International Orthopaedics
https://doi.org/10.1007/s00264-020-04871-9

ORIGINAL PAPER

The effects of systemic ozone application and hyperbaric oxygen therapy on knee osteoarthritis: an experimental study in rats

Onur Yılmaz[1] · Ali Bilge[1] · H. Yener Erken[1] · Tolgahan Kuru[1]

Received: 29 June 2020 / Accepted: 3 November 2020
© SICOT aisbl 2020

Abstract

Objective To evaluate the effects of systemic medical ozone (O_3) application and hyperbaric oxygen (HBO) therapy on surgically induced knee osteoarthritis (OA) in a rat model.

Materials and methods We performed anterior cruciate ligament transection (ACLT) in order to create experimental OA in the right knees of 27 male rats. The left knee joints of all rats were sham-operated without ACLT as the negative control group. The rats were randomly assigned into three groups: (1) control group, which received no treatment; (2) O_3 group, which received intraperitoneal 30 μg medical O_3; (3) HBO group, which received HBO therapy for 60 minutes twice a day. We sacrificed the rats on the tenth week after the operation. We evaluated the degree of OA using Mankin scores.

Results As a result of histopathological examination, the mean Mankin scores in the right knees with ACLT were 8.17 ± 2.12 in the control group, 6.22 ± 1.56 in the HBO group, and 4.72 ± 1.30 in the O_3 group. The differences between the O_3 group and the HBO group and the O_3 group and the control group were found to be statistically significant (p 0.001, p 0.039, respectively). There was no difference between the HBO group and the control group (p 0.086).

Conclusions The results of the present study show that systemic medical O_3 application was more effective than HBO therapy and may reduce development of cartilage damage and prevent OA formation.

Keywords Hyperbaric Oxygen · Osteoarthritis · Ozone

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by progressive cartilage degeneration, subchondral bone changes, osteophyte formation, and low-grade synovitis. The main physiopathology of OA is a dynamic process which is a result of disruption of the normal balance between destruction and construction of the joint cartilage and the subchondral bone [1, 2]. Changes in the articular cartilage occur as a consequence of this process. Additionally, all joint-related tissues such as subchondral and metaphyseal bone, synovium, ligaments, joint capsule, and joint-related muscles are affected by joint degeneration [1, 2]. Many proinflammatory mediators are released in the pathogenesis of OA including reactive oxygen species (ROS), such as nitric oxide (NO) and hydrogen peroxide (H_2O_2) [3]. The development and progression of OA are associated with constant exposure to oxidants, as in other degenerative diseases [3]. NO production in the early phase of OA can lead to chondrocyte apoptosis. Both events occur in the early phase of responses to cartilage injuries and are associated with the severity of cartilage degradation [4, 5]. Additionally, ROS tends to increase in OA patients in vivo, accelerating the breakdown of the cartilage matrix by inhibiting proteoglycan and collagen composition in the joint, leading to the apoptosis of cartilage cells [3–5].

In recent years, intra-articular medical O_3 therapy has been proposed as a treatment for OA due to its beneficial effects

Level of evidence: 2

✉ Onur Yılmaz
onurylmz52@gmail.com

Ali Bilge
alibilge498@hotmail.com

H. Yener Erken
yenererken@yahoo.com

Tolgahan Kuru
drtolgahankuru@gmail.com

[1] Faculty of Medicine, Orthopedics and Traumatology, Çanakkale Onsekiz Mart University, Canakkale, Turkey

Published online: 13 November 2020

such as increased oxygenation after neoangiogenesis, anti-inflammatory properties and its analgesic effect provided by antinociceptive system stimulation [6]. Intra-articular medical O_3 shows its anti-inflammatory effects by suppressing proinflammatory prostaglandin synthesis, inhibiting bradykinin release, and increasing the release of proinflammatory cytokine antagonists [7]. To the best of our knowledge, the present study is the first study in the literature evaluating the effects of systemically administered medical O_3 on preventing surgically induced OA in a rat model.

Hyperbaric oxygen (HBO) therapy has been used as a treatment of various orthopaedic diseases including soft tissue infections, acute traumatic ischemia, crush injury, compartment syndrome, problematic wounds, problematic skin grafts and flaps, refractory osteomyelitis, osteonecrosis, sports injuries, fracture healing, and nerve healing [8]. However, in the literature, there are limited number of preclinical studies which show the positive effects of HBO therapy on cartilage tissue [9–11].

The anterior cruciate ligament transection (ACLT) rat model used in our study is a surgical model that is frequently used in OA studies [12]. It is suitable for use in pharmaceutical studies because it makes for a fast but controlled OA model and is therefore also suitable for short-term studies. It does not cause chemical interaction and provides slower development of OA lesions compared with other surgical OA models [13]. Therefore, our models can simulate clinical effects and provide definitive experimental evidence for the prevention and treatment of OA animals such as rats. In addition, this experiment is easy to run and replicate in a short cycle and is suitable for active animals such as rats. Significant cartilage damage in the OA model created with ACLT can be accomplished two weeks after surgery [13].

The objective of the present study is to evaluate the effects of systemic medical O_3 application and HBO therapy on surgically induced knee OA in a rat model.

Materials and methods

Animals

Ethical approval was obtained from the Çanakkale Onsekiz Mart University Experimental Research Application and Research Center. The approval number is 2019/07-01. 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). We used 27 male Wistar albino rats weighing within a range of 350–450 grin our study. During the experimental procedure, all rats were housed under standard laboratory conditions with an artificial 12 hour 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. The rats were observed for seven days in the animal care laboratory to exclude any possibility of underlying disease before the experiment started.

Surgical technique and experimental design

The rats were equally and randomly divided into three groups including nine rats in each group: (1) OA group, (2) O_3 group, (3) HBO group. The rats were operated on following an intraperitoneal injection of ketamine HCl (30 mg/kg, Ketalar R, Eczacıbaşı, Istanbul, Turkey) and xylazine anaesthesia (10 mg/kg, Xylasinbio, Bioveta, Ankara, Turkey), as described by Williams et al. [14] with minor modifications [15]. After sterile preparation of the skin, we performed a parapatellar skin incision on the medial side of the right knee joint and then on the medial side of the patellar tendon. The patella was then dislocated laterally to provide access to the joint space, and the anterior cruciate ligament was transected while the knee joint was in flexion. A positive anterior drawer test confirmed complete transection of the anterior cruciate ligament. The joint was then irrigated with sterile saline solution in order to avoid secondary inflammation, and a suture was inserted. The left knee joints in all groups were sham-operated without transection of the anterior cruciate ligament and were accepted as the negative control group. Animal welfare guidelines were adhered to at all times.

Two weeks after the surgery, treatments were simultaneously started for the rats in the O_3 and HBO groups. No treatment was given to the rats in the OA group. Medical O_3 (Turkozone Blue S, Istanbul, Turkey) was obtained from the medical O_3 generator and was administered intraperitoneally at a concentration of 30 μg/ml and 2 ml volume once a day. This treatment protocol was applied every day for three weeks. HBO inhalation therapy was applied to the nine rats in the HBO group in the HBO therapy room (Barotech Medical Supplies Medical Products, Istanbul, Turkey) designed for experimental animals. HBO treatment was performed with 100% oxygen and discharged normal air. The pressure of the test chamber was gradually increased to 2.5 atmosphere absolute (ATA) within ten minutes, and the animals were applied 100% oxygen under 2.5 ATA pressure for the next 40 minutes. At the end of this period, the room pressure was gradually decreased to 1 ATA within ten minutes. Once the room pressure is at 1 ATA, the treatment is ended. This treatment protocol, which lasted 60 minutes in total, was applied to all nine rats in the HBO group twice a day for three weeks. All rats included in our study were sacrificed by cervical dislocation ten weeks after surgery, and both knee joints were removed to include the femur and tibia joint surfaces for evaluation. The knee joints removed were numbered by classifying the names and sides of the groups to which they

belonged and placed in pathology vessels containing 10% formalin solution for histological evaluation.

Histological evaluation

We harvested distal femurs including the joint surface from each animal. We fixed the specimens in 10% formaldehyde for 1 week. Following fixation, they were decalcified for five to seven days in 7% nitric acid. After the decalcification process, knee tissues passing through the femur medial condyle were taken from every animal, cut along the coronal plane, and placed in cassettes. After these samples were washed in stream to be purified from acid, they were subjected to routine tissue follow-up and 4-μm-thick sections were taken from paraffin blocks. Haematoxylin and eosin (H&E) and histochemical Toluidine Blue staining were performed. Sections were evaluated under a light microscope. Tissue samples were examined in order to evaluate cartilage structure, cellular structure, matrix staining (using Toluidine Blue), and smoothness of the joint surface. The findings (with H&E and Toluidine Blue) were evaluated using the classification described by Mankin et al (Table 1) [16]. Contrarily to Mankin's study, we stained the matrix using Toluidine Blue instead of Safranin-O, similar to the study by Hayami et al. [13]. Histopathological evaluations were performed by two independent observers who were blind to the study groups. Using this well-established method, the tissues were given scores ranging from 0 to 14, with 0 corresponding to a normal joint and 14 for a maximally degenerated joint [16]. The mean data of the two observers were used for calculating the Mankin score.

Table 1 The Mankin score

Parameters		Score
Structure	Normal	0
	Surfage irregularities	1
	Pannus and surfage irregularities	2
	Clefts to the transitional zone	3
	Clefts to the radial zone	4
	Clefts to calcified zone	5
	Complete lack of cartilage	6
Cellularity	Normal	0
	Pycnosis, hypercellularity	1
	Clusters	2
	Hypocellularity	3
Matrix structure (proteoglycan degradation) T. blue staining	Normal	0
	Slight reduction	1
	Moderate reduction	2
	Severe reduction	3
	No staining	4
Tidemark	Intact	0
	Destroyed	1

Statistical analysis

The data obtained in this study were analyzed with IBM Statistical Package for Social Sciences (SPSS) Statistics 22 software (SPSS Inc., Chicago, IL, USA). The normality of the distribution for the variables was tested with the Shapiro-Wilk Normality test. Nonparametric tests were used for variables without normal distribution. Continuous data without normal distribution were analyzed using the Kruskal-Wallis test and the Mann-Whitney *U* test. We used Kripppendorff's alpha value to evaluate the inter-rater reliability between the two independent observers who performed the histopathological evaluation. Kripppendorff's alpha < 0.67 was considered as weak, $0.67 \leq \alpha < 0.80$ as moderate, and ≥ 0.80 as excellent. Quantitative data were expressed as mean, standard deviation, minimum and maximum values. Confidence interval was 95%, *p* value less than 0.05 was considered statistically significant.

Results

Histological evaluation

When the cartilage structures from the left knees of the rats that were sham-operated and accepted as the negative control (arthrotomy only without ACLT) were examined, cartilage tissue was normal in most specimens except minor differentiations. We observed that these differentiations were generally limited to the surface; their shapes were normal in terms of their number and morphology; and there was no tissue loss in the joints. In general, we observed that the histology of the sham-operated left knees was normal and was not affected by the treatments applied in neither the O_3 nor the HBO groups (Figs. 1(a1), (a2) and 2a). However, in the right knees with ACLT of the OA group, we detected total cartilage loss and hypocellularity as well as the tidemark irregularities and significant reduction in the proteoglycan levels in the cartilage matrix (Figs. 1(b1), (b2) and 2b). We detected clefts extending to the middle layer and cell clustering and mild proteoglycan matrix depletion in the specimens of right knees with ACLT in the HBO group (Figs. 1(c1), (c2) and 2c). Right knees with ACLT in the O_3 group showed slight irregularity and pannus formation on the surface, mild reduction in proteoglycan, as well as some cell pycnosis in the cartilage. However, tidemark abnormalities were not detected (Figs. 1(d1), (d2) and 2d).

Average total Mankin scores in the left knees without ACLT were found to be 0.5 ± 0.61 in the OA group, 0.39 ± 0.48 in the HBO group, and 0.22 ± 0.44 in the O_3 group,

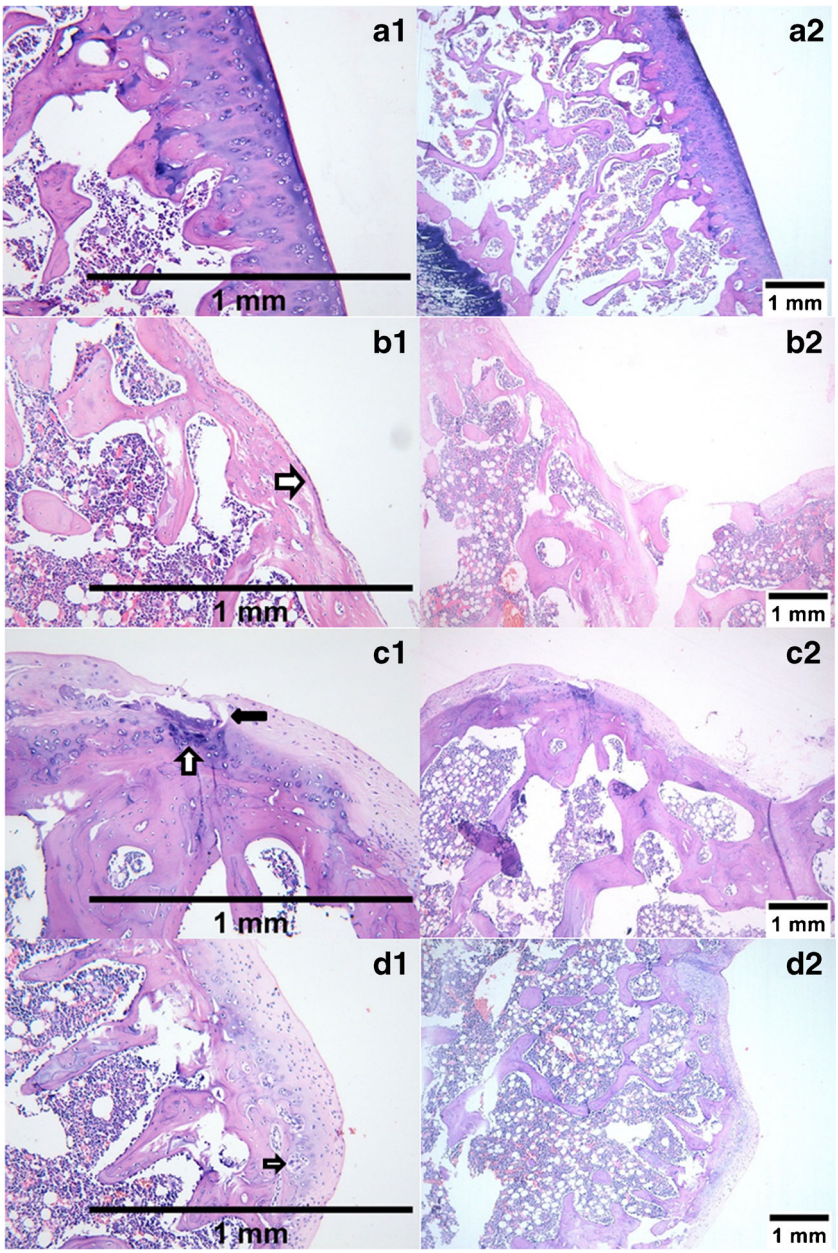

Fig. 1 Photomicrograph representing the histological structure of joint cartilage segments stained with routine haemotoxylen and eosin (H&E) staining. (a1) OA group, left knee with only arthrotomy without ACLT. Sections showing that the tidemark is preserved, the structure is normal, the joint surface is in normal appearance, and the cellularity is in normal range (H&E, × 10). (a2) Lower magnification of (a1) (H&E, × 4). (b1) OA group, right knee with ACLT. Sections showing right knee, total cartilage loss (white arrow), and hypocellularity are seen as well as tidemark irregularities (H&E, × 10). (b2) Lower magnification of b1 (H&E, × 4). (c1) HBO group, right knee with ACLT. Sections showing clefts extending to the middle layer (black arrow) and cell clustering (white arrow) (H&E, × 10). (c2) Lower magnification of (c1) (H&E, × 4). (d1) O_3 group, right knee with ACLT. Sections showing slight irregularity and pannus formation observed on the surface (black arrow) as well as some cell pycnosis in cartilage; tidemark abnormality was not detected (H&E, × 10). (d2) Lower magnification of (d1) (H&E, × 4)

respectively. When we compared sham-operated left knees of the OA, HBO, and O_3 groups, we found no statistically significant difference between the groups in terms of total Mankin scores (p 0.618). However, we found significant differences between right knees (ACLT) and left knees (arthrotomy only without ACLT) in each group and overall ($p < 0.001$).

Average total Mankin scores in the right knees with ACLT were found to be 8.17 ± 2.12 in the OA group, 6.22 ± 1.56 in the HBO group, and 4.72 ± 1.30 in the O_3 group, respectively. There was no statistically significant difference between the HBO and OA groups (p 0.086). However, there was a significant difference between the O_3 and HBO group and the O_3 and OA group (p 0.039, p 0.001, respectively). We detected an excellent consistency in the inter-rater reliability of the two independent observers (Krippendorff alpha = 0.991).

All subscores including cartilage structure, cellular abnormality, matrix staining, and tidemark continuity scores were lower in the O_3 group than other groups. These results are summarized in Table 2.

When we evaluated subscores more specifically, we determined no statistically significant difference in any of the parameters including cartilage structure (p 0.180), cellular abnormality (p 0.299), matrix staining (p 0.075), and tidemark integrity (p 0.539) between the OA group and the HBO group. In a statistical comparison between the OA group and the O_3 group, we found statistically lower scores in all but one of the parameters including cartilage structure (p 0.049), cellular

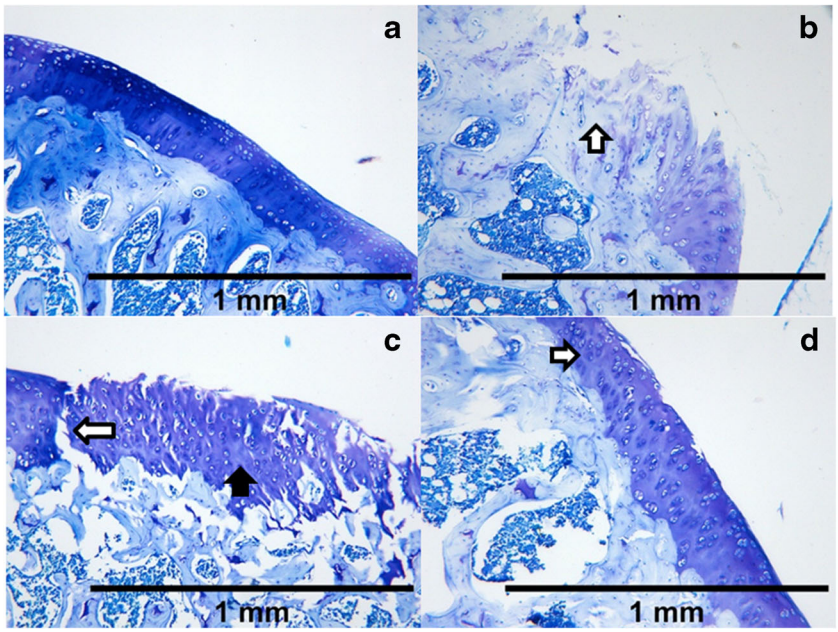

Fig. 2 Photomicrograph representing the histological structure of joint cartilage segments stained with Toluidine Blue staining. **a** OA group, left knee with only arthrotomy without ACLT. The structure and the joint surface is in normal appearance, and cellularity is in normal range. No proteoglycan depletion was observed. (Toluidine Blue, × 10). **b** OA group, right knee with ACLT. Sections showing significant reduction in proteoglycan levels in the cartilage matrix (white arrow); clefts extending to the calcified zone on the surface are notable (Toluidine Blue, × 10). **c** HBO group, right knee with ACLT. Sections showing clefts extending to the middle layer (white arrow) and cell clustering; mild proteoglycan matrix depletion (black arrow) was seen with Toluidine Blue staining (Toluidine Blue, × 10). **d** O_3 group, right knee with ACLT. Sections showing slight irregularities on the surface and mild reduction (white arrow) in proteoglycan (Toluidine Blue, × 10)

abnormality (p 0.017), matrix staining (p 0.007), and tidemark integrity (p 0.019). All parameters were statistically significant in the O_3 group. In the statistical comparison between the HBO group and the O_3 group, we found no statistically difference in none of the parameters including cartilage structure (p 0.341), cellular abnormality (p 0.337), matrix staining (p 0.458), and tidemark integrity (p 0.065).

Discussion

Our histological results in the OA group with ACLT including total cartilage loss, hypocellularity, tidemark irregularities, and significant reduction in proteoglycan levels in the cartilage matrix showed similar results with previous studies with successfully formed OA in a ACLT rat model [13, 14, 17–19].

O_3, which is known to have analgesic, anti-inflammatory, immunomodulatory, and trophic effects, is a gas with high reactivity since it is a strong oxidant agent [20]. When O_3 is administered intravenously, it dissolves in biological fluids and reacts with polyunsaturated fatty acids, antioxidants, reduced glutathione, and albumin. O_3 causes the oxidation of these compounds, leading to the formation of H_2O_2 and lipid oxidation products (LOP) [17]. Although O_3 is a strong oxidant molecule, it does not increase inflammation in tissues. On the contrary, O_3 acts as an effective modulating agent for biological oxidative stress. It reduces inflammation with a paradoxical

Table 2 Average Mankin scores and p values the right knees with ACLT in each group. (The values that are statistically significant differences were highlighted in italics)

	Groups					
	OA	HBO	O3	OA vs HBO (p values)	Ozone vs OA (p values)	Ozone vs HBO (p values)
Cartilage structure	3.39 ± 1.11	2.67 ± 0.71	2.33 ± 0.87	0.180	*0.049*	0.341
Cellular abnormality	2.22 ± 0.83	1.78 ± 0.97	1.27 ± 0.44	0.299	*0.017*	0.337
Matrix staining	1.67 ± 0.71	1 ± 0.71	0.78 ± 0.44	0.075	*0.007*	0.458
Tidemark continuity	0.89 ± 0.33	0.78 ± 0.44	0.33 ± 0.5	0.539	*0.019*	0.065
Total Mankin score	8.17 ± 2.12	6.22 ± 1.56	4.72 ± 1.3	0.086	*0.001*	*0.039*

effect while stabilizing antioxidant systems with a prooxidant effect [21]. O_3 can reduce the release of TNF-alpha (TNF-α), and other proinflammatory cytokines, by inhibiting the ROS-activated nuclear factor κB (NF-κB) pathway. This can prevent the inflammatory environment in the joint that damages the cartilage matrix and induces chondrocyte apoptosis in OA [22]. Zhao et al. showed that O_3 administered at doses of 40, 50, and 60 μg/ml reduced cell viability in a concentration-dependent manner in their in vivo study on rat chondrocyte cultures. Researchers have shown that the optimal O_3 dose for increasing cell viability and chondrocytes is 30 μg/ml. They have also shown that O_3 induces autophagy, which is considered to be an important mechanism of cell survival in various stress situations and plays an important role in cellular haemostasis [23]. In their experimental study on rats, Yu et al. reported that the application of O_3 at a dose of 35 μg/ml decreased the degeneration of articular cartilage caused by free oxygen radicals by increasing the superoxide dismutase (SOD) levels in the joint fluid and serum, while also decreasing the level of malonyl aldehyde (MAD). However, they reported that O_3 applied at a concentration of 70 μg/ml causes peroxidatic reactions in tissues and cells due to its strong oxidative effect, causing joint cartilage damage and a destructive effect on tissues [17]. Oladazimi et al. showed that activin-like kinase 5 (ALK5) expression increased in the OA group of their experimental study on rats. Researchers in that study found that when O_3, bone marrow-derived mesenchymal stem cell (MSC), and exercise training are applied individually, it could reduce the expression of matrix metalloproteinase 13 (MMP-13) and ALK5 genes. However, MSC and O_3 combined simultaneously with exercise training were found to be more effective [24]. Chen et al. reported that TNF-α and its receptors (TNF-R1 and TNF-R2) together mediate the pathology of local (joint) and systemic damage in arthritis. Their experimental study on rats showed that in arthritis, O_3 administration can reduce the synovium damage caused by TNF-α, causing a decrease in synovial TNF-α and TNF-R2 levels and an increase in TNF-R1 levels, inhibiting synovial cell proliferation and synovial cell apoptosis [25].

O_3 is a dose-dependent treatment agent in terms of its effect and properties and has a dose/effect ratio. For this reason, it is important to have a sensitive ozone generator (equipped with a well-standardized photometer, which allows us to determine the ozone concentration in real time) and to administer the optimal dose of O_3 while avoiding its toxicity. Although the known therapeutic dose range of ozone is between 10 and 80 μg/ml, optimal O_3 doses may be different for different medical applications [21]. On the other hand, the reported adverse effects of O_3 treatment in the literature are almost nonexistent and the reported side effects are complications due to application errors. Additionally, conditions such as glucose-6-phosphate dehydrogenase deficiency, early pregnancy (to exclude any mutagenic risk, although unlikely), use of angiotensin converting enzyme (ACE) inhibitors, hyperthyroidism, thrombocytopenia, severe cardiovascular instability, and allergy to O_3 can make this a risky treatment option. Apart from these conditions, O_3 is a very safe treatment option, with no official contraindications as stated in numerous studies [26].

Today, the use of HBO therapy is not included in the treatment guidelines of OA treatment [8]. The beneficial effects of HBO therapy on OA treatment has been shown in a limited number of experimental studies in the literature [9–11]. Yuan et al. showed that the cartilage defects they formed in rabbit knees healed at a higher rate in the HBO-treated group than in the group where HBO was not administered. Researchers have reported that HBO treatment reduces apoptosis in chondrocytes and increases proteoglycan synthesis, which is caused by the suppression of NO production [9]. In their study on rabbit knee chondrocyte cultures, Ueng et al. found that HBO prevented NO-induced apoptosis due to joint cartilage damagecaused by the increasing HSP 70 expression [10]. Although many experimental studies show the positive effects of HBO treatment on cartilage, in our study, we could not determine neither a beneficial nor harmful effect of HBO treatment. In our study, total and all subscores were lower in the HBO group compared with the OA group, but none of these differences was statistically significant.

In our study, total and all subscores of right knees with ACLT of the O_3 group showed statistically significantly lower scores than the HBO and OA group in our study. These results suggest that systemic application of O_3 may have a preventative effect on surgically induced OA in rats. In a recent experimental study, intraarticular ozone administration had been found to have preventative effects on surgically induced OA in rats [17]. To the best of our knowledge, this experimental study is the first study in the literature that evaluates the effects of systemic administration of O_3 on the development of surgically induced OA in a rat model. We think that the preventative effect of systemic O_3 on cartilage tissue, as seen in this study, might be due to O_3's anti-inflammatory and antioxidant effects. Also, the increase in the capacity of O_3 to clean ROS and reduce oxidative damage and apoptosis of cartilage cells can prevent cartilage matrix destruction.

The main limitation of our study is that we could only perform histopathological examination and we could not use other studies such as micro-CT, nanoindentation test, and microspectrophotometry which could have given further insight into cartilage tissue and the subchondral bone. As we could

use a limited number of rats in the study, we created the study plan using the left knees of the experimental groups as the negative control groups. However, it would be more appropriate to use a separate group as the negative control group in later studies in order to prevent interaction with the treatments applied. Additionally, the evaluation of ROS, such as NO and H_2O_2 in the joint fluid, could provide more detailed information about the effects of HBO therapy and systemic O_3 application.

In the present study, we found that the rats that were treated with systemic O_3 showed the lowest mean histopathological OA scores. The results of the present study show that systemic medical O_3 application was more effective than HBO therapy and may better reduce the development of cartilage damage and prevent OA formation. As this is a preliminary study, more research and further studies are needed to support these results and to clarify the mechanisms of these beneficial effects of O_3 on cartilage tissue.

Acknowledgments We would like to acknowledge Fatma Nur Yürüm and Aslı Muratlı MD for their support in their pathological examination.

Funding This work was supported by Çanakkale Onsekiz Mart University, The Scientific Research Coordination Unit, Project number: TTU-2019-3122.

Compliance with ethical standards

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).

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Ethical approval was given by the Çanakkale Onsekiz Mart University Animal Care and Ethics Committee for animal studies. Approval number: 2019/07-01

References

1. Buckwalter JA, Mankin HJ, Grodzinsky AJ (2005) Articular cartilage and osteoarthritis. Instr Course Lect 54:465
2. Goldring M, Goldring S (2010) Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis. Ann N Y Acad Sci 1192: 230–237. https://doi.org/10.1111/j.1749-6632.2009.05240.x
3. Ashkavand Z, Malekinejad H, Vishwanath BS (2013) The pathophysiology of osteoarthritis. J Pharm Res 7(1):132–138
4. Goldring SR, Goldring MB (2004) The role of cytokines in cartilage matrix degeneration in osteoarthritis. Clin Orthop Relat Res 427:S27–S36
5. Manoto SL, Maepa MJ, Motaung SK (2018) Medical ozone therapy as a potential treatment modality for regeneration of damaged articular cartilage in osteoarthritis. Saudi J Biol Sci 25(4):672–679
6. Bocci, V. (2002). Oxygen-ozone therapy: a critical evaluation. Springer Science & Business Media, Dordrecht
7. Shallenberger F, HMD A (2011) Prolozone™–regenerating joints and eliminating pain. J Prolother 3(2):630–638
8. Lindell K Weaver (2014). Hyperbaric oxygen therapy indications, thirteenth edn. The Hyperbaric Oxygen Therapy Committee Report. Best Publishing Company, Florida
9. Yuan LJ, Ueng SW, Lin SS, Yeh WL, Yang CY, Lin PY (2004) Attenuation of apoptosis and enhancement of proteoglycan synthesis in rabbit cartilage defects by hyperbaric oxygen treatment are related to the suppression of nitric oxide production. J Orthop Res 22(5):1126–1134
10. Ueng SW, Yuan LJ, Lin SS, Niu CC, Chan YS, Wang IC et al (2013) Hyperbaric oxygen treatment prevents nitric oxide-induced apoptosis in articular cartilage injury via enhancement of the expression of heat shock protein 70. J Orthop Res 31(3):376–384
11. Nagatomo F, Gu N, Fujino H, Okiura T, Morimatsu F, Takeda I, Ishihara A (2010) Effects of exposure to hyperbaric oxygen on oxidative stress in rats with type II collagen-induced arthritis. Clin Exp Med 10(1):7–13
12. Kuyinu EL, Narayanan G, Nair LS, Laurencin CT (2016) Animal models of osteoarthritis: classification, update, and measurement of outcomes. J Orthop Surg Res 11(1). https://doi.org/10.1186/s13018-016-0346-5
13. Hayami T, Pickarski M, Zhuo Y, Wesolowski GA, Rodan GA, Duong LT (2006) Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. Bone 38(2): 234–243
14. Williams JM, Felten DL, Peterson RG, O'Connor BL (1982) Effects of surgically induced instability on rat knee articular cartilage. J Anat 134(Pt 1):103
15. Galois L, Etienne S, Grossin L, Cournil C, Pinzano A, Netter P et al (2003) Moderate-impact exercise is associated with decreased severity of experimental osteoarthritis in rats. Rheumatology 42(5): 692–693
16. Mankin HJ, Dorfman H, Lippiello L, Zarins A (1971) Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J Bone Joint Surg Am 53(3):523–537
17. Yu B, Lin Q-R, Wang B-W, Zhu Q, He X-F (2010) Effect of intra-articular ozone injection on degenerative knee cartilage in rats. Int J Ozone Ther 9:14–20
18. Naito K, Watari T, Furuhata A, Yomogida S, Sakamoto K, Kurosawa H et al (2010) Evaluation of the effect of glucosamine on an experimental rat osteoarthritis model. Life Sci 86(13-14): 538–543
19. Jean YH, Wen ZH, Chang YC, Hsieh SP, Tang CC, Wang YH, Wong CS (2007) Intra-articular injection of the cyclooxygenase-2 inhibitor parecoxib attenuates osteoarthritis progression in anterior cruciate ligament-transected knee in rats: role of excitatory amino acids. Osteoarthr Cartil 15(6):638–645
20. Paoloni M, Di Sante L, Cacchio A, Apuzzo D, Marotta S et al (2009) Intramuscular oxygen-ozone therapy in the treatment of acute back pain with lumbar disc herniation: a multicenter, randomized, double-blind, clinical trial of active and simulated lumbar paravertebral injection. Spine 4(13):1337–1344
21. Bocci VA (2006) Scientific and medical aspects of ozone therapy. State of the art. Arch Med Res 37(4):425–435. https://doi.org/10.1016/j.arcmed.2005.08.006
22. Vaillant JD, Fraga A, Díaz MT, Mallok A, Viebahn-Hänsler R, Fahmy Z et al (2013) Ozone oxidative postconditioning ameliorates joint damage and decreases pro-inflammatory cytokine levels and oxidative stress in PG/PS-induced arthritis in rats. Eur J Pharmacol 714(1-3):318–324
23. Zhao X, Li Y, Lin X, Wang J, Zhao X, Xie J, Sun T, Fu Z (2018) Ozone induces autophagy in rat chondrocytes stimulated with IL-1β through the AMPK/mTOR signaling pathway. J Pain Res 11: 3003–3017. https://doi.org/10.2147/JPR.S183594

24. Oladazimi S, Azarbayejani MA (2020) Evaluation of ALK5 and MMP13 expression in the cartilage tissue of rats with osteoarthritis rats and effects of exercise training, ozone and mesenchymal stem cell therapies on expression of these genes. Med. Lab. J. 14(1):50–56. https://doi.org/10.29252/mlj.14.1.50
25. Chen H, Yu B, Lu C, Lin Q (2013) The effect of intra-articular injection of different concentrations of ozone on the level of TNF-α, TNF-R1, and TNF-R2 in rats with rheumatoid arthritis. Rheumatol Int 33(5):1223–1227. https://doi.org/10.1007/s00296-012-2529-7
26. Fernandez-Cuadros ME, Perez-Moro OS, Mirón-Canelo JA (2016) Could ozone be used as a feasible future treatment in osteoarthritis of the knee. Divers Equal Health Care 13(3):232–9

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.