Research Article / Araştırma Makalesi

Ozone vs Melatonin: The Therapeutic Effects In Alcoholic Liver Disease

Ozon vs Melatonin: Alkolik Karaciğer Hastalığında Terapötik Etkileri

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Abstract

Background: Alcoholic liver disease (ALD) is a worldwide health problem.Ozone and melatonin are agents that are widely used for their antioxidant properties in medical therapies.In this study, we aimed to evaluate the use of ozone and melatoninin ALD.
Materials and Methods: Forty-eight rats were used, and ethanol, melatonin, and ozone were administrated. Histopathological evaluation was performed and activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT) total antioxidant status (TAS), total oxidant status (TOS), and malondialdehyde (MDA)were determined in the blood samples, while TAS, TOS, and MDA parameters were measured in the liver tissue samples.
Results: There were statistically significant differences between the studied groups concerning ALT, AST activities and TAS, TOS, and MDA levels. Vascular congestion, hepatocyte damage, periportal inflammation, and microvesicular steatosis were detected inthe alcohol-induced toxicity group.
Conclusions: In the light of histopathological findings, we can claim that melatonin and ozone administrations are beneficial for alcohol-induced hepatotoxicity. However, ozone application is superior to melatonin.

Key words: Alcohol-Induced Disorders, Alcoholic Liver Diseases, Melatonin, Ozone, Oxidative Stress

Öz.

Amaç: Alkolik karaciğer hastalığı (ALD) dünya çapında bir sağlık sorunudur. Ozon ve melatonin tıbbi tedavilerdeki antioksidan özellikleri için yaygın olarak kullanılan ajanlardır. Biz bu çalışmada ALD'de ozon ve melatonin kullanımını değerlendirmeyi amaçladık.
Materyal ve Metod: Kırk sekiz sıçan kullanılmış ve etanol, melatonin ve ozon uygulanmıştır. Histopatolojik değerlendirme yapılmıştır ve kanda alanin aminotransferaz (ALT), aspartat aminotransferaz (AST), gama glutamil transferaz (GGT), total antioksidan kapasite (TAK), total oksidan kapasite (TOK) ve malondialdehit (MDA); karaciğer dokusunda TAK, TOK ve MDA parametreleri ölçülmüştür.
Bulgular: Çalışılan gruplar arasında ALT, AST aktiviteleri ve TAK, TOK ve MDA düzeyleri açısından istatistiksel olarak anlamlı fark vardı. Alkol kaynaklı toksisite grubunda vasküler konjesyon, hepatosit hasarı, periportal inflamasyon ve mikrovesiküler steatoz tespit edildi.
Sonuç: Histopatolojik bulgular ışığında melatonin ve ozon uygulamalarının alkole bağlı hepatotoksisite için faydalı olduğunu iddia edebiliriz. Bununla birlikte, ozon uygulaması melatoninden daha üstündür.

Anahtar kelimeler: Alkole Bağlı Bozukluklar, Alkolik Karaciğer Hastalıkları, Melatonin, Ozon, Oksidatif Stres

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Introduction

Alcoholic liver disease (ALD) is a common disease especially seen in western societies and may result in cirrhosis if the necessary interventions are not carried out in the process. Ethanol consumption causes alcoholic liver disease, whether excessive alcohol consumption or long term high consumption (1). It is known that alcohol causes oxidative stress in the living cells of the body, but in particular liver metabolism initiates a series of pathological processes including toxic protein aldehyde compounds, endotoxins, immunological activity and proinflammatory cytokine release. Histopathologically, steatosis, steatohepatitis and varying degrees of fibrosis are observed in this disease (2,3).

The liver is primarily responsible for the metabolic degradation of alcohol. As alcohol intake increases, pathophysiologically ethanol is destroyed by cytochrome P450 and acetaldehyde in liver creates toxic effects. The first step in the degradation of alcohol is oxidation to acetaldehyde. This process is primarily carried out by the enzyme alcohol dehydrogenase. The second step in the destruction of alcohol is the conversion of acetaldehyde to acetic acid, and acetaldehyde dehydrogenase (ALDH) is involved in this transformation. All these transformations cause oxidation and accumulate free oxygen radicals in the following processes. Increased free oxygen radicals cause liver disorders which are very difficult to reverse after a while (2-5).

Ozone therapy is a method that has been used clinically in various diseases including circulatory problems and wound treatment nowadays. Although ozone is not radical in its chemical structure, it is known as the third most powerful antioxidant substance after fluorine and persulphate (6). In the treatment of medical ozone, ozone gas is obtained with the help of a special device that allows mixing of oxygen with certain ratios (6). The mechanism in ozone therapy is expressed in its simplest form as activating antioxidant enzyme systems by creating a low level of oxidative effect in the organism (7,8).

Melatonin is secreted from the epiphysis and its secretion increases at night. Melatonin has been reported to eliminate the toxic effects of free radicals such as hydroxyl (OH), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), nitrosyl (NO), peroxynitric acid (ONOO), which can lead to serious oxidative damage and has protective effects against the harmful effects of the organism. (9-11). This strong antioxidant property of melatonin is thought to be due to its pyrrole ring and high lipophilic properties. The pyrrole ring in the structure is explained by a series of reactions while lipophilic property is explained by the fact that melatonin does not require any binding sites or a receptor (10,11).

Consequently, although alcoholic liver injury is multifactorial, oxidative damage is one of the main mechanisms. Therefore, in this study, we aimed to investigate the effects of ozone and / or melatonin -which are known to have antioxidant properties- on oxidative damage in rats with alcoholic liver injury.

Materials and Methods

The animals were obtained from the Ataturk University Medical Experimental Research and Application Center, and the study was approved by the Local Ethics Committee of Animal Experiments at Kafkas University (IRB number: KAU-HADYEK/2016-054). All animal procedures were performed according to the "Guide for the Care and Use of Laboratory Animals" principles (12). Study reporting was done per the CONSORT principles(13).

Forty-eight female Wistar albino rats, 4-6-weeks old, weighing 190-250g, were used in this study. The rats were housed in an animal room maintained at a temperature of 25°C with alternating light periods (12 hours light/12 hours dark). All animals were fed by ad-libitum. The rats were randomly divided into eight groups (n=6 per group). Table 1 gives detailed information about the experimental groups and the chemicals used.

Before the experiment, the animals were kept fasting overnight. The blood and tissue samples were taken immediately after performing cervical vertebra dislocation per ethical rules, and kept at -80˚C for biochemical analyses. On the other hand, the harvested liver tissues were fixed in 10% buffered formaldehyde solution to perform the pathological examinations.

At the end of the experiment, systemic necropsies of the rats were performed, and macroscopic findings were recorded. Later, the liver tissues were fixed in 10% buffered neutral formalin for 72 h, they were dehydrated in graded alcohol series, embedded in paraffin wax, 4 µm thick sections of paraffin blocks were obtained, and stained with hematoxylin & eosin. The sections were evaluated by light microscopy (Olympus BX46) for histopathological evaluation of the following parameters: vascular congestion, hepatocyte damage, periportal inflammation, and microvesicular steatosis. For scoring, the liver sections were inspected at 10 randomly selected areas with a 40X objective. Scoring was done semi-quantitatively using light microscopy on the preparations from each animal and were reported for vascular congestion, hepatocyte damage, and periportal inflammation parameters as follows: 0-none, 1-mild, 2-moderate, and 3-severe. On the other hand, microvesicular steatosis was scored as 0 (absent) and 1 (present).

The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), total antioxidant status (TAS), total oxidant status (TOS), and malondialdehyde (MDA) were determined in the blood samples, while TAS, TOS, and MDA parameters were measured additionally in the liver tissue.

Serum TAS level was determined using an automated measurement method, which was studied by Erel (18) and serum TOS level was determined using a novel automated measurement method, also suggested by Erel (19).In this analysis, oxidants oxidize the "ferrous ion-o-dianisidine complex" to "ferric ion." The oxidation reaction is boosted by glycerol molecules. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample.

The sample size calculation was based on the aspartate transaminase levels. As calculated by the GPower program (v. 3.1, Kiel University, Germany), to compare the eight groups with a common standard deviation of 0.12, an effect size of 0.6, alpha error of 5%, and a power of 80% with the one-way ANOVA test, a total sample size of 48 cases is necessary.

Statistical Analysis

Statistical differences between the groups were determined with the one-way ANOVA test. The differences were considered statistically significant when the p values were less than 0.05. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, version 19.0, IBM, Armonk, New York 10504, NY, USA).

Results

Histopathological examinations of the control group (Group 1) revealed mild to moderate vascular congestion due to the resection of liver tissues (Figure 1). In the toxicity group (Group 2), moderate to severe hepatocyte damage was detected in 3 rats, and focal moderate hepatocyte damage was found in 3 rats. On the other hand, mild periportal inflammation was observed in 3 rats, while severe periportal inflammation was observed in 2 rats (Figure 2). Microvesicular steatosis was observed in 5 rats at variable rates (Figure 3).

Findings of the control+ozone group (Group 3) were the same as the control group findings, where only vascular congestion was observed (Figure 4). However, mild vascular congestion in 5 rats and mild focal hepatocyte damage in one case were noted in the control+melatonin group (Group 4) (Figure 5). In the control+ozone+melatonin group (Group 5), all rats had varying degrees of vascular congestion, one rat had mild periportal inflammation and focal mild hepatocyte damage, and one rat had focal mild hepatocyte damage (Figure 6). In the toxicity+ozone group (Group 6), mild periportal inflammation in two rats, mild hepatocyte damage in one rat and focal hepatocyte damage in one rat, and microvesicular steatosis in four rats were observed (Figure 7). The histopathological findings of the toxicity+melatonin group (Group 7) were similar to the findings of Group 6 (Figure 8). In toxicity+ozone+melatonin group (Group 8), focal mild hepatocyte damage in one rat, moderate focal damage in two rats, and severe focal damage in one rat were detected. Additionally, mild periportal inflammation was observed in three rats (Figure 9) and microvesicular steatosis in four rats.

Table 1. Details of the study groups.

Group ID	Group Definition	Chemicals and Doses	Administered via	Duration
Group 1	Control group	--	--	--
Group 2	Toxicity group	7 g/kg/day ethanol	Oral gauge	14 days
Group 3	Control + Ozone	95% O_2 + 5% O_3 0.5mg/kg ozone	Intraperitoneally	14 days
Group 4	Control + Melatonin	2 mg/kg melatonin	Intraperitoneally	14 days
Group 5	Control + Ozone + Melatonin	95% O_2 + 5% O_3 0.5mg/kg ozone + 2 mg/kg melatonin	Intraperitoneally	14 days
Group 6	Toxicity + Ozone	7 g/kg/day ethanol 95% O_2 + 5% O_3 0.5mg/kg ozone	Oral gauge Intraperitoneally	14 days
Group 7	Toxicity + Melatonin	7 g/kg/day ethanol 2 mg/kg melatonin	Oral gauge Intraperitoneally	14 days
Group 8	Toxicity + Ozone + Melatonin	7 g/kg/day ethanol 95% O_2 + 5% O_3 0.5mg/kg ozone 2 mg/kg melatonin	Oral gauge Intraperitoneally	14 days

Table 2. The mean values and standard deviations of AST, ALT, and GGT measurements.

Groups	AST (U/L)	ALT (U/L)	GGT (U/L)
	Mean±SD	Mean±SD	Mean±SD
1	42.85+7.24	154.60+17.27	ND
2	48.90+3.36	137.28+14.05	ND
3	76.32+42.65	438.18+367.28	0.63+1.55
4	78.73+31.22	432.95+151.57	0.25+0.61
5	45.11+8.68	198.10+36.65	ND
6	34.88+4.21	124.28+19.42	ND
7	24.42+4.054	97.46+16.93	ND
8	28.68+5.58	115.08+14.08	ND

*ND: Non detected.

There was no significant difference between the groups regarding vascular congestion ($p>0.05$). No statistically significant difference was found between Group 1, Group 3, Group 4, and Group 5 regarding the histopathological parameters ($p>0.05$). In the analysis performed between the control group and the toxicity group, there was no statistically significant difference considering vascular congestion. However, hepatocyte damage ($p=0.003$), and periportal inflammation ($p=0.020$) and steatosis ($p=0.004$) were significantly increased in the toxicity group.

When Group 2 was compared to Group 6, Group 7, and Group 8; it was seen that hepatocyte damage and periportal inflammation were significantly higher in Group 2 than Group 6 ($p=0.017$, and $p=0.039$, respectively). A similar significance was noted regarding periportal inflammation between Group 2 and Group 7 ($p=0.039$). No statistical significance was found between Group 2 and Group 8

(p>0.05). And there was no difference between any experimental groupconcerning congestion and steatosis (p>0.05).

Table 3. Serum and tissue TAS, TOS, and MDA levels (values are given as Mean±Standard deviation- SD).

Groups	Serum TAS ($\mu molH_2O_2Eq$ v/L)	Serum TOS ($\mu molH_2O_2Eq$ v/L)	Tissue TAS ($\mu molH_2O_2Eq$ v/ g wet tis-sue)	Tissue TOS ($\mu molH_2O_2Eq$ v/ g wet tissue	Serum MDA ($\mu molH_2O_2Eq$ v/L)	Tissue MDA ($\mu molH_2O_2Eq$ v/g wet tissue)
1	1.53+0.28	10.73+5.29	17.58+2.05	40.05+3.97	1.59+0.24	7.74+3.89
2	1.38+0.07	5.29+1.31	20.64+0.42	37.78+3.10	1.40+0.08	8.60+0.77
3	1.30+0.21	12.60+4.03	16.40+1.26	40.51+5.65	1.45+0.12	11.41+4.00
4	1.35+0.22	10.11+3.48	15.06+0.47	37.27+2.70	1.50+0.15	9.55+3.31
5	1.28+0.12	9.14+1.40	15.55+1.73	36.94+1.11	1.70+0.23	11.78+2.47
6	1.45+0.33	10.48+1.71	16.65+1.34	40.95+1.86	1.72+0.19	13.10+4.93
7	1.42+0.08	20.18+5.06	16.20+1.32	38.06+1.88	1.66+0.10	14.17+2.14
8	1.42+0.02	16.84+3.08	17.49+0.88	39.92+1.03	1.43+0.06	9.10+1.97

The serum enzyme (AST, ALT, and GGT) levels are shown in Table 2.Significantly elevated AST levels were detected in Group 4 when compared to Group 1 (p=0.021). AST enzyme levels were also higher in Group 6, Group 7, and Group 8 when compared to Group 1 (p=0.042, p<0.001, and p=0.032, respectively). Also, there was a significant difference between Group 6 and Group 7 (p=0.001).

The serum ALT levels were significantly higher in Group 4 (p=0.001), Group 5 (p=0.025), Group 6 (p=0.017), Group 7 (p=0.000), and Group 8 (p=0.028) after the results matched with negative control results. On the other hand, there was a significant difference between Group 4 (p=0.001), Group 5 (p=0.004), Group 7 (p=0.001) when compared to Group 2. The significant differences between almost all experimental groups were detected.

The mean and standard deviations for GGT levels were "0" in some groups. Hence, no significance tests could be done for GGT levels.

Table 3 shows the mean values and standard deviations of serum and tissue TAS, TOS, and MDA levels of all experimental groups.Only Group 5 and Group 7 had statistically significant differences when the serum TAS values were evaluated (p<0.001).Significant differences were detected between Group 2 and the following groups: Group 1 (p=0.012), Group 4 (p=0.039), Group 6 (p=0.005), Group 7 (p<0.001), and Group 8 (p<0.001) regarding serum TOS levels. On the other hand, Group 3 was only significantly different from Group 7 (p=0.017). Statistically significant differences were also detected between Group 4 and Group 7 (p=0.002), Group 5 and Group 7 (p<0.001), Group 5 and Group 8 (p=0.001), Group 6 and Group 7 (p=0.001), and Group 6 and Group 8 (p=0.001).

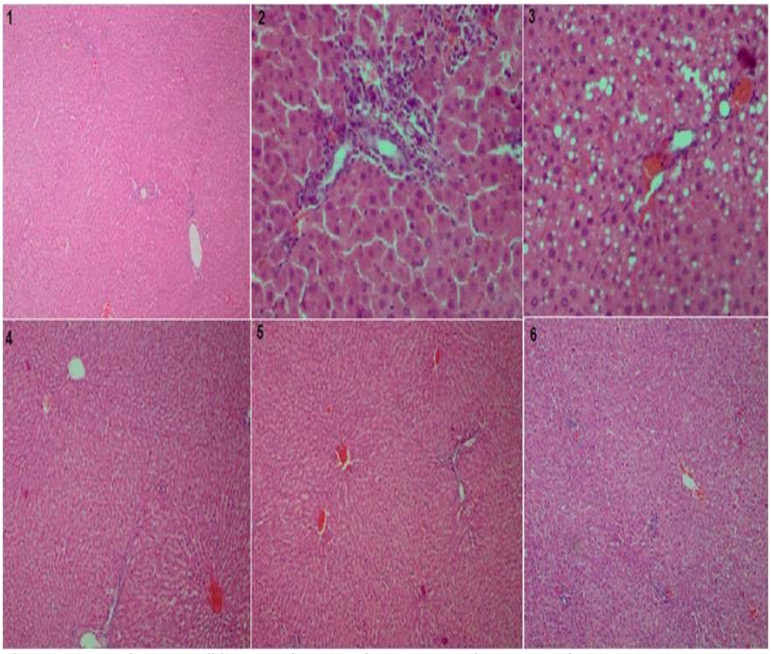

Figure 1: Control group, mild congestion vascular structures (H & E, 100x)
Figure 2: Toxicity group, periportal inflammation (H & E, 400x)
Figure 3: Toxicity group, microvesional steatosis (H & E, 400x)
Figure 4: Control+ ozone group, vascular congestion (H & E, 100x)
Figure 5: Control+ melatonin group, vascular congestion (H & E, 100x)
Figure 6: Control+ ozone+ melatonin group, hepatocyte injury and congestion (H & E, 100x)

When tissue TAS levels were evaluated, statistically significant differences were detected between the negative control and Group 2 (p<0.001) and Group 4 (p=0.015). Significant differences were also detected between Group 2 and Group 1 (p<0.001), Group 4 (p<0.001), Group 5 (p<0.001), Group 6 (p<0.001), Group 7 (p<0.001), Group 8 (p<0.001). Additionally, there was a difference between Group 5 and Group 6 (p=0.001), Group 8 (p=0.013) regarding tissue TOS levels.

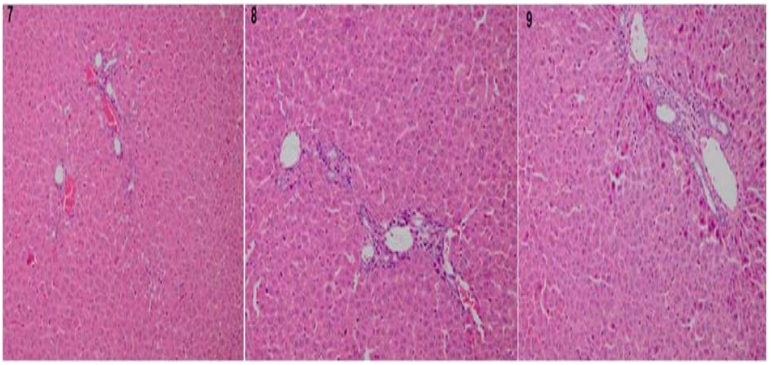

Figure 7: Toxicity + ozone group, hepatocyte injury and microvescular steatosis (H & E, 200x)
Figure 8: Toxicity + melatonin group, hepatocyte injury and periportal inflammation (H & E, 200x)
Figure 9: Toxicity + ozone + melatonin group, hepatocyte injury and periportal inflammation (H & E, 200x)

When the MDA levels were evaluated, it was seen that a significant decrease was detected in Group 6 (p=0.003) and Group 7 (p<0.001). A similar significance was determined between Group 2 and Group 8 (p=0.010). In addition, significantly increased MDA levels were detected in Group 6 (p=0.005) and Group 7 (p=0.001) when compared to Group 8.

The lowest tissue MDA levels were observed in Group 1, while the highest was detected in Group 7. Group 5 (p<0.001) and Group 7 (p=0.013) had decreased tissue MDA levels compared to Group 2. On the other hand, increased levels of MDA was detected in Group 7 (p= 0.017) compared to Group 4 and Group 8 (p=0.002).

Discussion

Alcoholic liver damage can be seen only with steatosis. However, cirrhosis and hepatocellular carcinoma show a continuous spectrum. Inflammation and oxidative damage are known to play acrucial role in the pathogenesis of this damage (14).This study demonstrated the effects of melatonin and ozone on the reduction of oxidative damage in alcoholic liver disease by evaluating serum enzyme levels, serum and tissue oxidant and antioxidant levels, and histopathological findings.

Quitting alcohol use is the backbone of therapy for patients with ALD, no matter at what stage. However, different drugs, chemical agents, and natural products that target specific pathways have been proposed for ALD treatment. Oxidative stress has a vital role in the pathogenesis of ALD.Manyin-vivo and in-vitrostudies with antioxidant agents were performed(2, 15, 16).

Ozone, melatonin and combined treatment options produced a statistically significant reduction of alcohol-induced liver damage as suggested by the serum transaminases levels. The transaminases ALT and AST are shown to be reliable markers of direct hepatocyte damage caused by alcohol or any other chemical. These enzymes are intracellular, and thus, can enter the circulation after toxicity-mediated cellular membrane damage(17). Our results showed that serum AST and ALT levels were decreased in the toxicity group after ozone, melatonin and combined treatment applications. Similar to our study, Hu et al. found that melatonin administration in alcohol-applied mice decreased AST and ALT values (18). In addition to this study, similar outputs have been available in the current literature about the decreased AST and ALT levels in hepatic toxicity groups after treatment with an agent(8, 19, 20). Therefore, wecan claim that in the hepatic toxicity model, ozone and melatonin cause either cell membrane stabilization or prevent its damage by free radicals. Typically high serum gamma-glutamyltranspeptidase (GGT) levels are expected in ALD, which were not measured in our study.

It was reported that some chemical agents such as melatonin increase the TASlevels in the organism by increasing antioxidant enzymes activity and decrease the TOSlevels by decreasing oxidative damage markers level (21, 22). On the other hand, decreased levels of the oxidative stress marker MDA revealed that a chemical, drug, or a natural compound positively modulated oxidative stress and strengthened the antioxidant system (23). The results of the present study in terms of TAS, TOS, and MDA activity are in contrast with the current literature. Most of the studies pointed out that melatonin or ozone treatment led to a significant decrease in the MDA levels in the toxicity groups and a significant increase in the SOD and GSH levels(20, 23, 24). Additionally, another study proved that CCL_4 increased the MDA and decreased the antioxidant enzyme levels in the toxicity group, while melatonin prevented MDA elevation in the CCL4-induced hepatotoxicity group (21).

When our results were evaluated, it was seen that MDA levels were lower in the toxicity groups than the treatment groups, the TOS activity was lower in the toxicity groups than the treatment groups, and the TAS activity was similar in the toxicity and treatment groups. Two questions can be raised at this point: “May ozone and melatonin treatments have additive effect concerning the tissue TOS levels?” and “Does alcohol haveadditive effects on serum levelsregarding oxidation?” In light of the biochemical results of our study, we can say that melatonin treatment increases the oxidative activity in the toxicity group. Thus, it has been concluded that, although the histopathological results suggest some benefit, the use of melatonin in terms of oxidation may worsen the prognosis. These conflicting findings need further elucidation.

Although several studies are evaluating the effects of some agents, drugs or chemicals on liver damage (23), limited publications are assessing the effects on liver morphology (25, 26). The damage that alcohol causes is characterized by vacuolar hepatic cell degeneration, microvesicular steatosis, and periportal inflammation. The ozone-alone treated group was histopathologically similar tothe control group. Besides, the only-melatonin treated group had similar effects with the only-ozone treated group. As to the literature, melatonin and ozone applications led to a histopathological recovery in the toxicity groups (8,18, 20, 26, 27). The same studies expressed that free radicals were also known to initiate inflammatory cell infiltration, thereby causing indirect liver injury (28). Becausemelatonincan scavenge free radicals,we may expect a decline in the inflammatory cell infiltration. Our histopathological results stated that ozone-alone administration was a better choice compared to melatonin-alone administration in preventing hepatocyte damage and periportal inflammation. Additionally, ozone therapy had similar effects with the melatonin treatment concerning microvesicular steatosis. It was detected that the combined administration of ozone and melatonin was less effective than single administrations.

Consequently, after our study there are many questions to

ask but the important one is: Apart from the antioxidant effect of ozone and melatonin, can there be different mechanisms that make ozone and melatonin therapeutic?

Limitations

Overall, the study has some limitations. The methodology of our study should be extended to cover other oxidant and antioxidant parameters. For a better assessment of the effects of ozone and melatonin administration, additional oxidant and antioxidant parameters such as SOD, CAT, LPO, and GSH should be evaluated. In addition, advanced histopathological techniques (additional and more complex staining techniques), as well as molecular methods, should be used to get more comprehensive data. Also, serum GGT levels are expected in ALD, which were not measured in our study.

Conclusion

Only melatonin treatment and combined ozone melatonin treatment had regulatory effects on the AST levels, while melatonin alone, ozone alone, and combined ozone+melatonin treatment had regulatory effects on the ALT levels.The TAS, TOS, and MDA levels were not parallel with current literature, however; administration of melatonin and/or ozone with alcohol significantly increased oxidant levels.In the light of histopathological findings, we can postulate that melatonin and ozone administrations are beneficial for alcohol-induced hepatotoxicity, however; ozone application seems to be superior to melatonin. The present study can speculate that the melatonin and ozone administration can be useful in clinics after confirmation by further studies.

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

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