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ORIGINAL ARTICLE

The impact of *ex vivo* ozone injection into the synovial fluid in patients with knee osteoarthritis: A controlled clinical trial

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ABSTRACT

Objectives: This study aimed to examine the effect of varying ozone doses on proinflammatory cytokine levels in the synovial fluid collected from individuals with knee osteoarthritis.

Patients and methods: The controlled clinical trial was conducted with 82 patients (61 females, 21 males; mean age: 63.1±10.0 years; range, 40 to 73 years) between 21 April 2023 and 20 May 2023. Synovial fluid samples were collected from the patients under ultrasound guidance and divided into three tubes, one of which was not injected with ozone, and the other two were injected with 10 and 30 gamma (γ) ozone, respectively. The total antioxidant status, total oxidant status, oxidative stress index, interleukin (IL)-1 beta (β), IL-6, and tumor necrosis factor-alpha (TNF-α) in the joint fluids were measured.

Results: The oxidative stress index and IL-1 β, IL-6, and TNF-α levels in the synovial fluid were lower at 10 and 30 γ compared to 0 and 10 γ, respectively. *In vitro* ozone injection at 30 gamma was more effective in reducing proinflammatory cytokines in the synovial fluid than that at 10 and 0 γ. Ozone injection into the pathological joint fluid was more effective in terms of total antioxidant status at 10 and 30 γ compared to 0 and 10 γ, respectively. No significant difference in total oxidant status was observed between the groups.

Conclusion: This study showed that *in vitro* ozone injection at 30 γ was more effective in reducing proinflammatory cytokines in the synovial fluid and in improving total antioxidant status than that at 10 and 0 γ. The results showed the potential significance of the ozone injection dosage in treating knee osteoarthritis.

Keywords: Knee osteoarthritis, ozone therapy, proinflammatory cytokines.

Knee osteoarthritis (OA) is characterized by the erosion of the joint cartilage, formation of osteophytes, subchondral sclerosis, synovitis, and functional impairment, which cause pain and restricted movement.[1] This condition stems from intricate interactions involving genetic, metabolic, biochemical, and biomechanical factors, coupled with secondary inflammation affecting synovial joints. These factors collectively contribute to the degradation of the cartilage, bone, and synovium. Inflammatory cytokines such as interleukins (IL)-1, -6, and -8, tumor necrosis factor-alpha (TNF-α), interferon-gamma (γ), and oxidative damage play pivotal roles in cartilage damage associated with knee OA.[2]

Although knee OA lacks a definitive treatment, several interventions, including nonpharmacological treatment, pharmacological treatment, and surgical treatment, aim to manage the symptoms and delay the disease progression to the advanced stage. Intra-articular steroids, hyaluronic acid, platelet-rich plasma, polynucleotide gel, and ozone injections are commonly used interventional treatment methods. Intra-articular injections are gaining increasing attention due to their direct effects on the target tissues and minimal side effects.[3] Ozone therapy is widely used in medicine worldwide. Numerous studies have confirmed the effectiveness and safety of ozone in treating musculoskeletal disorders.

In the last decade, many European centers have begun treating patients with knee OA with intra-articular ozone injections.[2]

Ozone therapy has been reported to induce interferon-β, diminish inflammatory mediators such as TNF-α, enhance tissue oxygenation, and augment superoxide dismutase activity, thus breaking down reactive oxygen species and decelerating the degenerative process. Moreover, ozone therapy has demonstrated favorable outcomes in terms of pain relief by providing an analgesic effect by inhibiting phosphodiesterase A2. Additionally, transforming growth factor-β has been observed to activate chondrocytes.[4] Furthermore, ozone therapy has not been reported to induce any acute or chronic toxicity.[2]

In the literature, intra-articular ozone injection has been administered at varying doses, and no standard dose has been recommended.[5,6] Furthermore, data on the optimal dosage of ozone injection and its corresponding level of efficacy is limited. While the literature has provided ample evidence of the impact of ozone therapy on biomarkers through blood assessments, there remains a dearth of information regarding its influence on synovial fluid. The measurement of biomarkers within synovial fluid serves as a direct reflection of the conditions within the articular cartilage and synovial membrane. These biomarker levels can serve as a foundational indicator for assessing the extent of synovitis and inflammation within the knee joint.[7-9] Therefore, it is crucial to evaluate the effects of ozone injection as a contemporary treatment modality aiming at elucidating the underlying mechanisms of disease pathogenesis in patients with knee OA.

Thus, this study aimed to investigate the effect of *in vitro* ozone injection on the levels of proinflammatory cytokines in the synovial fluid obtained by puncture from patients with knee OA-induced joint effusion. Additionally, the study aimed to determine the effect of varying ozone doses on synovial fluid to provide reliable and standardized data regarding the doses of intra-articular ozone injections commonly used in clinical practice.

PATIENTS AND METHODS

This controlled clinical trial, in concordance with the previous literature,[10] was conducted with 82 individuals (61 females, 21 males; mean age: 63.1±10.0 years; range, 40 to 73 years) with confirmed knee OA (Kellgren-Lawrence Grade 2 or 3) based on American College of Rheumatology criteria at the University of Health Sciences, Sultan 2. Abdulhamid Han Training and Research Hospital, Department of Physical Medicine and Rehabilitation between 21 April 2023 and 20 May 2023. Participants who experienced persistent knee pain (Visual Analog Scale pain score ≥4) for at least six months due to OA, had joint effusion on the affected knee, and were willing and able to comply with the study protocol, including follow-up appointments and procedures were included in the study. Based on the previous works in the literature,[11] participants with a history of ozone therapy sensitivity, knee infections in the past six months, recent knee surgeries or joint injections within three months, pregnancy or breastfeeding, and those with other chronic inflammatory joint conditions, autoimmune disorders, concurrent use of interfering medications, or any conditions compromising safety, compliance, or study validity were excluded. Randomization procedures were not employed, as the synovial fluid samples aspirated from each of the enrolled patients were divided into three distinct groups according to the ozone dose (0, 10, and 30 γ), with each group being allocated a portion of the samples. The flowchart of the study is presented in Figure 1.

Synovial fluid samples were collected from patients with effusion in their knee joint under sterile conditions by a joint puncture for *in vitro* studies. Ultrasonography was performed to examine the patients before the sample collection. The presence of synovial hypertrophy in patients during the ultrasound examination was recorded. Synovial fluid samples were collected using sterile 10 mL syringes under ultrasonographic guidance (Figure 2). Three sterile culture tubes containing equal amounts of synovial fluid, ranging between approximately 2 to 5 mL, were used for each patient. One tube contained only synovial fluid, and the other two contained synovial fluid and ozone gas (1 mL of ozone gas for every 1 mL of the synovial fluid).

The minimal recommended dosages for intra-articular ozone interventions in knee OA were established at 10 γ.[12] Nevertheless,

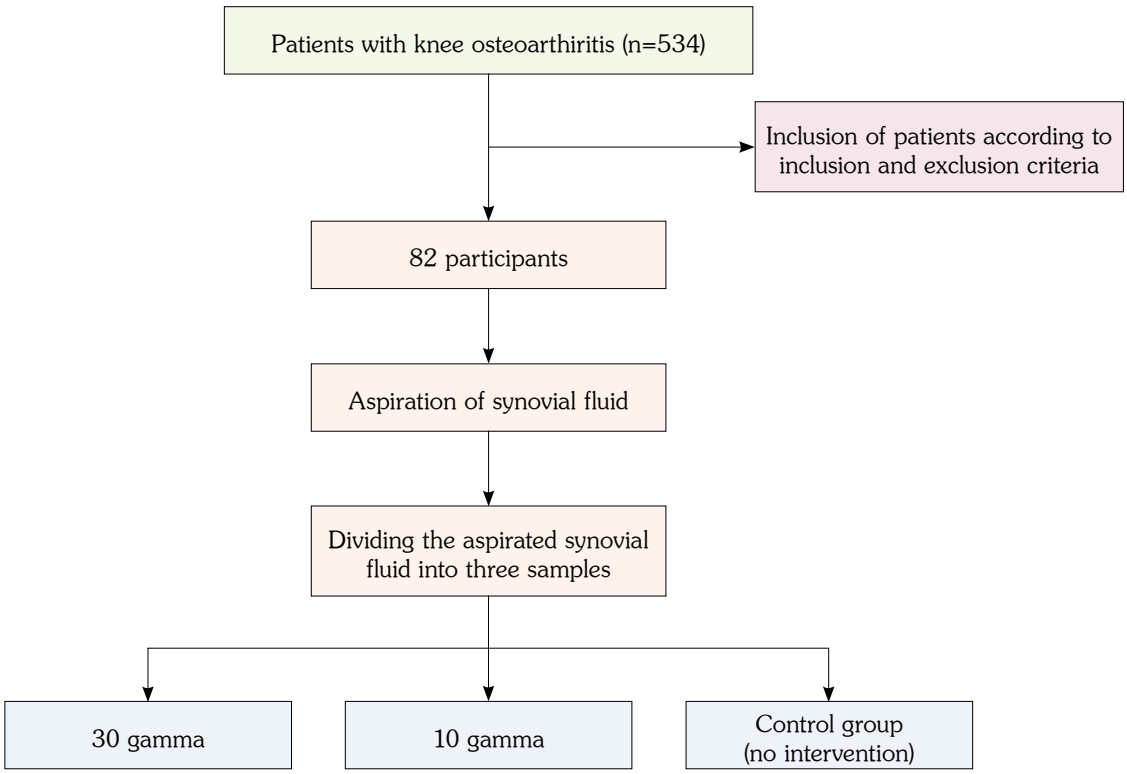

Figure 1. Flowchart of the study.

considering the findings from meta-analyses, applications at 30 γ doses were more prevalent.[13,14] Consequently, for the present study, dosages of both 10 and 30 γ were chosen for examination. The concentration of ozone injected into the tubes was either 10 or 30 γ. The synovial fluid samples were centrifuged at 500 g for 5 min using a centrifuge device Allegra X-15R (Beckman Coulter Allegra, Indianapolis, IN, USA). The upper portion of the samples was transferred to Eppendorf tubes and stored at −80°C for biochemical analyses.

Before conducting the biochemical analyses, synovial fluid samples (approximately 2 mL) were transferred to Eppendorf tubes and centrifuged at 1000 g for 5 min using the Beckman Coulter Allegra centrifuge. The supernatants were discarded, and 100 µL of the pellet was extracted and placed onto a slide. The slides were examined under a fluorescence microscope Eclipse Ts2 (Nikon, Tokyo, Japan) to obtain ×20 magnification images (Figure 3).

The total protein content in the synovial fluid samples was determined using a commercial

Figure 2. Ultrasound-guided investigation for the presence of synovial hypertrophy and aspiration of joint fluid.

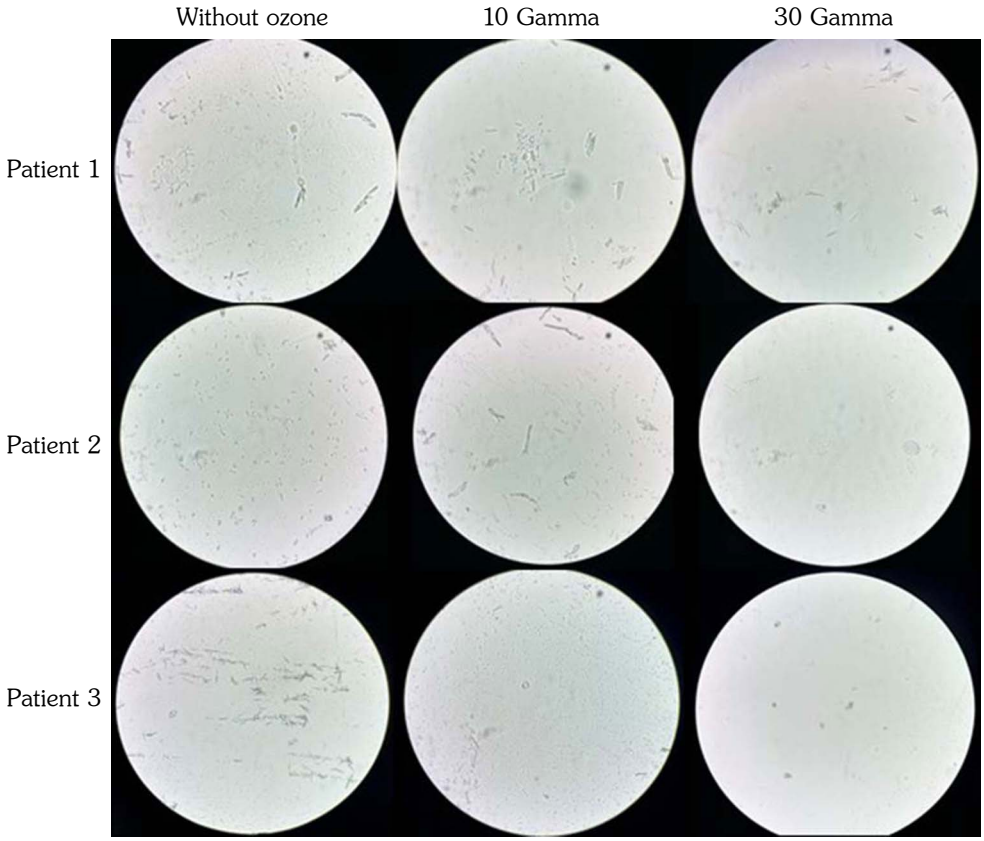

Figure 3. Images of joint fluid from three patients under a fluorescence microscope depicting a comparison between the fluid without exposure to ozone, with 10 gamma ozone, and with 30 gamma ozone (×20).

kit based on the Bradford method (Coomassie Plus Protein Assay; Thermo Fisher Scientific, Waltham, MA, USA) and measured at 595 nm.[15] Microgram quantities of protein were measured using a rapid and sensitive method. This method is based on the principle of protein-dye binding; when protein molecules bind to Coomassie dye under acidic conditions, the color changes from brown to blue.

The total antioxidant status (TAS) and total oxidant status (TOS) of the samples were measured using commercially purchased kits (Rel Assay Diagnostics, Mega Tıp, Gaziantep, Türkiye) according to the manufacturer's instructions. The oxidative stress index (OSI) was calculated using the following formula:[16] OSI (AU)= (TOS, μmol hydrogen peroxide equivalent/L)/(TAS, mmol Trolox equivalent/L). All calculated values were normalized to the total protein content measured in the synovial fluid.

Inflammatory biomarkers, IL-1 β (BT Lab, E0143Hu; Zhejiang, China), IL-6 (BT Lab E0090Hu), and TNF-α (BT Lab, E0082Hu), were measured in the synovial fluid by photometric methods with commercially purchased enzyme-linked immunosorbent assay kits. The inter-assay coefficient of variation was evaluated in triplicate (on three different dispensing cycles) in five different analytical runs using the serum of healthy subjects.

Statistical analysis

A power analysis was employed using G*Power version 3.1 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) to determine the minimum sample size required. In the power analysis conducted to assess sample size requirements for the three distinct groups, the following parameters were considered: an effect size (f) of 0.3, a desired statistical power (1-β) of 0.80, and a significance level (α) of 0.05.

Table 1. Demographic data of the patients

	Statistic		
Variables	n	%	Mean±SD
Age (year)			63.1±10.0
Body mass index			30.4±3.4
Sex			
Male	21	25.6	
Female	61	74.4	

SD: Standard deviation.

As a result of this analysis, the sample size was established to be 75.

Data analysis was performed using IBM SPSS version 25.0 software (IBM Corp., Armonk, NY, USA). Descriptive statistics were presented as mean±standard deviation for quantitative variables and as frequency and percentages for categorical variables. Categorical variables were compared using the chi-square test. The Friedman test was used for the nonnormal distribution of dependent variables among the three groups. A p-value <0.05 was considered statistically significant.

RESULTS

The mean body mass index of the patients was 30.4±3.4. In the context of the Kellgren-Lawrence grading system, 31 (37.8%) patients were classified as Stage II, while 51 (62.2%) patients were categorized as Stage III (Table 1).

When examining the presence of synovial hypertrophy on ultrasound and the IL-1β level in the synovial fluid (Table 2), a significant difference was observed (p<0.05). When synovial hypertrophy was positive, the IL-1β level in synovial fluid was significantly higher (90.2%) (Figure 5).

The intra-assay coefficient of variations was found to be 1.5% for TT&NT, 3.3% for TAS, and 2.7% for TOS. The interassay coefficient of variations was 2.1% for TT&NT, 3.2% for TOS, and 2.8% for TAS.

Table 2. Investigation of IL-1β levels and the presence of synovial hypertrophy on ultrasonography in the synovial fluid at 0 gamma

	0 gamma IL-1 beta level				
	45 pg/mL and below		45 pg/mL and above		
	n	%	n	%	
Synovial hypertrophy					
Negative	10	32.3	21	67.7	χ^2=6.504
Positive	5	9.8	46	90.2	p=0.011

IL: Interleukin.

Table 3. Comparison of variables according to ozone doses

	0 Gamma		10 Gamma		30 Gamma			
	Median	Min-Max	Median	Min-Max	Median	Min-Max	*p*	Difference
TAS	0.41	0.18-0.68	0.5	0.25-0.84	0.6	0.32-1.1	<.001	c>b>a
TOS	0.03	0.01-0.07	0.03	0-0.07	0.03	0-0.05	0.77	-
OSI	0.07	0.01-0.19	0.05	0-0.17	0.04	0.01-0.11	<.001	a>b>c
Interleukin beta	54.56	22.78-89.43	39.28	22.09-57.74	32.12	19.39-49.42	<.001	a>b>c
Interleukin 6	44.28	21.99-75.57	23.45	19.16-44.64	11.41	7.01-25.5	<.001	a>b>c
TNF-α	59.23	24.44-128.52	27.02	8.75-59.77	17.75	13.4-21.85	<.001	a>b>c

TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index; TNF-α: Tumor necrosis factor-alpha; a: 10 gamma; b: 10 gamma; c: 30 gamma data.

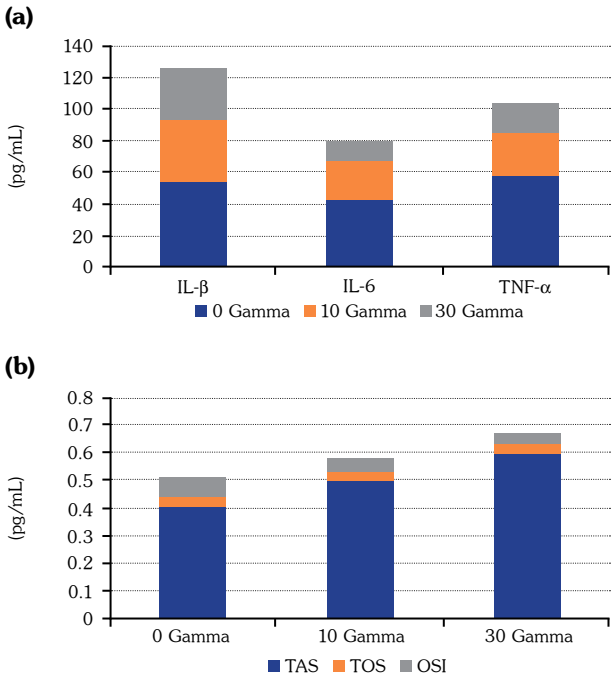

Figure 4. Comparison of **(a)** IL-1β, IL-6 and TNF-α, **(b)** TAS, TOS, and OSI measurements between groups.

IL: Interleukin; TNF-α: Tumor necrosis factor alpha; TAS: Total antioxidant status, TOS: Total oxidant status, OSI: Oxidative stress index

Statistically significant differences were observed in the measurements of TAS, OSI, IL-1β, IL-6, and TNF-α ($p<0.05$) (Table 3). The OSI, IL-1β, IL-6, and TNF-α measurements were lower at 10 γ than those at 0 γ and lower at 30 γ than those at 10 γ. *In vitro* ozone injection at 30 γ was more effective in reducing the proinflammatory cytokine levels in the synovial fluid than that at 10 γ and 0 γ ($p<0.05$) (Figure 4).

Furthermore, the measurements of TAS improved at 30 γ compared to 10 γ and at 10 γ compared to 0 γ. The data determined that the efficacy of *in vitro* ozone injection into the pathological joint fluid was statistically superior at 10 γ compared to 0 γ and at 30 γ compared to 10 γ. In TOS measurements, no statistically significant difference was observed between the groups ($p=0.774$).

DISCUSSION

The findings of this study suggests that *in vitro* ozone injection proved to be more effective in reducing inflammation in pathological synovial fluid, particularly concerning variables such as OSI, IL-1β, IL-6, and TNF-α, when administered at higher dose levels (10 γ to 30 γ) compared to lower dose levels (0 γ to 10 γ). The significance of this research lies in its pioneering exploration of the impact of ozone therapy on synovial fluid, as well as its endeavor to elucidate the dose-dependent nature of these effects.

In their article, Oliviero et al.[17] expounded that the therapeutic efficacy of ozone therapy emanates from the generation of reactive oxygen species and lipid oxidative products within the synovial fluid. The anti-inflammatory prowess of ozone therapy encompasses a spectrum of mechanisms, encompassing the suppression of

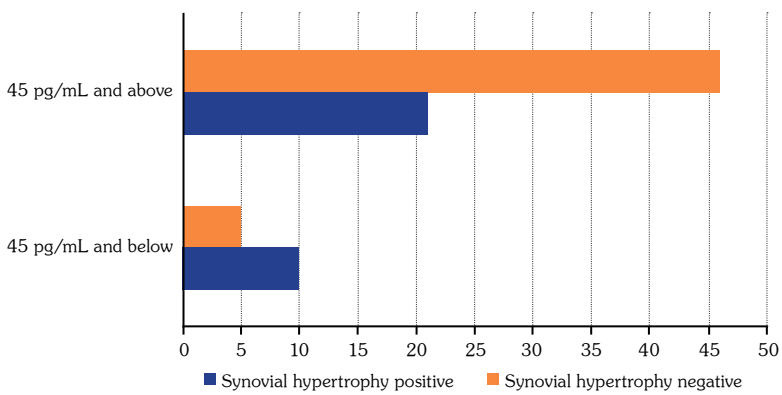

Figure 5. Investigation of IL-1β levels and the presence of synovial hypertrophy on ultrasonography in the synovial fluid at 0 gamma.

IL-1β: Interleukin 1 beta.

proteolytic enzyme release and the instigation of the release of soluble receptors, exemplified by IL-1 and akin soluble receptors, as well as antagonists equipped to effectively obstruct proinflammatory cytokines such as IL-1, IL-8, IL-12, IL-15, and TNF-α. Our study not only supported these findings but also showed that this effect varied in a dose-dependent manner.

A recent *in vivo* study by Fernández-Cuadros et al.[18] reported that intra-articular ozone reduced serum inflammation markers such as polymerase chain reaction, erythrocyte sedimentation rate, and uric acid and increased the minimum joint space of the medial and lateral components, as observed in radiological images. Hashemi et al.[11] also demonstrated comparable findings that applying intra-articular ozone injections in patients with knee OA resulted in a significant decrease in serum inflammatory cytokines at one, two, and six months after the procedure. In addition to the outcomes presented in prior investigations, our study provided insights into the impact of intra-articular ozone injection on inflammatory cytokines present within the synovial fluid. When considering the established influence of ozone therapy on serum inflammatory cytokines, alongside the novel revelation within our study regarding its effect on synovial fluid, ozone therapy may emerge as a dual-action therapeutic approach. This dual-action attribute may serve to elucidate its effectiveness and position it as a preferred treatment option for individuals with knee OA.

Bocci et al.[19] reported that low ozone doses might exhibit anti-inflammatory effects by decreasing the expression of cytokines and chemokines. However, high doses of ozone therapy may increase the expression of proinflammatory mediators, such as IL-1β and TNF-α, suggesting the potential for inducing proinflammatory effects at higher doses. In contrast to the previously mentioned outcomes, the results of our study revealed a statistically significant reduction in proinflammatory cytokine levels within the synovial fluid, accompanied by an enhancement in TAS within the synovial fluid, notably linked to the administration of high-dose ozone injections, in stark contrast to their low-dose counterparts.

This study has certain limitations. First, this is an *in vitro* study, and it is necessary to corroborate these findings with *in vivo* studies. The efficacy of ozone applications at various doses in the short, medium, and long term for daily activities and pain management could not be discerned as there was no direct application to patients. Therefore, further larger sample sizes and long-term patient follow-up studies are needed to evaluate the results. Nonetheless, this study boasts notable strengths, primarily in its ability to provide a quantitative elucidation of the *ex vivo* characteristics of ozone therapy on synovial fluid while also conducting a rigorous comparative analysis of the varying dosages' effectiveness.

In conclusion, *in vitro* applied ozone injection at 30 γ was found to be more effective in reducing proinflammatory cytokine levels in the synovial fluid and in improving TAS than that at 10 γ and 0 γ. These findings provide valuable insights into the potential use and significance of the dosage of ozone injection in treating knee OA. Further *in vivo* studies are also needed to confirm the findings and assess the efficacy of ozone applications in real-life situations.

Ethics Committee Approval: The study protocol was approved by the Health Sciences University Hamidiye Scientific Research Ethics Committee (date: 22.04.2022, no: 22/248). To ensure transparency, accountability, and scientific rigor, the clinical trial was duly registered with Clinical Trials under trial number NCT05824052. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patient Consent for Publication: A written informed consent was obtained from each patient.

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, design, critical review: E.A., H.B.; Control/supervision, writing the article: E.A., M.H.T., H.B.; Data collection and/or processing: S.A., E.M.G., S.Ö.; Analysis and/or interpretation: S.A., E.A., H.B.; Literature review: E.M.G., S.Ö.; References and fundings: M.H.T., S.Ö.; Materials: H.B., S.Ö., E.M.G.

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