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ORIGINAL ARTICLE

Efficacy of ozone therapy in the treatment of frozen shoulder in rats: An experimental study

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ABSTRACT

Objectives: This study aims to explore the therapeutic effects of medical ozone therapy on the frozen shoulder (FS) model and compares it with traditional corticosteroid treatments in rats.

Materials and methods: A total of 30 Spraque-Dawley rats aged 18 to 20 months weighing between 400 to 450 g were included in the study. The rats were randomly divided into three equal groups: a control group (C, n=10, FS model only), a corticosteroid treatment group (CST, n=10, FS model + intraarticular 0.5 mg/kg betamethasone), and an ozone treatment group (OT, n=10, FS model + intraarticular 1 mg/kg ozone). Frozen shoulder was induced via surgical immobilization, and treatments were administered intraarticularly. Outcomes were measured through histopathological and functional assessments.

Results: The CST and OT significantly reduced inflammation ($p<0.001$), capillary proliferation ($p<0.001$), fibroblastic proliferation ($p=0.002$), collagen type 3 staining ($p=0.022$), and mean capsular thickness ($p<0.001$), while improving the range of motion in all directions compared to the control group. Ozone therapy showed a comparable reduction in fibrosis and improvement in joint mobility to CST ($p=0.001$).

Conclusion: Ozone therapy effectively reduces fibrosis and improves mobility in an FS rat model, presenting a promising alternative to corticosteroids. However, further studies are still needed to elucidate the molecular mechanisms and optimize treatment protocols, underscoring the potential for future discoveries in this area.

Keywords: Frozen shoulder, corticosteroids, fibrosis, inflammation, ozone therapy.

Frozen shoulder (FS), which is also known as adhesive capsulitis, is characterized by active and passive shoulder movements are progressively restricted after spontaneous pain in the shoulder and heals by limiting itself over time.[1-3] It has an incidence of 2 to 5%, affecting women more frequently.[4] It is classified into primary (idiopathic) and secondary forms. Primary FS is often associated with diabetes mellitus, hypothyroidism, and parkinsonism, while a secondary FS results from trauma and long-term immobilization.[2-4]

Increasing evidence shows that FS involves a cascade of immune-mediated events triggered by chronic low-grade inflammation. Cytokines such as interleukin (IL)-6, IL-8, tumor necrosis factor-alpha (TNF-α) and tumor growth factor-beta1 (TGF-β1) play key roles, stimulating fibroblasts to transform into alpha-smooth muscle actin-positive (αSMA+) myofibroblasts. This transformation disrupts extracellular matrix (ECM) metabolism leading collagen deposition and joint capsule fibrosis.[1,5,6] Despite extensive research, the pathophysiological mechanisms remain incompletely understood, leaving no definitive treatment protocol.

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The applied treatment methods vary according to the stage of the disease. Pharmacotherapy and physiotherapy methods are at the forefront in the inflammatory phase, while surgical methods target advanced fibrosis.[2] Corticosteroids, although effective in early inflammation, show limited long-term benefits and carry risks of systemic and local complications such as tendon rupture, hyperglycemia, skin depigmentation, joint infection, periarticular calcification and subcutaneous atrophy, avascular necrosis and osteoporosis.[2,6]

Ozone therapy (OT), with its antioxidant and immunomodulatory properties, has shown promise in reducing fibrosis in experimental models. By activating pathways such as nuclear factor erythroid 2-related factor 2 (Nrf2), OT may address both inflammation and fibrosis with minimal side effects.[7,8] In this experimental study, we hypothesized that OT would reduce the development of fibrosis by suppressing inflammation and fibroblast activation, thereby limiting the loss of range of motion (ROM). We, therefore, aimed to evaluate the efficacy of OT in an FS animal model.

MATERIALS AND METHODS

All experimental procedures in this study were conducted in accordance with Directive 2010/63/EU of the European Parliament and Council, ensuring strict adherence to all ethical requirements concerning animal welfare. After our study was approved by the Local Ethics Committee for Animal Experiments of Başkent University, the experiments were started (date: 04.01.2023, Project no: DA22/30). Two researchers with an Experimental Animal Use Certificate performed all experiments under the supervision of veterinarians working at Başkent University Experimental Animal Production and Research Center.

The resource equation method was used in the power analysis before starting the study.[9] The results obtained determined that seven experimental animals in each group were sufficient for the experiments planned to be carried out in three groups. Considering the possible dropout that may occur during the experiment, a total of 30 Spraque-Dawley rats aged 18 to 20 months weighing between 400 to 450 g including 10 rats in each group were included in the study. The rats were randomly divided into three groups: a control group (C, n=10, FS model only), a corticosteroid treatment group (CST, n=10, FS model + intraarticular 0.5 mg/kg betamethasone), and an ozone treatment group (OT, n=10, FS model + intraarticular 1 mg/kg ozone).

Surgical procedure

Previous studies have shown that the FS model created in rats mimics FS disease seen in humans, both mechanically and histologically.[10-13] Therefore, a surgical FS model was created in this study, as previously described in the literature.[10]

The subjects fasted overnight before anesthesia. As an anesthetic method, intraperitoneal ketamine 60 mg/kg (Ketalar®; Pfizer PFE İlaçları, İstanbul, Türkiye) and xylazine 7 mg/kg (Rompun® 2%; Bayer Türk Kimya San. Ltd. Şti., İstanbul, Türkiye) were administered. Anesthesia was maintained at a level where response to pain was absent while the spontaneous breathing of the rats continued. After the induction of anesthesia, the left upper extremity of the rats was shaved, and the rats were placed in a prone position, fixing the other three limbs. Povidone-iodine was used for local antisepsis. All surgical procedures were performed by a single surgeon who previously established this surgical model. A skin incision of approximately 2 cm was made just below the glenohumeral joint, parallel to the humeral shaft. After the scapula was explored between the latissimus dorsi and trapezius muscle, two holes were made on the lateral edge of the scapula. The humeral shaft was, then, explored. The soft tissues, brachial artery, and radial nerve were preserved and prepared by dissecting to prevent additional soft tissue injury. The glenohumeral joint was, then, immobilized with two braided polyester sutures (No. 2/0, ETHIBOND EXCEL™, Ethicon, Inc., NJ, USA) through the holes in the scapula and humeral shaft. The surgical field was then irrigated using physiological saline at room temperature. After controlling bleeding, the subcutaneous tissues were sutured with absorbable suture (No. 3/0, coated Vicryl®, Ethicon, Inc., Belgium) and the skin with absorbable suture (No. 3/0, coated Vicryl®, Ethicon, Inc., Belgium). Intraperitoneal fentanyl 0.02 mg/kg was used to provide postoperative analgesia. Surgical infection prophylaxis was provided with intraperitoneally administered enrofloxacin 10 mg/kg (Baytril; Bayer Türk Kimya San. Ltd. Şti., İstanbul, Türkiye). Immediately after the procedure, the animals were allowed to engage in regular cage activity. They could walk and feed themselves, and no rehabilitation or activity restraints were applied.

After surgery, the glenohumeral joint was immobilized for eight weeks to wait for the development of adhesive capsulitis, as previously proven in different studies in the literature.[14,15]

At Week 8, the sutures fixing the scapula to the humerus were removed by surgical intervention through the same skin incision under general anesthesia.

Injection therapies

In the literature, different studies on rats have shown that the inflammatory process gives way to fibrosis in the third week of immobilization.[12,13] Therefore, in the third week of immobilization, injection applications were started. All injection applications were performed in the glenohumeral joint under fluoroscopic control using a 30-G fine needle. The CST group received a single dose of intraarticular 0.5 mg/kg betamethasone injection based on previous studies demonstrating its efficacy in reducing inflammation in animal models of FS without inducing systemic side effects.[15,16] Ozone gas was generated from pure oxygen using a medical ozone generator (Turkozone® Blue S Medical Ozone Generator, Istanbul, Türkiye), with the generator set to a concentration of 30 mg/mL. Due to the damaging effects of ozone on standard plastic syringes, specialized ozone-resistant syringes made of silicon-treated polypropylene were utilized. According to the literature, the therapeutic dose of ozone gas in rats ranges from 0.5 to 1 mg/kg. The ozone dose (1 mg/kg) was chosen in line with the therapeutic range (0.5 to 1 mg/kg) established in the literature, as it has been shown to be effective and safe in reducing fibrosis and inflammation in experimental studies.[17] This dosing ensures sufficient therapeutic effect while minimizing adverse reactions. The OT was administered four times at three-day intervals following the removal of sutures. The injections were given immediately to prevent ozone decomposition before administration.

Functional examination

After two weeks of suture removal, all three groups of rats were euthanized using 100 mg/kg of ketamine. The timing of euthanasia at Week 10 was selected based on the FS progression timeline documented in the literature. Adhesive capsulitis involves an initial inflammatory phase (Weeks 1-3), a fibrotic phase (Weeks 4-8), and a subsequent resolution phase (Weeks 9-12).[14,15] Euthanasia at Week 10 allowed us to capture the peak fibrosis stage, providing a robust platform to assess treatment effects on collagen deposition and joint mobility. The previous skin incision was reopened, and the shoulder joint was removed as a single unit for histopathological and functional examination of the shoulder joint and surrounding tissues. After the standard angle measurement template was drawn using a goniometer and fixed on the foam block, the scapula was placed on this template and fixed with pins. A standardized torque and rotational moment were created by hanging a brace on the intramedullary injector tip, and the possibility of fracture of the humerus was eliminated. When the shoulder joint was at 90 degrees, a 10-g weight was suspended from the junction of the humerus, and the needle with a rope and the addiction angle was measured, then the foam was rotated 180 degrees and the abduction angle was measured.

Histopathological examination

Histopathological evaluations, including capsule thickness and collagen type 3 staining, were performed by a single experienced pathologist using a standardized scoring protocol. To enhance consistency, all evaluations were repeated by the same pathologist, and the results were cross-verified for intra-observer reliability.

Histological examination

After the skin and subcutaneous tissue of the *en-bloc* specimens were excised, each specimen was placed in a separate container in a 10% neutral buffered formalin solution. The specimens were fixed in 10% buffered formalin for one week and then decalcified in 10% ethylenediaminetetraacetic acid/hydrochloric acid solution at pH 7.4 for four days. After decalcification, the specimens were embedded in paraffin blocks, and 5-μ-thick sections were obtained from the axillary pouch in the coronal plane. The specimens were stained with hematoxylin-eosin (H&E) for routine histopathology evaluation. The stained specimens were evaluated under a light microscope (Olympus BX51, Tokyo, Japan) for inflammation, capillary proliferation, fibroblast proliferation, collagen type 3 staining, the synovial surface structure of the recess axillaries and capsular thickness parameters. Evaluation under the light microscope was done as shown in Figure 1. The digital image taken at ×100 magnification was evaluated using the ImageJ software.

Immunohistochemical examination

The paraffin sections were initially dewaxed in three xylene solutions and then rinsed in graded ethanol to observe collagen type 3 using immunohistochemistry. Pepsin digestion was, then, carried out to expose the antigen. To deactivate endogenous peroxidase, the sections were subjected to 3% H_2O_2 treatment for 15 min at room temperature. Following a triple rinse with phosphate-buffered

Histopathological evaluation criteria	
Inflammation	No
	Mild
	Severe
Capillary proliferation	Capillary vessel count was averaged in two bilateral blind average (large magnification= ×40)
Fibroblastic proliferation	No
	Mild
	Severe
Collagen 3 staining	Mild
	Severe
Axillary pouch	Straight
	A few folds
	A lot of folds
Average capsule thickness (μ)	Measured as the perpendicular distance from the three thickest points in the axillary sac; the average value was taken

FIGURE 1. Histopathological evaluation criteria of frozen shoulder.

TABLE I
Descriptive data

	n	%	Mean±SD	Median	Range
Groups					
Control	9	32.14			
Corticosteroid	9	32.14			
Ozone	10	35.71			
Histopathologic examination					
Inflammation					
No inflammation	17	60.71			
Mild	8	28.57			
Severe	3	10.71			
Capillary proliferation			6.71±2.97	6	3-14
Fibroblastic proliferation					
No proliferation	9	32.14			
Mild	14	50.00			
Severe	5	17.86			
Collagen 3 staining					
Mild	17	60.71			
Severe	11	39.29			
Axillary pouch					
Straight	17	60.71			
A few folds	9	32.14			
A lot of folds	2	7.14			
Average capsule thickness (μ)			141.71±70.10	126.61	50.34-300.55
Range of motion (degree)					
Abduction			40±9.62	35	25-60
Internal rotation			47.86±8.54	50	35-65
External rotation			45±8.71	45	30-60

SD: Standard deviation.

saline (PBS) for 5 min, non-specific reactions were blocked by incubating the sections with PBS containing 5% normal goat serum for 10 min at room temperature. After another rinse in PBS, the sections were left to incubate overnight at 4°C with a monoclonal mouse anti-rat collagen type 3 antibody (Calbiochem, dilution 1:400) and, then, rinsed in PBS. Subsequently, the sections were exposed to a biotin-labeled goat anti-mouse immunoglobulin antibody for 30 min, followed by rinsing in PBS. The sections were then treated with peroxidase-labeled streptavidin for 20 min at room temperature. Finally, the chromogen, 3,3'-diaminobenzidine tetrahydrochloride, was used for the detection step. The sections were, then, counterstained with hematoxylin solution and rinsed with distilled water. Of note, PBS was utilized as a primary antibody for negative controls, and all slides were stained in one session.

Statistical analysis

Statistical analysis was performed using the SPSS version 25.0 software (IBM Corp., Armonk, NY, USA). The conformity of the variables to normal distribution was examined using the Shapiro-Wilk test. Descriptive data were expressed in mean ± standard deviation (SD), median (min-max) or number and frequency, where applicable. The Kruskal-Wallis test was used to evaluate non-normally distributed (non-parametric) variables among more than two groups. Differences between the groups were determined using the Dunn's Bonferroni test. Relationships between categorical variables were analyzed using the Fisher-Freeman-Halton exact test. In addition to p-values, effect sizes were calculated for pairwise comparisons to provide a measure of the magnitude of observed differences. Confidence intervals (95%) were also reported to enhance the interpretability of key outcomes. For non-parametric variables, effect sizes were calculated using eta-squared (η^2) values, while Cohen's d was applied for normally distributed variables. A p value of <0.05 was considered statistically significant results.

RESULTS

In this study, one rat from Group C and one rat from Group CST died due to wound infection. No complications, such as surgery-related infection, hematoma, or neurovascular injury, were observed

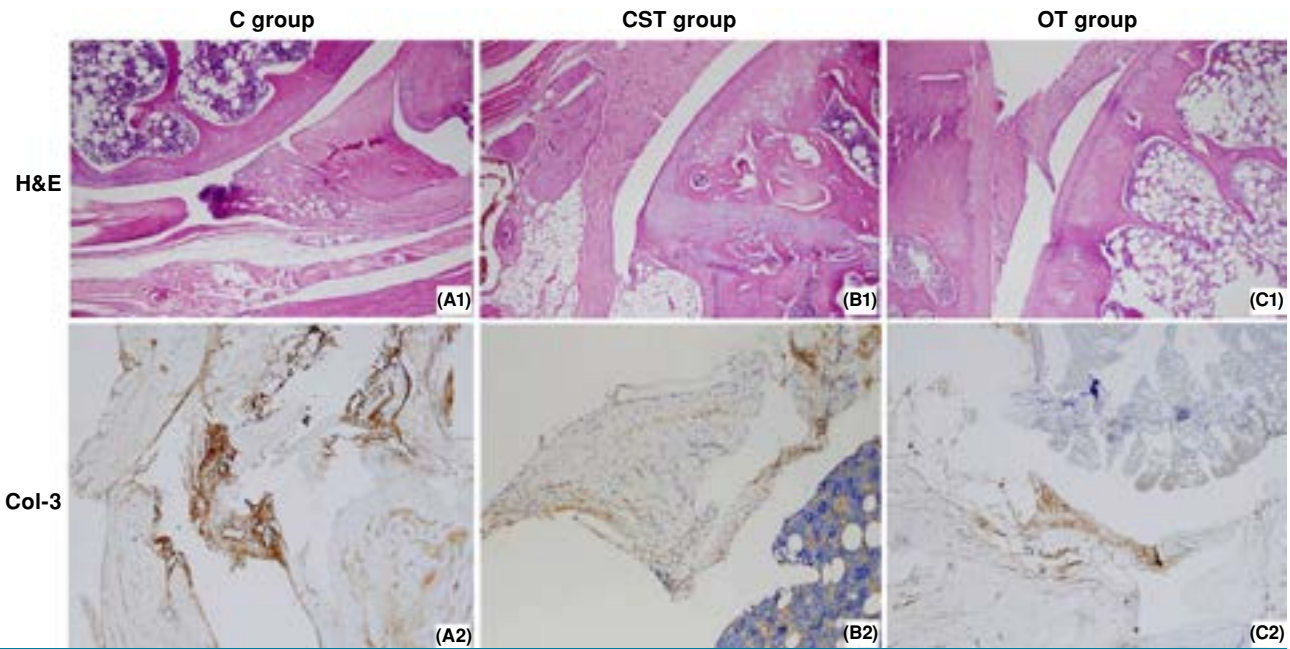

FIGURE 2. Histological analysis of the glenohumeral joint. (H&E, ×40). **(A1)** Shoulder in C group. **(A2)** Increased inflammation, fibroblastic proliferation, capillary vessel proliferation, and intense staining with immunohistochemical collagen type 3. **(B1)** Shoulder in CST group. Decreased capillary vessel proliferation is observed compared to the control group and no inflammation and fibroblastic proliferation is observed. **(B2)** Barely perceptible staining with immunohistochemical collagen type 3. **(C1)** Shoulder in OT group. Decreased capillary vessel proliferation, inflammation, and fibroblastic proliferation compared to the control group. **(C2)** Mild staining with immunohistochemical collagen type 3.
C: Control group; CST: Corticosteroid treatment group; OT: Ozone treatment group; Col-III: Collagen type 3 staining.

TABLE II

Comparison of histopathological data and joint range of motion values of the groups

	Control group					Corticosteroid group					Ozone group					
	n	%	Mean±SD	Median	Range	n	%	Mean±SD	Median	Range	n	%	Mean±SD	Median	Range	*p*
Histopathologic examination																
Inflammation																**<0.001**
No inflammation	1	11.11[a]				9	100.00[b]				7	70.00[b]				
Mild	5	55.56[a]				-	-[b]				3	30.00[a,b]				
Severe	3	33.33				-	-				-	-				
Capillary proliferation			10.22±2.22[a]	10[a]	7-14[a]			4.44±1.01[b]	4[b]	3-6[b]			5.6±1.51[b]	5[b]	4-9[b]	**<0.001**
Fibroblastic proliferation																**0.002**
No inflammation	-	-[a]				7	77.78[b]				7	70.00[a]				
Mild	6	66.67[a]				1	11.11[b]				2	20.00[b]				
Severe	3	33.33				1	11.11				1	10.00				
Collagen 3 staining																**0.022**
Mild	2	22.22[a]				7	77.78[a,b]				8	80.00[b]				
Severe	7	77.78[a]				2	22.22[a,b]				2	20.00[b]				
Axillary pouch																0.239
Straight	7	77.78				3	33.33				7	70.00				
A few folds	2	22.22				5	55.56				2	20.00				
A lot of folds	-	-				1	11.11				1	10.00				
Average capsule thickness (μ)			226.33±39.68	216.41	180.39-300.55[a]			90.14±28.13	86.	56.05-131.35[b]			111.95±42.85	106.65	50.34-169.15[b]	**<0.001**
Range of motion (degree)																
Abduction			31.11±4.86	35	25-35[a]			42.22±7.95	40	35-55[b]			46±8.76	45	35-60[b]	**0.001**
Internal rotation			41.67±5.00	40	35-50[a]			49.44±6.35	50	40-60[b]			52±10.06	52.5	35-65[b]	**0.020**
External rotation			36.67±2.50	35	35-40[a]			52.22±6.18	50	40-60[b]			46±8.10	45	30-55[b]	**0.001**

SD: Standard deviation; Different letters (e.g., a, b) in each row indicate significant differences between groups ($p<0.05$). Tests used: Kruskal-Wallis test, Fisher-Freeman-Halton Exact test.

in the other rats. Also, no complications related to the treatments applied in the groups occurred.

Descriptive data are given in Table I. Twenty-eight rats were included in the study. In the study, the majority of subjects showed no inflammation (60.71%), mild fibroblastic proliferation (50.00%), and mild collagen type 3 staining (60.71%). The axillary pouch was predominantly straight (60.71%) (Figure 2). The mean capsule thickness was 141.71±70.10 μm, with the ROM showing a mean abduction of 40±9.62 degrees, internal rotation of 47.86±8.54 degrees, and external rotation of 45±8.71 degrees.

Table II compares histopathological data and joint ROM values obtained from the study groups. Based on the histopathological evaluation of inflammation, no inflammation was observed in the CST group (n=9). In contrast, in the OT group, three subjects had mild inflammation, and seven subjects had no inflammation (Figure 3). The results showed that inflammation was significantly less in the CST and OT groups than in the control group. ($p<0.001$) Capillary proliferation in the control group was significantly higher than in the CST ($p<0.001$) and OT groups ($p=0.003$) (Figure 4). The results of fibroblastic proliferation showed that mild proliferation was observed in six subjects (66.67%) in Group C, while no fibroblastic proliferation was observed in seven subjects (77.78%) in Group CST and seven subjects (70%) in Group (Figure 5). Fibroblastic proliferation was statistically significantly less in group CST and OT ($p=0.002$). Mild staining with collagen type 3 was observed in seven subjects (77.78%) in

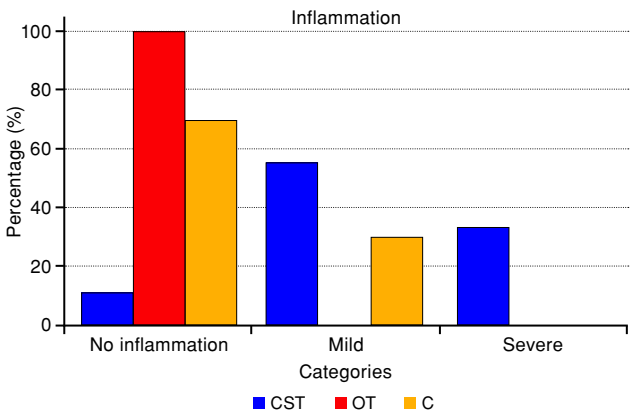

FIGURE 3. Percentage distribution of inflammation degrees among groups.
C: Control group; CST: Corticosteroid treatment group; OT: Ozone treatment group.

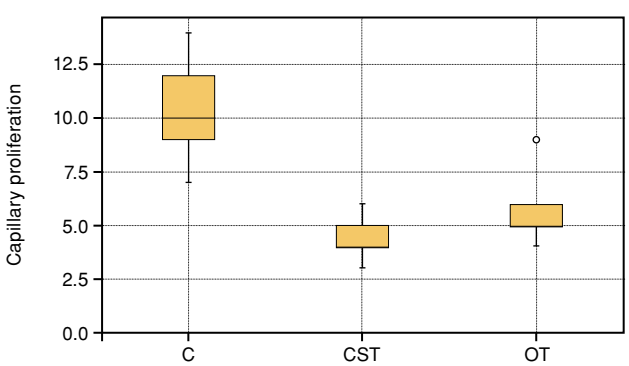

FIGURE 4. Distribution of capillary proliferation values according to different groups.
C: Control group; CST: Corticosteroid treatment group; OT: Ozone treatment group.

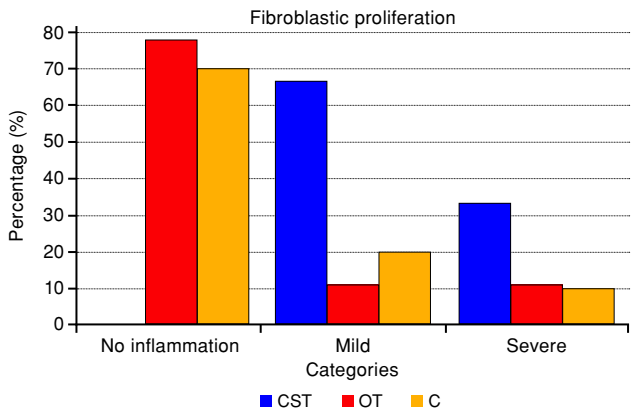

FIGURE 5. Percentage distribution of fibroblastic proliferation among groups.
C: Control group; CST: Corticosteroid treatment group; OT: Ozone treatment group.

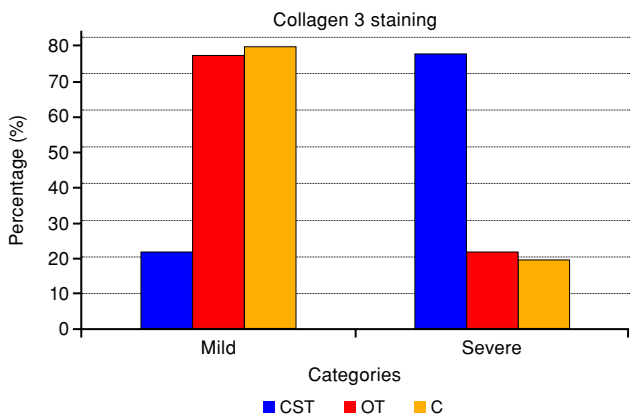

FIGURE 6. Percentage distribution of collagen type 3 staining among groups.
C: Control group; CST: Corticosteroid treatment group; OT: Ozone treatment group.

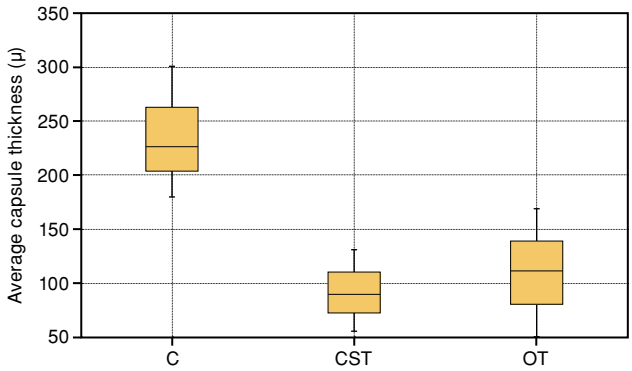

FIGURE 7. Distribution of average capsule thickness according to different groups.
C: Control group; CST: Corticosteroid treatment group; OT: Ozone treatment group.

the CST group and eight subjects (80%) in the OT group. In contrast, severe staining was observed in seven subjects (77.78%) in the C group (Figure 6). Staining with collagen type III was significantly lower in the CST and OT groups compared to the C group (p=0.022). The mean capsule thickness was significantly greater in group C than in the other groups (<0.001) (Figure 7).

Table II gives the statistical comparison of the values obtained from abduction, internal rotation, and external rotation measurements on the obtained samples. Abduction, internal rotation and external rotation measurements were significantly higher in the CST and OT groups than in the C group (p=0.025, p=0.001, p=0.032, p=0.025, p<0.001, and p=0.037, respectively) (Figure 8).

DISCUSSION

The search for new treatment methods in early diagnosis of FS, which is frequently seen in the clinic and characterized by shoulder pain and restriction of shoulder movements, and for preventing disease progression is still ongoing. Although progress has been made in elucidating disease pathophysiology with cellular and molecular studies in recent years, the transfer of possible agents that may be valuable in treatment to clinical use is quite limited.[1-3,5]

In the present study, we evaluated the efficacy of OT in an FS animal model. Our study results showed that intraarticular OT reduced inflammation, capillary proliferation, fibroblastic proliferation, and collagen type 3 amount with similar effectiveness to the most applied corticosteroid treatment in the clinic, thereby

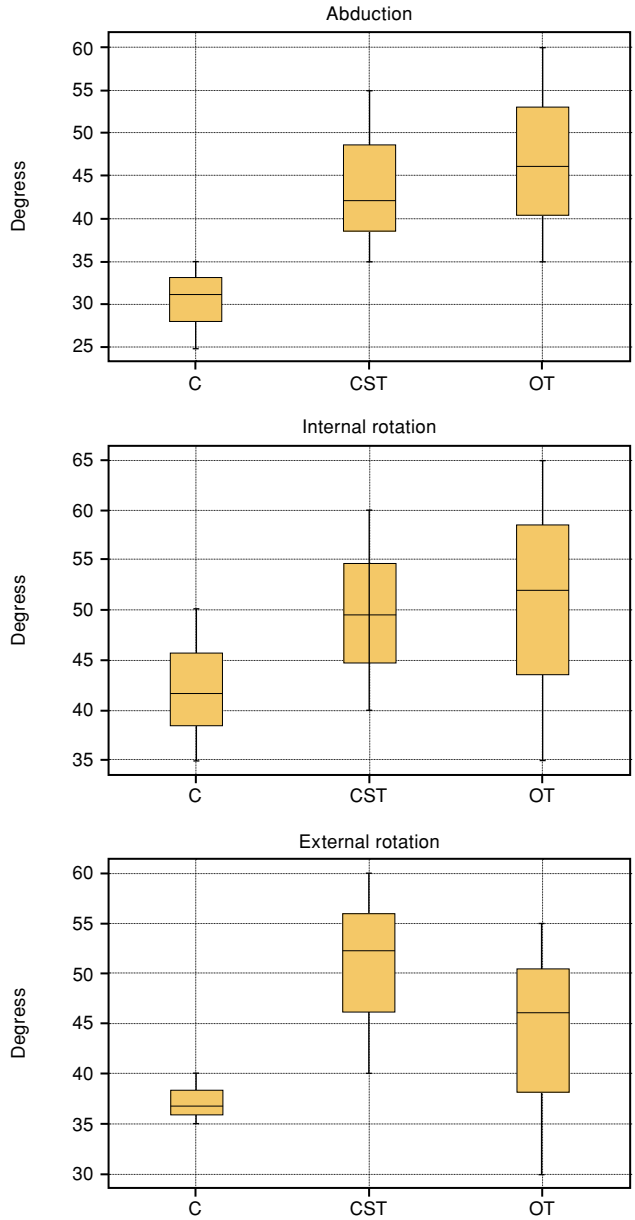

FIGURE 8. Distribution of abduction, internal rotation and external rotation values according to different groups.
C: Control group; CST: Corticosteroid treatment group; OT: Ozone treatment group.

reducing capsule thickness and increasing joint ROM.[18] While OT demonstrated a promising potential in reducing inflammation and fibrosis in FS, it is of utmost importance to acknowledge the rapid anti-inflammatory effects of corticosteroids, which make them a preferred choice in acute inflammatory scenarios. Corticosteroids act rapidly to suppress inflammation by targeting multiple inflammatory pathways, which can provide immediate symptom relief. This rapid

onset of action is particularly advantageous in patients presenting with severe pain and marked inflammation. However, the long-term use of these agents is limited by its side effects, such as tissue atrophy and systemic complications. In contrast, OT offers a more balanced approach with its antifibrotic and immunomodulatory effects, making it a promising alternative or adjunctive therapy in the chronic phases of FS.

Initially FS was categorized into four stages; however, it was later adjusted and condensed to three clinically relevant stages. Each stage is linked to distinct clinical and histopathological observations. During the early stages of FS, there is synovial hyperplasia caused by inflammation-induced hypervascularization and neurogenesis, as well as the proliferation of fibroblasts. As the condition progresses, the inflammatory process transitions to fibrosis, characterized by increased fibroblast activity and abnormal deposition of ECM.[2]

Increasing evidence suggests that FS is driven by chronic low-grade inflammation. Ozone therapy modulates this process by activating Nrf2-Keap1 signaling, enhancing the antioxidant response, and suppressing proinflammatory pathways, including cytokines like IL-6, TNF-α, and IL-1β.[1,5,8] Ozone therapy has also been shown to reduce the activity of advanced glycation end products (AGEs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling, both critical in FS progression.[19,20] These mechanisms likely contribute to the observed reduction in inflammation and fibrosis in our study, consistent with findings reported in the literature.[21,22] However, the absence of standardized OT dosing and application methods may explain its relatively lower efficacy compared to corticosteroids. Inflammation-related capillary proliferation is a common finding in FS studies, with increased CD34 and vascular endothelial growth factor (VEGF) expression observed in the synovium and joint capsule.[1,5] Systemic OT has been shown to significantly reduce VEGF levels in various models, including diabetic retinopathy and epidural fibrosis.[17,23] In this study, both corticosteroids and OT administered intraarticularly significantly reduced capillary proliferation. Local application of OT may enhance its retention and penetration into tissues, contributing to its effectiveness. Of note, TGF-β1 is a key cytokine in FS, inducing the fibrotic process by activating fibroblasts and promoting their differentiation into myofibroblasts. This leads to increased ECM production, with collagen type 3 replacing mature collagen type 1, and disruption of the matrix metalloproteinase and tissue inhibitor of metalloproteinases (MMP/TIMP) balance, resulting in excessive ECM deposition and fibrosis.[1,2,5] In addition, AGEs further exacerbate this imbalance by preventing collagen degradation through cross-linking.[1] Studies have demonstrated antifibrotic effects of OT in various models, including pyelonephritis, hepatic fibrosis, and renal interstitial fibrosis.[24-26] Our study results showed that intraarticular OT significantly reduced capsule thickness, similar to corticosteroid treatment, consistent with findings from previous animal models.[17] Frozen shoulder patients with restricted shoulder joint movements most frequently have restricted external rotation. Studies conducted on the FS model created in rats demonstrated that abduction angle was frequently restricted, unlike in humans.[10,13] Therefore, abduction angles are mainly evaluated in FS studies.[15,16,27] In their study, Çınar et al.[15] reported that oral and intraarticular corticosteroid treatment improved abduction angle compared to the control group. The most significant improvement was achieved in the group that received intraarticular injection. Similarly, our study showed a significant improvement in all joint ROM angles in the CST and OT groups compared to the control group, consistent with histopathological data.

According to previous studies and the present study, OT seems to be an effective treatment method for pathologies in which fibrosis plays a role in pathophysiology. In recent years, studies on possible treatment methods targeting the molecular mechanisms which play a role in the development of FS have gained momentum with the beginning of a better understanding of the pathophysiology. In these studies, it has been shown that subacromial collagenase injection, tetrandrine, calcitonin, relaxin-2, hyaluronic acid/pluronic F-127 hydrogel, and micro-ribonucleic acid (Mi-RNA)-211-5p applications can be effective in preventing fibrosis and preserving the ROM in the treatment of FS.[16,27-31] These studies were mostly conducted on experimental animals, and time is still needed for clinical application. On the other hand, OT is already used in many different areas in the clinic, particularly in musculoskeletal system pathologies such as low back pain, knee osteoarthritis, and lateral epicondylitis. Ozone therapy is administered under clinician supervision, has a low risk of side effects, and is easily accessible. This may

improve patient compliance, reduce costs, and increase treatment success in patients with FS. Low side effect profile and relatively straightforward application under clinician supervision of OT make it an accessible option for patients. Unlike corticosteroids, OT does not require stringent systemic monitoring, which can reduce healthcare costs and improve patient compliance. However, its clinical implementation faces challenges, including the availability of specialized equipment and training for clinicians. Additionally, the cost of ozone generators and syringes may pose barriers in under-resourced healthcare settings. Future studies should focus on optimizing OT protocols to enhance its cost-effectiveness and accessibility, particularly in comparison to corticosteroids, which remains more widely available. On the other hand, in clinical FS management, OT and CST may serve complementary roles. While CST is effective in rapidly controlling acute inflammation, OT may be integrated into treatment protocols to address chronic inflammation and prevent fibrosis progression. This combination approach may leverage the strengths of both therapies, optimizing outcomes while minimizing side effects associated with prolonged CST use. To illustrate, initiating CST in the early inflammatory phase followed by OT during the fibrotic phase may offer a holistic strategy for FS treatment.

Nonetheless, this study has some limitations. The main limitation is that it is an animal experiment and based on a rat model. The results from animal model experiments may not directly apply to clinical settings, as interspecies physiological variations can lead to differences in treatment responses and outcomes. Second, our study created a secondary FS model, and evaluating the primary FS is impossible. Third, the effects of different ozone doses and application methods (rectal, intraperitoneal, and periarticular) were unable to be assessed in this study. Moreover, although many studies have evaluated the impact of OT on different pathologies in rats, the standard dose is unknown. A notable limitation to this study is the absence of long-term follow-up data to assess the durability of improvements in ROM and fibrosis reduction. Frozen shoulder is a condition with a protracted natural history, and recurrence of fibrosis or deterioration in ROM over time could significantly impact treatment outcomes. Another limitation is that histopathological evaluations were performed by a single pathologist, which may introduce a degree of subjective bias. Although a standardized scoring system and repeated assessments were employed to minimize intra-observer variability, future studies should consider including multiple observers or using automated image analysis to further enhance reliability. Finally, while this study provides valuable insights into the effects of OT on FS, the absence of molecular data on key pathways such as TGF-β1 signaling and MMP/TIMP dynamics limits the mechanistic depth of our findings. These analyses were not performed due to budget constraints but represent an important area for future research to fully elucidate OT's antifibrotic mechanisms.

In conclusion, studies are ongoing to elucidate the pathophysiology of FS, which was defined more than a century ago as causing pain and progressive loss of ROM in patients' shoulders, and to determine the gold-standard treatment method. Our study results suggest that OT, which is increasingly used clinically in wound healing problems, ischemic diseases, chronic inflammatory diseases, and fibrotic pathologies, can be an effective treatment method for FS. However, future clinical studies are needed to elucidate better the mechanism of action of OT at the molecular level.

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

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