the control group (Table 3).

Groups	Necrosis		
	3rd day	7th day	14th day
Group 1	2.90 ± 0.10^{a}	2.50 ± 0.17^{a}	1.70 ± 0.21^{a}
Group 2	2.10 ± 0.10^{b}	1.90 ± 0.18^{b}	1.50 ± 0.17^{a}
Group 3	1.80 ± 0.13^{b}	1.60 ± 0.16^{b}	1.40 ± 0.16^{a}
Group 4	1.20 ± 0.13^{c}	0.90 ± 0.10^{c}	0.60 ± 0.22^{b}
	Cell infiltration		
	3rd day	**7th day**	**14th day**
Group 1	1.50 ± 0.17^{a}	0.30 ± 0.15^{a}	1.70 ± 0.21^{a}
Group 2	0.50 ± 0.17^{b}	2.00 ± 0.21^{b}	2.80 ± 0.13^{b}
Group 3	1.20 ± 0.13^{ac}	1.40 ± 0.16^{c}	1.40 ± 0.16^{a}
Group 4	1.90 ± 0.18^{ad}	1.30 ± 0.15^{c}	0.50 ± 0.17^{c}
	Hemorrhage and Congestion		
	3rd day	**7th day**	**14th day**
Group 1	1.20 ± 0.13^{a}	2.20 ± 0.13^{a}	0.70 ± 0.26^{a}
Group 2	1.50 ± 0.17^{a}	2.60 ± 0.16^{a}	0.20 ± 0.13^{a}
Group 3	0.30 ± 0.15^{b}	1.20 ± 0.13^{b}	1.40 ± 0.16^{b}
Group 4	0.20 ± 0.13^{c}	0.50 ± 0.17^{c}	0.20 ± 0.13^{a}

Table 1: The relation between groups regarding necrosis, cell infiltration, hemorrhage and congestion on the 3rd, 7th and 14th days.
The mean differences the values bearing different superscript letters within the same column are statistically significant (p<0.0001) (Mean ± SE)

Groups	3rd day	7th day	14th day
Group 1	242.2±2.34^{a*}	240.4±1.98^{a*}	220.6±2.95$^{a\neq}$
Group 2	238.6±3.56^{a*}	225.3±3.87^{b*}	205.5±3.23$^{b\neq}$
Group 3	228.3±2.87^{b*}	190.7±1.98$^{c\neq}$	186.2±2.67$^{c\neq}$
Group 4	194.3±2.56^{c*}	156.7±2.97$^{d\neq}$	135.6±1.56^{d*}

Table 2: The relation between groups regarding necrosis, cell infiltration, hemorrhage and congestion on the 3rd, 7th and 14th days.
The mean differences the values bearing different superscript letters within the same column are statistically significant (p<0.0001) (Mean ± SE)

Groups	3rd day	7th day	14th day
Group 1	35.21±1.43^{a*}	33.40±1.23^{a*}	32.21±1.38^{a*}
Group 2	33.24±2.15^{ab*}	32.42±1.83^{a*}	30.11±1.25^{a*}
Group 3	31.36±2.43^{b*}	29.74±1.80^{b*}	25.46±1.46$^{b\neq}$
Group 4	28.43±1.52^{bc*}	25.67±2.97$^{c\neq}$	19.93±1.82^{c*}

Table 3: Serum levels of TNF-α.
The letters a, b, c, and d show statistically significant differences in the same column.
**, # and ≠ icons show statistically significant differences in the same line*

Discussion

In this study, we investigated the efficacy of chrysin and bacitracin on burn healing in the rat model. We determined that on the 3rd and 7th days, the group to which bacitracin and chrysin administered separately had lower levels of necrosis compared to the control group. Although chrysin group had lower levels of necrosis, we could not detect any statistically significant differences between them. However, when bacitracin and chrysin administered together, necrosis levels were lower than that of single applications and control group on all days. Cell infiltration was found to be significantly lower than other groups on day 14 when bacitracin and chrysin were administered together. Congestion and hemorrhage were found to have the lowest levels on all days when bacitracin and chrysin were administered together. We determined that when chrysin was administered alone, it significantly lowered the levels of IL-1β all compared to the bacitracin and the control groups. However, when bacitracin and chrysin were administered together, they had the lowest levels of IL-1β serum levels in all days compared to the groups to which they were administered separately and the control group. We determined that when chrysin was administered alone, the serum levels of TNF-α significantly decreased on all days compared to the control group on days 7 and 14. However, when bacitracin and chrysin were administered together, they caused the lowest levels of TNF-α on the 7th and 14th days compared to groups to which they were administered separately and the control group. Our results suggested that chrysin was effective in the healing of burn wound, but it was more effective in terms of healing when combined with topical bacitracin pomade.

It is a known fact that burn causes increased vascular permeability, tissue edema, and excessive inflammation, blocking of capillaries, local tissue damage, leading to tissue ischemia and necrosis. Oxygen radicals emerging after burn increases tissue damage as well. Apart from the coagulation zone, which is also known as a necrotic zone where necrosis occurs, there is also a stasis zone, which is also called salvageable zone. It is a critical zone and it is affected adversely form the changes mentioned above[1].

Recovery in burn wounds is a complex process that begins with the infiltration of inflammatory cells after burning. These inflammatory cells are activated by the factors released during the burn event. By producing mediators such as cytokines (TNF-α, IL-1, IL-6, etc.) and some growth factors [(fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF)], inflammatory cells try to eliminate the cause of the damage. The products of arachidonic acid metabolism, such as lysosomal enzymes in neutrophils and macrophages, reactive oxygen metabolites, leukotrienes, and prostaglandins, contribute to inflammatory response. Since these products may lead to endothelial and tissue damage as well, they are able to intensify harm the burn area[8, 16-18].

Cytokines such as TNF-α, IL-1, and other inflammatory agents; cause damage in microcirculation and endothelial cells[19, 20]. In patients with burn injury; circulating levels of TNF-α and IL-1β increase[5]. IL-1 is a pleiotropic cytokine with various biological activities, including the regulation of the inflammatory response as a pyrogen, by inducing the maturation and activation of granulocytes and T and B cells by applying chemotactic activity[4]. TNF-α is a triggering cytokine that induces local and systemic sequelae and inducing humoral factors and plays a role in the development of a shock-like condition associated with thermal injury and sepsis[4]. It has been found that the production of TNF-α is effectively reduced by the application of antioxidant N-acetyl cysteine and necrosis of the stasis zone is prevented by this drug[21]. Also, the increase in TNF-α in burn patients is reported to be an indicator of poor prognosis[22]. In our study, we found that chrysin administration alone caused low levels of IL-1β and TNF-α serum levels, but when bacitracin and chrysin were administered together, IL-1β and TNF-α levels were found to be the lowest on all days compared to the other groups.

Flavonoids are naturally polyphenolic compounds found in vegetables, fruits and some beverages (tea, red wine)[23]. It has been reported that people take flavonoids at a dose of 1 g/day through various foods they consume[24].

Recent studies have shown that over 8000 flavonoid compounds are present in plants and they are formed by connecting in different ways of methoxyl and hydroxyl groups on the flavonoid skeleton[25]. Hesperidin, a member of the group of flavonoids, has been reported to have beneficial effects in the treatment of burns as well as anti-inflammatory, antioxidant and many other effects[22].

In one study, it was reported that chrysin which is a member of the group of flavonoids worsened burn wounds[26].

In our study, we found that chrysin was beneficial in healing the burn wound alone but was more useful when used with antibiotic pomades.

Burn wounds are sensitive to infection which is one of the factors detrimental to the healing of wounds. Thermal injuries cause immunosuppression as well as excessive inflammation[27]. Therefore, topical antibiotics are frequently used in the treatment of local burn wounds[9]. Moreover, because of their oily and moisturizing properties, topical pomades are very effective in supporting burn wound healing and re-epithelization[9]. In our study, we used bacitracin neomycin sulfate alone and with chrysin to investigate the effect of topical antibiotic pomade on burn wounds. We found that bacitracin administration alone was better than the control group. The best results were obtained with the use of chrysin and antibiotic pomade to heal the burn wound. Co-administration of chrysin and antibiotic pomade generated a stronger effect. To better understand the mechanisms of how chrysin heals burn wounds, more clinical studies are required.

Conclusion

We conclude that chrysin is effective in the treatment of burn wounds when used alone, but when combined with topical bacitracin pomade, it is more effective for healing.

References

1) Firat C, Samdanci E, Erbatur S, Aytekin AH, Ak M, et al. β-Glucan treatment prevents progressive burn ischaemia in the zone of stasis and improves burn healing: an experimental study in rats. Burns 2013; 39: 105-12.
2) Sener G, Kabasakal L, Cetinel S, Contuk G, Gedik N, Yeğen BC. Leukotriene receptor blocker montelukast protects against burn-induced oxidative injury of the skin and remote organs. Burns 2005; 31: 587-96.
3) Demling RH, Lalonde C. Systemic lipid peroxidation and inflammation induced by thermal injury persists into the post-resuscitation period. J Trauma 1990; 30: 69-74.
4) Cakir B, Yegen BC. Systemic responses to burn injury. Turk J Med Sci 2004; 34: 215-26.
5) Yamada Y, Endo S, Inada K, Nakae H, Nasu W, et al. Tumor necrosis factor-alpha and tumor necrosis factor receptor I, II levels in patients with severe burns. Burns 2000; 26: 239-44.
6) Meakins JL. Etiology of multiple organ failure. J Trauma 1990; 30: S165-8.
7) Lavaud P, Mathieu J, Bienvenu P, Braquet M, Gerasimo P, et al. Modulation of leucocyte activation in the early phase of the rabbit burn injury. Burns Incl Therm Inj 1988; 14: 15-20.
8) Turtay MG, Firat C, Samdanci E, Oguzturk H, Erbatur S, Colak C. Effects of montelukast on burn wound healing in a rat model. Clin Invest Med 2010; 33: E413-21.
9) Palmieri TL, Greenhalgh DG. Topical treatment of pediatric patients with burns: a practical guide. Am J Clin Dermatol 2002; 3: 529-34.
10) Hollman PC, Feskens EJ, Katan MB. Tea flavonols in cardiovascular disease and cancer epidemiology. Proc Soc Exp Biol Med 1999; 220: 198-202.
11) Sakagami H, Jiang Y, Kusama K, Atsumi T, Ueha T, et al. Induction of apoptosis by flavones, flavonols (3-hydroxyflavones) and isoprenoid-substituted flavonoids in human oral tumor cell lines. Anticancer Res 2000; 20: 271-7.
12) RiceEvans CA, Miller J, Paganga G. Antioxidant properties of phenolic compounds. Trends Plant Sci 1997; 2: 152-9.
13) Moon YJ, Wang X, Morris ME. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicol In Vitro 2006; 20: 187-210.
14) Zhang S, Qin C, Safe SH. Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell context. Environ Health Perspect 2003; 111: 1877-82.
15) Regas FC, Ehrlich HP. Elucidating the vascular response to burns with a new rat model. J Trauma 1992; 32: 557-63.
16) Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003; 9: 669-76.
17) Carmeliet P. Angiogenesis in health and disease. Nat Med 2003; 9: 653-60.
18) Schwacha MG. Macrophages and post-burn immune dysfunction. Burns 2003; 29: 1-14.
19) Rodríguez-Bigas M, Cruz NI, Suárez A. Comparative evaluation of aloe vera in the management of burn wounds in guinea pigs. Plast Reconstr Surg 1988; 81: 386-9.
20) St John RC, Dorinsky PM. Immunologic therapy for ARDS, septic shock, and multiple-organ failure. Chest 1993; 103: 932-43.
21) Deniz M, Borman H, Seyhan T, Haberal M. An effective antioxidant drug on prevention of the necrosis of zone of stasis: N-acetylcysteine. Burns 2013; 39: 320-5.
22) Oguzturk H, Ciftci O, Taslidere A, Turtay MG, Gurbuz S, et al. Investigating the therapeutic effects of flavonoid-structured hesperidin in acute burn traumas. Fresenius Environmental Bulletin 2018; 27: 5446-54.
23) Hollman PCH, Katan MB. Absorption, metabolism and health effects of dietary flavonoids in man. Biomed Pharmacother 1997; 51: 305-10.
24) Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. J Nutr 2000; 130: 2073S-85S.
25) Hodek P, Trefil P, Stiborova M. Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. Chem Biol Interact 2002; 139: 1-21.
26) Park BK, Lee S, Seo JN, Rhee JW, Park JB, et al. Protection of burn-induced skin injuries by the flavonoid kaempferol. BMB Rep 2010; 43: 46-51.
27) Uygur F, Evinc R, Urhan M, Celikoz B, Haholu A. Salvaging the zone of stasis by simvastatin: an experimental study in rats. J Burn Care Res 2009; 30: 872-9.

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Corresponding Author:
Muhammet Gokhan Turtay MD, Prof
Email: mgturtay@gmail.com
(Turkey)