Botafarma Sağlık Ürünleri Ticaret ve Sanayi Limited Şirketi

Mofex içerikli Rapider Bariyer krem isimli preparatın deri üzerindeki fizyolojik ve mekanik etkisi üzerine yapmış olduğumuz preklinik çalışmalar ve klinik gözlem sonuçları Ek'te rapor halinde sunulmuştur.

Saygılarımızla
Botafarma Sağlık Ürünleri
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GİRİŞ

Fiziksel, kimyasal, termal radyasyon ve cerrahi nedenlere bağlı veya kendiliğinden gelişen doku bütünlüğünün bozulması **yara** olarak isimlendirilir[1]. Hücre ve dokular zedelendiğinde yaralanmış dokunun bütünlüğünün yeniden oluşturulması için temel olarak üç fazda gelişen biyolojik bir süreç başlar. Bu fazlar, karışık ve çeşitli dokularla organize etkileşim oluşturan erken dönem cevabı, epitel yenilenmesi ve cilt altı dokuların onarımı şeklinde gelişen enflamasyon, hücre sayısında artışın meydana geldiği proliferasyon ve iyileşen dokunun sağlamlaşma süreci olan yeni oluşum (remodeling) fazlarıdır[2].

Yara iyileştirici etkinin belirlenmesi amacıyla fare, sıçan ve kobay gibi küçük boyutta memeli hayvanlar tercih edilir. Hayvanlar üzerinde mekanik veya termal travma yoluyla spesifik yaralar oluşturulur[3].

Cilt yüzeyinde belirli bir alanda oluşturulan insizyon, eksizyon veya yanık yara modellerinde belirli aralıklarla fotoğraflanan yara alanları AutoCAD gibi alan ölçmeye yarayan bilgisayar programları kullanılarak ölçülür[4]. Ölçülen alanlardan hareketle yara kontraksiyonu hesaplanır. Alanda meydana gelen küçülme yara iyileşmesinin görsel ifadesidir.

Yara gerilim kuvvetinin ölçülmesi, insizyon tipi yara oluşturulmuş deney hayvanları üzerinde yapılan deneyleri kapsamaktadır. Hayvanların sırt kısımlarında steril cerrahi bistüri ile kesi yarası oluşturulduktan sonra yara kenarları cerrahi dikişle birleştirilir. Test numunelerinin yara gerilim kuvveti üzerindeki etkisi, belirlenen bir süre sonra dikişlerin alınması ve yara alanlarının çıkarılarak tensiometre adı verilen yara kuvvetini ölçen cihazlarla ölçülür. İyileşen dokudaki yırtılma kuvvetinin yüksek olması yaranın sağlamlığının göstergesidir. Bu da dokudaki kolajen oluşumu hakkında bilgi verir[5]. Kolajen, yaranın hızlı bir şekilde iyileşmesi için gerekli olan önemli bir ekstraselüler matriks proteinidir. Yara alanındaki kolajen miktarının tespit edilmesi için kolajeni oluşturan aminoasitlerden olan hidroksiprolinin dokudaki miktarı ölçülür[6].

Bu araştırmada, "**Rapider Bariyer Krem**" isimli preparatın deriden nem kaybı üzerindeki etkisi incelenerek, **yara oluşumunu engelleyici/geciktirici** etkisinin araştırılması amacıyla preklinik çalışmaların yapılması planlanmıştır. Bu amaçla kremin deriden nem kaybı üzerindeki etkisi incelenerek, yara oluşumunu engelleyici/geciktirici aktivitesi *in vivo* deney modeli olan çizgisel insizyon yara modeli ve akabinde histopatolojik incelemeler yapılarak araştırılmıştır. Deney sonuçları kendi içerisinde pozitif ve negatif kontrol grupları kullanılarak istatistiksel olarak değerlendirilmiş ve anlamlı sonuçlar kayıt altına alınmıştır.

MATERYAL VE METOT

Deney Hayvanları

Deneylerde Kobay Firması Deney Hayvanları Araştırma Laboratuvarı'ndan temin edilen 160-180 g ağırlığında erkek Sprague&Dawley sıçanlar kullanıldı. Hayvanların ortama adapte olabilmesi için deneye başlamadan önce laboratuvar şartlarında en az üç gün bekletildi. Bu bekleme süresince hayvanlar standart pellet yem ve su ile beslendiler, 12 saat aydınlık 12 saat karanlık uygulaması yapılan laboratuvarda oda sıcaklığında barındırıldılar. Deneylerde her grupta altı hayvan kullanıldı.

Test numunesi

Botafarma Sağlık Ürünleri Tic. San. Ltd. Şti. tarafından gönderilen ve preparat üzerindeki içeriği aşağıda belirtilen "**Rapider Bariyer Krem**" isimli bitmiş ürün test numunesi olarak kullanıldı.

Rapider Bariyer Krem İçerik Bilgisi
Deionized water
Mofex
Lanovaseline
Gliseril Monostearat
Cera alba
Setilstearil Alkol
Shea Butter
Jasmine oil
Trietanolamin,
Methyl p-hydroxybenzoate
Propil p-hidroksibenzoat
Butilhidroksitolüen
Alfa tokoferol

DENEY PROTOKOLÜ

Kullanılan Madde, Solvan ve Cihazlar

Biyolojik aktivite deneylerinde kullanılan madde, solvan ve cihazlar ve bunların temin edildiği firmalar aşağıdaki tabloda verilmiştir.

Tablo 1. Deneylerde kullanılan madde ve solvanlar

Madde/Solvan/Cihaz	Temin edildiği firma
Alfazine	Alfasan International B.V., Hollanda
Cerrahi ipek iplik	Huaiyin Medical Instruments
Hematoksilen	Sigma-Aldrich 517-28-2
Işık mikroskobu	Nikon Eclipse C Analiz Sistemi
Ketasol	Richter Pharma
Tensiometre	Zwick/Roell Z0.5, Germany

Çizgisel İnsizyon Yara Modeli

Çizgisel insizyon yara modelinde deney süresince uygulanan merhemlerin kolajen yapımı ve yara gerilimini arttırıcı etkisi, Lodhi ve ark. ile Suguna ve ark.'nın yöntemi kullanılarak değerlendirilmiştir[7,8].

Sıçanlara intraperitonal yolla 0.05 cc Ksilazin (%2 Alfazine®) ve 0.15 cc Ketamin (%10 Ketasol®)enjeksiyonu ile genel anestezi yapıldı. Sırt kısımlarının orta hattından 2 cm uzaklıkta bistüri ile iki adet 5 cm'lik çizgisel insizyon yarası oluşturuldu. Cerrahi ipek iplikle eşit aralıklarla 3 adet dikiş atıldı. 10 gün boyunca günde bir defa 500'er mg merhem formülasyonları haricen yaralara uygulandı. 11. gün sonunda dikişler alındı. 11. gün hayvanlar yüksek doz anestezi ile öldürüldü. Yara oluşturulan bölgeler yara kenarlarının 2'şer cm uzağından cerrahi makasla kesildi. Yaralardan biri histopatolojik incelemeler için ayrıldı. Diğer yaranın tensiometre ile gerilim kuvveti ölçüldü[9].

Çizgisel insizyon yara modelinde yüzde gerilme kuvveti hesaplanırken aşağıdaki formül kullanılmıştır.

$$\% \text{ Gerilme kuvveti} : \frac{(T-NK)}{NK} \times 100$$

NK: Tedavi edilmeyen grup

T: Test numunesi uygulanan grubun gerilme kuvveti ortalaması

Resim 1. Tensiometre (Yara Gerilme Kuvvetinin Ölçümü)

Deney grupları

Deney grupları aşağıda Tablo 2'de sunulmuştur.

Tablo 2. Deney grupları

Grup	Uygulama yöntemi
İnsizyon (Negatif Kontrol (NK))	Deney hayvanlarının sırtlarında 5 cm uzunluğunda sadece insizyon yarası oluşturulmuş ve tedavi uygulanmamıştır.
İnsizyon +RapiderBariyer Krem	Deney hayvanlarının sırtlarında 5 cm uzunluğunda insizyon yarası oluşturulmuş ve 10 gün boyunca Rapider Bariyer Krem ile tedavi edilmiştir.
RapiderBariyer Krem + İnsizyon + Rapider Bariyer Krem	Deney hayvanlarının sırtlarına 10 gün boyunca Rapider Bariyer Krem uygulanmış akabinde 5 cm uzunluğunda insizyon yarası oluşturulmuş ve 10 gün boyunca Rapider Bariyer Krem ile tedavi edilmiştir.
Sağlam deri	Deney hayvanlarına herhangi bir uygulama yapılmamıştır.

Histopatolojik İncelemeler

Histopatolojk incelemeler için uygun büyüklükteki örnekler %10'luk tamponlu formaldehitte tespit edildi, rutin doku takibine alındı ve parafinde bloklandı. Parafin bloklardan alınan 5 µm kalınlığındaki kesitler lama transfer edilerek hematoksilen-eozin (HE) ve Van Gieson (VG) boyaları ile boyandı. Işık mikroskobu (Nikon Eclipse Ci bağlı Kameram® Dijital Image Analiz Sistemi) altında incelenen dokularda epidermal ve dermal re-modelleme hafif (+), orta (++) ve şiddetli (+++) olmak üzere derecelendirildi. Epidermis re-epitelizasyon veya ülser; dermis ise fibroblast proliferasyonu, mononüklear ve/veya polimorfonüklear hücreler, neovaskülarizasyon ve kollojen birikimi yönünden incelenerek epidermal ve/veya dermal re-modelleme evrelendirildi. Van Gieson boyanmış kesitlerde kollojen birikimi ortaya kondu [10,11].

İstatistiksel Değerlendirmeler

Deney sonuçları değerlendirilirken tek yönlü "ANOVA" testini içeren "Instat" (Windows) istatistik programı ve Students-Newman-Keuls posthoc testi kullanıldı.

Kontrol grubu ile karşılaştırılan deney sonuçlarındaki istatistiksel belirginlik aşağıdaki şekillerle ifade edildi:

* : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$

SONUÇ

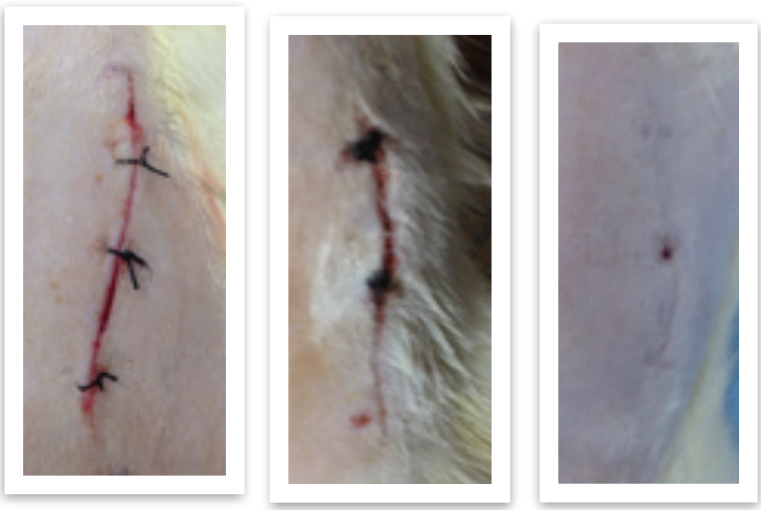
Fiziksel, kimyasal, termal radyasyon ve cerrahi nedenlere bağlı veya kendiliğinden gelişen doku bütünlüğünün bozulması yara olarak tanımlanır. Yara iyileşmesi kendiliğinden gelişen fizyolojik bir süreçtir. Deride yaranın oluşması ve iyileşmesi süreçlerinde derideki nem oranının çok önemli bir faktör olduğu yapılan çalışmalarla ortaya konmuştur. Cilt üzerinde nem kaybına karşı **bariyer oluşturan** ürünler hem yara oluşumunu geciktirmekte hem de oluşmuş yaraların iyileşme sürecini kısaltmaktadır. Özellikle derideki açık yaraların enfeksiyon ve komplikasyonlara açık olması nedeniyle mümkün olduğu kadar hızlı ve herhangi bir iz bırakmadan, düşük maliyet ile iyileşmesini sağlayabilecek maddelere ihtiyaç duyulmaktadır. Bu amaçla gerek sentetik moleküller gerekse de biyolojik kaynaklı ürünlerden yararlanmak üzere kapsamlı bilimsel araştırmalar yürütülmektedir. Özellikle bitkisel ürünler halk arasında, kolay sağlanabilmesi ve yan etki oluşturma düzeylerinin düşük olması nedeniyle yara iyileştirici amaçla yaygın olarak tercih edilmektedir [12].

Çalışmada **Rapider Bariyer Krem** deriden nem kaybı üzerindeki etkisi incelenerek, yara oluşumunu **engelleyici/geciktirici** aktivitesi sıçanlar üzerinde çizgisel insizyon deney modeli kullanılarak yara alanındaki kolajen oluşumu ve kapanan yaranın sağlamlığı değerlendirilmiştir. Çizgisel insizyon yara modelinde "**Rapider Bariyer Krem**" formülasyonunun insizyon yarası oluşturulduktan sonra 10 gün boyunca uygulanması ile %50.57; 10 gün boyunca krem uygulanıp insizyon yarası oluşturulduktan sonra yine 10 gün boyunca tekrar krem uygulanması suretiyle yapılan işlemde %75.72 oranında yara kuvvetini arttırdığı tespit edilmiştir (Tablo 2). A***yrıca Rapider Bariyer Krem + İnsizyon + Rapider Bariyer Krem uygulamasının hiçbir işlem yapılmamış sağlam deriye yakın bir yara gerilme kuvveti gösterdiği de tespit edilmiştir.***

Tablo 3. "Rapider Bariyer Krem" formülasyonunun çizgisel insizyon deney modeli üzerinde-ki etkileri

Materyal	**Yara gerilme kuvveti ± O.S.H (%Yara gerilme kuvveti)**
İnsizyon (Negatif Kontrol (NK))	10.46±1.99
İnsizyon + Rapider Bariyer Krem	15.75±1.86 **(50.57**)**
Rapider Bariyer Krem + İnsizyon + Rapider Bariyer Krem	18.38±1.72 **(75.72***)**
Sağlam deri	27.32±2.42

* : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$; *** : $p < 0.001$; O.S.H.:Ortalama Standart Hata
Yüzde yara gerilme kuvvetleri: Negatif kontrol grubuna gore kıyaslandı.



0. gün **5. gün** **11. gün**

Resim 2. İnsizyon yara modeli (0., 5. ve 11. gün fotoğrafları)

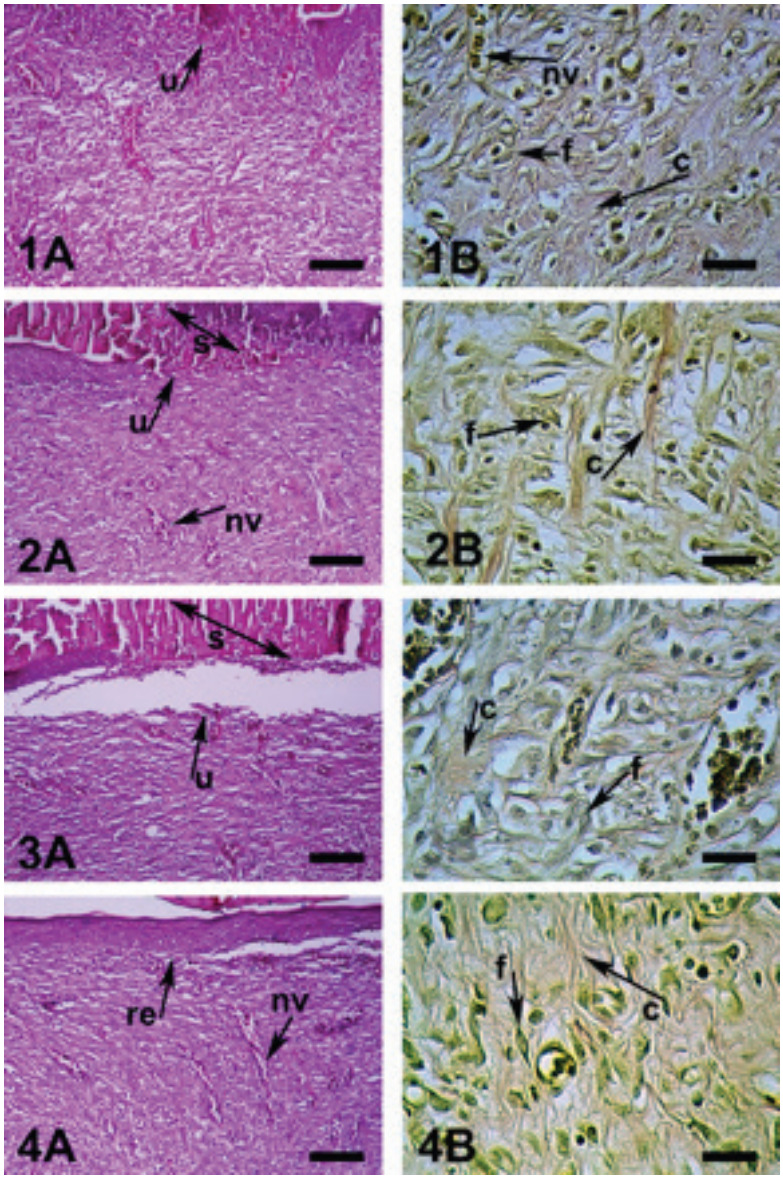
Histopatolojik incelemeler sonucunda remodeling (yeni oluşum), özellikle re-epitelizasyon "Rapider Bariyer Krem" formülasyonu ile tedavi edilen dokularda tespit

edilmiştir (Tablo 3). Histopatolojik incelemede, iyileşme sırası fazlardan inflamasyon, proliferasyon ve remodelleme göz önünde tutulduğunda en iyiden en kötüye Sağlam deri , Rapider Bariyer Krem + İnsizyon + Rapider Bariyer Krem , İnsizyon + Rapider Bariyer Krem ve İnsizyon (Negatif Kontrol (NK)) grupları olarak sıralanmıştır (Resim 3 ve Tablo 4).

Tablo 4. Test numuneleri ile tedavi edilen yara dokularının hayvanlarda yara iyileşmesi ve epidermal/dermal re-modelleme'nin histopatolojik karakteristikleri*

Gruplar	Yara İyileşmesine İlgili Veriler								İyileşme Evreleri		
	S	U	RE	FP	CD	MNC	PMN	NV	I	P	R
İnsizyon (Negatif Kontrol (NK))	++/+++	++/+++	-/+	++/+++	++	++/+++	+/++	++/+++	++	++/+++	-/+
İnsizyon + Rapider Bariyer Krem	+++	+++	-	+++	++/+++	++/+++	++	+++	++	+++	-
Rapider Bariyer Krem + İnsizyon + Rapider Bariyer Krem	++	+/++	-/+	++	+/++	++	-	++/+++	+/++	++/+++	-/+
Sağlam deri	+	-	++	++	+/++	+	+	++	+	++	++

*S: Yara kabuğu, U: Ülser, RE: Re-epitelizasyon, FP: Fibroblast proliferasyonu, CD: Kollojen birikimi, MNC: Mononüklear hücre, PMN: Polimorf hücre, NV: Neo-vazkülarizasyon, I: Yangısal evre, P: Proliferatif evre, R: Re-modelleme evresi.



Resim 3. Deney numuneleri ile tedavi edilen yara dokularının histopatoloji sonuçları

1. Rapider Bariyer Krem + İnsizyon + Rapider Bariyer Krem 2. İnsizyon + Rapider Bariyer Krem, 3. İnsizyon (Negatif Kontrol (NK)), 4. Sağlam deri A: Hematoksilen-eozin ile boyanmış epidermis ve dermis; B: Van Gieson ile boyanmış dermis. A kodlu resimlerin orijinal büyütmesi x 100 olup büyütme barı 100 µm, B kodlu resimlerin orijinal büyütmesi x 400 olup büyütme barı 25 µm. Bilgiler her gruptaki 6'şar hayvanın ortalamasını yansıtacak şekilderdir. s: Yara kabuğu , re: Re-epithelizasyon, u: Ülser, f: Fibroblast, c: Kollajen, nv: Neovazkülarizasyon.

Sonuç olarak;

"**Rapider Bariyer Krem**" isimli müstahzar üzerinde çizgisel insizyon yara modeli ile yapılan deneyde deri üzerindeki **yara oluşumunu engellediği/geciktirdiği tespit edilmiştir**. Bu etkinin kuvvetle muhtemel mekanizmalarından birisi "Rapider Bariyer Krem"in deri üzerinde mekanik bir bariyer oluşturması ve bu suretle ciltteki nem kaybını azaltarak yara oluşumunu geciktirdiği/engellediği şeklinde yorumlanabilir. "Rapider Bariyer Krem"in aynı zamanda oluşmuş yarada enfeksiyon riskini de azaltarak yara iyileşmesini hızlandırdığı yapılan bu deneyde gösterilmiştir.

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ORIGINAL ARTICLE

The beneficial effects of *Momordica charantia* (bitter gourd) on wound healing of rabbit skin

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Momordica charantia **(MC; bitter gourd) is a traditional herbal commonly used for its antidiabetic, antioxidant, contraceptive and antibacterial properties. In the current study, the authors aim to observe the topical effect of MC cream on the wound-healing process in rabbits. Moreover, they compare the healing potential with conventional creams used therapeutically. Towards this aim, 28 New Zealand rabbits were divided into four groups and excision wounds (7 cm^2) were made on their backs. Open wound dressing was carried out daily for 28 days among the experimental groups with the application of dekspanthenol (Bepanthen®; BP group, *n* = 7), nitrofurazon (Furacin®; FR group, *n* = 7) and olive oil extract of MC (MC group, *n* = 7). No application was made to the control group. At the end of day 28, areas of the skin with initial wound area were *en bloc* dissected and prepared for histopathological and stereological analysis. Inflammatory cells were abundant in the control group and cream application led to a decrease in the number of these cells, especially in the MC group. The highest number of fibroblasts was detected in the MC group. Furthermore, the MC group displayed the highest fractions of epidermis to papillary dermis, fibroblasts to reticular dermis and collagen fibres to reticular dermis. The MC group also presented a high density of blood vessels, moderate density of collagen fibres and mature fibroblasts. The BP group showed better epithelialisation compared with the FR group, but the latter provided more effective reorganisation of the dermis. Different cream supplements caused healthy and fast wound healing according to untreated controls and the results show that administration of the MC extract improves and accelerates the process of wound healing in rabbits in comparison with the BP and FR extracts.**

Key words: Momordica charantia, dekspanthenol, nitrofurazon, wound healing, histopathology, stereology, rabbit

Introduction

In developing countries across the world, 80% of population continues to use traditional medicine for their primary medical problems. Therefore, recent research has focused on the scientific evaluation of traditional drugs made from plants. In this study, the authors evaluate the use of one such plant, *Momordica charantia* (MC), as a medicine (1,2).

MC, a climber belonging to family Cucurbitaceae, is commonly known as bitter melon in English, *karela* in Hindi and *kudret narı* in Turkish (3). The plant is oblong, 15–20 cm long, pendulous and orange in colour when mature or green or whitish when unripe and the pulp is blood-red or scarlet after dehiscence (4). It is grown in the natural environment of India, Asia and South America in tropical and subtropical climate zones. MC is widely used around the world because of antidiabetic properties (5–8). It is used as a vegetable and also as a traditional ingredient for treating various conditions such as the common cold, fever, helminths, rheumatism and wounds (4). It has also been used as a laxative, an antioxidant and antispasmodic and cholinergic agents (9).

In Turkey, it is used externally on wounds and consumed orally to resolve stomach complaints caused by peptic ulcers (4,7). A commonly used MC preparation is the oil extract of fruits in Turkish folk medicine (3).

Recent studies on the pharmacological properties of MC have revealed several biological activities including antidiabetic, anti-lipidemic, antibacterial, antiviral and anticancer activities (10–13). However, only a few studies have focused on its wound healing effects. Further, none of these studies have compared MC's would healing effects with other medical treatments using unbiased stereological methods.

In this study, the authors investigate the wound healing effects of MC and compared the effects with those of (Bepanthen® (BP), Furacin® (FR)) treatment by using stereological methods and histopathological evaluation. The study shows that the oil extract of MC is comparable with BP and FR in terms of re-epithelialisation, vascularisation, proliferation of dermal fibroblasts, inflammation and collagen production.

Materials and methods

Drugs and chemicals

Plant material, olive oil (Sıhhat® drinkable olive oil; acidity 0.25–0.30%), dekspanthenol (BP) and nitrofurazon (FR) were applied externally on the wound.

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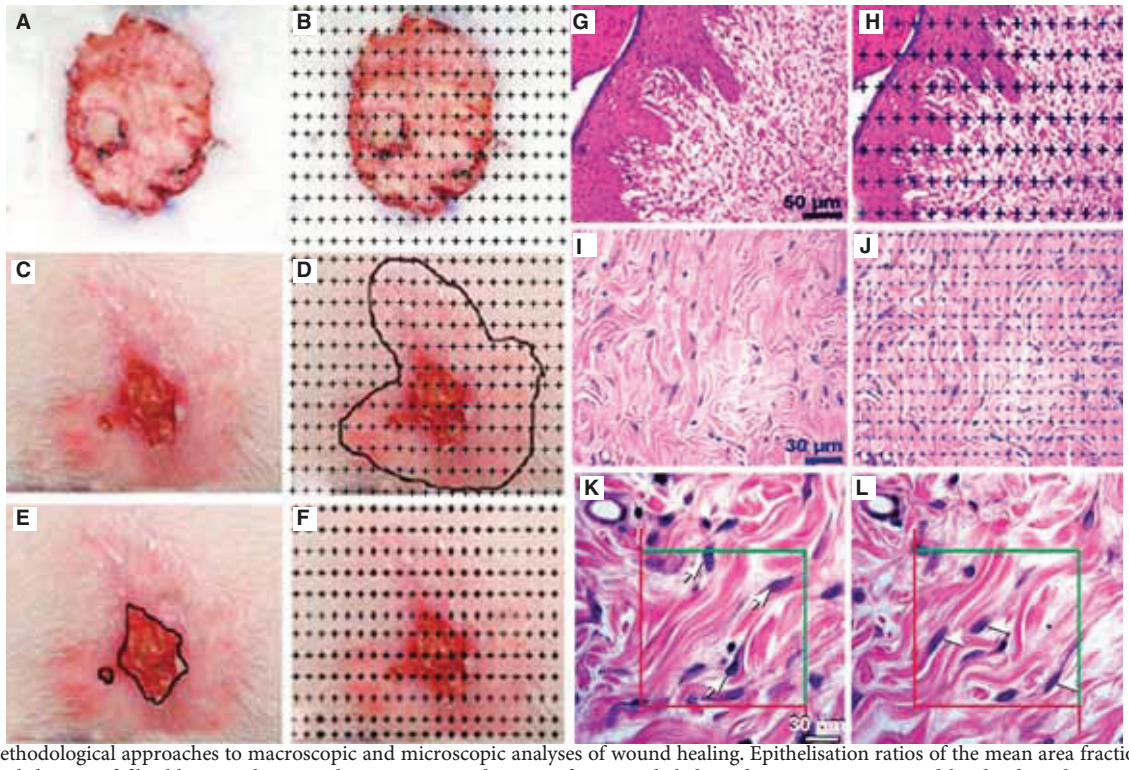


Figure 1. Methodological approaches to macroscopic and microscopic analyses of wound healing. Epithelisation ratios of the mean area fractions of layers and numerical density of fibroblasts in the wounds were estimated. Ratio of non-epithelialisated areas were estimated by dividing the wound area still uncovered with epidermis by the total lesion area (A, B). Rates of re-epithelisation areas were estimated by dividing the wound area covered with epidermis by the total lesion area (C, D). Wound contraction data were obtained by dividing the wound area at the beginning of the study by the lesion area still uncovered with epidermis (E, F). Also in the current study, the mean area fractions of epidermis to dermis (G, H) collagen fibres and fibroblasts to reticular dermis (I, J) were estimated. All surface area fractions were estimated with point counting grids (B, D, F, H and J) and numerical density of fibroblasts were estimated with unbiased counting frames (K, L). Arrows and arrow heads indicate countable fibroblasts, whose nuclei are seen in reference sections (K or L) but not in look-up section (K or L); each disector particle that is completely inside in the unbiased counting frames or superimposed with its including edges, H&E staining.

Preparation of plant material

Mature MC fruits were purchased from a garden in Çarşamba, Samsun, Turkey in July 2010. The fruits were identified by the Botanical Institutes, Ondokuz Mayıs University, Samsun, Turkey, where a voucher specimen is maintained. Five hundred gram of MC was added into 500 g of olive oil. Approximately 30 days later (when MC was completely dispersed in the olive oil), the oil was filtered with a fine sieve and centrifuged; only the oil was prepared for use. This oil was stored at -80°C until use.

Animals

Male New Zealand rabbits (2.5–3 kg) used in the study were obtained from the Animal Laboratory of Ondokuz Mayıs University. The animals were maintained in standard laboratory conditions. Food was withdrawn 24 h before the experiment, but the animals were allowed free access to water. Experiments were conducted in accordance with the recommendations from the Declaration of Helsinki and the internationally accepted principles in the care and use of experimental animals. The protocols used were approved by Ondokuz Mayıs University's Ethic Committee for Animal Use.

Experimental procedure

The 28 New Zealand rabbits were divided into four groups and excision wounds (7 cm^2) were made on their backs. Open wound dressing was carried out daily for 28 days among the experimental groups with dekspanthenol (BP group, $n = 7$), nitrofurazon (FR group, $n = 7$) and olive oil extract of MC (MC group, $n = 7$). No application was made to the control group.

At the beginning of the study, the rabbits were anesthetised with ketamine (5 mg/kg, i.p.) and xylazine (2 mg/kg, i.p.), their backs were shaved bilaterally and full-thickness excision wounds (7 cm^2) were made. The wounds were not sutured or covered or healed by second intention. The experimental groups received a daily supplementation of different agents: dekspanthenol (BP), nitrofurazon (FR) and olive oil extract of MC. Open wound dressing was carried out with the application of these creams and the MC extract on the wounds of the rabbits. The creams were applied twice daily in the morning and evening for 28 days, starting from the day of wounding. The day on which the wound was made was considered as day 0. At the end of the day 28, the initial wound areas of the skin were *en bloc* dissected and prepared for stereological and histopathological analysis. The wounds were then closed primarily and the rabbits were not sacrificed.

Stereological histopathological procedures

Stereological analysis

In this study, stereological analyses were made on macroscopical and microscopical views of the skin samples. Macroscopical analyses include surface areas of the wound still uncovered with epidermis, the total lesion area and the wound area covered with epidermis. Their fractions showed different values as explained below. Also, microscopical analyses contain the surface area fraction of epidermis to papillary dermis, fibroblasts to reticular dermis, collagen fibres to reticular dermis and numerical density of fibroblasts in reticular dermis.

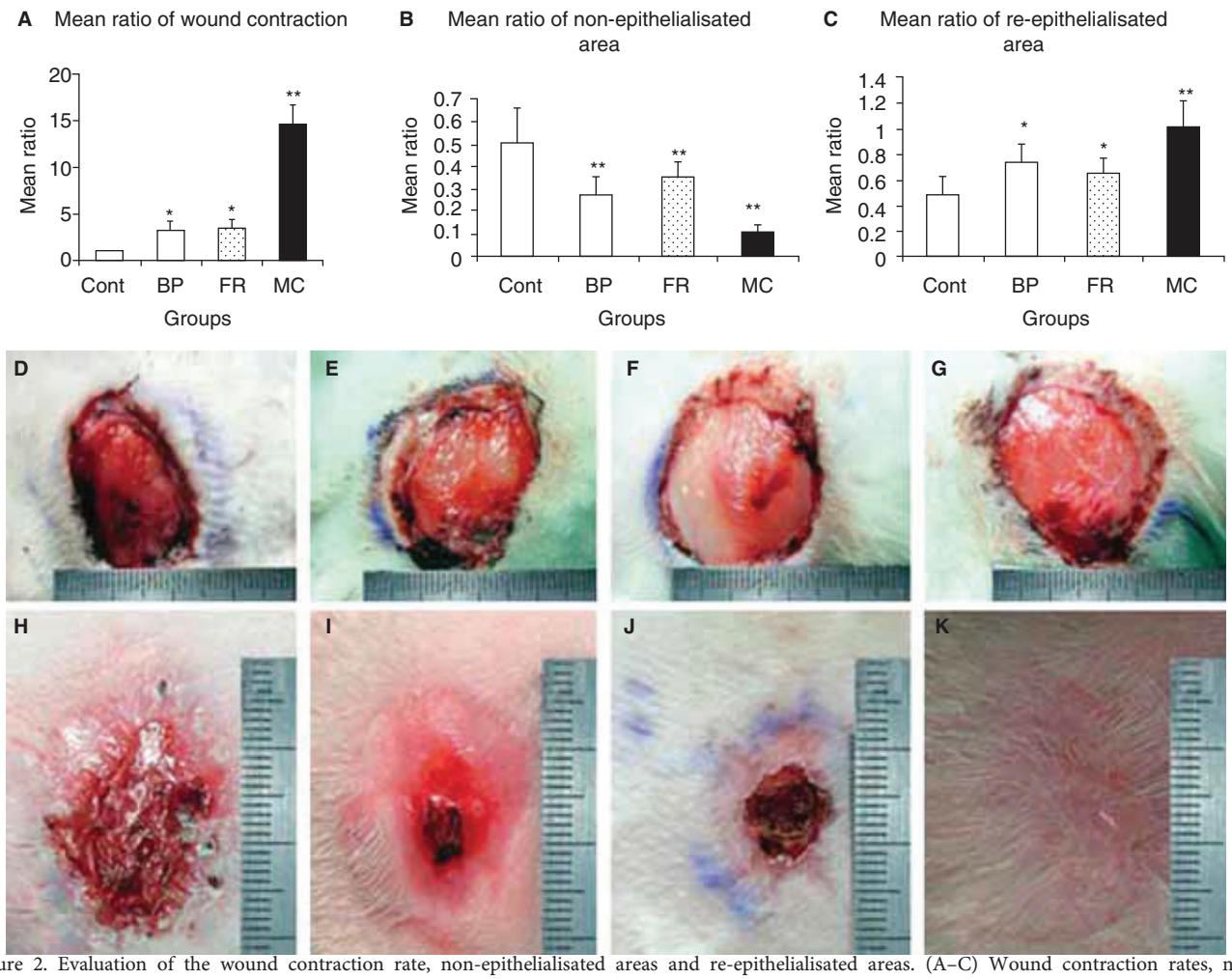


Figure 2. Evaluation of the wound contraction rate, non-epithelialised areas and re-epithelialised areas. (A–C) Wound contraction rates, non-epithelialised areas and re-epithelialised areas; (D–G) wound areas on the first day of wounding; (H–K) wound areas on day 28 of wounding. The areas of total wound (D–G), non-epithelialised wound (still uncovered by neoepidermis) (H–J) and re-epithelialised wound (H–K) are obviously seen. (*) and (**) indicate 0.05 and 0.01 significance levels, respectively.

Macroscopic analysis

To evaluate wound contraction and epithelialisation rates, photographs of all wounds and a metal ruler were taken with a digital camera (Fujifilm Finepix S2800, Tokyo, Japan) at the first and last days of the study. On these photographs, the margins of all wounds, epithelialisated and non-epithelialisated areas were traced. The areas were then estimated with a point counting grid using the Stereoinvestigator software (Stereoinvestigator version 9.2, Microbrightfield, Colchester, VT, USA) (14–16). Rates of non-epithelialisated areas were estimated by dividing the wound area still uncovered with epidermis by the total lesion area. Wound contraction data were obtained by dividing the wound area at the beginning of the study by the lesion area still uncovered with epidermis. Rates of re-epithelialisated areas were estimated by dividing the wound area covered with epidermis by the total lesion area. Mean ratios of the groups and standard error of mean (SEM) values of all groups were used (Figure 1).

Microscopic analyses

Surface area fraction of epidermis to papillary dermis, fibroblasts to reticular dermis and collagen fibres to reticular dermis. On the basis of a pilot study, the authors decided to select every 40th section from the consecutive sections (17). Then, the point counting technique was applied to the light microscopic images for the stereological estimation of surface area fraction of epidermis to dermis, fibroblasts to dermis and collagen fibres to reticular dermis (18,19). Two different point counting test grids were used for the estimation of the sectioned areas, as shown in Figure 1. These grids were used to estimate the layers of skin (a/p = 6.25 μm^2) and mean area fraction of both fibroblasts and collagen fibres (a/p = 6.25 μm^2). The point density of the counting grids was set to obtain an appropriate coefficient of error (CE) for the interested areas in the images of these serial sections (18–20). CE and coefficient of variation (CV) were estimated according to Gundersen and Jensens' formula (20). The test grids with systematic arrays of points were randomly placed in the computerised stereology software (Stereoinvestigator 9.2, Microbrightfield).

Each area of interest in the skin sections was estimated with the following formula (16):

$$\text{Interested area} = (a/p)\cdot\Sigma P$$

where (a/p) represents the area of each point on the point counting grid, and ΣP, the total number of points hitting the sectioned surface area.

Numerical density of fibroblasts

In brief, the authors obtained approximately 80 serial sections from each skin sample. The ratio of section sampling was 1/8. Dissector pairs were taken from the tissue at known intervals,

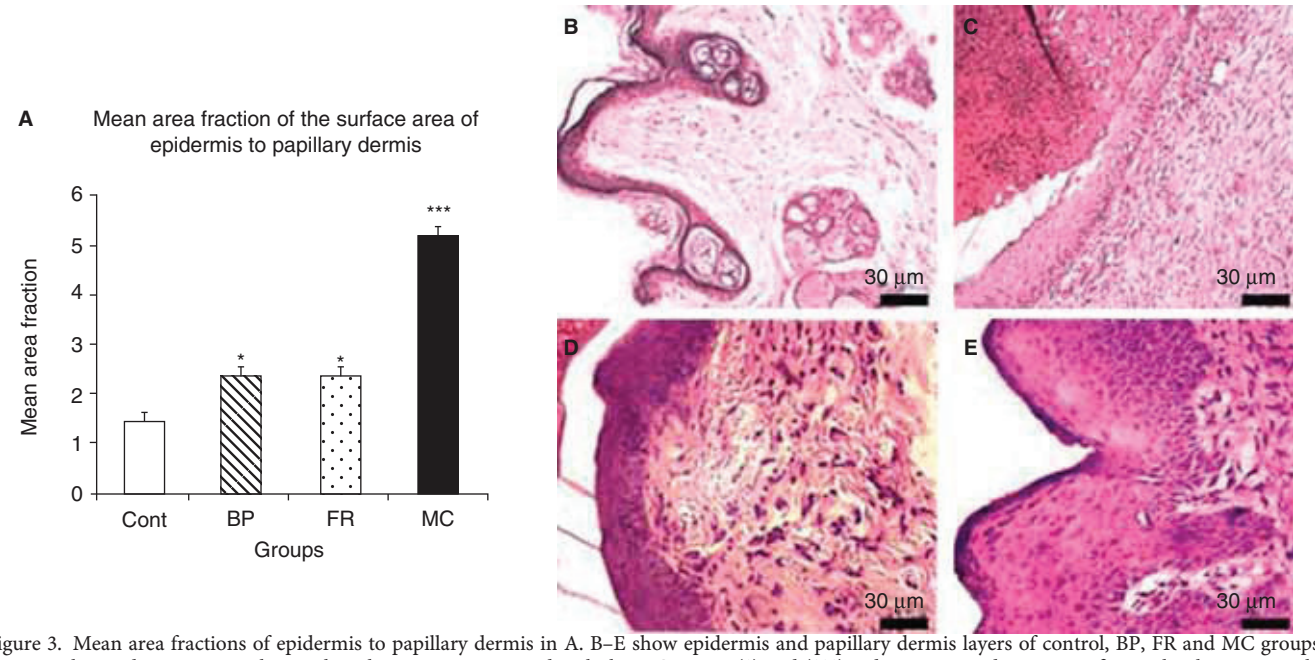


Figure 3. Mean area fractions of epidermis to papillary dermis in A. B–E show epidermis and papillary dermis layers of control, BP, FR and MC groups, respectively. Epidermis is very thin in the other groups compared with the MC group. (*) and (***) indicate 0.05 and 0.001 significance levels, respectively, H&E staining.

until the tissue sample was exhausted. Two consecutive sections were mounted on the same slice. Photographs of adjacent sections were taken with a digital camera (Leica DM 4000 B, Tokyo, Japan) at a magnification of 480×. Unbiased counting frames of size 10 cm^2 (real size of the frame was 441×10^{-6} cm^2) were superimposed on these photographs. Nuclei of fibroblasts in the reference section and not the look-up section were counted. To increase the dissector particle number, that is, the nuclei number, the role of the sections were changed. An unbiased counting frame was placed on the reference and the look-up sections on the screen of the PC to perform the counting according to the dissector counting method. The bottom and the left hand edges of the counting frame are considered to be the exclusion lines together with the extension lines. Other boundaries of the frame and the top-right corner were considered to be inclusion points and any particle that hit these lines or was located inside the frame was counted as a dissector particle (21,22).

The mean numerical density of fibroblasts in per mm^3 was estimated using the following formula:

$$Nv_{(FC)} = \Sigma Q^{-}_{(FC)} / t.a$$

where $Nv_{(FC)}$ denotes the numerical density of the fibroblast cells; $\sum Q^{-}_{(FC)}$, the total number of fibroblast cells; t, the thickness of the section and a, the counting frame area (Figure 1).

Light microscopy

Skin tissues were kept in 10% formalin for fixation. On the fourth day following fixation, the samples were exposed to routine histological procedures and embedded in paraffin blocks. Each paraffin-embedded tissue block was serially sectioned using a Leica RM2125RT microtome (Leica, Germany). Sections of 5 µm were mounted on glass slides and one series of sections were stained with H&E and another with Mallory's trichrome so as to evaluate the general histological structure and collagen contents, respectively. Then, all slides were photographed on the PC screen using a light microscope (Leica, Japan) with a digital colour camera attachment.

Histological score

The Abramov's histological scoring system was used in this study (12). Each parameter was evaluated independently and assigned Abramov's assessment score system. Acute and chronic inflammatory infiltrates, the amount of granulation tissue, and collagen deposition were graded as 0 (none), 1 (scant), 2 (moderate) or 3 (abundant). The fibroblast maturation of granulation tissue was graded as 0 (immature), 1 (mild maturation), 2 (moderate maturation) or 3 (fully matured). Fibroblast maturation was evaluated as to nuclear composition and cytoplasm. Neovascularisation was graded as 0 (none), 1 (up to five vessels per 40× high magnified area, HMA), 2 (6–10 vessels per HMA) or 3 (more than 10 vessels per HMA). Two independent histologists performed the histological examination and applied the scoring system in a blind manner.

Statistical analysis

The Bonferroni test was used for multiple comparisons. Inter- and intra-observer variabilities were calculated using Cohen's K test. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) for Windows 13.0 (SPSS, Inc., Chicago, IL, USA). p-Values of less than 0.05 were considered as statistically significant. All data are shown as means ± SEM (standard error mean).

Results

Macroscopical results

Twenty-eight days after wounding, the percentage of wound healing was higher in the MC group than in the control, BP and FR groups ($p < 0.01$) (Figure 2). In addition, wound healing in the BP and FR groups was better than that in the control group ($p < 0.05$) (Figure 2). There was no difference between the BP and FR groups (Figure 2). According to macroscopical evaluation, the ratio of the non-epithelialisated area was least and that of the re-epithelialisated area was highest in the MC group ($p < 0.01$). However, the non-epithelialisated site was higher in the FR group compared with the BP group and re-epithelisation rates were higher in the FR group compared with the BP group ($p > 0.05$). Additionally, the non-epithelialisated area was also highest in the

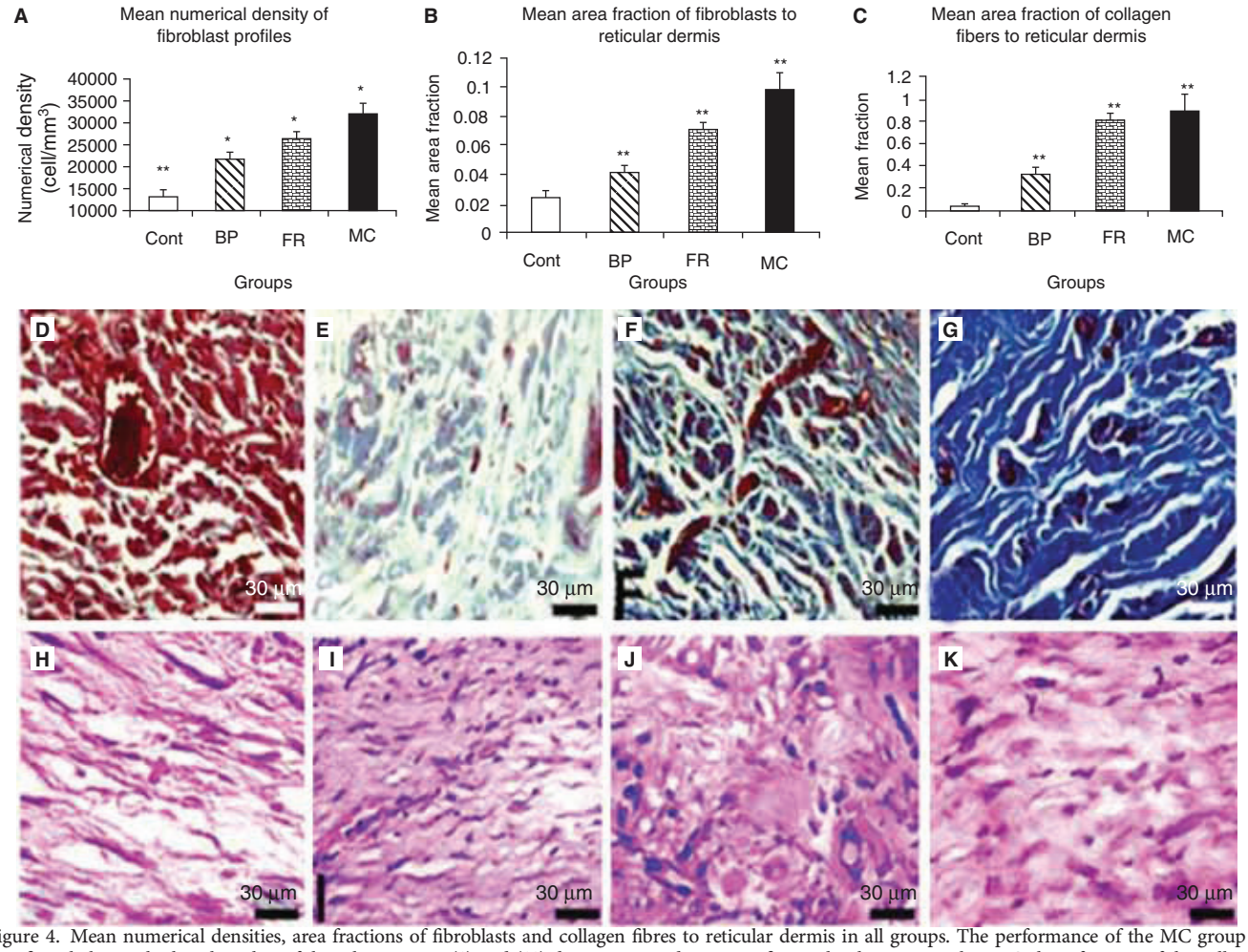


Figure 4. Mean numerical densities, area fractions of fibroblasts and collagen fibres to reticular dermis in all groups. The performance of the MC group is significantly better, higher than that of the other groups. (*) and (**) denote 0.05 and 0.01 significance levels, respectively. D–G show features of the collagen fibres. The most mature and thick bundles of the collagen in the MC group were examined with Mallory's trichrome staining. Fibroblast profiles of the reticular dermis from all groups are shown in H–K with H&E staining. The highest numbers of fibroblast profiles in the reticular dermis are seen in the MC group.

control group ($p < 0.01$) (Figure 2). Wound healing and re-epithelisation rates were compared with each other in all the groups. Also non-epithelialisated and re-epithelialisated areas were inversely proportional to each other.

Microscopical results

Mean area fraction of epidermis to papillary dermis

The mean area fraction of epidermis to papillary dermis in the MC group was significantly higher than that of the other groups ($p < 0.001$). Also, this fraction significantly increased in the BP and FR groups in comparison with the untreated controls ($p < 0.05$), but no significant difference between the BP and FR group was found in terms of the fraction ($p > 0.05$; Figure 3).

Mean numerical density of fibroblasts

The mean numerical density of fibroblasts in the MC group was significantly higher than that of the other groups ($p < 0.05$). Also, this value significantly increased in the BP and FR groups in comparison with the untreated controls ($p < 0.01$) and a significant difference was detected between the BP and FR groups in terms of the value ($p < 0.05$) (Figure 4).

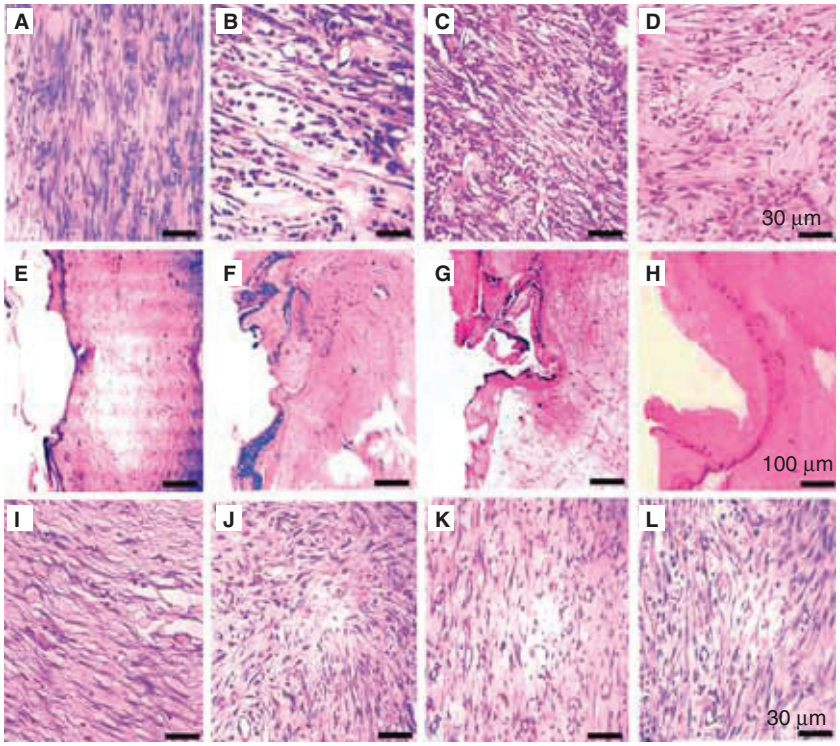
Mean area fraction of fibroblasts and collagen fibres to reticular dermis

Mean area fractions of fibroblasts and collagen fibres to reticular dermis in the MC group were significantly higher than those of the other groups ($p < 0.01$). These fractions increased significantly in the BP and FR groups in comparison with the untreated controls ($p < 0.01$) and such differences were also detected between the BP and FR groups in terms of the fractions ($p < 0.01$; Figure 4).

Histopathological scoring

Histopathological scores of all groups are summarised in Figure 5. The acute inflammation score for the treated groups was less than that of the control group, and there was a significant difference between the groups on day 28 post-application ($p < 0.05$). Further, there was a significant difference between the groups in terms of chronic inflammation ($p < 0.05$). The amount of granulation tissue in the treated groups on day 28 was higher than that of the control group and there were significant differences among the groups concerning the amount of granulation tissue formation ($p < 0.05$). Fibroblast maturation in the treated groups on day 28 was higher than in the control group; there was a significant difference between groups ($p < 0.05$). Collagen deposition in the treated groups (Figure 4) gradually increased by day 28, there was a significant difference among the groups in terms of collagen deposition ($p < 0.05$). A significant difference was found between the groups regarding neovascularisation ($p < 0.05$). Histological comparisons of wounds for all groups are shown in Figure 5.

Scores	Cont	BP	FR	MC
Acute inflammation	2.8 ± 0.7	1.9 ± 0.6	1.5 ± 0.37	0.7 ± 0.21
Chronic inflammation	0.5 ± 0.15	1.6 ± 0.42	2.4 ± 0.86	1.2 ± 0.24
Amount of granulation tissue	1.1 ± 0.5	2.6 ± 0.81	2.1 ± 0.49	1.8 ± 0.38
Fibroblast maturation	1.5 ± 0.23	2.0 ± 0.65	2.3 ± 0.76	2.7 ± 0.88
Collagen deposition	1.2 ± 0.12	1.9 ± 0.51	2.2 ± 0.44	2.6 ± 0.31
Neovascularization	1.0 ± 0.3	1.3 ± 0.21	1.5 ± 0.36	1.8 ± 0.47



Light microscopical results

In the present study, histopathological evaluation was conducted with a light microscope. In the skin samples of the controls, the inflammatory infiltrate and scar formation were abundant. This group presented a reduced number of fusiform fibroblast-like cells, thin epidermis and leaks in the dermal papillary. After cream application, the authors detected hyperkeratinisation and scar formation in the BP group. In the FR group, keratinisation was less than that of the BP group and irregular and rare collagen fibres were seen. In both the BP and FR groups, oedema, fibrin clots and haemorrhage were seen. In the MC group, a thick epidermis and collagen bundles and more fibroblast profiles, capillaries and macrophages were found, as compared with other groups (Figure 5).

Discussion

MC, a member of the Cucurbitaceae family, is a plant grown throughout the world for use as a vegetable. The unripe fruit has also been used in developing countries in traditional medicines for healing microbial infections, sluggish digestion, intestinal gas, inflammation, fever and wounds (5). Immunosuppressive and immunostimulating activities of MC or its constituents have also been reported (23).

In the present study, a full-thickness excisional cutaneous wound was created in animals given supplements of different wound care creams (dekspanthenol (BP), nitrofurazon (FR) and MC olive oil extract) and in untreated control animals. The effect of the supplements was evaluated with macroscopical and microscopical pictures using stereological techniques and histopathological methods. In the present study, it was shown that MC olive oil extract has a more positive effect on wound healing when compared with the dekspanthenol, nitrofurazon and untreated control groups.

Wound healing is a complex and sequential process that occurs in three overlapping stages: inflammation, cell proliferation and tissue remodelling, and results in scar tissue formation (24). A large number of biological mediators control the molecular and cellular processes that comprise wound healing (25). During wound healing, fibroblasts secrete an extracellular matrix (in the form of

collagen, which ultimately forms the bulk of the mature scar) that brings the edges of a wound together. Besides wound contraction, re-epithelialisation is also required to completely close the wound. In addition, angiogenesis is a crucial step that allows the delivery of nutrients to granulation tissue components (24).

Quantitative analysis of the size, shape and number of objects could be done by stereological techniques. It is well known that when they are properly used, it does play an important role in validating and rejecting experimental hypotheses in biological researches that had previously been done, since the results of these techniques are not only accurate but also efficient and more reliable than other *ad hoc* quantitative analyses (17–22).

According to the stereological results, the mean area fractions of epidermis to papillary dermis; fibroblasts to reticular dermis and collagen fibres to reticular dermis of the MC group was significantly higher than that of the other groups. Further, the mean numerical density of fibroblasts in the MC group was significantly higher than that of the other groups. These findings indicate that MC performs better epithelisation, fibroblast maturation and collagen remodelling in comparison with dekspanthenol and nitrofurazon. Further, in terms of connective tissue remodelling, the nitrofurazon showed more positive effects in fibroblast maturation and collagen synthesis as compared with dekspanthenol.

In the present study, the treated groups of rabbits showed a decrease in the number of infiltrated cells at the end of day 28. The epithelialisation was found to be greater in the treated groups than in the non-treated group. The histological pictures of the treated group with topical MC extract showed a well-formed epidermis. The skin of rabbits had a normal epithelium, thereby indicating the protective role of MC extract on skin. If the histology of the wound, after any type of oxidative damage, shows rapid epithelialization which could be considered a positive sign for regeneration (26). In the present study, the authors found that the treated groups, especially the MC group, showed an increased granulation tissue and neovascularisation compared with the control group indicating MC extract was particularly beneficial in the healing process of wounds.

Although bitter melon has been shown to possess a wound-healing property (5,27) in the literature, there is not sufficient number of study to demonstrate the effects of MC on wound healing. But, the results of few studies are very promising as the present study. For this reason, in the literature there are no studies that have proven the mechanism of MC on wound healing. Prasad et al. investigated the effects of MC on the different types of wounds and they found that the level of hydroxyproline which is basic building block of collagen synthesis significantly increased in MC-treated groups (27). Also MC has useful effects on wound healing by reducing inflammation. At this point, we would like to state on a study that was conducted by Özbakıs and co-workers. They did a chemically induced of wound on rat stomach and compared the effect of MC with famotidine and olive oil on wound healing. They reported that MC was more effective than the others in wound healing by reducing inflammation (4). Kuri et al. investigated the chemical composition of MC and found that MC is a very rich source in terms of essential amino acids (28). However, neither unbiased quantitative methods were used nor effects of the MC compared with any medication in previous studies (4,27,28).

In conclusion, this study showed that the oil extract of MC significantly contributed to wound healing and the results suggested that the extract markedly stimulates the epithelialisation, neovascularisation and proliferation of fibroblasts. And also it suppressed the inflammation and provided rapid improvement of wounds.

Finally, the findings suggest that potential usefulness of the extract for tissue regeneration and in the absence of any deleterious side effects being known, the MC extract can be used safely for treating wounds.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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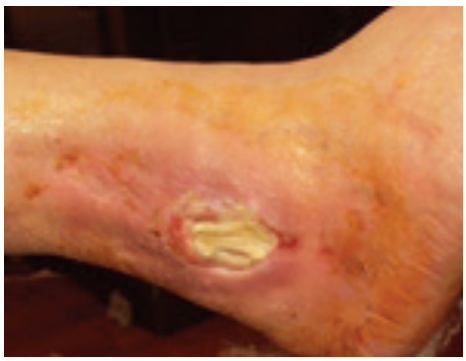
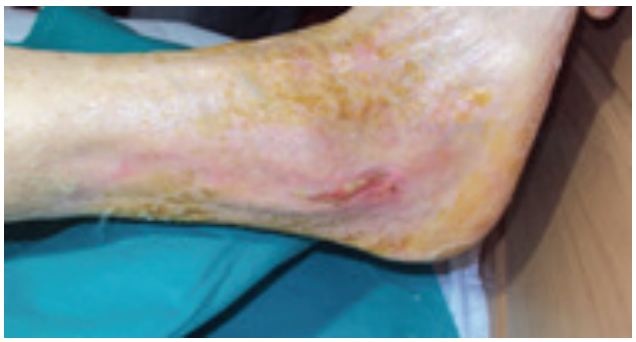
KLİNİK GÖZLEMLER

VAKA 1 : 80 Yaşında tip 1 diabet hastası ,dolaşım bozukluğu mevcut
Diabetik ayak yara tedavisinde RAPİDER BARİYER KREM Kullanımı



pansuma öncesi



pansuman sonrası (5 ay kullanım)

VAKA 2: 40 yaşında cerrahi operasyon gecirmiş hasta dekubit oluşmuş
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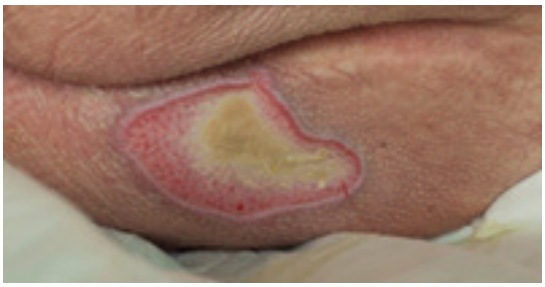

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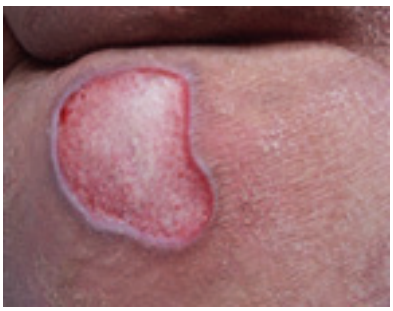
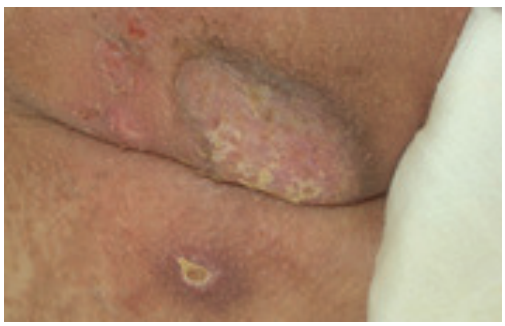
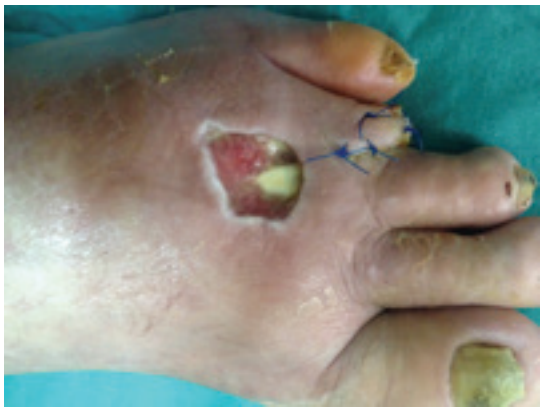


pansuman sonrası (10.gün)



pansuman sonrası (21. gün)

**VAKA 3 : 50 yaşında diabet hastası bir parmak ampute olmuş
ayak üstünde yara açıldı ve RAPİDER BARİYER ile pansuman edild**



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