EXPERIMENTAL STUDY

# Effect of hyperbaric oxygen therapy when combined with fresh and frozen platelet-rich plasma on chronic wound healing in rats

**Nevra Seyhan, M.D., Sinan Öksüz, M.D.**

Department of Plastic Reconstructive and Aesthetic Surgery, Gülhane Training and Research Hospital, Ankara-*Türkiye*

## ABSTRACT

**BACKGROUND:** Hyperbaric oxygen therapy (HBOT) has an added dimension to the armamentarium of treating complicated chronic wounds. The purpose of this study is to investigate the effects of HBOT on chronic wounds when combined with fresh and frozen platelet-rich plasma (PRP).

**METHODS:** Rats were divided into two main groups containing 18 rats in each group as HBOT received and non-received. Each group was divided into three subgroups as Group 1: Control, Group 2: Fresh, and Group 3: Frozen PRP applied. For PRP preparation, 10 rats were used. Histologic parameters including fibroblast, collagen fibers, lymphocytes, and vessels were evaluated by Clemex Vision Lite 3.5; wound sizes were evaluated by ImageJ digital analyzing program.

**RESULTS:** In HBOT received group, the number of fibroblasts, collagen fibers, lymphocytes, and vessels in all fresh and frozen PRP applied and control subgroups were significantly higher than hyperbaric oxygen non-received group ($p<0.05$). In HBOT received group, wound surface area measurement values of control, fresh, and frozen PRP applied groups at 5-10-15 days were lower than HBOT non-received group.

**CONCLUSION:** HBOT accelerates wound healing when combined with both fresh and frozen PRP. Frozen PRP is as effective as fresh form to be considered as an alternative in clinical setting.

**Keywords:** Chronic wound; fresh; frozen platelet-rich plasma; hyperbaric oxygen therapy.

## INTRODUCTION

Hyperbaric oxygen therapy (HBOT) is known as intermittently inhaling 100% oxygen inside a chamber at pressures >1 atmosphere absolute (ATA). This therapy has been widely used to treat soft-tissue infection, osteomyelitis,[1] gas embolism,[2] compartment syndrome,[3] carbon monoxide poisoning,[4] and osteoradionecrosis[5] since the 1950s.

The role of hyperbaric oxygen (HBO) in wound healing is multifactorial. Oxygen consumption is increased as leukocytes migrate to the wounded area. Accumulation of high oxygen at the wound site increases fibroblast activity, collagen deposition, and collagen synthesis and stimulates neovascularization.[6]

Platelet-rich plasma (PRP) is composed of various types of growth factors including platelet-derived growth factor-BB (PDGF-BB), epidermal growth factor, transforming growth factor (TGFβ), and vascular endothelial growth factor (VEGF). All of them have important role in wound healing improvement through different mechanism of action.[7,8] PRP is undoubtedly the most widely used serum for obtaining these growth factors. Containing high concentrations of growth factors make PRP having potential to improve wound healing. PRP is known with its positive effects on chronic wounds.[9] It can be used in fresh form and it can be frozen.

The purpose of this study is to investigate the effects of HBOT, fresh PRP, and frozen PRP on chronic wounds both

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Address for correspondence: Nevra Seyhan, M.D.
Gülhane Eğitim ve Araştırma Hastanesi, Plastik Rekonstrüktif ve Estetik Cerrahi Kliniği, Ankara, Türkiye
Tel: +90 312 - 304 54 09 E-mail: drnevraseyhan@hotmail.com

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alone and when combined. Our hypothesis is that the combination therapies will potentiate their therapeutic effect. An experimental ischemic rat model is designed for this purpose because ischemia adversely affects wound healing and causes chronic wound formation.

## MATERIALS AND METHODS

### Experimental Groups and Wounding Procedures

The study protocol was approved by animal ethical committee for animal experiments. We established chronic wound healing model on rats to examine the effects of PRP and HBO. We used the model previously described by Schwarz[10] on the back of the rats. Totally, 46 Wistar rats were used in this study. To obtain fresh and frozen forms of PRP, 10 rats were used as donors. Animals were divided into two main groups as Group A (HBO non-received) and Group B (HBO received). Each group was divided into three subgroups as Group 1: Control, Group 2: Fresh PRP applied, and Group 3: Frozen PRP applied. The result of sample size calculation determined six animals in each subgroup.

After anesthetizing the animals with intraperitoneal injection ketamine (90 mg/kg) and xylazine (10 mg/kg), the surgical area was completely shaved and disinfected before skin excision. After planning a flap 10x4 cm dimension in size, the skin was incised. The flap was raised by dissecting the areolar tissue at the level of the panniculus carnosus and deep fascia of the rat's dorsal musculature as far as the interscapular base. The flap was repositioned in its original position and was sutured with 4–0 monofilament nylon single stitches at 0.5 cm intervals (Fig. 1a). We waited for 3 days for occurrence of ischemia on the flap then six wounds were created 6 mm diameter with a punch biopsy instrument (Acu-Punch, Acuderm Inc., Lauderdale, FL, USA) (Fig. 1b).

To achieve consistency and overcome ischemic gradient; fresh PRP application was adjusted cranially localized wounds; frozen PRP was applied to the middle localized wounds. Caudally localized wounds were the control. Only Group B received HBO therapy in combination with PRP appliance.

### Preparation of Fresh and Frozen PRP

Donor rats (n=10) were anesthetized and their blood was collected by cardiac puncture and taken into acid citrate dextrose tubes for the preparation of fresh and frozen forms of PRP. The blood was centrifuged at 1000 rpm for 10 min. Subsequently, the red blood cell layer formed at the lowest level, the buffy coat (containing the platelets, leukocytes, and a few erythrocytes) in the middle, and platelet poor plasma at the top. A pipette was used to aspirate the plasma fraction to the level of the interphase zone and transferred to another tube. One milliliter of PRP was obtained from 10 ml blood.

Before freezing 6% DMSO (dimethyl sulfoxide), 5 µl is added to 5 ml supernatant according to our cryopreservation protocol and kept at −80°C for 1 day. Before use thawed at 37°C and diluted with 0.9% NaCl. Both fresh and frozen PRP are activated by %10 CaCl2 before appliance.

### HBOT Application

HBO sessions were conducted using a hyperbaric chamber with a 10 rat capacity. The sessions were conducted daily six animals at a time, and began on the 1st post-operative day after creation of the wounds and continued for 7 days for 90 min duration at 2.4 ATA in each session.

The beginning of the session included a compression period and ended with decompression period.

### Histopathological Analysis

At the end of the experiment, all rats were sacrificed by decapitation and the wound tissues were excised. Stained specimens were investigated by a Nikon Eclipse E400 light microscope. For each specimen, the same area was photographed using a Nikon Coolpix 5000 photograph attachment.

The photograph of Nikon micrometer microscope slide was also taken during the procedure. All photographs were then transferred into PC environment and analyzed using Clemex Vision Lite 3.5 Image analysis program (Clemex Technologies, Quebec, Canada) (Fig. 2). The length was calibrated by com-

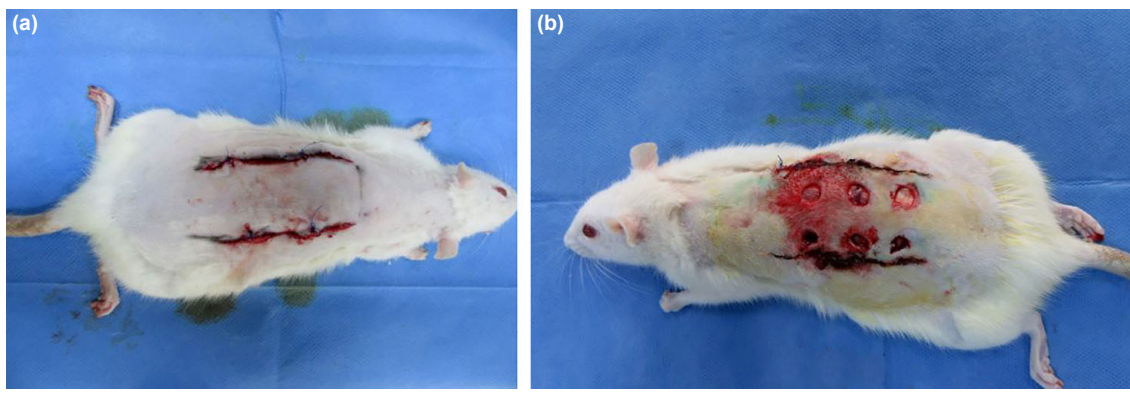


**Figure 1. (a)** Bipedicle flap raised and sutured to its original position for creation chronic ischemic wound. **(b)** Full-thickness wounds created on the cephalic, central, and caudal parts of the flap are seen.

paring the photograph of specimen with the photograph of Nikon micrometer microscope slide, which was taken under the same magnification. 7,924,450.5 $\mu m^2$ areas was designated with using Clemex Vision Lite 3.5 image analysis program; then, vessels, fibroblasts, collagen fiber, and lymphocytes were marked with the same Image analysis program in 7,924,450.5 $\mu m^2$ area. Damaged cells were not evaluated. The marked cells were counted automatically with the same program. The obtained data were recorded for statistical analysis.

## Wound Surface Area Measurement

For the evaluation of the wound size, photographs of the wounds were taken by digital camera at 5-10-15 days of the experiment. Wound sizes were measured with ImageJ digital analyzing program. The picture of the created 6 mm diameter wound before the treatments applied is calculated by the same analyzing program and estimated as 10,000 piksel.

## Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics are presented as means and standard deviation. The Chi-square test was used for categorical variables, and parametric t-tests were used for the measurement of wound surface. The Mann-Whitney U-test was also used to establish the effect of PRP and HBO treatment on wound surface area.

The Kruskal–Wallis test was used to compare the significance between the control group and the two different treatment groups. Using Bonferroni correction, the interactions were controlled. The recovery of the wound after the treatments was charted using SPSS module. All $p<0.05$ were accepted as statistically significant.

# RESULTS

In HBO received group, the number of fibroblasts, collagen fiber, lymphocytes, and vessels in all fresh and frozen applied and control subgroups were significantly higher than HBO non-received group ($p<0.05$) (Fig. 3). In both HBO therapy received and non-received groups, the number of fibroblasts, collagen fiber, lymphocytes, and vessels of fresh and frozen PRP applied groups were significantly higher than the control group. In both HBO therapy received and non-received groups, there was no statistically significant difference between fresh and frozen PRP applied groups in terms of histologic parameters (Table 1).

In HBO therapy received group (Table 2 and Fig. 4), wound surface area measurement values of control, fresh, and frozen PRP applied subgroups at 5-10-15 days were lower than HBO therapy non-received group ($p<0.05$) (Table 3 and Fig. 5).

In HBO therapy non-received groups, there was no statistically significant difference between fresh and frozen PRP applied groups at day 15. In HBO therapy received groups, there

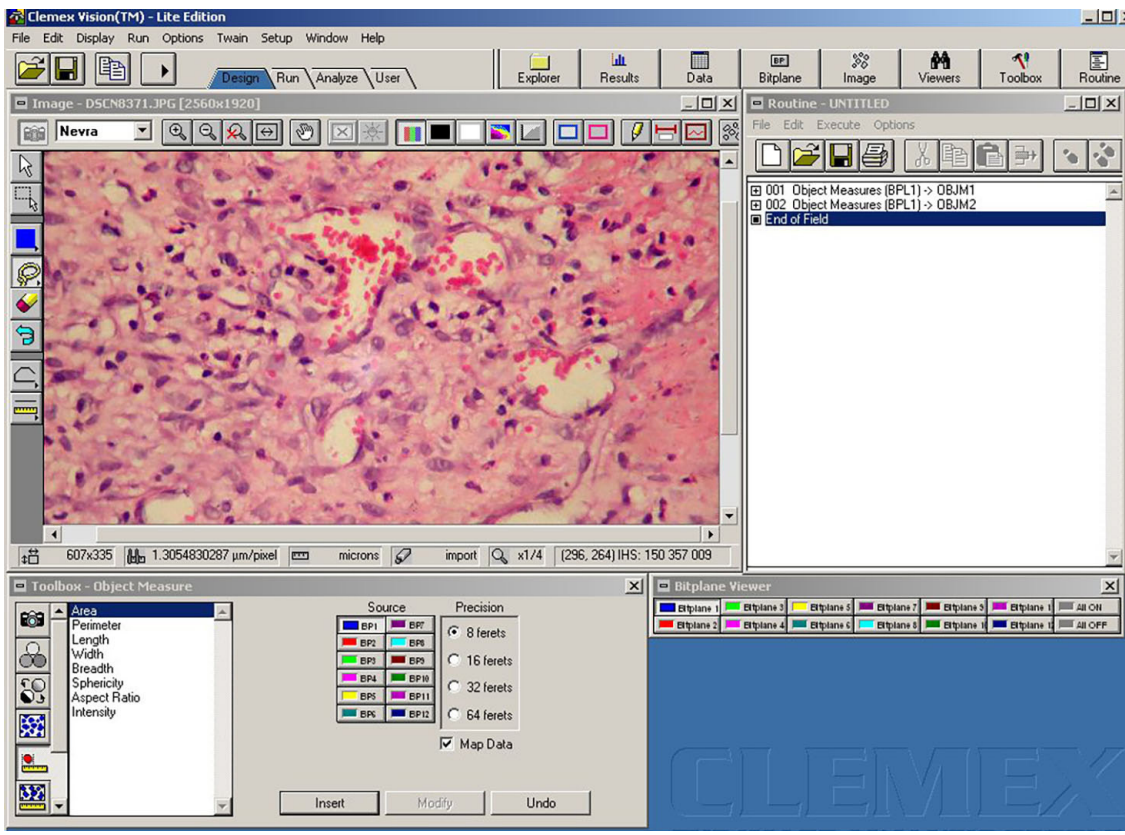


**Figure 2.** The view of Clemex Vision Lite software image analyzing program.

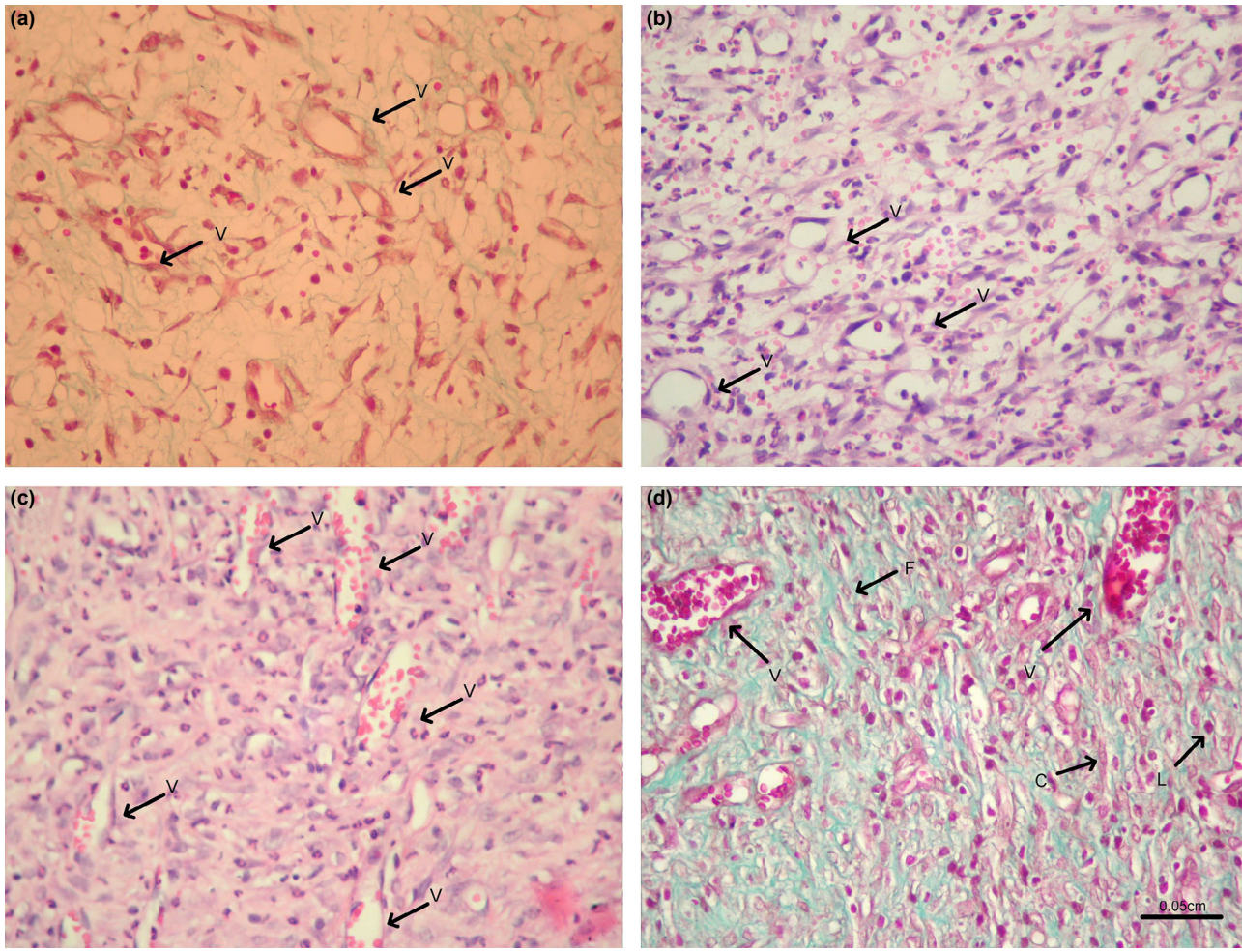


**Figure 3.** Histopathological assessment (the arrows symbolizes; V: Vessel, F: Fibroblast, C: Collagen, and L: Lymphocyte). Vessels are more predominant in Group B2 and B3 when compared to Group A2 and A3 indicating neovascularization. **(a)** HBO non-received fresh PRP applied group (Group A2), **(b)** HBO non-received frozen PRP applied group (Group A3). **(c)** HBO received fresh PRP applied group (Group B2). **(d)** HBO received frozen PRP applied group (Group B3).

**Table 1.** The mean values of the histopathological parameters evaluated

| | HBO Non-Received Group | | | HBO Received Group | | |
|---|---|---|---|---|---|---|
| | **Control (Mean±SD)** | **Fresh PRP (Mean±SD)** | **Frozen PRP (Mean±SD)** | **Control Mean±SD** | **Fresh PRP Mean±SD** | **Frozen PRP Mean±SD** |
| Fibroblast | 5.38±1.18 | 8.63±1.18<br>$\chi^2$=14.159* p=0.001***<br>Mean difference=1-2. 1-3**** | 8.88±0.99 | 7.88±0.83 | 15.13±3.09<br>F=31.570** p<0.001<br>Mean difference=1-2. 1-3 | 17.50±2.97 |
| Collagen fiber | 4.63±0.74 | 8.88±1.35<br>$\chi^2$=15.747 p<0.001<br>Mean difference=1-2. 1-3 | 8.13±1.45 | 5.88±1.35 | 15.63±2.20<br>$\chi^2$=17.780 p<0.001<br>Mean difference=1-2. 1-3 | 18.00±1.30 |
| Lymphocyte | 5.13±0.83 | 8.13±1.55<br>F=13.049 p<0.001<br>Mean difference=1-2. 1-3 | 8.13±1.55 | 7.63±1.50 | 14.25±2.60<br>F=22.520 p<0.001<br>Mean difference=1-2. 1-3 | 14.50±2.67 |
| Vessels | 4.63±0.74 | 7.75±1.28<br>$\chi^2$=15.179 p=0.001<br>Mean difference=1-2. 1-3 | 7.88±0.99 | 6.88±1.64 | 13.75±1.58<br>F=60.800 p<0.001<br>Mean difference=1-2. 1-3 | 15.38±1.68 |

HBO: Hyperbaric oxygen; PRP: Platelet rich plasma; SD: Standard deviation.

**Table 2.** The mean values of wound surface areas of hyperbaric oxygen therapy received group at 5-10-15 days of the experiment

| Wound surface area | Control (A) MD±SD | Fresh PRP (B) MD±SD | Frozen PRP (C) MD±SD | Total | Main effect | | Interaction effect |
|---|---|---|---|---|---|---|---|
| | | | | | Time | Group | |
| 5. day (1) | 7787.12±1114.04 | 3773.50±741.35 | 3264.63±887.90 | 4941.75±2248.12 | F=159.57* | F=129.919 | F=3.177 |
| 10. day (2) | 5974.88±861.39 | 1837.13±697.42 | 1781.88±590.37 | 3197.96±2122.20 | p<0.001** | p<0.001* | p=0.023 |
| 15. day (3) | 3230.13±1135.00 | 182.25±50.33 | 186.13±56.40 | 1199.50±1595.36 | $\eta^2$=0.941 | $\eta^2$=0.925 | $\eta^2$=0.232 |
| Total | 5664.04±2158.13 | 1930.95±1601.13 | 1744.20±1412.77 | | | | |
| **Source of difference for interaction (Group x Time) for time** | | | | | | | |
| **Pairwise comparison**** **(Time)** | | **Pairwise comparison**** **(Time)** | | **Pairwise comparison**** **(Time)** | | | |
| Control 1-2, 1-3, 2-3 p<0.001 | | Fresh PRP 1-2, 1-3, 2-3 p<0.001 | | Frozen PRP 1-2, 1-3, 2-3 p<0.001 | | | |
| **Source of difference for interaction (Group x Time) for group** | | | | | | | |
| **Pairwise comparison**** **(Group)** | | **Pairwise comparison**** **(Group)** | | **Pairwise comparison**** **(Group)** | | | |
| Time=5 A-B, A-C p<0.001 | | Time=10 A-B, A-C p<0.001 | | Time=15 A-B, A-C p<0.001 | | | |

*F test statistics value. **The mean difference is significant at the 0.05 level (p<0.05). ***Simple effects analysis with Bonferroni adjustment were used.
PRP: Platelet rich plasma; MD: Median; SD: Standard deviation.

was no statistically significant difference between fresh and frozen PRP applied groups at day 10–15 (Fig. 6).

## DISCUSSION

Dealing with non-healing chronic wounds is interesting for researches so many studies are going on this area.[11,12] The rich growth factor profile of PRP makes it to have a potential for easy and cost-effective treatment of a variety of wound types.[13]

PRP may freshly be isolated from blood and can also be refrigerated. Cryopreservation of platelets with 6% DMSO and storage at −80°C increases their shelf life from 7 days to 2 years.[14] Cryopreservation methods reduce the potential for

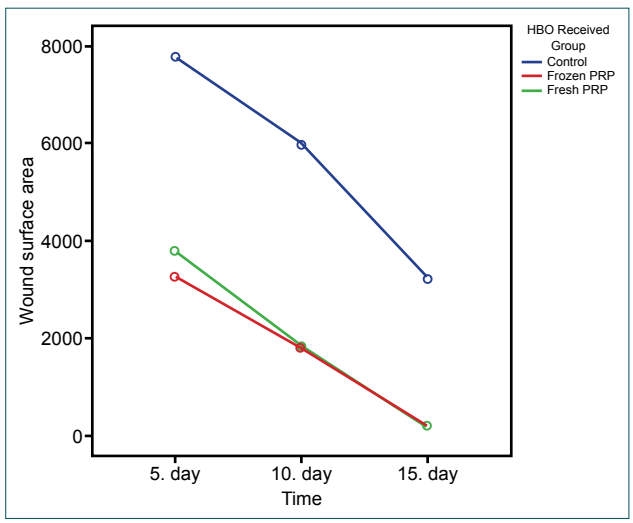


**Figure 4.** The graphic demonstrates wound surface area measurements on 5-10-15 days in HBO received group.

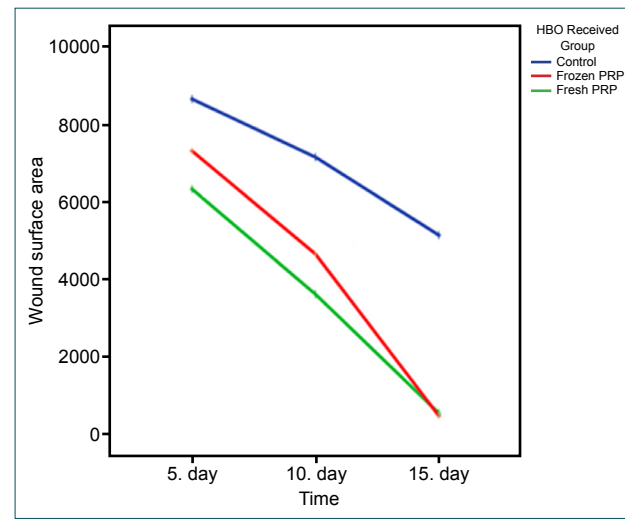


**Figure 5.** The graphic demonstrates wound surface area measurements on 5-10-15 days in HBO non-received group.

**Table 3.** The mean values of wound surface areas of hyperbaric oxygen therapy non received group at 5-10-15 days of the experiment

| Wound surface area | Control (A) MD±SD | Fresh PRP (B) MD±SD | Frozen PRP (C) MD±SD | Total | Main effect | | Interaction effect |
|---|---|---|---|---|---|---|---|
| | | | | | Time | Group | |
| 5. day (1) | 8652.50±618.31 | 6327.75±669.65 | 7312.75±658.95 | 7431.00±1154.20 | F=1052.91* | F=93.211 | F=9.971 |
| 10. day (2) | 7154.63±528.34 | 3614.50±1069.42 | 4652.62±914.07 | 5140.58±1729.48 | p<0.001** | p<0.001 | p<0.001 |
| 15. day (3) | 5140.75±810.97 | 548.13±177.26 | 477.00±74.99 | 2055.29±2275.80 | $\eta^2$=0.991 | $\eta^2$=0.899 | $\eta^2$=0.487 |
| Total | 6982.62±1600.52 | 3496.79±2512.12 | 4147.45±2960.72 | | | | |

**Source of difference for interaction (Group x Time) for time**

| Pairwise comparison*** (Time) | Pairwise comparison*** (Time) | Pairwise comparison*** (Time) |
|---|---|---|
| Control 1-2, 1-3, 2-3 | Fresh PRP 1-2, 1-3, 2-3 | Frozen PRP 1-2, 1-3, 2-3 |
| p<0.001 | p<0.001 | p<0.001 |

**Source of difference for interaction (Group x Time) for group**

| Pairwise comparison*** (Group) | Pairwise comparison*** (Group) | Pairwise comparison*** (Group) |
|---|---|---|
| Time=5 A-B, A-C, B-C | Time=10 A-B, A-C | Time=15 A-B, A-C |
| p<0.001 | p<0.001 | p<0.001 |

*F test statistics value. **The mean difference is significant at the 0.05 level (p<0.05). ***Simple effects analysis with Bonferroni adjustment were used.
PRP: Platelet rich plasma; MD: Median; SD: Standard deviation.

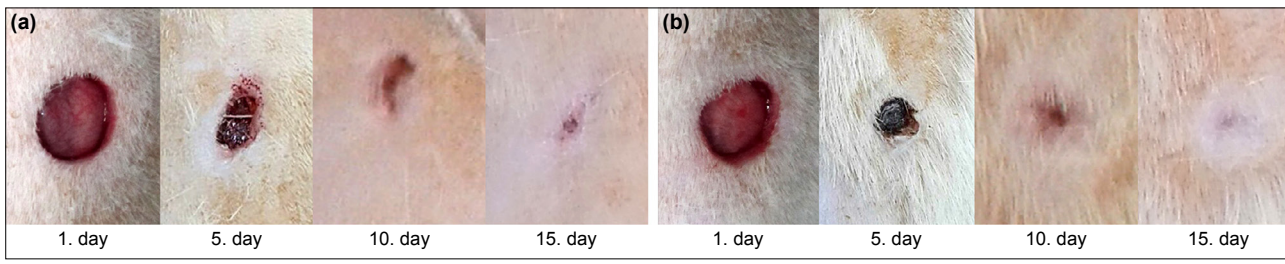


**Figure 6.** Photographs of the wound surface areas evaluated by ImageJ digital analyzing program. The picture is showing wound healing of the created defects on the back of the rats according to the days of the experiment in HBO received fresh **(a)** and frozen PRP **(b)** applied groups.

bacterial proliferation.[15] Cold storage preserves platelets physiological properties.[16]

The beneficial effects of PRP freezing were proven in many studies.[17,18] Our results are in accordance with them. Frozen PRP was found to be as effective as fresh PRP on wound healing in our experiment. The potential for keeping the specialties of platelets and extension of the time for their therapeutic use without interfering with their ability of delivering growth factors makes freezing methods particularly suitable for enhanced wound care.

Frozen form of PRP may be considered as an alternative to fresh form for appliance to the patients with chronic wounds. With the aim of ulceration treatment, PRP preparation and application will be difficult to prepare in adequate amounts and time may not coincide with adjunct therapies such as HBO. Therefore, it would be useful if PRP could be isolated beforehand and stored until use. This would save patients from undergoing multiple traumatizing injection cycles.

Non-healing wounds are those which fail to heal within a reasonable time and its consequence is ulcer formation. HBO promotes wound healing by counteracting tissue hypoxia and is a valuable adjunct in the management of ischemic, infected, and non-healing wound.[19,20] Ueno et al.[21] found HBO effective in treating chronic wounds when used in combination with conventional standard therapy. HBOT has certain potential to promote ulcer healing and reduce amputation rate in patients with ischemic diabetic foot ulcers.[22]

HBOT stimulates growth factors such as TGFβ which play a role in regulation of cell proliferation, differentiation, apoptosis, and induction of intimal thickening; PDGF that is a major mitogen for connective tissue cells and VEGF involving particularly in angiogenesis. HBOT increases the oxygen gradient between the center and periphery of the wound thus creating a strong angiogenic stimulus.[23,24] Angiogenic activities of PRP are modulated by the stimulatory pro-angiogenic factor VEGF and increase in angiogenesis contributes to acceleration of healing in areas of poor vascularization.[25]

Vessels were more predominant in HBO received fresh and frozen PRP applied groups which were detected by Clemex Vision Lite 3.5 Image Analysis program proving the efficiency of HBO through the process of angiogenesis.

Beside ulceration; HBO and PRP combination were conducted on bone healing with an improvement of bone regeneration.[26,27]

Wound closure is one of the most important significant parameters in assessing chronic wound healing. For this reason, in our study design, we have taken the photographs of the wounds for following wound closure rate. The decrement in wound surface area was more significant in HBO received group.

Non-healing wounds are a major concern both for patients and health-care professionals. Solutions must be sought for overcoming this serious problem that causes a substantial financial burden for the health-care system. Since the number of patients with critical limb ischemia is increasing in many countries, treatment of chronic wounds cost great amount of money from the budget of governments. Increased amputation rates, lengthening hospital stay, and money spent on expensive dressing materials all contribute to high expense rates in dealing with these patients.

The limitation of this study is that animal methodologies are not ideal study settings for successful translation into clinical practice. For obtaining successful results in the treatment of chronic wounds and decrease financial cost; well-designed controlled clinical studies must be planned for assessment cost-effectiveness of combination therapies.

## Conclusion

We found experimentally HBO treatment accelerated wound healing when combined with both fresh and frozen PRP. Frozen PRP is as effective as fresh form to be considered as an alternative in clinical setting.

**Ethics Committee Approval:** This study was approved by the Gülhane Training and Research Hospital Animal Experiment Ethics Committee (Date: 24.04.2018, Decision No: 18/13).

**Peer-review:** Externally peer-reviewed.

**Authorship Contributions:** Concept: N.S.; Design: N.S.; Supervision: N.S.; Fundings: N.S.; Materials: N.S.; Data: N.S.; Analysis: N.S.; Literature search: S.Ö.; Writing: N.S.; Critical revision: S.Ö.

**Conflict of Interest:** None declared.

**Financial Disclosure:** The authors declared that this study has received no financial support.

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DENEYSEL ÇALIŞMA - *ÖZ*

## Hiperbarik oksijen tedavisinin taze ve donmuş trombosit zengin plazma ile kombinasyonunun sıçanlarda kronik yara iyileşmesine etkisi

**Dr. Nevra Seyhan, Dr. Sinan Öksüz**

Gülhane Eğitim ve Araştırma Hastanesi, Plastik Rekonstrüktif ve Estetik Cerrahi Kliniği, Ankara

AMAÇ: Hiperbarik oksijen tedavisi (HBOT), komplike kronik yaraların tedavisine yeni bir boyut eklemiştir. Bu çalışmanın amacı, hiperbarik oksijen tedavisinin, taze ve donmuş trombosit zengin plazma (TZP) ile kombine edildiğinde kronik yaralar üzerine olan etkisini araştırmaktır.
GEREÇ VE YÖNTEM: Sıçanlar, her grupta 18 sıçan olacak şekilde hiperbarik oksijen tedavisi alan (Grup B) ve almayan (Grup A) olarak iki ana gruba ayrıldı. Her ana grup Grup 1: Kontrol, Grup 2: Taze TZP uygulanan, Grup 3: Dondurulmuş TZP uygulanan olmak üzere üç alt gruba ayrıldı. TZP hazırlamak için 10 adet sıçan kullanıldı. Fibrobastlar, kollajen lifler, lenfositler ve damarları içeren histolojik parametreler Clemeks Vision lite 3.5, yara yüzey alanları Image J dijital analiz programları ile değerlendirildi.
BULGULAR: HBOT alan grupta (Grup B) fibrobast, kollajen lif, lenfosit ve damar sayıları tüm taze TZP, donmuş TZP uygulanan ve kontrol alt gruplarında HBOT almayan gruptan (Grup A) anlamlı olarak yüksekti ($p<0.05$). HBOT alan grupta (Grup B) yara yüzey alanı ölçüm değerleri kontrol, taze ve donmuş TZP uygulanan alt gruplarda 5–10–15. günlerde HBOT almayan (Grup A) gruptan düşüktü.
TARTIŞMA: HBOT; taze ve donmuş TZP ile kombine edildiğinde yara iyileşmesini hızlandırmaktadır. Dondurulmuş TZP, taze formu kadar efektiftir ve klinik uygulamalarda bir alternatif olarak düşünülebilir.
Anahtar sözcükler: Hiperbarik oksijen tedavisi; kronik yara; taze ve donmuş trombosit zengin plazma.

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