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Comparison of the effect of stem cell, platelete rich plasma and ovarium folicular fluid on burn stasis zone (Experimental study)

Kök hücre, plateleten zengin plazma ve ovaryum follikül sıvısının yanık staz zonuna etkisinin karşılaştırılması (deneysel çalışma)

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SUMMARY

Objective: The basic aim in the treatment of second-degree burns is to prevent progressive cell death, and so treatments are directed at the zone of stasis. In this study, We were aimed to investigate the healing effects of using Mesenchymal Stem Cells (MSCs), Ovarian follicular fluid (OFS) and Platelet-rich plasma (PRP) on burn stasis zone in an experimental burn model.

Method: Forty rats were divided randomly into four groups. Burns were created in each group according to the comb burn model. The control group received no treatment; the mesenchymal stem cell [MSC] group received MSC; the platelet-rich plasma [PRP] group received PRP; and the ovarian follicular fluid [OFF] group received OFF subcutaneously on days 1, 3 and 5. On days 1 and 21, all rats were photographed and the burn sites were calculated. At the end of day 21, all rats were sacrificed, the dorsum containing the created burns were excised, and the epithelialization, collagen amount, fibroblast density, inflammatory cell density and VEGF amounts were evaluated histopathologically.

Results: The groups were assessed based on the burn site healing rate and histopathological scoring. Healing was faster in the MSC group [$p<0.005$], PRP group [$p>0.005$] and OFS group [$p<0.005$] than in the control group. When the treatment groups were compared with each other, the best healing was observed in the MSC, PRP and OFF groups, respectively.

Conclusions: MSC, PRP and OFF were found to have a positive effect on burn healing, with MSC being the most efficient method among the three, followed by PRP and OFF, respectively, which were found to provide a faster healing than the control group.

Keywords: Burn, mesenchymal stem cells, platelet-rich plasma, ovarian follicule fluid

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ÖZET

Amaç: İkinci derece yanık tedavisinde en temel mantık staz zonundaki progresif hücre ölümünü engellemektir. Bunun için yapılan tedaviler staz zonuna yöneliktir. Bu çalışmada deneysel yanık modelinde Mezenkimal Kök Hücre(MKH), Ovarian follikül sıvısı(OFS) ve Plateletten zengin plazma(PRP) kullanımının yanık staz zonuna olan iyileştirici etkilerini araştırmak amaçlandı.

Yöntem: 40 adet sıçan rastgele 4 gruba ayrıldı. Her gruba comb yanık modeline göre yanık oluşturuldu. Kontrol grubuna hiçbir tedavi uygulanmadı. mezenkimal kök hücre (MKH) Grubuna; MKH, plateletten zengin plazma(PRP) grubuna; PRP ve ovaryum folikül sıvısı (OFS) grubuna; OFS'nı 1., 3. ve 5. günlerde subkutan uygulandı. 1. Gün ve 21. Gün tüm sıçanların fotoğrafları çekilerek yanık alanları hesaplandı. 21. Gün sonunda ratlar sakrifiye edilerek yanık oluşturan sırt bölgeleri eksize edilip histopatolojik olarak epitelizasyon oluşumu, kollogen miktarı, fibroblast yoğunluğu, inflamatuar hücre yoğunluğu ve VEGF miktarı değerlendirildi.

Bulgular: Yanık alanı iyileşme oranı ve histopatolojik skorlamaya göre gruplar değerlendirildi. MKH grubu($p<0.005$), PRP grubu($p>0.005$), ve OFS grubunda($p<0.005$), kontrol grubuna göre daha hızlı iyileşme sağlandığı görüldü. Tedavi grupları kendi arasında karşılaştırıldığında en iyi iyileşme sırasıyla MKH, PRP ve OFS gruplarında olduğu görüldü.

Sonuç: MKH, PRP ve OFS'nin yanık iyileşmesinde olumlu etkisi olduğu görülmüştür. Bu üç yöntem arasında en etkili yöntem MKH'dir. Sonrasında sırasıyla PRP'nin etkili olduğunu ve OFS'nin kontrol grubundan daha hızlı iyileşme sağladığı tespit edilmiştir.

Anahtar sözcükler: Yanık, mesenkimal kök hücre, plateletten zengin plazma, ovarium follikül sıvısı

INTRODUCTION

Burn wound is an injury to the skin and its layers that can be caused by encounters with fire, steam, hot fluids, hot solids, electricity, chemical substances, radiation, etc.. The severity of a burn depends on the duration of exposure, the temperature and concentration of the burning substance, and the resistance of the tissue [1].

Jackson classified burn wounds under three distinct categories based on damage severity and the resulting blood flow changes [2]. The cells at the center of the point at which the thermal agent contacts the skin, as well as the surrounding extracellular matrix proteins, become denatured. This is referred to as the "coagulation zone", while the deeper zone on the periphery of the coagulation zone, where the damage is slight and many cells remain initially viable, is known as "the zone of stasis". The tissue in the zone of stasis maintains its viability immediately after the burn trauma; however, the reduced blood flow associated with edema, prolonged inflammation, the formation of free oxygen radicals, the collection of cytotoxic cytokines in the environment and hypercoagubility, result in tissue necrosis within 48 hours, contributing to an increase in the width and depth of the burn [3,4]. Patients must be given supportive treatment immediately if the cells in the zone of stasis are to maintain viability. Under the appropriate conditions, the cells in the zone of stasis will regain their viability within one week [5]. The zone surrounding these two regions is known as the "zone of hyperemia", where blood flow is increased due to reactive vasodilatation and there is a hyperemic appearance. This zone is likely to recover on its own within a few days.

Mesenchymal stem cells (MSCs) have the ability to differentiate between the cells involved in wound healing and epithelization, as well as the effects that increase vascularization and migration to the wound site, by secreting growth hormones. Studies showed that MSCs promising results regarding anti-inflammatory effects and also preventing burn wound healing[6].

Platelets are the main source of the growth factor complexes that play a key role in the wound healing process, and so the use of platelet-rich plasma (PRP) for therapeutic purposes has become popular, being a plasma fraction derived from autologous blood that contains platelets above the reference line [7].

Ovarian follicular fluid (OFF) is routinely acquired during oocyte aspiration from the hyper-stimulated preovulatory follicles of women undergoing infertility treatment at *in vitro* fertilization centers. Previous investigations have identified all of the cytokines that are involved in wound healing in ovarian follicular fluid [8-14].

Literature contains numerous studies investigating the minimization of tissue damage and the acceleration of healing in the zone of stasis in burn injuries, although there has been no study to date comparing MSC, OFS and PRP in this regard. The present study addresses this gap in literature with a comparison of the three methods. This is the first study to compare the effects of MSC, PRP and OFF in the treatment of burn wound healing.

MATERIAL AND METHODS

Experimental Animals

Approval for the study was granted by the Cumhuriyet University Experimental Animals Local Ethics Committee, dated 09.10.2017, No: 65202830-050.04.04-90. The study was conducted in the Experimental Animals Laboratory of Cumhuriyet University, with full adherence to the principles laid out in the Experimental Animals Ethics Committee guidelines. The study included 46 female Wistar albino rats, weighing 200–250 g and aged four months. The rats were kept in metal cages with a mat below at a room temperature of 22–24°C and a humidity of 55% in a 12-hour light and 12-hour dark environment, with water and food provided *ad libitum*. Of the total, six rats were used for the PRP preparation, and the remaining 40 rats were divided into four equal groups (**Table 1**). The control group received no treatment; the mesenchymal stem cell [MSC] group received MSC; the platelet-rich plasma [PRP] group received PRP; and the ovarian follicular fluid [OFF] group received OFF subcutaneously on days 1, 3 and 5. At the end of day 21, the rats were euthanized using 200 mg/kg pentothal sodium, and tissue samples were collected.

Table 1: Experimental groups

Groups	Treatment Type (1., 3., 5. days)
Control group	Untreated group
MSC group	subcutaneous MSC applied to the stasis zone
PRP group	subcutaneous PRP applied to the stasis zone
OFF group	subcutaneous OFF applied to the stasis zone

Table 2: Subjects in the groups; Epithelialization, Inflammatory Cell, Fibroblast, Collagen, VEGF and Burn Healing Percentage Minimum, Maximum, Mean and Standard Deviation.

	Groups	Minimum	Maximum	Mean	Standard Deviation	p
Epithelialization	Control	1	2	1.70	0.48	<0.001
	PRP	2	3	2.20	0.42	
	OFF	2	3	2.60	0.52	
	MSC	3	4	3.60	0.52	
Inflammatory Cell	Control	2	3	2.70	0.48	<0.001
	PRP	2	3	2.40	0.52	
	OFF	2	3	2.20	0.42	
	MSC	1	2	1.30	0.48	
Fibroblast	Control	2	3	2.20	0.42	<0.001
	PRP	2	3	2.70	0.48	
	OFF	2	3	2.60	0.52	
	MSC	3	4	3.80	0.42	
Collagen	Control	1	2	1.20	0.42	<0.001
	PRP	2	3	2.30	0.48	
	OFF	3	4	3.30	0.48	
	MSC	3	4	3.80	0.42	
VEGF	Control	3	4	3.80	0.42	<0.001
	PRP	2	3	2.70	0.48	
	OFF	2	3	2.20	0.42	
	MSC	2	3	2.20	0.42	
Burn Healing Percentage	Control	24.28	55.38	40.39	10.39	<0.001
	PRP	13.82	25.73	19.99	5.12	
	OFF	21.67	46.67	30.81	9.07	
	MSC	11.98	23.23	15.47	3.97	

Burn Creation

Anesthesia was achieved with 40 mg/kg Ketamine and 10 mg/kg Xylazine, and the dorsum of each rat was shaved. A brass plate consisting of four rows (10×20 mm) and three interspaces (5×10 mm) (**Figure 1 A, B**) was made for the experiment. The plate was placed in boiling water (100°C) for three minutes, and after the appropriate administration of antiseptic, was held in contact with the dorsum of the rat at the midline for 30 seconds with no pressure other than gravity, in accordance with the burn model described by Regas and Ehrlich in 1992 [15]. The procedure resulted in four second-degree burns (10x20 mm) on the hairless dorsum of the rats and three interspersed zones of stasis (5x20 mm) (**Figure 1 C**).

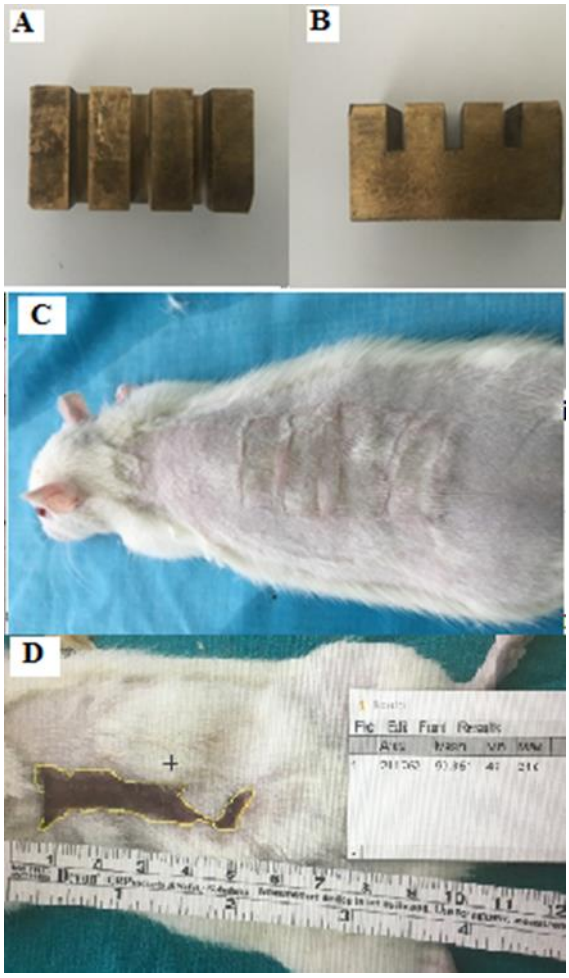

Figure 1. A: Brass plate (viewed from the top), **B**: Brass plate (side view), **C**: View immediately after burn creation, **D:** Calculation of burn area through photoanalysis

Supply and Application of Stem Cells

The adipose-derived stem cells to be administered to the group 2 rats were prepared by the Cumhuriyet University Stem Cell and Gene Therapies Research Center, in accordance with the protocol defined by Ogawa et al. The frozen flasks were thawed and controlled with a light microscope, and the preparation was diluted to create 10^5 cells for each rat in the stem cell group, to be injected subcutaneously into the zone of stasis on days 1, 3 and 5.

Preparation and Administration of PRP

The PRP to be administered to the group 3 rats was prepared in the Cumhuriyet University Biochemistry Laboratory. A total of 8 cc of blood was collected using an intracardiac injector from two rats at a time. The blood was placed into prepared BD Vacutainer[r] tubes containing 1.5 ml

anticoagulant acid citrate dextrose and centrifuged for eight minutes at 450 g; the supernatant was collected and then centrifuged again for eight minutes at 850 g. The buffy coat was collected using an 18-gauge pipette. A blood count was made using a hemogram device, and approximately 1 million platelets were separated for each animal through dilution and administered subcutaneously into the zone of stasis on days 1, 3 and 5.

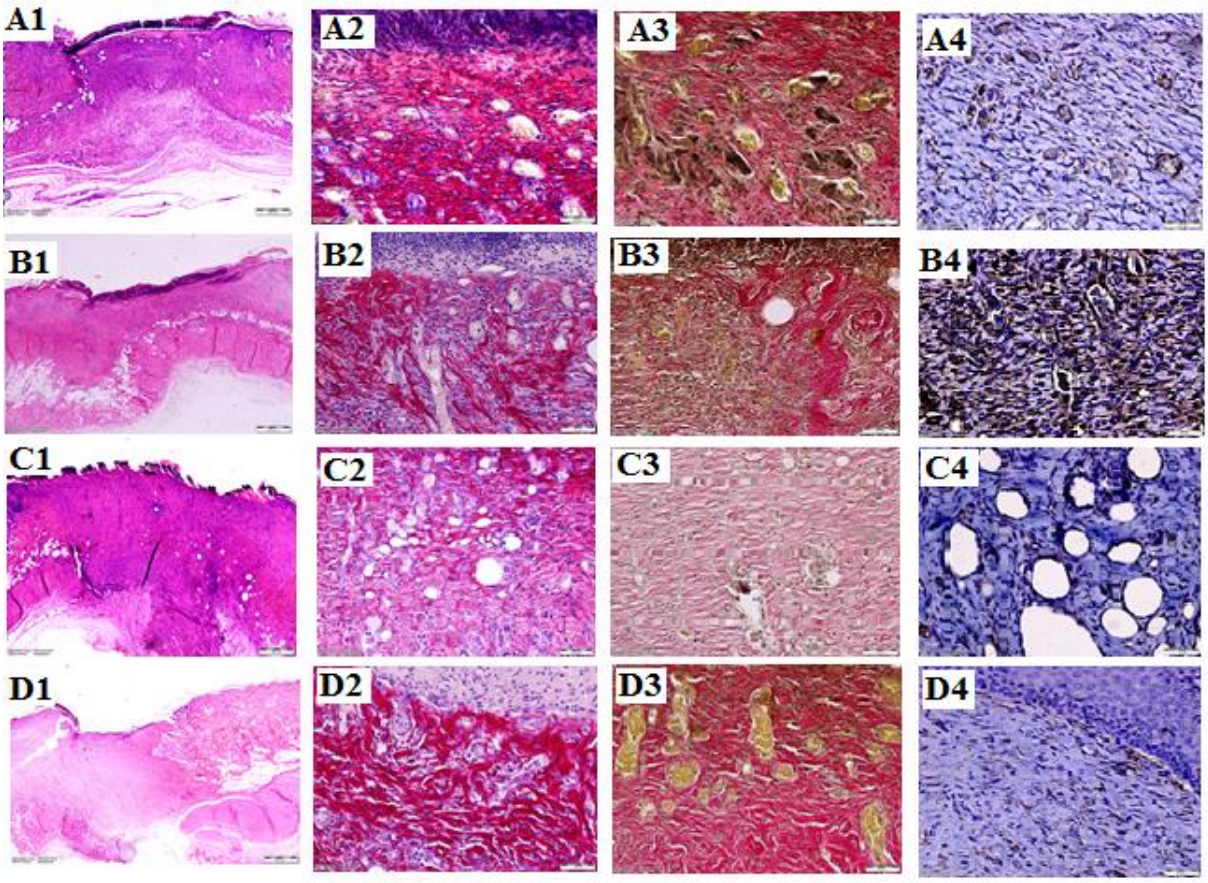

Figure 2: Graph of mean epithelization, fibroblast, inflammatory cell count, collagen and VEGF amount

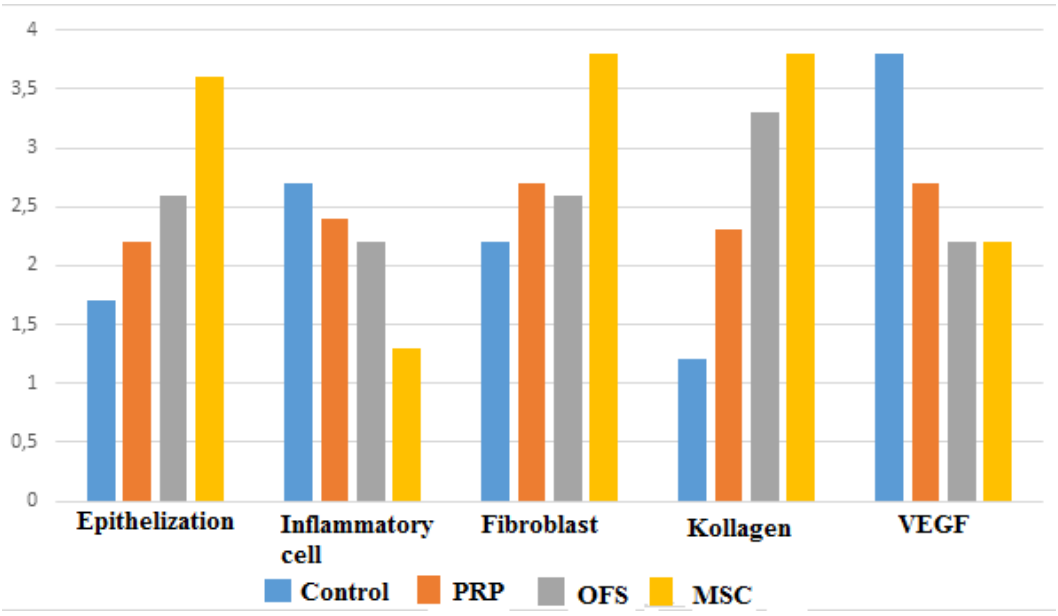

Figure 3: Graph of average burn area healing ratio

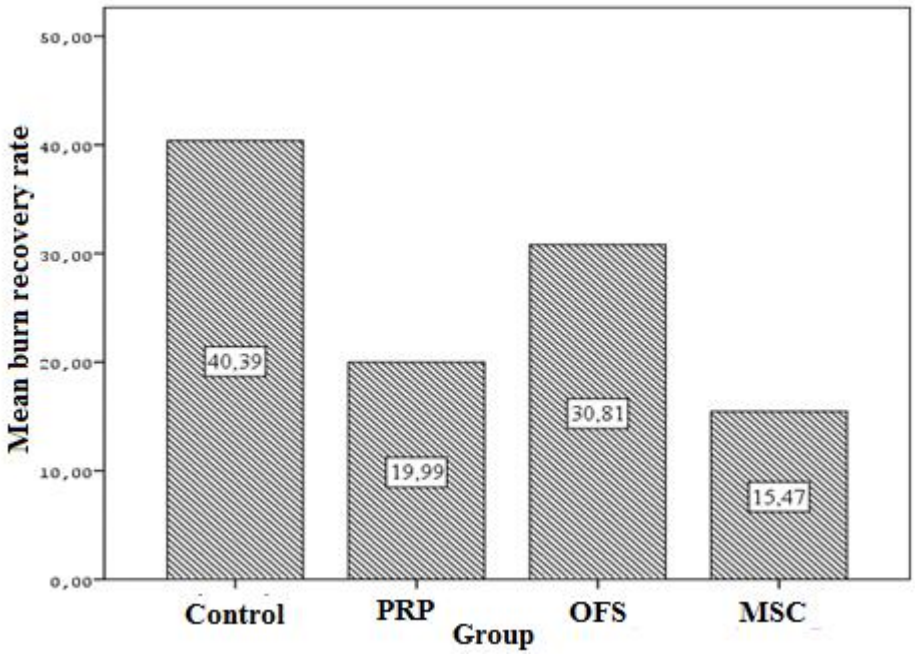

Figure 4: A: Control group**, B:** OFF group**, C:** PRP group**, D:** MSC group. **1:** H&E staining 40X, **2:** Sirius Red, staining 40X, **3**: Van Gieson staining 40X, **4**: VEGF staining 40X.

Acquisition and Administration of Ovarian Follicular Fluid

OFF was collected from the preovulatory follicles of women undergoing treatment in the Assisted Reproductive Treatment Center of the Cumhuriyet University Medical Faculty Hospital. After centrifuging the follicular fluid for 10 minutes at 1000 g, the supernatant was collected using an 18-gauge pipette and injected subcutaneously into the zone of stasis on days 1, 3 and 5 [16].

Comparison of Burn Sites through the Photoanalysis Method

After the burn was created and on day 21, a guide ruler was placed on the dorsum of each rat and photos were taken at a 50-cm distance using a digital camera (Canon sx440ıs, Canon Inc. Tokyo - Japan). The measurement of the area for the ImageJ (National Institutes of Health, USA) program was read from the guide ruler, and the burn site was calculated accordingly (**Figure 1 C**).

For the histomorphological examination, scar tissue taken from the rats was fixed in 10% buffered formaldehyde and embedded in paraffin, from which sections 4–5 µm thick were taken. The sections were deparaffinized and hydrated in descending ethyl alcohol series. The tissue was then placed in distilled water and stained with hematoxylin-eosin (H&E), and then photographed to allow an overall assessment of the wound healing site, and an evaluation of epithelization range, inflammatory cell quantities, and fibroblast and blood vessel density. Van Gieson and Sirius Red staining were then carried out for all groups to ascertain the collagen fiber amount. The deparaffinized and rehydrated sections were then treated with 3% hydrogen peroxide for 10 minutes at room temperature for tissue endogenous peroxidase inactivation in an immunohistochemical evaluation of VEGF density at the healing site. Antigen retrieval was achieved through boiling for 15 minutes in a microwave in an EDTA buffer with a pH of 8.5, and the antigen was twice washed with PBS (pH: 7.2–7.6). The non-specific binding sites were blocked with Ultra V Block (Thermo Scientific, Cheshire, UK) for 20 minutes, then incubated with the primary antibody VEGF (Thermo Scientific, Cheshire, UK) for 1.5 hours at 36°C, and then twice re-washed with PBS and incubated with a biotinylated secondary antibody
(Thermo Scientific, Cheshire, UK) for 20 min at 36° C. After incubation, the tissues were twice washed with PBS, and the procedure was repeated with streptavidin-HRP (Thermo Scientific, Cheshire, UK). To visualize the antibody-stained areas, the DAB chromogen (Thermo Scientific, Cheshire, UK) was applied for 5 minutes, after which contrast staining with H&E was carried out, with a light microscope (Olympus BX51, JAPAN) used for visualizations. All assessments were made by two different histopathologists and scored semiquantitatively (Scoring: none, 0; mild, 1; moderate, 2; distinctive, 3; severe, 4).

Statistical Assessment

Burn healing percentages were measured and calculated using the pathological scoring and photoanalysis method, and the data were analyzed using the SPSS 23 software package. The results of the analysis, carried out to establish the method differences between groups were analyzed at a 95% confidence interval. Since the number of rats in the groups met the condition n<30, no test of normality for the variables was required. "Being non-parametric" was taken into consideration in all difference testing and correlation analyses. A Kruskal-Wallis H test was used to ascertain whether the data varied from group to group. In cases of statistical significance, a Mann-Whitney U test was used to establish which groups produced the difference. Co-variances between variables were analyzed using Spearman's correlation test.

RESULTS

Throughout the 21-day experimental period, no systemic infection or mortality was seen in any of the rats. The intact tissue and the burn site were found to be visibly distinguishable from each other within the first 24 hours of the creation of the burn in the groups.

DISCUSSION

Burn injuries have been associated with high morbidity and mortality. In addition to physical trauma, burns can also cause psychological trauma due to such complications as scarring and prolonged treatment. There are numerous approaches to the treatment of burns, although currently, the most commonly recognized treatment is tangential excision and grafting [17].

Microthrombi occur in the zone of stasis, where apoptosis can be observed within the first few days due to ischemia-reperfusion damage. The cells in the zone of stasis can recover with appropriate treatment, [18] while the zone of hyperemia can recover on its own. Previous studies have shown cytokines and growth factors to regulate and accelerate wound healing [19,20].

Mesenchymal stem cells are multipotent cells that can be found in several tissues, such as bone marrow, adipose tissue, dermis, brain and spleen[21]. Mesenchymal stem cells can differentiate into several cells that have been exposed to trauma. Our bodies contain stem cells that reside in the basal layer of the epidermis and that play a role in wound healing [22]. MSCs accelerate epithelization and neovascularization through such growth factors as EGF and VEGF that they secrete [23]. Bone marrow or adipose tissue can be used to derive MSC, although adipose tissue possesses contains more stem cells than bone marrow, and deriving stem cells from adipose tissue is easier[24]. Shumakov et al. (2003) first identified MSCs as aiding healing in deep burn injuries, reporting MSCs to have a faster healing rate than embryonic fibroblasts and the control group, and to be better in promoting the formation of granulation tissue and neovascularization [25]. Falanga et al. reported that an MSC application within fibrin spray accelerated wound healing in humans [26]. In Lu et al.'s double-blind, randomized clinical trial involving humans, and comparing autologous bone marrow-derived MSC and bone marrow-derived mononuclear cell injections in diabetic feet, found ulcer healing to be statistically and significantly better in the MSC group than in the mononuclear cell group [27].

There have been several studies reporting the intravenous or intradermal application of MSCs to improve the cutaneous wound healing of acute incisional and excisional wounds, diabetic ulcers, radiation ulcers, and acute or chronic skin injuries in humans and animals, including burn injuries [23, 28-30]. In the present study, the application of adipose-derived MSCs was found to diminish the burn site in burn wounds in rats, and provided a significantly higher degree of healing ($p<0.05$).

The primary function of platelets is known to be hemostasis in the initial phase of wound healing; however, platelets are also involved in all subsequent wound healing phases [31]. According to Peavy and Su, the secretion of growth factors and other molecules starts 10 minutes after platelet activation, and more than 95% of growth factors are secreted after one hour [32, 33]. These growth factors take part in various wound healing processes, including chemotaxis, cell adhesion, mitogenesis, proliferation and angiogenesis [33-36]. Furthermore, platelets have been associated with antimicrobial effects, in addition to their pain-relieving effects [31,37]. An experimental study by Henderson et al. treated pigs with full-thickness burns with PRP gel, and found a significant increase in fibroblastic proliferation and angiogenesis, while no increase was noted in re-epithelization [38]. A randomized controlled clinical trial by Kazakoz et al. reported that the application of PRP to an acute wound resulted in distinctively faster wound healing [39].

Liu et al. conducted a randomized controlled clinical trial involving 68 patients with deep second-degree burns, comparing two wound dressing groups, using sulfadiazine cream and autologous platelet-rich gel, and reported that the dressing was less frequent, the healing rate of the

wound site was higher, and the clinical presentation was better in the group treated with platelet-rich gel [40].

A porcine study by Singer et al. compared PRP application with the tangential excision and grafting method. The authors concluded that a single topical PRP cream application two days after burn creation did not improve epithelization or scarring in a full-thickness porcine burn wound, regardless of the timing of the excision and graft. They further concluded that PRP was ineffective in wound healing in their study in a single application and topical administration [41].

In the present study, MSC and PRP produced statistically and significantly better healing than the control group in terms of burn injury epithelization and wound contraction [$p<0.05$]. The comparison of the MSC and PRP groups revealed statistically and significantly greater wound healing in the former, as was the case also in the present study. The various findings regarding the effects of PRP on burns and burn healing, and the variability in the application methods and application frequencies in literature, make the interpretation of the findings even more complex [42]. New studies of PRP applications and the optimum frequencies are required.

Studies in literature have shown ovarian follicular fluid to contain several growth factors and mediators, including EGF, TNF-α, IL-1β and IL-6 [8, 9, 11,12, 14, 43]. These growth factors and mediators are molecules that are known to affect wound healing; however, there is still a lack of studies in literature investigating their effects in burn wounds. The only study to date analyzing the effect of OFF on wound healing was conducted by Ayhan and Aral [44] in 2007 as a Master's thesis. The authors found the wound healing process to be highly accelerated in the group administered OFF when compared to the sham group and the embryonic stem cell group, with a proven pluripotent effect. In the present study, wound healing was significantly faster in the OFF group than in the control group, but significantly slower than in the MSC group [$p<0.05$]. This difference may be attributable to two reasons, one being the use of embryonic stem cells in the study by Ayhan and Aral, while adipose tissue-derived stem cells were used in the present study; and the other being the use of fewer stem cells in the study by Ayhan and Aral (100,000) than in the present study.

This study has two limitations. The first limitation of the present study relates to the acquisition of MSC and PRP from different rats and OFF from humans. Using larger experimental animals and autologous MSC, PRP and OFF for each subject may affect the results. The second limitation is the study's comparison of MSC, PRP and OFF in various doses and in combination with each other, while the third limitation is the small sample size of 10 for each group.

CONCLUSION

The present experimental burn model study identified some positive effects of mesenchymal stem cells, platelet-rich plasma and ovarian fluid on wound healing. A comparison of these three methods revealed MSS to be the most efficient. This was followed by PRP, and, although not as effective as the other methods, OFF, which led to faster healing than in the control group. The efficacy of OFF, which has to date not been used for the treatment of burn injuries, needs to be demonstrated through prospective randomized studies. Further studies are required to establish a standard MSC therapy and clinical approach.

Conflict of interest

The authors declare no conflict of interest.

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