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ORIGINAL ARTICLE

The effects of systemic ozone application and hyperbaric oxygen therapy on avascular necrosis of the femoral head: An experimental study in the vascular deprivation of the rat femoral head model

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ABSTRACT

Objectives: This study aims to assess the effects of systemic medical ozone (O_3) application and to compare its effects with hyperbaric oxygen (HBO) therapy for preventing avascular necrosis of the femoral head in a rat model.

Materials and methods: A total of 50 male Wistar albino rats were divided into six groups including five rats in each control and sham-operated control group and 10 rats in the remaining four groups: *(i)* control group, *(ii)* sham-operated control group, *(iii)* avascular necrosis group, *(iv)* intraperitoneal ozone given avascular necrosis group, *(v)* HBO therapy given avascular necrosis group, and *(vi)* intraperitoneal ozone and HBO given avascular necrosis group. We surgically induced osteonecrosis by cutting the ligamentum teres and placing a tight ligature around the femoral neck. At Week 11, we harvested femoral heads bilaterally from each animal and performed a macroscopic, histological evaluation and histomorphometric, immunohistochemical analysis.

Results: The intertrabecular mesenchymal cell ratio was substantially higher in the O_3 group than that of all other groups in the histological evaluation ($p<0.05$). Group O_3 had also significantly more CD31-positive stained new vasculature than other groups, with the exception of the HBO therapy group, according to the immunohistochemical analysis ($p<0.05$).

Conclusion: The results of this experimental study suggest that the application of medical ozone alone may have a positive effect on new vessel formation and the repair process and may be more beneficial than HBO therapy and HBO+O_3 therapy in the vascular deprivation of the rat femoral head model.

Keywords: Avascular necrosis, femoral head, hyperbaric oxygen, osteonecrosis, ozone.

Avascular necrosis of the femoral head (AVNFH) is a type of osteonecrosis which causes progressive deterioration in the bone structure of the femoral head and, as a result, serious deterioration and deformities in the structure of the hip joint, thereby causing hip osteoarthritis.[1] Although there are many traumatic and non-traumatic pathological conditions in its etiology, it can also be seen as idiopathic in many cases.[1,2] Many studies have been conducted in the literature to develop treatment options to prevent hip osteoarthritis, which occurs due to necrosis in the bone tissue of the femoral head structure and the collapse of the femoral head, and the progressive deterioration of the hip joint structure.[3,4]

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Medical ozone (O_3) is obtained by interacting with high electricity voltage with the help of a pure oxygen generator. There are clinical studies in the literature showing the effectiveness of topical application of medical O_3 on osteonecrosis of the jaw associated with bisphosphonate usage. In these clinical studies, the debriding effect of O_3 on necrotic bone tissues has been shown as the potential mechanism of action.[5-7] Medical ozone is an agent known to have anti-inflammatory effects due to suppressing pro-inflammatory prostaglandin synthesis, inhibiting the release of bradykinin, and increasing the release of pro-inflammatory cytokine antagonists.[8] Its use in osteoarthritis is still a treatment that is under investigation. Although many studies have been conducted in the literature, there is still no clear consensus on the method of application and dosage.

Hyperbaric oxygen (HBO) therapy is used by applying 100% oxygen in a closed chamber with high pressure.[9,10] The HBO therapy has been widely used in the treatment of several orthopedic conditions, including limb ischemia, crush injury, compartment syndrome, infections of the soft tissues, wounds-skin grafts and flaps' healing problems, osteomyelitis, bone avascular necrosis, sports injuries, fracture healing, and nerve healing.[11] It is also believed to have beneficial effects on AVNFH and is widely used in clinical practice; however, its mechanism of action is still uncertain.[12,13] Most of the studies in the literature are clinical studies, and there are only a few experimental studies showing the effect of HBO application on AVNFH.[14,15]

Due to its debriding effect of O_3 on necrotic bone tissues in biphosphate-induced jaw osteonecrosis and its increasing effect on microcirculation in the joint,[5-8] we hypothesized that systemic medical O_3 application could also be effective in preventing AVNFH. In the present study, we, therefore, aimed to assess the effects of systemic O_3 application and to compare its effects with HBO therapy for preventing AVNFH in a rat model.

MATERIALS AND METHODS

In this study, a total of 50 male Wistar albino rats (3- to 6-month-old, 220 to 275 g) were used. The study protocol was approved by Çanakkale Onsekiz Mart University Experimental Research Application and Research Center (date: 27.12.2019, no: 2019/10-0188816). All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (revised 1985).

During the study, we kept all rats in the same laboratory, under the same conditions. During the experimental procedure, all rats were housed under standard laboratory conditions with an artificial 12-h light/dark cycle. They were caged individually under a controlled temperature (22±1°C) and relative humidity, and were allowed free access to food and water in polycarbonate units *ad libitum*. The rats were observed for seven days in the animal care laboratory to exclude any possibility of underlying disease before the experiment started.

We randomly divided the rats into six groups: five rats each in the control and sham-operated control group, and 10 rats each in the remaining four groups. The groups were as follows: *(i)* control (C) group (no surgery was performed), (*ii)* sham-operated control (SC) group (only skin incision and closure were done), *(iii)* avascular necrosis (AVN) group, *(iv)* intraperitoneal O_3 given AVN group (O_3 group), *(v)* HBO therapy given AVN group (HBO group), and *(vi)* intraperitoneal O_3 and HBO given AVN group (HBO+O_3 group). We induced bilateral AVNFH using the previously described method.[16] We surgically induced osteonecrosis by cutting the ligamentum teres and placing a tight ligature around the femoral neck. All rats included in our study were sacrificed by cervical dislocation 11 weeks after surgery and bilaterally femoral heads harvested from each animal and performed a macroscopic, histological evaluation and histomorphometric, immunohistochemical analysis.

Surgical technique

We administered anesthesia by using the method of Williams et al.[17] We made a longitudinal skin incision over the greater trochanter. Then, we dissected and separated the gluteal muscles from the bone. We cut the joint capsule and dislocated the femoral head (Figure 1a). To induce AVNFH, we cut the ligamentum teres and placed a tight 3-0 Vicryl suture around the femoral neck (Figure 1b). We, then, reduced the femoral head to the acetabulum and repaired the gluteal muscles and skin with 4-0 sutures. We performed the same procedure bilaterally on each rat. In the SC group, we did not transect the joint capsule and did not apply a ligature to the femoral neck. Group C did not receive any surgery. Additionally, we applied enrofloxacin 5 mg/kg/day as antibiotic prophylaxis for three days to all groups that received surgery.

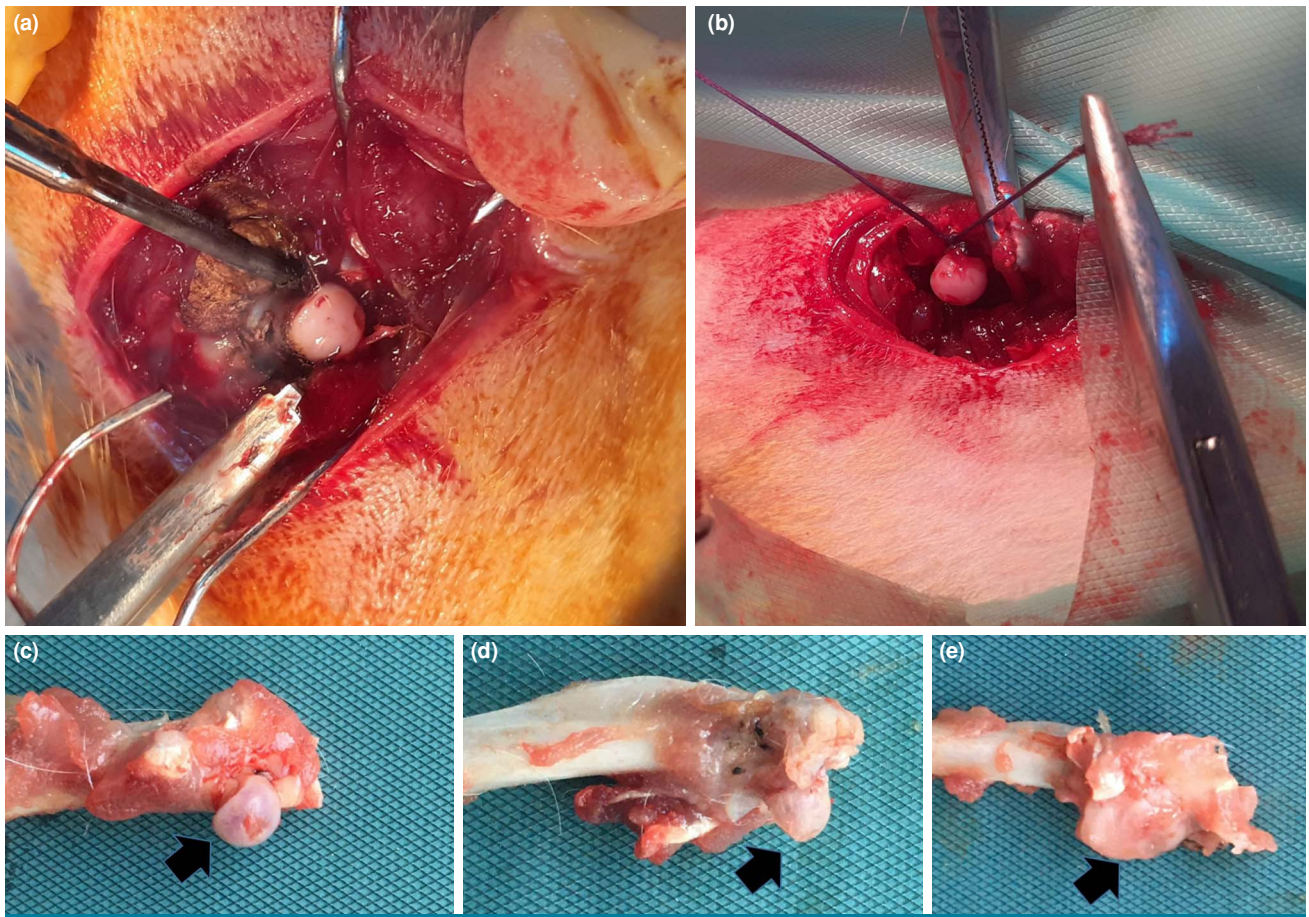


FIGURE 1. (a) Dislocation of the femoral head **(b)** placement of the ligature around the femoral neck. **(c-e)** Classification of macroscopic changes as: normal, loss of sphericity, and head collapse (shown with arrows).

On postoperative Day 3, we started treatments for the rats in the O_3, HBO, and HBO+O_3 groups. The rats in the C, SC, and AVN groups received no therapy.

Intraperitoneal O_3 application and HBO therapy

We applied 30 μg/mL dose of medical O_3 obtained from a medical O_3 generator (Turkozone Blue S, Istanbul, Türkiye) intraperitoneally to rats as 1 mL volume/day. For a duration of 10 weeks, we followed this therapy regimen every other day.

The HBO inhalation therapy was applied to the rats in the HBO and HBO+O_3 groups in the HBO therapy room (Barotech Medical Supplies Medical Products, İstanbul, Türkiye) designed for experimental animals. The HBO therapy was performed with 100% oxygen and discharged normal air. The pressure of the test chamber was gradually increased to 2.5 atmospheres absolute pressure (ATA) within 10 min and the animals were applied 100% oxygen under 2.5 ATA pressure for the next 40 min. At the end of this period, the room pressure was gradually decreased to 1 ATA within 10 min. Once the room pressure reached 1 ATA, the treatment was ended. This treatment protocol, which lasted 60 min in total, was applied to the rats in the HBO and HBO+O_3 groups twice a day for 30 days.

Histological and immunohistochemical evaluation

We harvested femoral heads bilaterally from each animal and evaluated them macroscopically before histological preparation. Using a frontal plane cut, we fixed each femoral head in 40% formalin, decalcified it in 7% nitric acid, and then embedded it in paraffin. A microtome was used to cut five-micron serial cross-sections. Photomicroscopy using Primo Star (Carl Zeiss Microscopy GmbH, Germany) light

microscope at 4× magnification was used to capture images of 14 femoral head slides from each of the AVN, O_3, HBO, and HBO+O_3 groups, as well as five slides from each of the C and SC groups. The slides were stained with hematoxylin and eosin (H&E). Morphometric measurements were performed using the ImageJ version 1.46r software (National Institutes of Health, Bethesda, MD, USA) after light microscope images were input. The trabeculae area (bone volume = BV) and the total area (total volume = TV) of the femur head as a whole were measured to compute the BV/TV ratio.[16,18-20]

The epiphyseal plate and articular cartilage structures in the whole femoral head were evaluated as absent, irregular or regular, and the cartilage thickness was measured from three different regions and averaged in each structure.[20,21] The collapse in the articular space was evaluated as none, <50% and ≥50%. The percentages of hematopoietic cells, adipocytes and mesenchymal cells in the intertrabecular area were calculated semi-quantitatively by examining the entire area of all femoral heads at 10x magnification under a light microscope.[14]

We performed immunohistochemical staining using CD31 to investigate angiogenesis. Five femoral head slides each from the C and SC groups and six slides each from the AVN, O_3, HBO, and HBO+O_3 groups were examined with a light microscope at 10× magnification. The CD31-positive stained vessels were counted in five areas in each slide.[16] Measurements with images and analysis of H&E and CD31 stained slides were performed by two researchers blinded to the groups. All sections were photographed with Axiocam 503 color camera using Zeiss Axio Scope A1 Light Microscope and ZEN 2 software (blue edition) (Carl Zeiss Microscopy GmbH Göttingen, Germany).

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). Continuous data were expressed in mean ± standard deviation (SD) or median (min-max), while categorical data were expressed in number and frequency. The normality of the distribution for the variables was tested using the Shapiro-Wilk test. Non-parametric tests were used for variables without normal distribution. The Kruskal-Wallis and Mann-Whitney U tests were used to assess continuous data that was not normally distributed. A p value of <0.05 was considered statistically significant with 95% confidence interval (CI).

RESULTS

Gross evaluation

After we harvested the femoral heads, we evaluated them macroscopically before the histological preparation. We classified macroscopic changes as normal, loss of sphericity, and head collapse (Figure 1c-e). Two researchers who were blinded to the groups and each other evaluated the specimens and classified the macroscopic changes. If there was any disagreement between them, a third physician made the final decision. Gross evaluation results according to the groups are summarized in Table I.

Histomorphometric analysis

1) *Articular cartilage thickness:* Considering measurements with ImageJ, the cartilage thicknesses of the C and SC groups were found to be significantly higher than all other groups (C group *vs.* AVN, O_3, HBO, HBO+O_3 p=0.033, p=0.026, p=0.005, p=0.008, respectively, SC group *vs.* AVN, O_3, HBO, HBO+O_3 p=0.016, p=0.016, p=0.002, p=0.004, respectively). The articular cartilage thickness did not differ statistically significantly between the C and SC groups. When the O_3, HBO, and HBO+O_3 groups were compared, the highest cartilage thickness was found to be in the O_3 group, but the difference was not statistically significant (Figure 2a-f).

TABLE I
Numbers and percentages of macroscopic findings of the femoral heads in each group

	AVN		Ozone		HBO		HBO + Ozone	
	n	%	n	%	n	%	n	%
Normal	8	40	11	55	7	35	4	20
Loss of sphericity	5	25	4	20	8	40	5	25
Head collapse	7	35	5	25	5	25	11	55

AVN: Avascular necrosis; HBO: Hyperbaric oxygen.

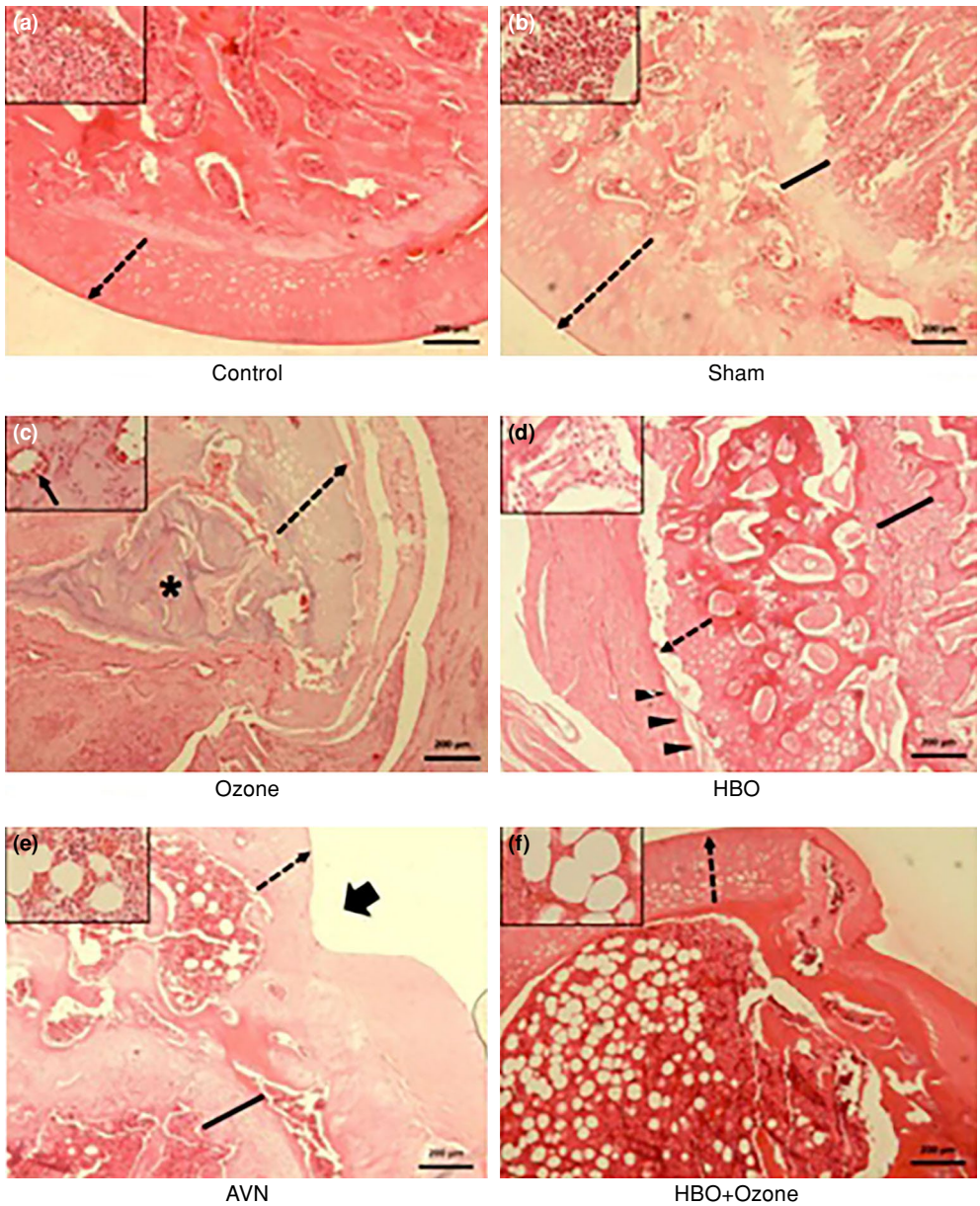


FIGURE 2. (a, b) Hematopoietic cells in the control and sham groups, Regular and thick articular cartilage in the control and sham groups (dashed arrows), epiphyseal cartilage in sham group (line). (H&E staining, 50×. Insets; 200×). **(c, d)** Vascularization (thin arrow) and endochondral ossification (asterisk) in the ozone, epiphyseal cartilage (line) and collapse in the articular space (triple arrows) in the HBO groups. Dashed arrows show irregular and thin articular cartilage. (H&E staining, 50×. Insets; 200×). **(e, f)** Intertrabecular area adipocytes, irregular and thin articular cartilage in the AVN and HBO+O_3 groups, depressed articular cartilage (thick arrow), epiphyseal cartilage (line) in the AVN group. (H&E staining, 50×. Insets, 200×).
HBO: Hyperbaric oxygen; AVN: Avascular necrosis; O_3: Ozone.

2) *Epiphyseal plate thickness:* Considering measurements with ImageJ, the epiphyseal plate thicknesses of the AVN, HBO, and HBO+O_3 groups were found to be significantly lower than the C and SC groups (C group *vs.* AVN, HBO, HBO+O_3 p=0.008, p=0.024, p=0.00001, respectively, SC group *vs.* AVN, HBO, HBO+O_3 p=0.037, p=0.031, p=0.00001, respectively). Only the epiphyseal plate thickness of the O_3 group was significantly higher than the HBO+O_3 group (p=0.009).



FIGURE 3. Representative photographs of the microscope images of H&E-stained femoral heads taken for histomorphometric measurements with ImageJ. Femoral head collapse in the HBO group (triple arrows).

AVN: Avascular necrosis; HBO: Hyperbaric oxygen.

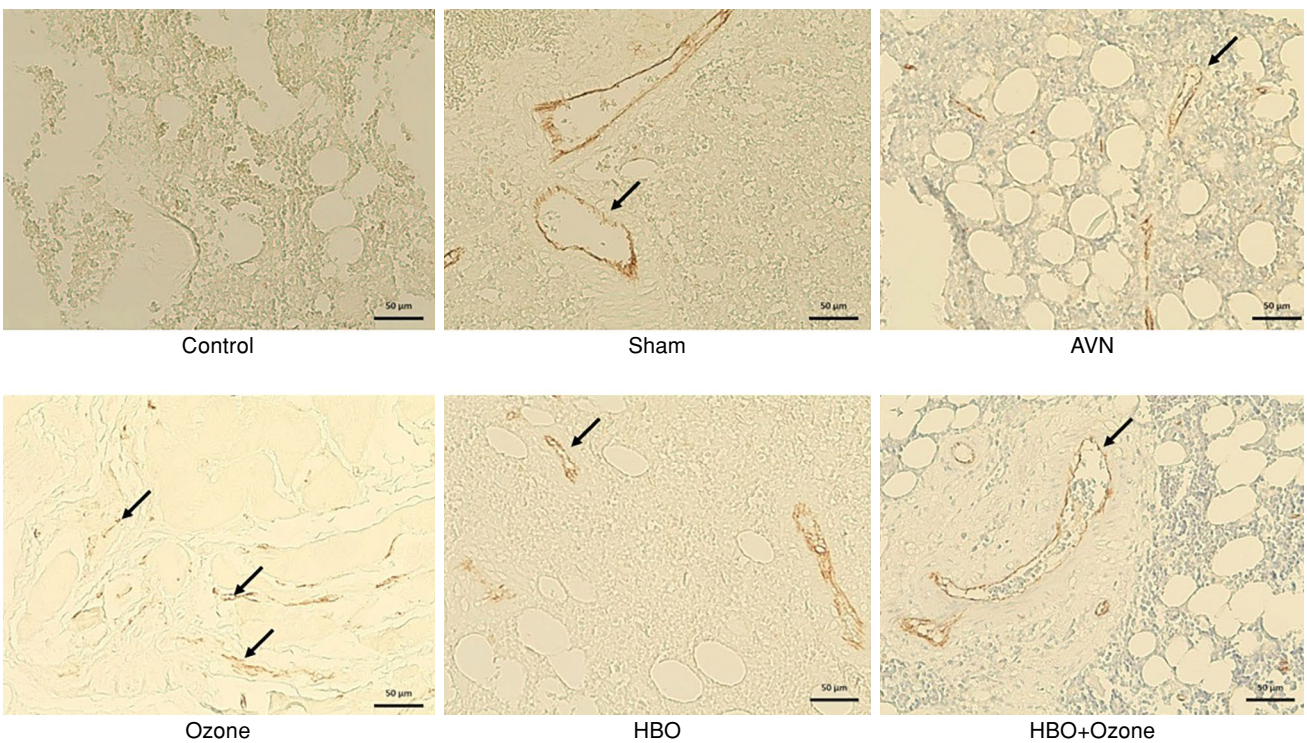


FIGURE 4. CD31 immunostaining; ozone and HBO groups showed the highest number of vessels stained with CD31 (arrows), respectively. (200×, Bar = 50 µm).

AVN: Avascular necrosis; HBO: Hyperbaric oxygen.

TABLE II

Histological evaluation, histomorphometric and immunohistochemical analysis results

	Control	Sham	AVN	O_3	HBO	HBO+O_3
Articular cartilage thickness (µm), Mean±SD (median)	219.35±15.4 (226.25)	256.75±51.4 (238.75)	161.34±112.6 (160.62)	182.73±149.4 (158.12)	95.41±84 (114)	90±93.5 (81.25)
Epiphyseal plate thickness (µm), Mean±SD (median)	127.1±5.32 (128.5)	110.9±30.97 (126.87)	46.07±56.02 (30.62)	91.7±90.8 (67.5)	52.7±94.23 (0)	12.86±30.13 (0)
Trabecular bone volume (mm^2), Mean±SD (median)	1.75±0.48 (1.74)	0.81±0.31 (0.8)	0.92±0.4	0.83±0.41 (0.82)	1.03±0.58 (0.95)	0.87±0.55 (0.9)
Total volume (mm^2), Mean±SD (median)	3.15±0.72 (3.1)	1.94±0.29 (1.9)	2.46±0.66 (2.5)	2.42±1.11 (2.45)	2.62±1.39 (2.42)	2.88±1.54 (2.7)
Bone volume/total volume ratio (%), Mean±SD (median)	55.7±6.9 (56.1)	41.4±15.85 (36.9)	38.3±13.83 (42.98)	36.35±16.93 (36.37)	42.25±16.9 (37.5)	35.63±21.69 (31.25)
Hematopoietic cell ratio (%), Mean±SD (median)	98.4±2.07 (99)	93±7.58 (95)	31.78±22.83 (25)	27.1±28.93 (25)	40±21.83 (50)	41.4±23.8 (40)
Adipocyte cell ratio (%), Mean±SD (median)	1.6±2.3 (2.30)**	2±2.73 (0)**	36.78±28.79 (35)	10.71±18.5 (0)**	34.28±25.6 (40)	46.07±27.04 (45)
Mesenchymal cells and fibroblasts (%), Mean±SD (median)	0	0	25±32.52 (5)	57.86±43.7 (75)*	22.86±24 (5)	13.21±17.93 (0)
Articular cartilage regularity, n (%)						
Absent	0	0 (0)	2 (15)	3 (20)	5 (35)	6 (45)
Irregular	0	2 (40)	7 (50)	9 (65)	9 (65)	5 (35)
Regular	5 (100)	3 (60)	5 (35)	2 (15)	0	3 (20)
Epiphyseal plate regularity, n (%)						
Absent	0	0	7 (50)	5 (35)	9 (65)	11 (80)
Irregular	0	1 (20)	2 (15)	6 (45)	5 (35)	3 (20)
Regular	5 (100)	4 (80)	5 (35)	3 (20)	0	0
Collapse in the articular space, n (%)						
>50%	0	0	5 (35)	8 (60)	10(70)	11 (80)
<50%	0	0	4 (30)	3 (20)	4 (30)	1 (5)
None	5 (100)	5 (100)	5 (35)	3 (20)	0	2 (15)
Number of the vessels stained with CD31 (Mean±SD)	2.4±2.19	11.60±4.037	15.5±11.07	38±11.7***	24.5±15.02	7.5±5.68

AVN: Avascular necrosis; O_3: Ozone; HBO: Hyperbaric oxygen; SD: Standard deviation; * Intertrabecular mesenchymal cells and fibroblasts ratio of the O_3 group was significantly higher compared to all other groups (O_3 group *vs.* Control, Sham, AVN, HBO, HBO+O_3 p values (p=0.008, p=0.008, p=0.035, p=0.037, p=0.011 respectively); ** Intertrabecular adipocyte cell ratios of the Sham, Control, and O_3 groups were found to be significantly lower than all other groups (O_3 group *vs.* AVN, HBO, HBO+O_3 *p* values (p=0.012, p=0.001, p=0.001 respectively), Control group *vs.* AVN, HBO, HBO+O_3 *p* values (p=0.021, p=0.021, p=0.004 respectively), Sham *vs.* AVN, HBO, HBO+O_3 *p* values (p=0.021, p=0.020, p=0.004 respectively); *** The number of CD31+ stained vessels in the Ozone group was significantly higher than the control, sham, AVN and HBO+O_3 groups (O_3 group *vs.* Control, Sham, AVN, HBO+O_3 *p* values (p=0.006, p=0.006, p=0.025, 0.004 respectively); µm: micrometer, mm2: square millimeter.

3) *BV/TV (bone volume/total volume) ratio:* Considering measurements with ImageJ, no significant difference was found between the groups in terms of BV/TV ratios (p=0.175).

Histological evaluation

1) *Intertrabecular area content:* According to microscopic examination of the slides stained with H&E, hematopoietic cell ratios in the intertrabecular areas of the C and SC groups were significantly higher than all other groups (C group *vs.* AVN, O_3, HBO, HBO+O_3 p=0.001, p=0.004, p=0.001, p=0.001, respectively, SC group *vs.* AVN, O_3, HBO, HBO+O_3 p=0.001, p=0.004, p=0.001, p=0.002, respectively). Intertrabecular adipocyte cell ratios of the SC, C, and O_3 groups were found to be significantly lower than all other groups (p=0.021, p=0.021, p=0.004, respectively, SC group *vs.* AVN, HBO, HBO+O_3 p=0.021, p=0.020, p=0.004, respectively, O_3 group *vs.* AVN, HBO, HBO+O_3 p=0.012, p=0.015, p=0.001, respectively). Intertrabecular mesenchymal cell ratio of the O_3 group was significantly higher compared to all other groups (O_3 group *vs.* C, SC, AVN, HBO, HBO+O_3 p=0.008, p=0.008, p=0.028, p=0.037, p=0.011, respectively).

2) *Articular cartilage regularity:* The femoral head articular cartilage was mostly considered "regular" in the C and SC groups, most of the articular cartilages in the AVN group was rated "undulating" or "depressed". Irregular articular cartilage was observed in the other groups as well (Figure 2a-f).

3) *Epiphyseal plate regularity:* Various amounts of irregularity, or the absence of epiphyseal plate was observed in all groups, except for Groups C and SC.

4) *Collapse in the articular space:* Complete or incomplete collapse of the femoral heads was observed in all groups, except for Groups C and SC (Figures 2a-f and 3).

Immunohistochemical analysis

Groups O_3 and HBO had the highest number of vessels stained with CD31. In comparison to the C, SC, AVN, and HBO+O_3 groups, the O_3 group had a substantially greater number of CD31-positive stained vessels (p=0.006, p=0.006, p=0.025, and p=0.004, respectively). Between the O_3 and HBO groups, there was no significant change in the quantity of CD31-positive stained vessels (p=0.148) (Figure 4). Histological evaluation, histomorphometric and immunohistochemical analysis results are summarized in Table II.

DISCUSSION

In our experimental study, AVNFH formation was successfully achieved in the rat model. Our histological results in the O_3 group with AVNFH showed us an increase in the proportion of intertrabecular mesenchymal cells and fibroblasts and a significant decrease in the levels of adipose tissue formation. We believe that this is due to the fact that O_3 treatment acts as a biological oxidative stress active modulator by stabilizing oxidant systems and promoting antioxidant activity as reported in the literature.[7]

Cell death is an evident part of hypoxia in osteonecrosis. It has been shown that the ability to proliferate and form colonies is increased in the mesenchymal stromal cells that are exposed to hypoxia. In the hypoxic region, mesenchymal stromal cells are capable of proliferation and differentiation. Osteonecrosis appears to be associated with an increase in mesenchymal stromal cell adipogenesis.[22] Mesenchymal cells can develop into a range of mesenchymal tissues, including cartilage, fat, muscle, tendon, and bone marrow stroma. Human bone marrow mesenchymal cells which have been mechanically stimulated appropriately have been demonstrated to prevent adipogenic differentiation and increase osteogenic differentiation.[23] Soon after osteonecrosis formation, intertrabecular mesenchymal cells and fibroblasts move from the bone-cartilage junction, epiphysis, and metaphyseal tissues into the necrotic tissue. In the intertrabecular area, fibrous tissue and new vessels develop due to the development and change. Dead bone fragments are removed by macrophages which come from these arteries. Mature osteoblasts formed from mesenchymal cells enable the deposition of new, immature bone on dead bone tissue. This immature new bone turns into mature lamellar bone and the intertrabecular area is filled with hematopoietic and adipocyte cells.[24] Considering the fact that the decrease in hematopoietic cells and the increase in adipocyte cells is one of the indicators of osteonecrosis formation,[22-26] our results indicate that O_3 application alone may have an effect on converting the formation of adipocyte cells into mesenchymal cells and may have a positive effect in the healing process. In the immunohistochemical analysis performed with CD31 staining, vascular staining could not be demonstrated in Group C. Additionally, the O_3 group showed higher vascular

staining patterns compared to all other groups. Statistical analysis revealed that the O_3 group had more new vessels than the other groups except for HBO. In this respect, the O_3 group did not show a statistically significant difference, although they had more new vessels than the HBO group. The findings also indicate that the application of O_3 by itself may improve the development of new vessels and the healing process.

Medical ozone is a gaseous chemical that has analgesic, anti-inflammatory, and immunomodulatory properties in addition to being a potent oxidant agent.[27] When administered systemically, it dissolves in the circulation and combines with antioxidants and polyunsaturated fatty acids to generate lipid oxidation products and H_2O_2.[28] Of note, O_3, although being a potent oxidant molecule, paradoxically decreases inflammation in tissues by stabilizing antioxidant systems and promoting oxidant activity. As a result, it functions as a biological oxidative stress active modulator.[29] It is significant at this stage, as it inhibits the nuclear factor-kappa B (NF-κB) pathway, which is activated by reactive oxygen species (ROS), thereby lowering the release of tumor necrosis factor-alpha (TNF-α) and other pro-inflammatory cytokines.[30] Considering that TNF-α is a cytokine that inhibits fracture healing in osteoblastic cells, bone morphogenetic protein (BMP)-related bone formation, and BMP-dependent alkaline phosphatase activity, it can be speculated that inhibition of TNF-α has an important place in the normal bone formation process.[3] The mechanism of systemic O_3 application in converting the formation of adipocyte cells into mesenchymal cells, which we observed in the present study, may be due to the inhibiting effect on TNF-α production. In contrast, in our study, HBO+O_3 group showed the highest percentage of femoral head collapse, adipocyte cell ratio, and lowest mesenchymal cell and fibroblast ratio and least number of new vessel formation. We believe that the reason why the combined therapy of ozone and HBO showed the worst results in terms of prevention of femoral head collapse and enhancement of angiogenesis and mesenchymal tissue formation may be due to the neutralizing effect of 100% oxygen application with high pressure on O_3 in the systemic circulation.

The strong oxidative properties of O_3 can reduce inflammation and prevent the process leading to fibrosis with certain concentrations and application protocols.[31] However, the biological effects of O_3 are dose-dependent and, while it exhibits therapeutic properties at low to moderate levels, it carries a potential risk of toxicity at high doses. This is because excessive ROS formed at high doses can lead to cellular damage and inflammation. Therefore, determining the optimal dose and application protocols is of utmost importance. In our experimental study, we applied O_3 at a dose of 30 μg/mL. Zhao et al.[32] reported that the optimum dose of O_3 to increase cell viability and chondrocytes was 30 μg/mL, while O_3 administered at doses of 40, 50 and 60 μg/mL decreased cell viability in a concentration-dependent manner. Yu et al.[33] reported that O_3 administration at a dose of 35 μg/mL reduced the degeneration of articular cartilage caused by free oxygen radicals, but O_3 administered at a concentration of 70 μg/mL caused peroxidatic reactions in tissues and cells due to its strong oxidative effect, causing articular cartilage damage and a destructive effect on tissues.

Nonetheless, there are some limitations to this study. First, the evaluation methods of the present study only consist of histological, histomorphometric, and immunohistochemical analyses and the lack of radiological evaluations including micro-computed tomography, measures of the mineral content and density of bone, and *in vitro* cell cultures might be considered the main limitations. Second, since this is a short-term study, there are still questions to be answered: Will long-term application of systemic O_3 continue to have beneficial effects and if there is a change in the dose and application frequency of the systemic O_3, how will these changes alter the current results?

In conclusion, our study findings suggest that, in the vascular deprivation of the rat femoral head model, systemic O_3 treatment alone is more advantageous than HBO therapy and HBO+O_3 therapy. As this is a preliminary study, however, future studies evaluating the effects of systemic O_3 application with different doses and frequencies are warranted to better demonstrate the potential beneficial effects and effect mechanism of this treatment.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Idea/concept, literature review, writing the article: H.Y.E., O.Y.; Design: O.Y.; Control/supervision: H.Y.E., T.K.; Data collection and/or processing: M.İ.G., A.G.B., Y.A.; Analysis and/or interpretation, critical review: O.Y., T.K.; References and fundings, materials: M.İ.G., A.G.B., Y.A.

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