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# The current analysis of the effect of hyperbaric oxygen therapy on the frostbitten tissue: Experimental study in rabbits

**Research Article**

Fatih Uygur[1*], Nurettin Noyan[1], Celalettin Sever[1], Tuna Gümüş[2]

[1] *Department of Plastic and Reconstructive Surgery and Burn Unit, Gülhane Military Medical Academy and Medical Faculty, Haydarpasha Training Hospital, 34668 Istanbul, Turkey*

[2] *Department Of Undersea and Hyperbaric Medicine, Kasimpasha Military Hospital, 34440 Istanbul Turkey*

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**Abstract:** Many experimental studies have been performed and the mechanism of hyperbaric oxygen therapy on the frostbitten tissue has not been elucidated. In this study, we evaluated the effect of hyperbaric oxygen therapy on the frostbitten ears of rabbits in an experimental animal model by examining the concentrations of thromboxane $A_2$ (as thromboxane $B_2$ – Tx $B_2$) and of prostaglandin $I_2$ (PG $I_2$) (as 6-keto-prostaglandin $F_1\alpha$ – PG $F_1\alpha$) in tissues, and by counting the numbers of inflammatory cells (neutrophils and mast cells- MC) Hyperbaric oxygen therapy (HBO) at 2.5 ATA for 90 minutes twice daily for fourteen days to rabbits, the ears of which were subjected to frostbite, decreased presence of inflammatory cells (mast cells -75%; neutrophils -40%) and increased prostaglandin $I_2$ (PG $I_2$) (as 6-Keto-PGF1$\alpha$) in the involved skin. Thromboxane $A_2$ (TxA2) (as Tx $B_2$) was unaffected. Our results revealed that an inflammatory process was the underlying cause of frostbite injury and that hyperbaric oxygen therapy was active in pathological situations involving an inflammatory process in frostbite.

**Keywords:** *Hyperbaric Oxygen Therapy • Frostbite • Arachidonic acid metabolism*



## 1. Introduction

Frostbite is an injury due to failure of normal protective mechanisms against thermal environment results in localized tissue temperature falling. This injury seems to be frequent for outdoor winter activities and homelessness [1].

There are several therapeutic modalities on frostbite injury. Antithromboxane agents like aloe vera and methimazole or antiinflammatories like defibrotide are a few examples. Among these modalities, hyperbaric oxygen therapy was effective. Macroscopic and other changes had been reported in studies on hyperbaric oxygen on frostbite injury. But the effects of hyperbaric oxygen on cellular or inflammatory mechanisms had not been clearly elucidated.

In this study, we investigated the effect of hyperbaric oxygen therapy on frostbitten rabbit ear skin by examining the levels of arachidonic acid metabolism.

## 2. Material and Methods

The experiment was approved by the Committee for Ethical Animal Experiments, the Gülhane Military Medical Academy Ethics Committee (Ankara, Turkey.) New Zealand white rabbits, each weighing 1.5 to 2.5 kg, were obtained from a university animal research institution and were used at the experiment. Anesthesia was induced with ketamine hydrochloride (80 mg/kg) and xylazine (9 mg/kg) intramuscularly. A total of 20 rabbits were used: 10 rabbits were randomly allocated for the

* *E-mail: fatihuygur@hotmail.com*

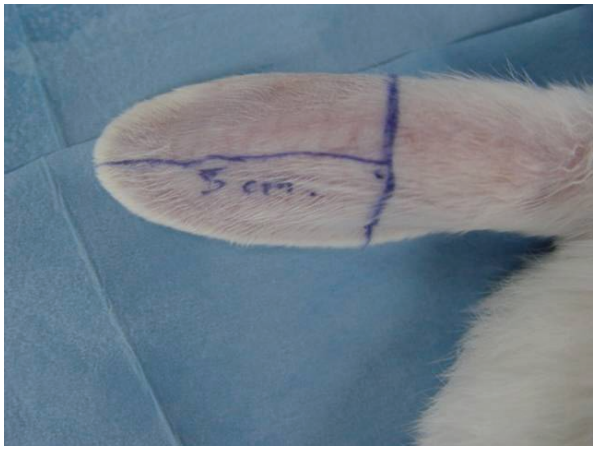


**Figure 1.** Markings of rabbits ear.

**Figure 2.** Application of frostbite injury.

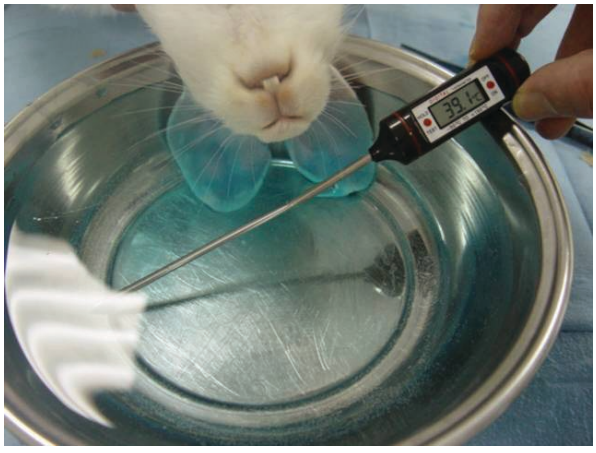


**Figure 3.** Rapid Rewarming after injury.

control group and 10 rabbits to the study group. The animals received normal daily care, were fed standard chow and tap water ad libitum.

Both ears of the rabbits in both groups were marked 5 cm proximally from the tip perpendicular to the ear long axis (Figure 1). A digital thermometer was placed into ethanol solution. Temperature was measured before the frosting procedure (basal values). Afterward, the ear was dipped, up to the mark, into a container filled with 90% (volume/volume) ethanol at -25°C (Figure 2). Temperature was recorded every 15 seconds for 3 minutes. At the end of this time , the ear was removed from the ethanol and embedded at rapid warming serum at nearly 40°C (Figure 3), and its temperature was recorded every minute. Body (rectal) temperature was recorded for the duration of the procedure. Afterward, the same procedure was applied to the opposite ear.

The study group was treated with hyperbaric oxygen 1 hour after the injury at 2.5 ATA for 90 minutes twice daily for fourteeen days while the control group had no therapy.

Skin samples (2 cm width) were taken on the fourth day from one ear of each rabbit. Each skin sample was divided into two parts: one for histologic analysis (fixed in buffered formalin), and the other for biochemical analysis (stored at -80°C).

The skin of the opposite ear was not preserved and was used as a sample donor to show ear skin survival by the third week after frostbite injury. The surviving skin area was compared as a percentage. Also similar skin samples for biochemical and histologic analysis were also obtained from rabbit ears in control group.

### 2.1. 6-Keto Prostaglandin $F_1\alpha$ and Thromboxane $B_2$ Analysis

Prostaglandin $I_2$ and thromboxane $A_2$ were measured by enzyme-linked immunoassay of their stable metabolites; 6-keto-prostaglandin $F_1\alpha$ and thromboxane $B_2$, according to the method of Powell.

### 2.2. Histologic Analysis of Polymorphonuclear Leukocytes and Mast Cells

After embedding into paraffin, skin samples were diced into 5-µm sections and stained with hematoxylin-eosin and toluidine blue. The number of polymorphonuclear leukocytes per microscope field (magnification, 400×) was determined in slides stained with hematoxylin and eosin, compared to mast cells which were determined on slides stained with toluidine blue.

### 2.3. Skin Survival Measurement

The surviving skin areas were measured on the ears, which were preserved for histologic and biochemical analysis, and expressed as percentage area (Figures 4,5).

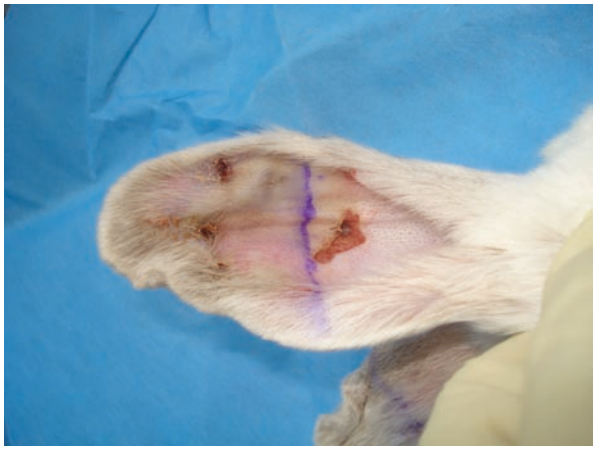

**Figure 4.** Necrosis ratio in study group.

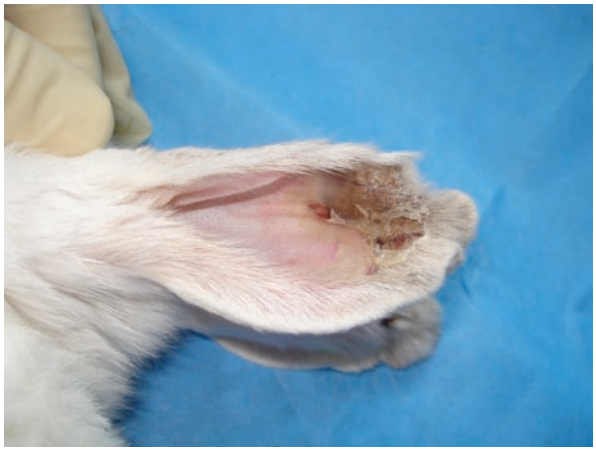

**Figure 5.** Necrosis ratio in control group.

### 2.4. Statistical Analysis

The groups were compared by Mann-Whitney U test. The differences between the groups were considered statistically significant when $p$ was <0.05.

## 3. Results

### 3.1. General features

The mean body weights of the 3 groups of rabbits were not statistically different. The rectal temperature of rabbits did not change throughout the experiment (data not shown). Edema and hyperaemia were observed on the first day of frostbite injury. Edema decreased by the 4th day. Some rabbits developed bullae on their ears and serous leakage was seen. Dry necrosis was present after the 5th day. Part of the necrotic tissue was detached by the third week.

**Table 1.** Tromboxane- $B_2$ and 6-KetoPG1α levels in Pg/g wet tissue.

| Pg/g wet tissue | Tromboxane $B_2$ | 6-Keto PGF1α |
|---|---|---|
| CONTROL | 4312 ± 491 | 9807 ± 697 |
| STUDY | 4478 ± 895 | 21087 ± 3299* (+115%) |

* = $p < 0.05$

**Table 2.** Count of Mast Cells and Neutrophiles per field.

| Number/field | MAST CELL | NEUTROPHIL |
|---|---|---|
| CONTROL | 4.8 ± 0.5 | 86.3 ± 3.6 |
| STUDY | 1.2 ± 0.4* (- 0.75%) | 51.9 ± 2.9* (-40%) |

* = $p < 0.001$

**Table 3.** Rate of skin survival.

| Rate (% area) | Skin Survival |
|---|---|
| Study | 36.21 ± 8.1 |
| Control | 54.37 ± 7.93* (+%39) |

* = $p < 0.05$

### 3.2. 6-Keto-PGFI α and TxB 2 analysis

TxB2, increasing in frostbitten skin, was not affected by HBO treatment. While there was not a statistically difference between groups on tromboxane $B_2$ levels; an increased level of 6-Keto-PGF1α was significantly different in the treatment group compared to the control group (Table 1).

### 3.3. Histological analysis of PNL and MC

MC number increased in frostbitten skin, but was decreased by 75% (P<0.001) by HBO treatment. PNL number, increased in frostbitten skin, was also decreased by 40% ($p$ <0.001) by HBO treatment (Table 2).

### 3.4. Skin survival measurements

HBO treatment improved skin survival by 39% besides this improvement was statistically significant in comparison to untreated frostbitten group ($p$ <0.05) (Table 3).

## 4. Discussion

There have been several studies on frostbite animal models, and some experiments used rabbits ears or rats legs [18,19]. Some authors prefered to use rabbit ear as a model [2]. In our study we prefered to use rabbit ear as we accept rabbit ear composing of thin layer of skin and cartilage and, this has a advantage of consisting as less different tissue components as possible which may diseffect the results.

Also there have been many studies on frostbitten tissue experimentally about the physiopathogenesis of frostbite [2]. It is revealed that frostbite injury causes

direct cellular damage and death and the second one is progressive tissue ischemia [3-9]. Acute effects of frostbite can be seen as formation of extracellular ice crystals. By changing the osmotic gradient which results in cellular dehydration it directly injures cellular membranes [10-11]. It has been observed that the rate of cooling results in intra or extracellular ice crystals [12]. With the increasing rate of cooling intracellular freezing which causes severe cell damage occurs; if the rate is not high extracellular ice crystals which cause transmembrane osmotic shift resulting in intracellular dehydration occurs. This process usually cause to failure of intracellular homeostasis due to change of protein and lipid conformation and change in pH and tissue electrolytes levels [16-18]. The microvascular injury effects endothelial cells and basement membranes, it has marked vasodilation, inflammatory reaction, and tissue edema effects. The ischemia caused by freezing is initially followed by complete vascular reflow. After the frostbite injury number of polymorphonuclear leukocytes increases and activates [19]. Prevention of leukocyte adherence and aggregation had been found beneficial in experimental frostbite injury [20]. Inhibition of release of vasoactive agents expressed by mast cells by decreasing the cell number may also decrease the tissue edema in frostbite [21].

In the literature there are few studies on tissue levels of arachidonic acid metabolism on frostbite [2,19,22]. The levels of PG $I_2$ and Tx $A_2$ (as their metabolites) had been found increased similar to previous clinical and experimental studies [23]. It has been suggested that a disturbance in the balance of prostaglandin $I_2$ - thromboxane $A_2$ might be of pathophysiologic significance in certain disorders [24] because vasoconstricting and platelet aggregating effects could be more prominent than vasodilating and antiplatelet activities, as shown in burns that are similar to frostbite injury [25-26]. Increase level of prostaglandin $I_2$ (6-keto-prostaglandin $F_1\alpha$) in frostbitten skin could be a defensive tissue response to thromboxane $A_2$ (thromboxane $B_2$) rise, maintaining the 6-keto-prostaglandin $F_1\alpha$ thromboxane $B_2$ balance. The prostaglandin $I_2$ increase could be considered a defensive mechanism, because prostaglandin $I_2$ has membrane-stabilizing activity [27].

Antithromboxane agents like aloe vera and methimazole as treatments have been used in the belief of thromboxane is responsible for this pathologic condition [19,28]. Secondary to excessive thromboxane $A_2$ production the progressive ischaemic necrosis has been observed, which changes the normal balance between prostacyclin and thromboxane.

HBO treatment is indicated in wide variety of diseases [29]. Some of the effects of hyperbaric oxygen therapy can be explained with the vasoconstruction due to hyperoxy, blood flow to tissues is reduced but by the increased level of dissolved oxygen in the plasm oxygenization to tissues are increased. Also by decreasing the intersitisiel pressure it helps to reduce the edema caused by hypoxia and ischemia. It inhibits bacterial exotoxines. Lactate which is like a stimulus for kollagen production can be obtained in hyperoxic state. The effects of hyperbaric oxygen on edema reducing is well known, but tissue levels of arachidonic acid metabolism and number of inflammatory cells by this therapy hadn't been observed yet. By maintaning the balance of PG $I_2$/Tx $A_2$ vasoconstricting and platelet aggregating effect of Tx $A_2$ may be reduced. This may help in wound healing of the frostbitten area and reduce the necrosis ratio. Also by preventing the increase of inflammatory cell, edema may be reduced. Further studies may be beneficial on the hyperbaric oxygen therapy with frostbitten tissues due to the many effects of this therapy method which can be studied on.

In conclusions, our results revealed that an inflammatory process was the underlying cause of frostbite injury and that hyperbaric oxygen therapy was active in pathological situations involving an inflammatory process in frostbite.

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