Article

TOWARDS THE OPTIMIZATION OF OZONE THERAPY IN KNEE OSTEOARTHRITIS: FACTORS INFLUENCING TREATMENT OUTCOMES

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ABSTRACT

The aim of this study is to determine the optimal ozone concentration for achieving the longest-lasting therapeutic benefits in ozone therapy for osteoarthritic knees. Additionally, we aim to assess the duration of effectiveness following each intra-articular ozone administration and, based on this, determine the appropriate injection frequency. Moreover, investigate whether the therapeutic efficacy of each ozone injection follows the circadian rhythm, which could necessitate selecting a specific time for administration. Methods: The study was conducted in two phases. In the first phase, patients who had undergone intra-articular ozone injections and achieved complete symptomatic relief were categorized into three groups based on the ozone concentration administered. In the second phase, the included patients were divided into four groups based on the time interval after injection at which they reported the maximum therapeutic effect (1-6 days, 7-14 days, 14-21 days, and 21-28 days). We also evaluated whether the ozone concentration influenced the timing of peak efficacy. Additionally, the impact of the administration time on therapeutic outcomes was examined by allocating patients into three different time-of-day groups for ozone injection. Findings from the first phase indicate that an ozone concentration of 30 µg provides the longest-lasting therapeutic effect following intra-articular administration, with a mean duration of 2.17 years. The second phase demonstrated that the optimal therapeutic effect following a single injection occurred at approximately 21 days, regardless of the ozone concentration used. Furthermore, results suggest that the time of day of administration does not significantly affect therapeutic efficacy. Our study suggests that medical ozone therapy provides a longer-lasting therapeutic effect compared to other commonly used intra-articular injection methods for osteoarthritic knees. Despite the fact that ozone, upon contact with biological fluids, ceases to exist as an independent entity within less than a minute, the therapeutic benefits of even a single injection peak around three weeks post-administration. This observation can be explained by ozone's biochemical and biological mechanisms of action and supports the recommendation of administering treatment every three to four weeks. Although literature suggests that biological factors influenced by ozone may follow a circadian pattern, our study did not confirm this association. Consequently, ozone therapy can be performed at any time of day with consistent therapeutic outcomes.

KEYWORDS: *ozone therapy, osteoarthritis, intra-articular injections, therapeutic efficacy, circadian rhythm*

INTRODUCTION

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Osteoarthritis (OA) of the knee is a prevalent degenerative joint disorder primarily affecting elderly individuals. It is characterized by the progressive degeneration of articular cartilage, leading to pain, stiffness, and a loss of joint function. The etiology of knee OA is multifactorial, involving both known and idiopathic causes. Identifiable causes include post-traumatic injuries, congenital abnormalities, and malalignment of the knee in varus or valgus deformities (1, 2).

The prevalence of knee OA increases with age, affecting approximately 40% of individuals by the age of 70. Women are more frequently affected than men, suggesting a potential hormonal or biomechanical predisposition specific to women (1, 2).

Articular cartilage is primarily composed of type II collagen, proteoglycans, chondrocytes, and water. In a healthy joint, articular cartilage maintains a dynamic equilibrium between the synthesis and degradation of its components, ensuring structural integrity and function. However, in OA, this balance is disrupted due to the overexpression of matrix metalloproteinases (MMPs), a group of degradative enzymes responsible for the breakdown of collagen and proteoglycans (1, 2).

In the early stages of OA, chondrocytes attempt to counteract cartilage degradation by secreting tissue inhibitors of metalloproteinases (TIMPs) and increasing proteoglycan synthesis. However, this compensatory mechanism proves insufficient in preventing disease progression. The resulting imbalance leads to a net loss of proteoglycans despite their increased synthesis, an increase in water content within the cartilage matrix, and a disorganized collagen network. These changes contribute to the progressive loss of cartilage elasticity and resilience (1, 2).

Macroscopically, these pathological alterations manifest as cartilage softening, cracking, fissuring, and eventual erosion of the articular surface. Over time, these degenerative changes lead to joint space narrowing, osteophyte formation, subchondral bone sclerosis, and chronic inflammation, all of which contribute to the clinical symptoms of knee OA. Understanding the molecular mechanisms underlying OA progression is essential for developing targeted therapeutic strategies aimed at preserving joint function and delaying disease progression.

Ozone therapy is a globally recognized medical intervention known for its broad therapeutic benefits, cost-effectiveness, and minimal side effects when administered according to established protocols. These protocols include those outlined by the Italian Federation NUOVA FIO (Art. 6-L. 8 March 2017, n. 24), the Madrid Protocol established by the International Scientific Committee of Ozone Therapy in the Madrid Declaration (2nd Edition, 2015), and the Low-Dose Ozone Concept, which provides treatment strategy guidelines (3).

Ozone therapy typically involves the administration of a carefully selected concentration of ozone (O_3) mixed with oxygen (O_2). Ozone is generated on-site from pure oxygen using specialized medical devices, ensuring precise concentration control. Despite the existence of standardized guidelines, variations in treatment protocols persist. For instance, recommended ozone concentrations for subcutaneous injection range from 2-5 μg/mL O_3/O_2 (without local anesthesia) to 10-15 μg/mL O_3/O_2 (with local anesthesia), according to Viebahn, while other protocols suggest concentrations such as 3 μg/mL O_3/O_2 (SIIOT), 8-10 μg/mL O_3/O_2 (NUOVA FIO), 10 μg/mL O_3/O_2 (Russian protocols), and 5-20 μg/mL O_3/O_2 (Madrid Declaration). This variability has resulted in confusion among practitioners, underscoring the need for further standardization of protocols.

Once administered, ozone dissolves in the extracellular fluid, where it reacts with antioxidants, polyunsaturated fatty acids (PUFAs), proteins, and carbohydrates, leading to the production of hydrogen peroxide (H_2O_2) and 4-hydroxy-2-nonenal (4-HNE). H_2O_2 enters erythrocytes, activating glycolysis and transiently increasing ATP and 2,3-bisphosphoglycerate (2,3-DPG). The increase in 2,3-DPG shifts the oxyhemoglobin dissociation curve to the right, facilitating the release of oxygen into ischemic tissues (3, 4). This mechanism is particularly relevant for improving microcirculation in contracting muscles, ligaments, tendons (e.g., partial supraspinatus tendon ruptures), and trigger points.

Simultaneously, 4-HNE, generated from PUFA peroxidation, circulates throughout the body, accumulating in chronically inflamed tissues, and enters cells under oxidative stress conditions. It interacts with Keap1, releasing nuclear factor erythroid 2-related factor 2 (Nrf2), which is normally bound to Keap1. The release of Nrf2 enables its translocation into the nucleus, where it binds to antioxidant response elements (AREs) on gene promoters, inducing the expression of over 230 protective genes. These include genes encoding for glutathione (GSH), GSH reductase, GSH transferase, GSH peroxidases, thioredoxin, thioredoxin reductase, NADPH, NAD(P)-quinone oxidoreductase 1 (NQO1), and heme-oxygenase (5). Additionally, Nrf2 is involved in glucose homeostasis and metabolic reprogramming, redirecting anabolic pathways, inhibiting lipogenesis, and improving insulin sensitivity, thereby offering potential benefits in metabolic disorders such as obesity and diabetes (6-10).

Beyond metabolic regulation, ozone therapy has demonstrated effects on hormonal balance, modulating cortisol, aldosterone, and thyroid-stimulating hormone (TSH) levels, contributing to systemic homeostasis (11-15). Specifically, an increase in cortisol levels has been observed following ozone autohemotherapy, which stabilizes within an individual's normal physiological range over time (11).

Ozone therapy also plays a significant role in neurological function and pain management. Ozone selectively activates transient receptor potential ankyrin 1 (TRPA1) ion channels while sparing transient receptor potential vanilloid 1 (TRPV1) channels, a critical distinction for the treatment of neuropathic pain (16). In knee joint structures, nociceptors of the TRPV1 type are abundant (17).

Additionally, ozone promotes neural cell migration and proliferation, as demonstrated in studies involving Schwann cells following sciatic nerve transection and in neural stem cells. At a concentration of approximately 11 μg/mL, ozone significantly accelerated cell migration (18, 19). Furthermore, ozone therapy reduces oxidative stress and apoptosis (via downregulation of caspase-3), limits gliosis (via downregulation of GFAP), and enhances neurogenesis (by upregulating Ki-67 expression), thereby supporting its neuroprotective potential (20-22).

At the immunological level, ozone exerts anti-inflammatory and immunomodulatory effects, inhibiting the synthesis of matrix metalloproteinases (MMPs), nitric oxide (NO), prostaglandin E2 (PGE2), interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interferon-beta (IFN-β). Concurrently, ozone enhances the production of anti-inflammatory cytokines, such as interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-13 (IL-13), transforming growth factor-beta (TGF-β), and insulin-like growth factor-1 (IGF-1) (23).

The effectiveness and safety of ozone therapy are influenced by individual responses to oxidative stress, making the personalization of ozone dosage crucial. Spectroscopic techniques, including intrinsic fluorescence, circular dichroism, SDS-PAGE, and dynamic light scattering (DLS), have been utilized to evaluate hemoglobin modifications following ozonation. These analyses demonstrated that exposure to ozone at inappropriate concentrations can result in hemoglobin oligomerization, potentially leading to detrimental effects. Based on experimental findings, it is essential to determine a tailored ozone concentration to ensure the safety and efficacy of autohemotherapy (24, 25).

To date, there is considerable variation in the recommendations within the literature regarding the intervals between ozone therapy sessions and the different concentrations used, as suggested by various authors. The following intervals have been proposed in different studies:

- twice a week: one study administered a 20 ml ozone-oxygen mixture (ozone concentration of 20 μg/ml) via intra-articular injection through the inferomedial approach, with therapy lasting for six weeks and administered twice a week (26);
- once a week: another study reported administering a 10 ml ozone injection at a concentration of 15 μg/ml once a week for four weeks (27);
- every 15 days with dextrose and somatotropin: a separate study incorporated ozone therapy along with dextrose and somatotropin, with sessions conducted every 15 days, using 10 ml of ozone at 25 μg/ml (28);
- three-monthly intra-articular injections of 10 ml ozone at 30 μg/ml vs. 40 mg methylprednisolone: a study comparing the effects of these two treatments found an 80% success rate in the ozone group compared to a 60% success rate in the corticosteroid group after three months of follow-up. The authors also noted that when these injections are administered simultaneously, the outcomes are superior compared to each treatment individually (29).

Currently, no statistical study has suggested or indicated that a specific timing or frequency of ozone therapy sessions could optimize the results. This gap in the existing literature highlights the need for further research to establish an optimal regimen for ozone therapy.

Despite the compelling scientific evidence supporting ozone therapy, the lack of universally standardized protocols remains a challenge, leading to variations in clinical practice. Establishing consensus guidelines based on controlled clinical trials is crucial to optimizing therapeutic outcomes and ensuring reproducibility.

MATERIALS AND METHODS

A total of 283 patients were included in this study, comprising 128 patients in Step 1 and 155 patients in Step 2. The cohort consisted of 90 females and 38 males in Step 1, and the same gender distribution was maintained across Step 2. The patients had a clinical diagnosis of knee OA, and they were treated with intra-articular ozone therapy as part of their routine clinical care. The study was conducted over a period of 30 years, with patient follow-up and data collection ensuring the long-term analysis of treatment outcomes. The inclusion criteria required patients to have symptomatic knee OA and to be eligible for ozone therapy based on their clinical assessment. Exclusion criteria included pregnancy, active

infection at the injection site, significant comorbidities that might interfere with the study (e.g., uncontrolled diabetes), and prior allergic reactions to ozone or any of the components used in the procedure.

Methods

The treatment protocol involved subpatellar injections of O3/O2, typically performed without ultrasound guidance in normal-weight patients and under ultrasound (US) guidance in obese patients. The ultrasound guidance was provided using a Sonosite® TURBO X system with a linear transducer (HFL50xp, 15-6 MHz), and all procedures were performed by the same physician, one of the authors. The ozone generator used was an Alnitec (CR, Italy), capable of producing ozone concentrations ranging from 1 to 100 μg/ml, with continuous variability and precise control monitored by a high-precision infrared photometer.

The needles used for injections were generally BD Microlance 30G x 1-1/2 or 27G x 1-1/2, with 23G x 2-3/4 needles employed in obese patients when necessary. Injections were routinely performed without local anesthesia, although in rare instances, for sensitive patients, a 2% preservative-free buffered lidocaine (Demo Pharmaceutical, Athens, Greece) was used. The lidocaine (1/2-1 ml) was diluted to a 0.5% concentration and administered to the overlying skin and underlying fascia at the injection site for patient comfort. Following the injection, patients reported a brief sensation of bruising lasting 1-2 minutes; no adverse reactions were observed. Each patient received 15 ml of the medical ozone treatment (O3/O2 mixture) per injection.

Statical analysis

Statistical analysis of the results was achieved using the three-way ANNOVA method.

RESULTS

Our approach was conducted in two distinct stages: Step 1: the clinical outcomes of 128 patients with knee OA were analyzed to determine the optimal ozone concentration that provides the longest-lasting symptom relief following treatment completion. Over the past 30 years, we have treated a substantial number of OA patients with intra-articular ozone injections. A total of 128 patients (90 females and 38 males) were followed up efficiently. By evaluating their outcomes in relation to the ozone concentration administered, we determined that a concentration of 30 μg of ozone appeared to optimize the duration of therapeutic effects. Concentrations exceeding 30 μg did not yield additional benefits in terms of treatment duration, suggesting that higher doses are unnecessary. In fact, the same therapeutic effect was achieved with either 30 μg of ozone or concentrations between 31-40 μg. However, concentrations below 30 μg were associated with a shorter duration of symptom relief (Fig. 1). Regarding treatment longevity, the average duration of therapeutic effects was found to be 2.17 years.

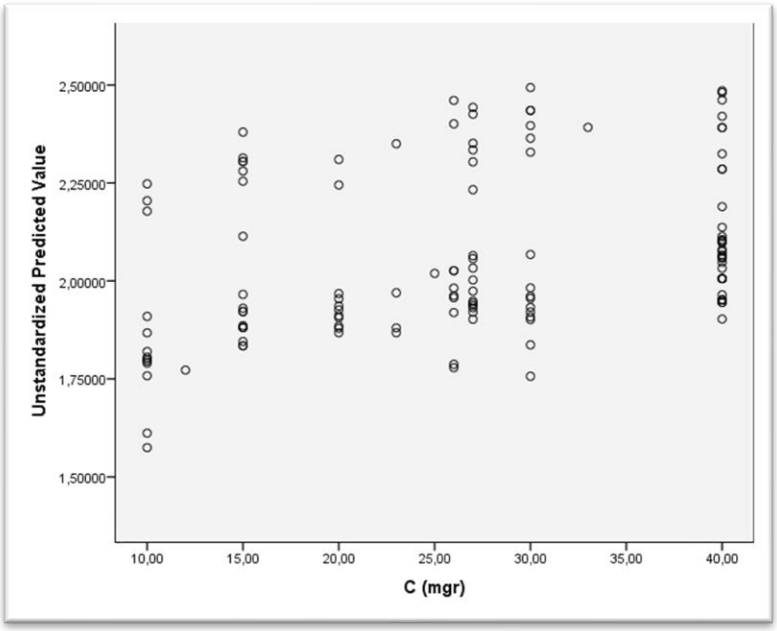

Fig. 1. *Predicted values of the duration of the therapeutic outcome in relation to the concentration of ozone.*

Step 2: A total of 155 patients were studied and divided into three time-based groups to evaluate the effects of circadian rhythms on the efficacy of ozone therapy. Given that certain biological factors fluctuate throughout the day, this study aimed to determine whether the timing of ozone administration influenced therapeutic outcomes.

Additionally, this study investigated the effects of delayed treatment sessions by dividing patients into four groups based on the number of days they delayed their treatment, as some patients were unable to attend their scheduled weekly appointments. Four different ozone concentrations were administered. Patients were asked to report when they experienced peak improvement following ozone injections, allowing us to assess the optimal therapeutic window and the impact of delayed administration on the effectiveness of treatment.

A three-way ANOVA was conducted on a final sample of 155 patients, with the dependent variable being the percentage of improvement and the independent variables including the time of day (1 = 9:00 - 11:00, 2 = 11:00 - 14:00 & 16:30 - 21:00), the days after therapy (1-5, 6-15, 16-25, 25+), and the concentration of ozone (25, 30, 35, and 40) (Table I).

Table I. *A three-way ANOVA regarding the % of improvement after ozone therapy.*

Source	Type III Sum of Squares	df	Mean Square	F	p	Partial Eta Squared
Corrected Model	47613.281[a]	33	1442.827	2.091	.002	.363
Intercept	103668.067	1	103668.067	150.243	.000	.554
time	2203.928	2	1101.964	1.597	.207	.026
days	12553.535	3	4184.512	6.064	.001	.131
concentration	3660.468	3	1220.156	1.768	.157	.042
time * days	5003.369	6	833.895	1.209	.307	.057
time * concentration	1815.501	5	363.100	.526	.756	.021
days * concentration	2874.721	7	410.674	.595	.759	.033
time * days * concentration	2550.167	6	425.028	.616	.717	.030
Error	83490.589	121	690.005			
Total	370775.000	155				
Corrected Total	131103.871	154				

a. R Squared = .363 (Adjusted R Squared = .189)

The main effects of time, $F(2, 121) = 1.597$, $p = .207$, $\eta p^2 = .026$, and concentration, $F(3, 121) = 1.768$, $p = .157$, $\eta p^2 = .042$, were not statistically significant. Similarly, the two-way interactions of time * days, $F(6, 121) = 1.209$, $p = .307$, $\eta p^2 = .057$, time * concentration, $F(5, 121) = .526$, $p = .756$, $\eta p^2 = .021$, and days * concentration, $F(7, 121) = .595$, $p = .759$, $\eta p^2 = .033$, were not significant. The three-way interaction of time * days * concentration, $F(6, 121) = .616$, $p = .717$, $\eta p^2 = .030$, also failed to reach statistical significance.

However, the main effect of days was statistically significant, $F(3, 121) = 6.064$, $p = .001$, $\eta p^2 = .131$. A post hoc Bonferroni test revealed that patients who received their initial dosage more than 25 days prior (M = 70.29%, SD = 28.64%) showed greater improvement than those who received their treatment after 16-25 days (M = 45.23%, SD = 27.19%, p = .045), 6-15 days (M = 34.77%, SD = 27.66%, p = .000), and 1-5 days (M = 28.25%, SD = 18.87%, p = .000). These percentages of improvement are illustrated in Fig. 2.

In Fig. 3 and 4, there is an indication that the maximum percentage of improvement was achieved regardless of the time of day that the ozone dosage was administered, as well as for ozone concentrations of 25 or 30. However, in Fig. 5 and 6, the available data do not support this claim conclusively.

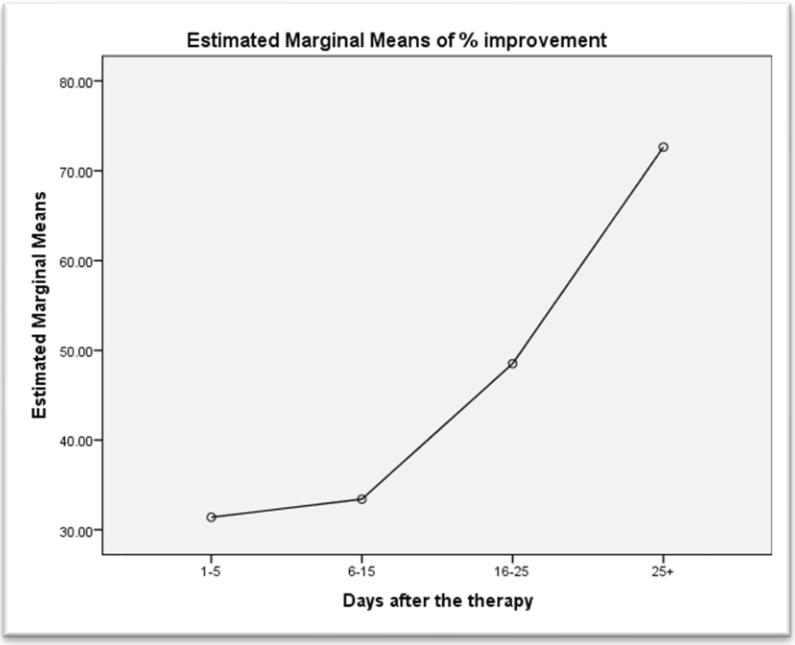

Fig. 2. *% of improvement in relation to the days after the therapy.*

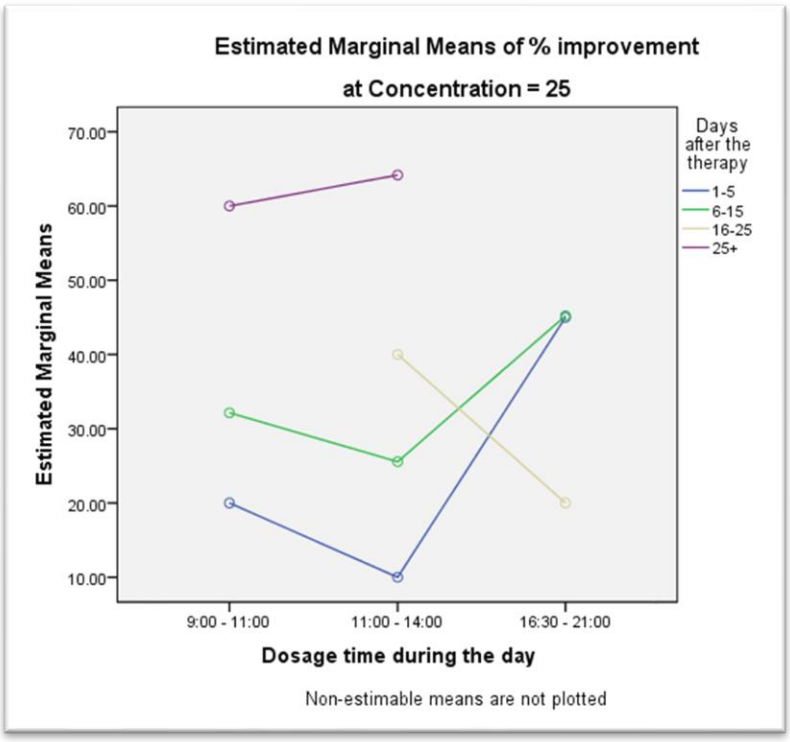

Fig. 3. *% of improvement in relation to the days after the therapy and the time during the day in the case of concentration equal to 25μg.*

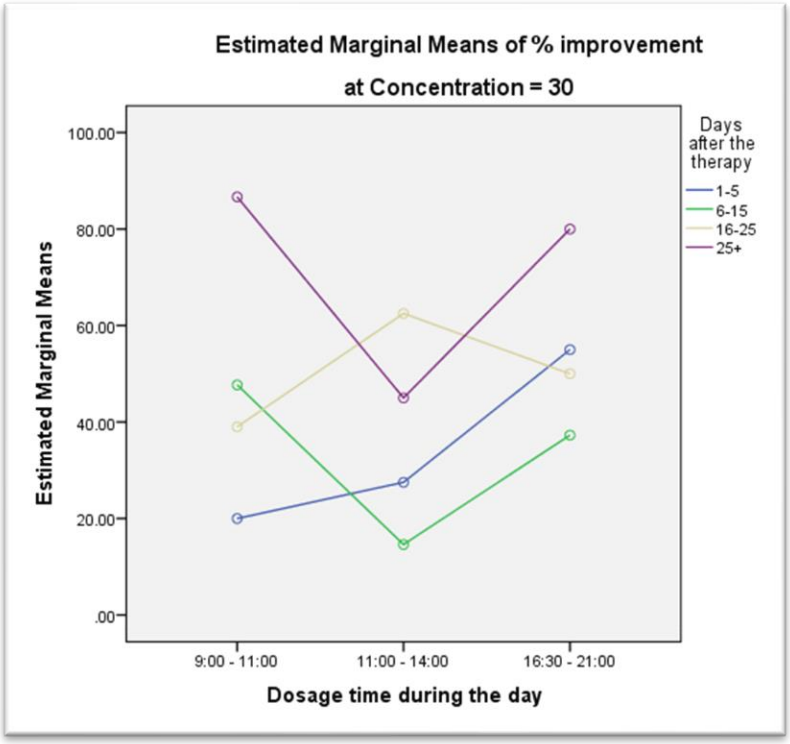

Fig. 4. *% of improvement in relation to the days after the therapy and the time during the day in the case of concentration equal to 30μg.*

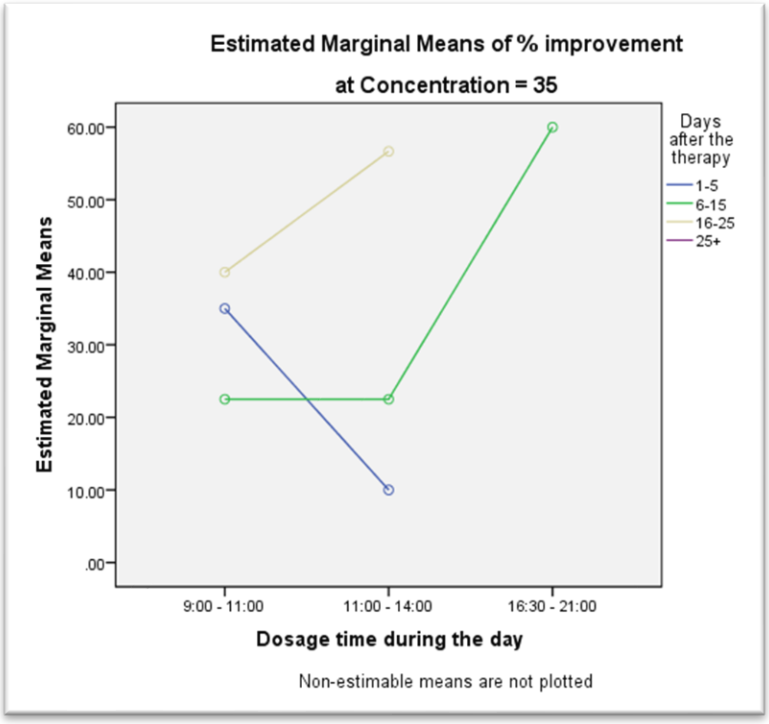

Fig. 5. *% of improvement in relation to the days after the therapy and the time during the day in the case of concentration equal to 35μg.*

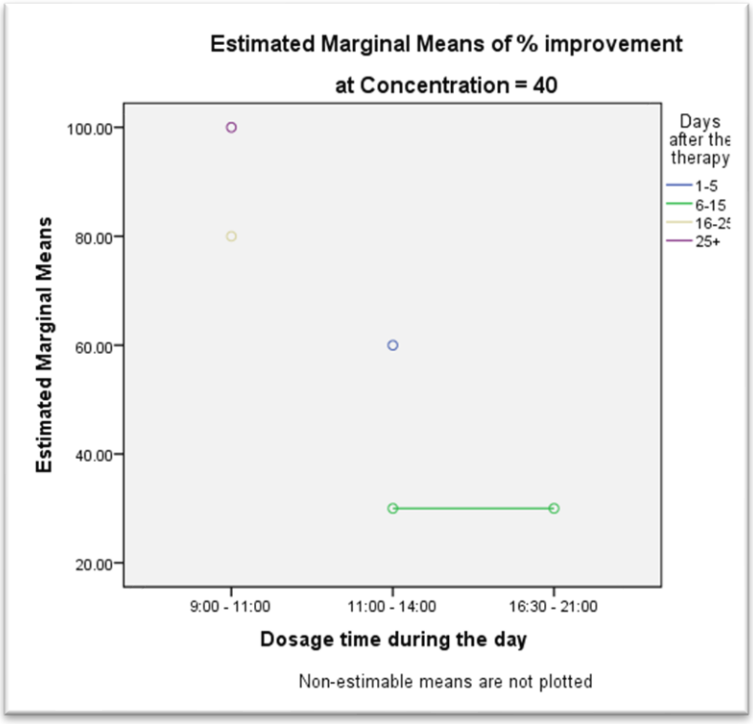

Fig. 6. *% of improvement in relation to the days after the therapy and the time during the day in the case of concentration equal to 40μg.*

DISCUSSION

Reactive oxygen species (ROS) are natural products of cellular metabolism, playing essential roles in physiological processes. The circadian rhythm, a system of internal 24-hour cycles that regulate various physiological functions, has become increasingly recognized for its role in immune system function, particularly in relation to inflammatory diseases such as arthritis. Circadian rhythms are not only present in immune cells but also significantly influence their activity, shaping immune responses based on the time of day.

Recent research has demonstrated that key immune system factors, such as antioxidants, pro-inflammatory cytokines, and molecular pathways like NF-κB and Nrf2, follow distinct circadian patterns. For instance, regulatory T (Treg) cells, which play a crucial role in immune tolerance and inflammation modulation, show non-rhythmic activity patterns under normal conditions. However, systemic signals can induce rhythmic activity in these cells, thus influencing the course of chronic inflammatory conditions, including arthritis. This finding highlights the importance of considering time-of-day factors when modulating immune responses, potentially leading to new therapeutic strategies for inflammatory diseases (30).

Moreover, certain antioxidant enzymes exhibit significant diurnal variation. Enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), catalase, superoxide dismutase (SOD), along with uric acid and peroxiredoxins (Prxs), reach their peak levels during the morning. In contrast, molecules associated with oxidative stress, including melatonin, plasma thiols, lipid peroxidation, and ascorbic acid, show elevated concentrations in the evening. These diurnal fluctuations reflect a complex interplay between oxidative stress and circadian rhythms, influencing cellular responses to ROS throughout the day (31).

The circadian rhythm also affects the balance between pro-inflammatory and anti-inflammatory cytokines. Research has found that pro-inflammatory cytokines are typically elevated during the body's rest phase, usually at night, while anti-inflammatory cytokines increase during periods of activity, generally during the day. This suggests that the body’s natural rhythms contribute to the regulation of immune responses, ensuring proper inflammation management during both rest and active phases (32). Thus, understanding the interplay between circadian rhythms and immune system function could optimize therapeutic interventions for inflammatory diseases.

The transient nature of ozone and its oxidative products must be carefully considered. Ozone itself disappears within seconds, while hydrogen peroxide (H_2O_2), a key messenger in oxidative stress, persists in plasma for about two

minutes. On the other hand, lipid oxidation products such as 4-hydroxynonenal (4-HNE) are more stable and serve as persistent messengers (33). These oxidative products play significant roles in signaling pathways involved in inflammation and immune modulation. Ozone therapy has been explored as a treatment for OA due to its ability to regulate oxidative stress, modulate cytokine levels, and promote antioxidant defences (34, 35).

In clinical settings, ozone therapy has demonstrated positive outcomes when applied intra-articularly. Ozone acts as a bioregulator, modulating redox balance, cytokine levels (e.g., IL-8, TNF-α, TGF-β1, PDGF), and promoting antioxidant responses (36, 37). Additionally, ozone enhances oxygenation, reduces inflammation via inhibition of NF-κB activation, and stimulates ATP production, thus aiding cell survival and reducing cartilage degradation (38, 39). Ozone's effects on oxidative stress and inflammatory pathways make it a valuable tool for treating OA, where excessive ROS and inflammatory cytokines contribute to cartilage and synovial fluid degradation (40).

Ozone therapy, with its complex biochemical actions, has been shown to induce oxidative stress in a controlled manner, stimulating a variety of biological processes. When blood is exposed to ozone, H_2O_2 initially increases rapidly but then slows down as ozone is depleted. At therapeutic concentrations, ozone causes only a transient decrease in plasma antioxidant capacity, which is rapidly restored due to the efficiency of the redox system (41, 42). The therapeutic window for ozone has been well-established between 10 and 80 μg/mL, ensuring a controlled oxidative stress response that is effective yet non-toxic (3). Studies indicate that doses between 20 and 40 μg/mL of ozone trigger an acute oxidative stress response, activating various signalling pathways, including the NF-κB and Nrf2 pathways, and modulating immune and antioxidant responses.

Ozone's impact on immune modulation is particularly significant, as it can activate white blood cells (WBCs) through NF-κB signaling. At therapeutic doses, ozone downregulates NF-κB signaling, reducing inflammation, but excessive oxidative stress can upregulate NF-κB, leading to heightened inflammation and tissue damage (7, 43). The ability of ozone to finely regulate NF-κB activation makes it an effective therapy for conditions characterized by immune dysregulation, such as chronic infections, autoimmune diseases, and cancer (44, 45). Additionally, ozone activates Nrf2, enhancing antioxidant defences by promoting the expression of antioxidant enzymes such as heme oxygenase-1 (HO-1), SOD, and catalase (46, 47). This mechanism strengthens the body's ability to resist oxidative damage and inflammation, which is especially beneficial in chronic inflammatory conditions like rheumatoid arthritis.

In our previous studies, the safety and efficacy of ozone therapy in OA were examined in rat models. The results showed that medical ozone, when injected intra-articularly, effectively modulated inflammation and enhanced tissue repair. Our experiment with Wistar rats demonstrated that ozone therapy significantly improved cartilage repair when administered at early stages of OA (48). Furthermore, our research indicated that the most significant therapeutic effect occurred when ozone was administered during the first stage of cartilage degeneration, with diminishing effects observed at later stages (5). These findings suggest that ozone therapy is most effective when administered early in the disease process, supporting its use as a therapeutic option in the management of OA.

Our clinical studies on the use of ozone therapy in 128 patients with osteoarthritic knees further corroborated these findings. Patients treated with intra-articular ozone injections at a concentration of 30 μg/mL reported prolonged symptom relief, with an average symptom-free period of 2.17 years. This duration surpassed the typical relief provided by hyaluronic acid injections, which usually last for about six months. Our statistical analysis also revealed that concentrations above 30 μg/mL did not provide any additional benefits in terms of treatment duration, suggesting that higher concentrations may be unnecessary and that a dose range of 25-30 μg/mL is optimal for achieving long-term therapeutic effects (3, 49).

In a follow-up study involving 155 patients, we investigated the potential impact of the timing of ozone injections on treatment outcomes. The results showed that the effectiveness of ozone therapy remained consistent across different time intervals during the day, eliminating the need for adjustments based on circadian rhythms. This finding emphasizes the stability and consistency of ozone's therapeutic effects, regardless of the time of administration. Furthermore, the optimal ozone concentration for maximum therapeutic efficacy was confirmed to be within the 25-30 μg/mL range (50-52).

Our study also addressed the timing of subsequent ozone injections, highlighting that the greatest improvement was observed in the period 25+ days post-injection, suggesting that subsequent treatments are more effective when administered during the peak of improvement. This finding also aligns with the regenerative nature of ozone therapy, similar to prolotherapy, which works through the promotion of tissue repair (53). However, further studies with larger patient samples and more detailed biochemical validation are necessary to standardize ozone therapy protocols and optimize patient outcomes.

In conclusion, ozone therapy presents a promising therapeutic approach for managing OA, with its ability to regulate oxidative stress, modulate cytokine production, and promote tissue repair. Our research underscores the

importance of ozone concentration and treatment timing in optimizing therapeutic outcomes. While ozone therapy's effects are relatively stable across different times of day, the optimal concentration for prolonged symptom relief remains within the 25-30 μg/mL range. Further research is needed to refine treatment protocols and establish standardized guidelines for ozone therapy in clinical practice.

CONCLUSIONS

ROS formed during OA activates NF-κB pathways by increasing its translocation into the nuclei and this causes the activation of intracellular inflammation pathways such as IL-1β, IL-6, TNF-α and COX-2 which then open the apoptotic cascade. O_3 can inhibit apoptosis and degradation of the cartilage matrix by inhibiting the activation of NF-κB, resulting in cell survival (54).

The ozone concentration typically recommended by leading authors as the safest and most effective therapeutic range varies between 10 and 80μg. However, almost all empirical studies utilize concentrations between 20 and 40μg for knee OA treatment. The optimal concentration for each patient is likely to differ, as it depends on the balance between inflammatory cytokines, hormones, NF-kB, and Nrf2, which must exceed a certain threshold to counteract antioxidants; these levels vary individually. In our statistical analysis, a concentration of 30μg appears to yield the most long-term benefits, and therefore, we recommend this dosage. Additionally, our study suggests that medical ozone therapy provides a longer-lasting (2.17 years) therapeutic effect compared to other commonly used intra-articular injection methods for osteoarthritic knees.

Despite the fact that ozone ceases to exist as an independent entity within less than a minute upon contact with biological fluids, different authors recommend varying treatment intervals, ranging from twice per week to a monthly injection, alone or in combination with other intra-articular agents. In our statistical analysis, the therapeutic benefits of even a single injection peak approximately 25 days post-administration. This observation should be further investigated in studies focusing on the biochemical and biological mechanisms of ozone's action and supports the recommendation of administering treatment every three to four weeks.

Although literature suggests that the biological factors influenced by ozone follow a circadian pattern, our study did not confirm this association. Consequently, ozone therapy can be performed at any time of the day with consistent therapeutic outcomes.

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