

Spermiogram Parameter Alterations in Acute Liver-Damaged Rats Following *Lavandula Angustifolia* Oil and Medical Ozone Therapy

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Abstract

BACKGROUND/AIMS: In animal experiments, carbon tetrachloride (CCl₄) is commonly used to produce hepatotoxicity because it induces oxidative stress. Increased production of reactive oxygen species, resulting in oxidative stress, has detrimental effects on several cellular components. *Lavandula angustifolia* oil has been recognized as an indirect antioxidant that boosts the activity and expression of antioxidant enzymes. Medical ozone therapy, which uses appropriate concentrations of ozone, is being investigated for its potential to treat and prevent hepatotoxicity and other conditions. The effects of medical ozone, lavender, and the combined effects of both therapies on sperm morphology and count in hepatotoxic rats were examined in this study.

MATERIALS AND METHODS: CCl₄ was used to cause acute hepatotoxicity in Wistar rats and sperm cells were obtained from - extracted epididymal structure. Then, the Spermac Kit (FertiPro Spermac Stain) was used to analyze sperm morphology after medical ozone, lavender, and combined treatment.

RESULTS: Adding lavender to the treatment increased sperm count ($p < 0.001$), irrespective of medical ozone. However, the sperm count is unaffected when lavender and medical ozone are combined ($p > 0.05$). However, The total number of defects in the head, neck, and tail varies among the three groups' sperm morphologies.

CONCLUSION: Only the rats who received *Lavandula angustifolia* oil treatment showed an increase in spermatozoa, suggesting that medical ozone may counteract *Lavandula*'s effects. Therefore, this study is crucial to understanding the relationship between medical ozone and lavender oil.

Keywords: Carbon tetrachloride, *Lavandula angustifolia* oil, medical ozone, semen analysis, sperm quantification, morphology of sperm, hepatotoxicity

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INTRODUCTION

Hepatocellular toxicity, a significant medical concern, often results from exposure to various xenobiotics and can lead to severe liver damage.¹ In recent years, therapeutic strategies have focused on the antioxidant and anti-inflammatory properties of medicinal plant derivatives to mitigate such damage by scavenging reactive oxygen species (ROS) and enhancing endogenous antioxidant defenses.² In animal research carbon tetrachloride (CCl₄) is commonly used to induce hepatotoxicity by generating oxidative stress within the organism. An increase in ROS causes oxidative stress, which negatively affects cell metabolism and can result in a variety of diseases.³ Prevention and treatment of oxidative stress remain significant challenges in modern medicine. One well-known dangerous chemical that causes poisoning, especially in the liver, is CCl₄. The enzyme cytochrome P450 2E1 (CYP2E1) is responsible for the biotransformation of CCl₄, which produces free radicals such as chloroform and hexachloroethane. The trichloromethylperoxy moiety reacts with nucleic acids, proteins, and lipids covalently attached to biological macromolecules. Protein production in liver cells is mediated by ribosomes, which stop translating due to ribonucleic acid hypomethylation. Therefore, oxidative stress in the organism impairs liver enzyme function.⁴ When the production and removal of ROS are out of equilibrium, oxidative stress occurs. Important biological constituents, such as proteins, lipids, and nucleic acids, are adversely affected by increased ROS production.⁵ Many diseases, such as cancer and cardiovascular conditions, are influenced by oxidative stress in their onset and progression.⁴ Essential oils have been used since ancient times because of their multiple benefits and medicinal properties.⁵ With the increase in use of essential oils throughout the late twentieth and twenty-first centuries, and an increase in its use at the outset, this field became known as aroma science therapy.⁶

Lavender oil, derived from *Lavandula angustifolia*, is a potently perfumed oil obtained from a shrub-like plant of the family Lamiaceae. Lavender oil is used as a sedative in pain management, and as an analgesic, anti-inflammatory, antifungal, and antispasmodic agent; it is also used to lower blood pressure. It also aids wound healing and reduces anxiety.⁷ Moreover, *Lavandula angustifolia* has calming effects on the central nervous system; is anticonvulsant, spasmolytic, antioxidant, and antimicrobial; and inhibits mast cell degranulation.⁸ It has been established that lavender oil acts as an indirect antioxidant by enhancing the production and activity of antioxidant enzymes, hence raising the natural several studies have shown that lavender oil exhibits antioxidant properties by increasing the production and activity of antioxidant enzymes.^{9,10} *Lavandula angustifolia* oil has demonstrated significant hepatoprotective effects, primarily by reducing oxidative stress and inflammation.¹

Medical ozone therapy is the controlled administration of ozone gas at appropriate dosages, despite ozone being widely acknowledged as detrimental. Researchers have identified a promising strategy for disease prevention and therapy. The observation that ozone decreases oxidative stress suggests its potential application in preventing and treating hepatotoxicity. The medical benefits of ozone therapy require small quantities and a delicate balance among variables, including the number of antioxidants that react with ozone.¹¹ Medical ozone therapy is being investigated for its potential benefits in the treatment of various disorders. Moreover, Valacchi and Bocci¹² showed that applying ozonated water to skin wounds accelerated wound healing in rats.

Male infertility significantly contributes to infertility in couples, with impaired sperm motility being a primary factor.^{13,14} Current medical and surgical interventions often fail to reverse compromised semen parameters, underscoring the need for alternative therapeutic strategies. This highlights the critical importance of exploring novel pharmacological agents and natural compounds that can enhance sperm quality parameters.¹⁴ Essential oils, derived from various plants, have demonstrated potential to improve sperm parameters, including motility and vitality, and offer a promising avenue for further investigation.^{13,15} Specifically, studies have shown that certain essential oils, such as those from oregano and sage, can positively influence sperm physiology by improving motility and vitality.^{13,15,16} Among these, *Lavandula angustifolia* oil has garnered attention for its antioxidant and antimicrobial properties, which may mitigate the deleterious effects of ROS on spermatozoa during preservation. The protective effect of lavender oil extends to improving various spermatological parameters during cryopreservation, thereby enhancing the overall quality of frozen-thawed semen. Given its potential, further exploration into the specific mechanisms by which *Lavandula angustifolia* oil enhances sperm viability and motility, especially under cryogenic conditions, is warranted. Furthermore, medical ozone, known for its potent antimicrobial and oxidative properties, is a promising candidate for mitigating bacterial contamination and enhancing sperm quality during semen preservation.¹⁷

This study aims to investigate the individual effects of *Lavandula angustifolia* oil and of medical ozone on key sperm quality parameters, including viability, motility, and the acrosome reaction, under controlled experimental conditions. This investigation will also examine the chemical characterization of the essential oils to better understand their bioactive components and the mechanisms by which they affect sperm morpho-functional parameters. The purpose of this research was to examine the effects of medical ozone and lavender, alone and in combination, on the sperm count and morphology in hepatotoxic rats. Notwithstanding these drawbacks, medical ozone treatment remains important; hence, the uniqueness of this study is demonstrated by the lack of research combining medical ozone therapy with lavender oil rather than applying it to essential oils or “ozonated oils”. This study aimed to examine whether the morphology and quantity of sperm in hepatotoxic rats were influenced by lavender oil, medical ozone therapy, or their combined (synergistic) application. This is among the first studies to demonstrate an association between medical ozone therapy and lavender oil in the literature.

MATERIALS AND METHODS

Study Design and Ethical Approval

Acute hepatotoxicity Model of Wistar Rats: Male Wistar rats weighing 250-500 g and 8-12 weeks of age were chosen randomly for the study. The 4 main experimental groups were: control, *Lavandula angustifolia* oil, medical ozone, and combined treatment (*Lavandula angustifolia* oil and medical ozone); each group included 7 randomly assigned animals. This research was authorized by the Maltepe University Animal Experiments Local Ethics Committee (approval no: 2023.06.03, date: 29.06.2023; informed consent: as procedures were carried out on animals, patient approval was not obtained). The Maltepe University Experimental Animals Research Unit bred and cared for all the rats, and the study employed a prospective design.

Hepatotoxicity in rats was induced by intraperitoneal injection of 0.2 mL/kg of CCl_4 daily for 10 days.⁴ CCl_4 -induced liver damage is shown in Figure 1. Following CCl_4 administration, 400 mg/kg Lavandula angustifolia oil was threatened by gavage every other day for 14 days to Lavandula angustifolia oil group.¹⁸ The medical ozone group received intraperitoneal injections of medical ozone (1 mg/kg) for seven cycles.¹¹ In the final experimental group (combined therapy), animals received 400 mg/kg of Lavandula angustifolia oil by gavage for 14 days and 1 mg/kg of medical ozone by intraperitoneal injection on seven occasions, every other day. On days when medical ozone was not administered, a sham procedure was performed to induce comparable stress in rats (Table 1). Finally, 100 mg/kg of ketamine was used to euthanize the animals, and their epididymides were removed from the testicular tissue in all groups at the conclusion of the treatment regimen.

Sperm Collection: The extracted epididymal tissue was ruptured with an insulin needle in phosphate-buffered saline and incubated for ten minutes in a 37 °C incubator. Following this procedure, samples were obtained from the incubators, diluted 1:10, and used to prepare smears that were then fixed.

Sperm Count: A light microscope with a 20X objective and a Makler camera were used to count sperm in the 1 mL sample pellet. The sperm count was taken to determine the spermatozoa concentration (10^6 sperm/mL).

Sperm Morphology: Spermac Kit (FertiPro Spermac Stain) was used to stain the smear preparations following fixation. The stained samples were examined under a light microscope. According to the Rat Sperm Morphological Evaluation Guide H.L. Science.(2000), at least two hundred sperm were counted and examined for head, neck, and tail abnormalities.^{19,20}

Statistical Analysis

The software packages IBM SPSS Statistics 26.0 and GraphPad Prism 8 (Dotmatics) were used for all statistical evaluations. For sperm counts, Kolmogorov-Smirnov and Shapiro-Wilk normality distribution tests were performed, and the Tukey HSD test was used to compare groups. The data was provided as the mean \pm standard deviation. Statistical significance was defined as $p < 0.05$. The differences between groups were displayed in figures using the symbols *for $p < 0.01$, **for $p < 0.05$, and ***for $p < 0.001$. The sperm morphology parameters (head, neck, and tail) were analyzed using two-way analysis of variance and were presented as mean values (upper limit; lower limit). Multiple comparisons of sperm morphology were performed using the Tukey HSD TEST.

Table 1. The chart illustrates the induction of acute liver damage with CCl_4 , followed by therapy with Lavandula angustifolia oil, medical ozone, and combination therapies

Days	Procedure applied
1-10	CCl_4 treatment
11	Lavandula angustifolia oil + medical ozone/medical ozone
12	Lavandula angustifolia oil
13	Lavandula angustifolia oil + medical ozone/medical ozone
14	Lavandula angustifolia oil
15	Lavandula angustifolia oil + medical ozone/medical ozone
16	Lavandula angustifolia oil
17	Lavandula angustifolia oil + medical ozone/medical ozone
18	Lavandula angustifolia oil
19	Lavandula angustifolia oil + medical ozone/medical ozone
20	Lavandula angustifolia oil
21	Lavandula angustifolia oil + medical ozone/medical ozone
22	Lavandula angustifolia oil
23	Lavandula angustifolia oil + medical ozone/medical ozone
24	Lavandula angustifolia oil

CCl_4 : Carbon tetrachloride.

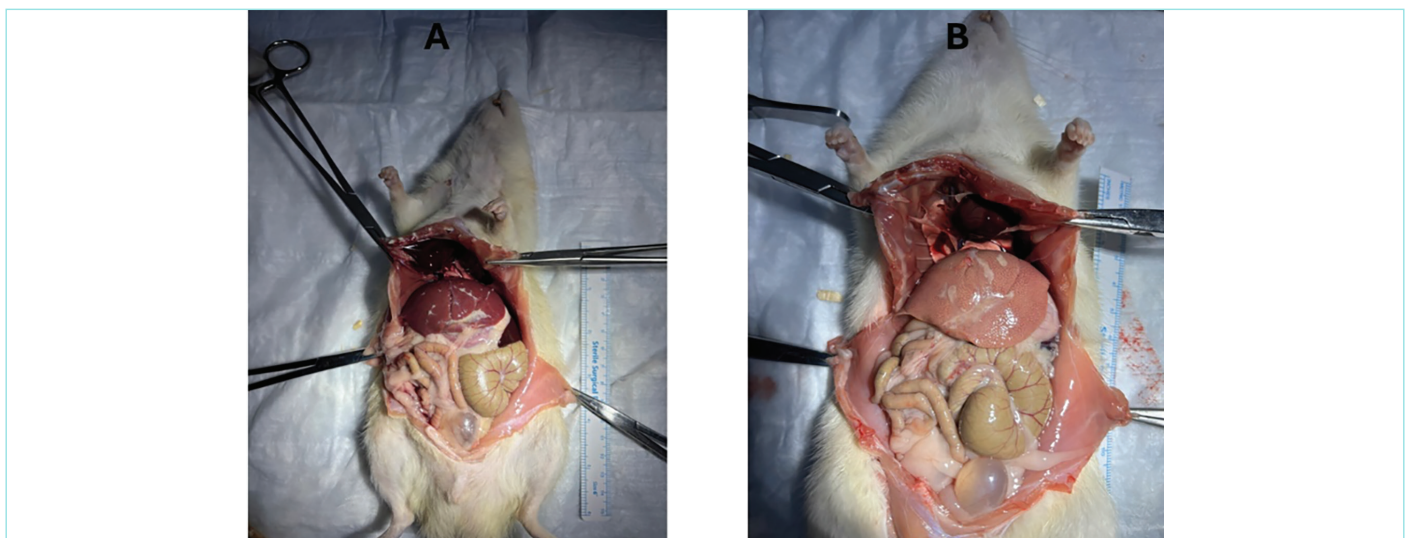


Figure 1. The impact of CCl_4 treatment on liver after 10 days A) Healthy liver B) Acute toxic liver.

CCl_4 : Carbon tetrachloride.

RESULTS

Sperm Morphologies After Treatments

When examining sperm morphology for abnormalities, the three groups differ in the total numbers of head, neck, and tail abnormalities. Anomalies of the head, neck, and tail in the experimental groups were presented as mean (upper limit-lower limit). A statistically significant difference was observed between Lavender *angustifolia* oil-treated (12; 8.198-17.234) and medical ozone-treated (14.5; 10.289-20.049) groups ($p < 0.001$) for sperm head anomalies. Furthermore, for neck anomalies, there was a statistically significant difference between the Lavender *angustifolia* oil-treated group (68; 61.248-74.073) and the medical ozone-treated group (65; 58.163-71.271) ($p < 0.001$). Moreover, statistically significant differences in sperm tail anomalies were observed between groups treated with Lavender *angustifolia* oil (20; 15.045-26.086) and those treated with medical ozone (20.5; 15.486-26.626) ($p < 0.001$). Nevertheless, there was no discernible difference between the control group (16.5; 11.997-22.266/54; 47.082-60.767/29.5; 23.614-36.159 respectively) and the Lavandula *angustifolia* oil group, the medical ozone group, and the combination group (10; 6.567-14.941/60.5; 53.588-67,016/29.5; 23.614-36.159 respectively; $p > 0.05$; Figure 2A, B, and C).

Sperm Counts After Treatments

The findings indicate that Lavender *angustifolia* oil (24.25 ± 4.683), when incorporated into the treatment regimen for rats with liver impairment, both in combination (21.88 ± 8.692) and medical ozone (22.88 ± 7.936) significantly elevated sperm count compared to the

control group (17 ± 3.586 ; $p < 0.001$). However, compared with the control group, the combination of lavender and medical ozone did not affect sperm count ($p > 0.05$; Figure 3).

DISCUSSION

The available conventional therapies for drug-induced liver diseases are frequently insufficient and carry substantial adverse effects, necessitating the exploration of novel, efficacious therapeutic agents.²¹ In this context, natural compounds (e.g., from *Lavandula angustifolia* oil) and therapeutic gases (e.g., medical ozone) present promising avenues for investigation due to their documented biological activities.²² The liver plays a crucial role in metabolism, making it the primary target of toxicity induced by a range of drugs, xenobiotics, and oxidative stress.²³ Many medicinal plants are known to protect against CCl_4 -induced liver injury in animal models, demonstrating their potential as hepatoprotective agents.²⁴ Essential oils, extracted from aromatic plants, are increasingly recognized for their medicinal potential, particularly their antioxidant and antigenotoxic properties, which are crucial for mitigating hepatocellular damage.²⁵ *Lavandula angustifolia* oil, in particular, has demonstrated significant antioxidant activity that can counteract oxidative stress implicated in various forms of hepatic injury.^{26,27} Its constituents have been shown to modulate antioxidant enzymes and xenobiotic-metabolizing pathways, offering a multi-faceted approach to ameliorating liver damage.²⁸ The presence of compounds such as 1,8-cineole in *Lavandula angustifolia* oil has been shown to restore liver cell function and integrity, consequently reducing the leakage of serum aminotransferases and enhancing antioxidant enzyme activity.¹ Additionally, the essential oil of *Lavandula angustifolia*

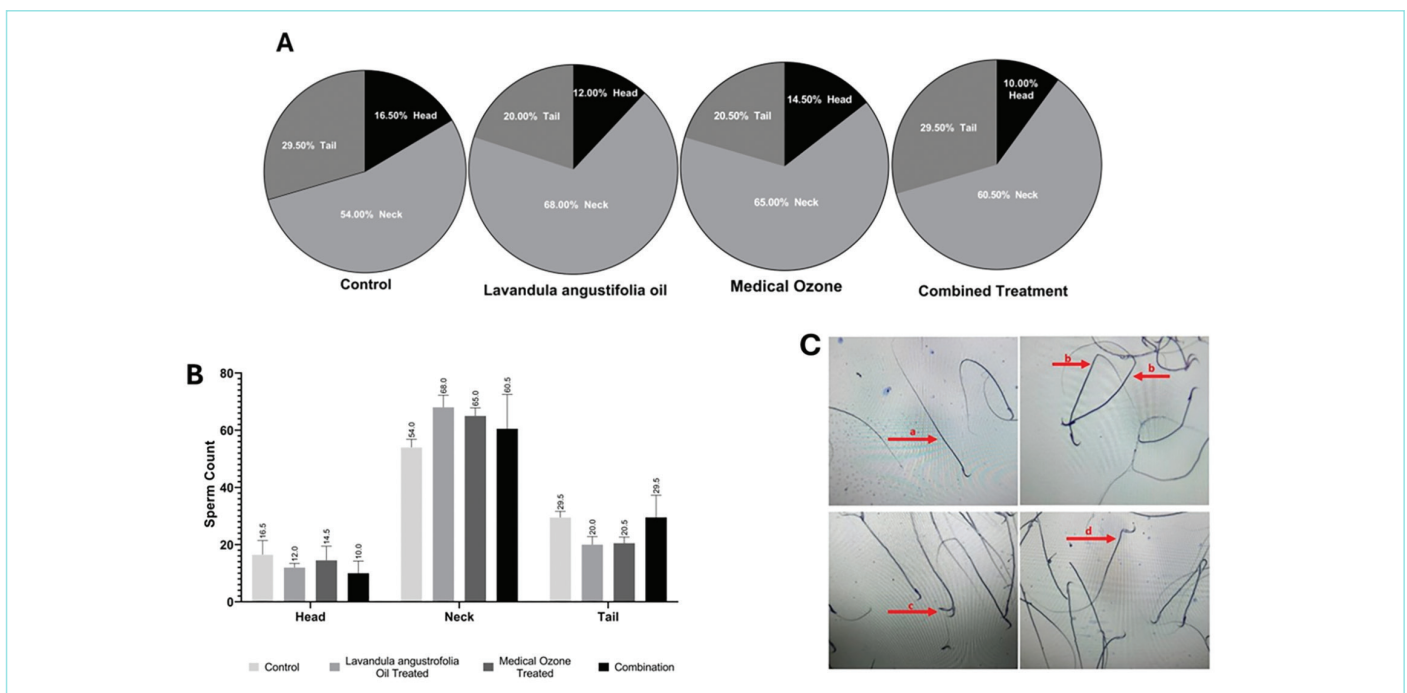


Figure 2. The impacts of *Lavandula angustifolia* oil, medical ozone and combined treatment (*Lavandula angustifolia* oil and medical ozone) in the sperm samples of CCl_4 induced hepatotoxic rats. A. Sperm morphology abnormalities (head, neck, and tail) between groups. B. Anomalies of the head, neck, and tail are observed across groups. C. Rat sperm morphologies: normal morphology (a), bent tail (b), double head (c), and flattened head (d). The control group did not differ from the other groups ($p > 0.05$).

CCl_4 : Carbon tetrachloride.

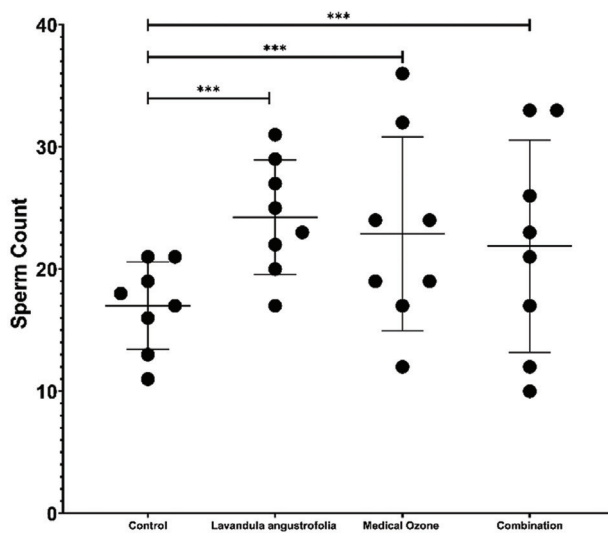


Figure 3. The sperm count was affected by the treatments, and the control group and the lavender-treated group. Sperm count differed among treatments; the control and lavender-treated groups differed ($p < 0.001$).

has demonstrated anti-inflammatory properties, significantly reducing levels of interleukins interleukin (IL)-1 and IL-8, and of nuclear factor kappaB, which are key mediators in inflammatory hepatocellular injury.²⁹

The therapeutic application of medical ozone has emerged as a complementary strategy to mitigate hepatocellular injury because of its immunomodulatory and oxidative preconditioning effects.³⁰ Medical ozone, through its controlled oxidative stress, can induce adaptive responses that upregulate endogenous antioxidant enzymes and modulate inflammatory pathways, thereby conferring hepatoprotection.³¹ This preconditioning effect, often termed ozonated autohemotherapy, can activate the nuclear factor erythroid 2-related factor 2 pathway, leading to enhanced synthesis of antioxidant enzymes, such as heme oxygenase-1 and glutathione reductase. The transient and controlled oxidative stimulus provided by medical ozone also promotes immune modulation and improves microcirculation, both of which are crucial for attenuating hepatic I/R injury and enhancing tissue repair.²⁸ *Lavandula angustifolia* oil ameliorates oxidative stress by increasing glutathione levels and reducing concentrations of ROS, malondialdehyde (MDA), and oxidized glutathione.^{1,26} Medical ozone acts indirectly by triggering an Nrf2/Keap1/ARE-mediated antioxidant response, which upregulates transcription of genes encoding various antioxidant and detoxification enzymes, including heme oxygenase-1, NADH:quinone oxidoreductase-1, and glutathione-synthesizing enzymes.^{32,33} This indirect activation by ozone, distinct from direct antioxidant supplementation such as intravenous glutathione, involves the generation of ozonides and hydrogen peroxide (H_2O_2) as signaling molecules.³⁴ These reactive species subsequently interact with biomolecules, leading to the activation of adaptive cellular defense mechanisms.³⁵ This controlled induction of mild oxidative stress by ozone is believed to enhance the overall antioxidant capacity of the cell, preparing it to better withstand subsequent, more severe oxidative insults.³⁶

The synergistic interplay between the direct antioxidant properties of *Lavandula angustifolia* oil and the redox bioregulatory effects of

medical ozone could therefore offer a comprehensive strategy for protecting hepatocytes against various toxic insults by simultaneously neutralizing free radicals and bolstering intrinsic cellular defenses.^{26,37} Linalool, a key component of *Lavandula angustifolia* oil, activates the Nrf2 signaling pathway, which is crucial for reducing ROS during inflammatory processes and thereby enhancing the cellular antioxidant defense system.³⁸ This activation of Nrf2 by linalool contributes to the overall antioxidant and anti-inflammatory effects observed with *Lavandula angustifolia* oil thereby protecting against cellular damage.³⁷ Similarly, medical ozone, through its hormetic action, specifically targets the Nrf2-Keap1-ARE system via ROS-mediated signaling, thereby inducing a potent antioxidant and pro-survival response within hepatocytes.³⁹ This induction of the Nrf2 pathway by both *Lavandula angustifolia* oil components and medical ozone - albeit through potentially distinct initial mechanisms - ultimately converges to enhance cellular detoxification and antioxidant capacities and to safeguard hepatocellular integrity.⁴⁰ Because activation of Nrf2 is a key mechanism of both *Lavandula angustifolia* oil components and medical ozone, their combined application may synergistically upregulate antioxidant and detoxification pathways, enhancing protection against hepatocellular insults. Specifically, the regulation of Nrf2 by ozone, a relationship first described in the 21st century, highlights its role in modulating antioxidant mechanisms, like the effects observed with low-dose ozone and Nrf2 activation.⁴¹

In the present research, we adopted a liver model mediated by CCl_4 , which is one of the most widely used xenobiotics to cause tissue damage mediated by lipid peroxidation in animal model studies. This study aimed to investigate how lavender, medical ozone, and their synergistic combination affected sperm count and morphology in rats with hepatotoxicity. Initially, we demonstrated that *Lavandula angustifolia* oil without medical ozone enhanced sperm count in rats with hepatic impairment when administered to treat organ damage. The use of *Lavandula angustifolia* oil during treatment results in an increase in sperm count; this effect is reversible. The inactivation of *Lavandula angustifolia* oil raises the possibility that, when combined

with medical ozone, the oil's effect may be suppressed. Souli et al.⁴² evaluated the protective effects of *Rosmarinus officinalis* essential oil (ROEO) and *Lavandula stoechas* essential oil (LSEO) in preventing reproductive impairment and oxidative stress in male diabetic rats induced by alloxan exposure. They discovered that ROEO and LSEO, in addition to preventing alloxan-induced increases in body weight, relative reproductive organ weights, testosterone levels, and loss of sperm quality, had a substantial antioxidant capacity. Conversely, they demonstrated that the administration of alloxan was associated with an oxidative stress status measured by elevated levels of H₂O₂ and MDA, as well as decreased levels of sulfhydryl group content and antioxidant enzyme activities of the testis, epididymis, and sperm include catalase, SOD, and glutathione peroxidase. Notably, in rats with alloxan-induced diabetes, ROEO and LSEO therapy substantially prevented oxidative damage to the male reproductive organ systems.⁴³ In the literature, studies report hepatoprotective and renoprotective effects of Lavender essential oils against malathion-induced oxidative stress in young male mice, showing that LSEO protect the rat liver and kidney, an effect due in part to their antioxidant properties. However, Tuşat et al.,²⁰ in an experimental torsion model, investigated the effect of ozone therapy on testicular damage resulting from I/R injury. They found that the ozone-treated rats had lower histopathological tissue levels, serum ischemia-altered albumin, total oxidant status, and oxidative stress index than the torsion/detorsion group.²⁰ Research indicates that medical ozone therapy may have beneficial effects on histopathological and biochemical findings in the reproductive systems of rats.

Study Limitations

Despite these strengths, there are a few limitations that should be noted. Sperm quality was mainly assessed by concentration and morphology, while motility and viability were not evaluated. Due to the length of rat sperm tails, motility is difficult to assess; however, including these parameters could provide a more complete understanding of treatment effects. Secondly, the study did not evaluate additional parameters such as histopathological examination at testicular and hepatic levels or inflammatory pathways. Consequently, the current findings serve as preliminary data in this regard; further studies with larger sample sizes are needed to elucidate the potential biological basis of the significant interaction observed between medical ozone and lavender oil treatments.

CONCLUSION

The literature contains a limited number of articles on medical ozone therapy; therefore, the novelty of this study is indicated by the lack of studies that combine medical ozone therapy with *Lavandula* oil, as opposed to applying medical ozone therapy to essential oils, or "ozonated oils". This investigation will critically evaluate the efficacy and safety profiles of "*Lavandula angustifolia*" oil and medical ozone in preclinical models of liver injury, thereby contributing to the development of novel therapeutic strategies. Although the sperm counts of rats treated with medical ozone alone and those treated with *Lavandula angustifolia* oil plus medical ozone did not differ significantly, our results showed that only rats treated with *Lavandula angustifolia* oil alone showed an increase in sperm counts, indicating that medical ozone may counteract the effects of *Lavandula angustifolia* oil. Our research is among the earliest to demonstrate a connection between medicinal ozone and lavender oil in the literature. Further research is

required to determine the effectiveness of medical ozone treatment and lavender oil on spermogram parameters in rats with liver impairment.

MAIN POINTS

- Lavender oil increased sperm count in rats with carbon tetrachloride-induced liver injury.
- Medical ozone therapy alone did not improve sperm count.
- Combined lavender oil and ozone showed no synergistic benefit.
- Ozone treatment may counteract lavender's positive effect on spermogram parameters.
- Sperm morphology was not significantly altered by combined therapy.

ETHICS

Ethics Committee Approval: This research was authorized by the Maltepe University Animal Experiments Local Ethics Committee (approval no: 2023.06.03, date: 29.06.2023).

Informed Consent: As procedures were carried out on animals, patient approval was not obtained

Footnotes

Authorship Contributions

Surgical and Medical Practices: N.Ö.K., Ç.Ö., İ.Z.T., E.A., N.A., Concept: N.Ö.K., Ç.Ö., Design: N.Ö.K., Ç.Ö., Data Collection and/or Processing: N.Ö.K., Ç.Ö., İ.Z.T., E.A., N.A., Analysis and/or Interpretation: N.Ö.K., Ç.Ö., İ.Z.T., E.A., N.A., E.Ç., Literature Search: N.Ö.K., Ç.Ö., E.Ç., Writing: N.Ö.K., Ç.Ö., İ.Z.T.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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