

# Detection of chlorite, chlorate and perchlorate in ozonated saline

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**Abstract.** Medical ozone is used to treat various diseases, including numerous pathologies associated with chronic pain. Chronic pain may be treated by systemic administration of ozone, with ozonated autohemotherapy (OAH) being the commonly used method. In the clinic, intravenous infusion of ozonized saline has been used to treat various diseases. Compared with OAH, ozonized saline infusion is less technically demanding and causes minimal damage to veins. However, it has been indicated that ozone may oxidize saline and generate toxic substances, and therefore, the safety of ozone treatment has been questioned. In the present study, the potential chemical compounds produced from ozone and saline, including chlorite, chlorate and perchlorate, were examined at various time-points with ion chromatography-mass spectrometry (IC-MS). A control group (pure oxygen group) and an ozone group were included in the present study. Two subgroups were included within each group: A saline bottle (made from polypropylene) subgroup and an ozone-resistant blood transfusion bag [made from medical polyvinyl chloride, di(2-ethyl) hexyl phthalate plasticized] subgroup. For the ozone group, 100 ml saline and 100 ml medical ozone at various concentrations (20, 40 or 60  $\mu\text{g}/\text{ml}$  in pure oxygen) were injected into the saline bottle or blood bag, and for the control group, 100 ml of pure oxygen was injected into the saline bottle or blood bag. The presence and the content of chlorite, chlorate and perchlorate were determined at different time-points (3, 6 and 15 days after mixing) by IC-MS. Chlorate was detected in the ozone groups at three time-points and its

content increased as the ozone concentration and the reaction time increased. Under the same conditions (the same ozone concentration and the same incubation time), the chlorate content ( $0.90\pm 0.14$ - $7.69\pm 0.48$   $\mu\text{g}/\text{l}$ ) in the blood bag subgroup was significantly lower than that in the saline bottle subgroup ( $45.23\pm 6.14$ - $207.6\pm 15.63$   $\mu\text{g}/\text{l}$ ). However, chlorite and perchlorate were not detected at any time-point in the two groups. In addition, in the control group (pure oxygen group), chlorite, chlorate and perchlorate were not detected at any time-point. These results indicate that ozone reacts with saline to produce chlorate. Ozone may also react with the polypropylene saline bottle to increase the chlorate content in the bottled solution. Due to a lack of toxicology studies of chlorate in blood, it remains elusive whether ozonated saline and chlorate at the range of  $0.90\pm 0.14$ - $7.69\pm 0.48$   $\mu\text{g}/\text{l}$  has any toxic effects. The potential toxicity of chlorate should be considered when ozonated saline is used for clinical infusions.

## Introduction

Increasing evidence has indicated that ozone therapy is effective for treating numerous types of diseases featuring chronic pain, including osteoarthritis (1-4), neck and shoulder pain (5), lower back pain (6-9), myofascial pain syndrome (10), fibromyalgia (11), complex regional pain syndrome (12), zoster-associated pain (13,14) and other diseases, such as intractable headache and cardiovascular diseases (15,16). Medical ozone is administered in a flexible manner and may be applied externally or orally. It may also be administered locally or intravenously. Ozonated autohemotherapy (OAH) is widely used in the treatment of chronic pain (14,16,17). It has been reported that OAH alleviated pain of patients with post-herpetic neuralgia (14), intractable headache (16), as well as hyperuricemia and gout (17). Moderate concentrations of ozone (10 or 50  $\mu\text{g}/\text{ml}$ ) have been reported to increase deformability of red blood cells (18) and OAH is able to ameliorate renal ischemia-reperfusion injury in patients with kidney injury (19).

The specific mechanisms of action of ozone treatment in various diseases remain to be further studied. It is thought that the effects of ozone treatment are based on its strong

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oxidative and anti-pathogenic effects and immune regulation ability, as well as the increase of oxygen supply and reduction of oxidative stress (12,20-27). For instance, ozone was proven to relieve pain by causing the following effects: i) Release of endorphins (26); ii) inhibition of the activation of microglia via adenosine monophosphate (AMP)-dependent protein kinase signaling (28); iii) inhibition of the expression of purinergic receptors P2X3 and P2X7 in the spinal dorsal horn (29); iv) promoting phosphodiesterase 2A-cyclic guanosine monophosphate/cyclic AMP/NF $\kappa$ B-p65 signaling (30); v) inhibition of autophagy (31,32); vi) reduction of the expression of pro-inflammatory/pro-apoptotic caspases (33); and vii) increase of oxygen supply in tissues and cells (22).

OAH comprises the drawing of blood and blood transfusion through a steel needle, which causes vascular damage. To prevent infection, the blood transfusion operation requires strict aseptic conditions in the operating room. Furthermore, OAH is time-consuming and cumbersome. Therefore, clinicians are exploring novel techniques for systemic ozone delivery. It has been proposed that ozonated saline infusion may be able to replace OAH. Compared to OAH, ozonized saline infusion is easy to perform and does not require the drawing of blood or blood transfusions. Furthermore, the requirements for the operating room are easy to fulfill and the cost is relatively low, making ozonated saline infusion a possible alternative to OAH therapy. Compared to the OAH, the ozonated saline is more convenient and more operable, however there is controversial about clinical application of ozonated saline. A comparison of the two types of infusion is presented in Table I.

Intravenous infusion of ozonated saline has been used in the clinic to treat a variety of diseases. A clinical study suggested that intravenous infusion of ozonated saline contributes to the elimination of macrophages from wounds, mainly through regulating genetically programmed cell death (apoptosis), which has a significant role in the inflammatory process (34). Intravenous infusion of ozonated saline may improve symptoms of ischemia and hypoxia in the lower limbs of patients with occlusive atherosclerosis by stabilizing lysosomal hydrolyase activity (35). Intravenous infusion of ozonized saline may also reduce the viscosity of blood and the aggregation of red blood cells, as well as enhance the deformability of red blood cells (36).

Animal studies have indicated that intravenous injection of 5 ml/kg ozonized saline bubbled with 4  $\mu$ g/ml ozone in dogs increased the number of polymorphonuclear neutrophilic leukocytes, as well as their ability to capture bacteria for 2 days. In addition, this treatment led to an increase in the adaptability and compensatory capacity of the body (37). Intravenous infusion of ozonated saline may relieve liver injury induced by CCl<sub>4</sub> via its effects on reactive oxygen species (ROS) and the Kelch-like ECH-associated protein 1/nuclear factor, erythroid 2-like 2/ARE signaling pathway in rats (38).

With the development of ozonated saline infusion, it has been suggested that ozonated saline may contain toxic substances, including hypochlorite, chlorite, chlorate or even perchlorate (39). However, it was reported that ozone interacts neither with Na<sup>+</sup> nor with Cl<sup>-</sup> and no sodium hypochlorite or other chlorine-containing oxygen ions were detected (40). The possible reaction mechanisms include the following: i) Chloride ions in normal saline are first oxidized by ozone to

form chlorine atoms, initiating a chain reaction. The chlorine atoms are then oxidized by ozone to form harmful substances including chlorite, chlorate and perchlorate; ii) chloride ions are directly oxidized by ozone to form hypochlorite and oxygen, and hypochlorite is then oxidized by ozone to form chlorite, chlorate and perchlorate (39). These suggestions bring the clinical safety of ozonated saline infusion therapy into question (41).

In the present study, the safety of ozonated saline that may be used for intravenous infusion therapy was investigated. Ion chromatography-mass spectrometry (IC-MS) was used to determine the presence and content of chlorite, chlorate and perchlorate in ozonated saline at various time-points following preparation.

## Materials and methods

**Materials.** The following products were used in the present study: Medical ozone generator (Medozon compact, Herrmann Apparatebau GmbH); Dionex ICS5000<sup>+</sup> ion chromatograph with EGC eluent autogenerator (Thermo Fisher Scientific, Inc.); AB 4000 QTRAP triple-quadrupole mass spectrometry system with electrospray ion source and Analyst 1.6.2 workstation (AB Sciex API 4000 Qtrap; AB Sciex LLC); Dionex Ion Pac AS16 anion analysis column (Thermo Fisher Scientific, Inc.); C18 solid-phase extraction (SPE) column (Agela Technologies); ozone-resistant blood bag (S-200; Sichuan Nigale Biomedical Co., Ltd.); bottled saline (Sichuan Kelun Pharmaceutical Co., Ltd.); and ozone-resistant syringe (Shenli).

**Preparation of ozonated saline.** Ozone was produced with a medical ozone generator and various groups were set up. Ozone concentrations were set as 0 (100% oxygen), 20, 40 and 60  $\mu$ g/ml in oxygen. Ozone and saline were mixed at a volume ratio of 1:1. Using a 50-ml anti-oxidation syringe (Fig. 1A), 100 ml ozone of varying concentrations in oxygen, and 100 ml saline were drawn and mixed in saline bottles (Fig. 1B) or ozone-resistant blood transfusion bags (Fig. 1C). Ozonated saline was prepared and stored in room temperature without protection from light. On the 3rd, 6 and 15th day after the preparation, chlorite, chlorate and perchlorate in the ozonated saline were detected. The saline bottles and the blood bags had all been produced with the same batch numbers (E218092707 and 180808, respectively).

**Confirmation of chromatographic conditions.** A Dionex ICS5000<sup>+</sup> ion chromatograph system (Thermo Fisher Scientific, Inc.) was used. The mobile phase was KOH, the flow rate was set to 0.25 ml/min and the separation was performed on a Dionex Ion Pac AS16 analysis column (250x2 mm; Thermo Fisher Scientific, Inc.), and attached a Dionex Ion Pac AS16 anion guard column (50x2 mm; Thermo Fisher Scientific, Inc.). The column temperature was maintained at 25°C during the operation. The injection volume was 25  $\mu$ l. A 2-mm ASRS 500 anion suppressor (Thermo Fisher Scientific, Inc.) was used with an external water mode.

**Confirmation of mass spectrometry conditions.** An AB 4000 Qtrap triple-quadrupole mass spectrometer was used for mass

Table I. Comparisons of OAH and ozonated saline infusion.

Treatment	Blood drawing and transfusion	OD of puncture needle	Infusion environment	Operational difficulty	Patient acceptance	Treatment duration	Cost	Dispute in the medical field <sup>a</sup>
OAH	Yes <sup>b</sup>	20 G	Clean area for operation	High	Difficult	Relatively long	Relatively high	No
Ozonated saline infusion	No	24 G	Low requirement	Low	Easy	Relatively short	Relatively low	Yes

<sup>a</sup>Bocci *et al*, J Pharm Pharmacol 64 (4): 482-489, 2012. <sup>b</sup>Hu *et al*, J Pain Res 11: 1637-1643, 2018. OD, outer diameter; OAH, ozonated autohemotherapy.

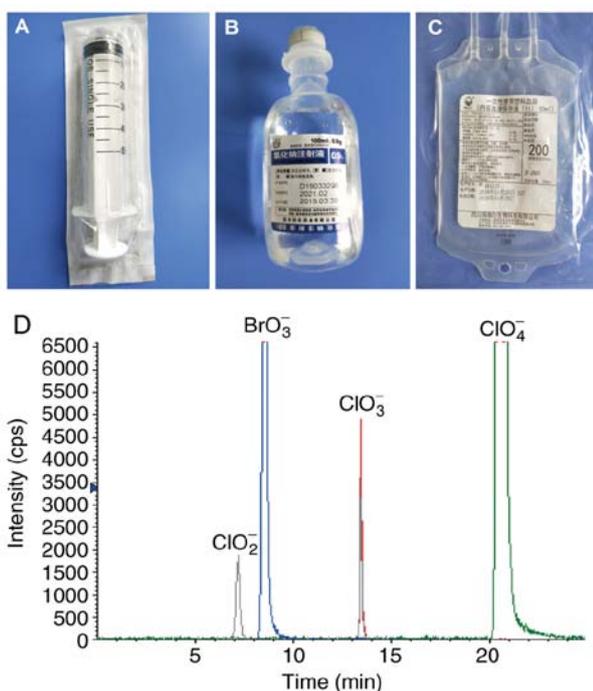


Figure 1. Materials used for the preparation of ozonated saline. IC-MS was used to detect chlorite, chlorate and perchlorate. (A) Ozone-resistant 50-ml syringes were used to inject varying concentrations of ozone and saline; (B) saline bottles that were used as the container for the ozone and saline solutions in the saline bottle subgroup; (C) ozone-resistant blood bags that were used to hold the ozone and saline solutions in the blood bag subgroup. (D) IC-MS results of 5 µg/l chlorite, chlorate and perchlorate standards. The retention times of 7.2, 13.5 and 20.8 min corresponded to chlorite, chlorate and perchlorate, respectively. IC-MS, ion chromatography-mass spectrometry; cps, counts per second.

spectrometry. The ion source was set as electrospray negative ion mode. The source temperature was 500°C. Further parameters were as follows: Ion spray voltage, -4,500 V; sprayer 1 (GS1), 206.84 kPa; GS2, 344.74 kPa. The transitions were used in multiple-reaction monitoring (MRM) mode. The collision gas was set to the medium mode. The surface heating system was set to open and the scan time was 200 msec.

**Selection of column and elution gradient.** The Dionex Ion Pac AS16 anion analysis column (250x2 mm) and the corre-

sponding guard column may rapidly and accurately detect trace amounts of perchlorate and other highly excited anions in multiple aqueous samples (42), and were therefore selected for the present study. In this experiment, 3, 25 and 45 mmol/l mobile phase (KOH) were used to analyze the separation effect of chlorite, chlorate and perchlorate.

**Optimization of mass spectrometry.** A total of 5 ml of 1 mg/l chlorite, chlorate and perchlorate standard solution were prepared and a needle pump was used to continuously inject at a flow rate of 10 µg/min. First, a primary precursor ion scan (Q1MS mode) was performed. Under the selected mass spectrometry conditions, chlorite, chlorate and perchlorate demonstrated a good response. After the precursor ion was determined, the solution was subjected to a two-stage scan (Q1MI mode). The product ions with the highest response value were selected separately. The declustering potential (DP) value was optimized, and a three-stage mass spectrometry fragment scan (MS2 mode) was then performed, and the collision energy (CE) value was optimized. Finally, the solution was analyzed in MRM mode. Detected ion pairs and optimized DP and CE voltage values for chlorite, chlorate and perchlorate are presented in Table II.

**Confirmation of linear association, detection limit and quantitation limit.** A total of five standard solutions (developed by the Ministry of Agriculture Environmental Protection Research Institute, Tianjin, China) with various concentrations (1, 2, 5, 10 and 20 µg/l) were accurately prepared and measured under optimized IC-MS conditions, as described above. Linear regression calculations were performed using the peak area (y-axis) of the analytes and the corresponding mass concentration (x-axis, µg/l) to obtain a linear regression equation and a linear correlation coefficient (R). This was used to determine the detection limit of an ion chromatographic peak at a signal-to-noise ratio (S/N)=3 and quantitation limit of peak at S/N=10. The results indicated that chlorite, chlorate and perchlorate had a good linear correlation in the corresponding mass concentration range and the linear correlation coefficient was >0.999. The detection limits of chlorite, chlorate and perchlorate were 0.2, 0.5, and 0.01 µg/l, respectively. The quantitation limits of chlorite, chlorate and perchlorate were 0.7, 1.5 and 0.05 µg/l, respectively.

Table II. Mass spectrometry conditions.

Analyte	Detected ion pairs (m/z)	DP (V)	CE (V)
Chlorite	66.9/50.8	-80	-20
Chlorate	82.9/66.9	-50	-30
Perchlorate	98.9/82.9	-60	-30

m/z, ratio of mass to charge; DP, declustering potential; CE, collision energy.

*Evaluation of precision and recovery rate.* Samples were accurately weighed using 1, 2, 5, 10 and 20  $\mu\text{g/l}$  of single standard solution and spiked recovery and precision tests were performed. The average recovery of each analyte was between 83.5 and 106.2%, and the relative standard deviation ( $n=6$ ) was between 3.7 and 5.8%.

*Sample detection.* The chloride ions in the sample solution were removed using a C18 SPE column at a flow rate of 3 ml/min and the concentrations of chlorite, chlorate and perchlorate in the sample were then measured using the method described above.

*Statistical analysis.* Statistical analysis was performed using Prism (v.7.0; GraphPad Software, Inc.) or SPSS 19.0 (IBM Corp.). Values are expressed as the mean  $\pm$  standard deviation. Statistical analysis between the groups of varying concentrations or containers was performed using repeated-measures two-way analysis of variance (ANOVA) tests followed by Sidak's multiple-comparisons tests. Statistical analysis of differences within the same concentration group at different time-points were performed using repeated-measures two-way ANOVA followed by Tukey's multiple-comparisons tests.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*Chlorite, chlorate and perchlorate presented a good separation effect.* Dionex Ion Pac AS16 anion analysis columns (250x2 mm) demonstrated a good separation effect on the chlorite, chlorate and perchlorate. An improved separation of chlorite, chlorate and perchlorate was achieved at the low mobile-phase concentration of 3 mmol/l KOH under the experimental conditions. The retention time of 7.2 min corresponded to chlorite, 13.5 min corresponded to chlorate and 20.8 min corresponded to perchlorate (Fig. 1D).

*Chlorite was not detected in the ozone-saline or oxygen-saline solution.* A total of five standard solutions (developed by the Ministry of Agriculture Environmental Protection Research Institute, Tianjin, China) with different concentrations (1, 2, 5, 10 and 20  $\mu\text{g/l}$ ) were added from low to high concentration for the spiked recovery test. Recovery rates on the 3rd day were 96.0-105.4%, those on the 6th day were 88.5-103.5% and those on the 15th day were 84.0-102.6%. The mass spectrometry results indicated that chlorite was not detected

on the 3rd, 6 and 15th day in either the ozone-saline or oxygen-saline solutions.

*Chlorate was detected in the ozone-saline subgroups at the three time-points.* The five standard solutions with different concentrations (1, 2, 5, 10 and 20  $\mu\text{g/l}$ ) were added from low to high concentration for the spiked recovery test. The recovery rates on the 3rd day were 88.5-106.0% and those on the 6th day were 83.5 and 106.2%. The recovery rates on the 15th day were 88.0-102.2%.

From the 3rd day to the 15th day after the ozonated saline was prepared, chlorate was detected in the blood bag subgroup (Fig. 2A and B) and the saline bottle subgroup (Fig. 2C and D). As time increased, the chlorate content in the two subgroups increased. There was no significant difference in the chlorate content between the 3rd and the 6th day in the blood bag and the saline bottle subgroups. However, on the 15th day, the chlorate content in the ozone-saline blood bag subgroup was significantly increased at all ozone concentrations compared with the corresponding levels on the 3rd and 6th days (Fig. 2B). In the saline bottle subgroup, the chlorate content on the 15th day was only significantly increased compared with that on the 3rd and 6th day in the same group (20  $\mu\text{g/ml}$  ozone). No significance was observed for the 0, 40 and 60  $\mu\text{g/ml}$  ozone groups across the time-points examined (Fig. 2D).

On the 3rd, 6 and 15th day, for the blood bag subgroup (Fig. 2B) and the saline bottle subgroup (Fig. 2D) at the same time-points, the chlorate concentration increased with increasing concentrations of ozone.

Under the same conditions (at the same time-points and the same ozone concentration), in the ozone-resistant blood bag subgroup, the chlorate content in the ozonated saline was significantly lower than that in the saline bottle subgroup (Fig. 3).

*Perchlorate was not detected in the ozone-saline or oxygen-saline solution.* The Five standard solutions with different concentrations (1, 2, 5, 10 and 20  $\mu\text{g/l}$ ) were added from low to high concentration for the spiked recovery test. The recovery rates were 86.6-101.0% on the 3rd day, 89.4-105.0% on the 6th day and 83.6-102.0% on the 15th day. The mass spectrometry results indicated that perchlorate was not detected on the 3rd, 6 and 15th day in either the ozone-saline or oxygen-saline solutions.

## Discussion

The results of the present study indicated that ozone is able to oxidize chloride ions in saline to form chlorate and the chlorate content gradually increased with time. In addition, higher ozone concentrations result in increased chlorate levels. Importantly, ozone may react with its container and thereby increase the levels of toxic substances in the ozone solution.

In the present study, when 100 ml of 20-60  $\mu\text{g/ml}$  ozone was mixed with an equal volume of saline, the content of chlorate in the ozonated saline was between  $0.90 \pm 0.14$  and  $207.6 \pm 15.63$   $\mu\text{g/l}$ . A low level ( $< 0.4$   $\mu\text{g/l}$ ) of chlorate was detected in the absence of ozone in the blood bag, this could be a result of detection errors of the machine, or from the material of the blood bag. To the best of our knowledge, no

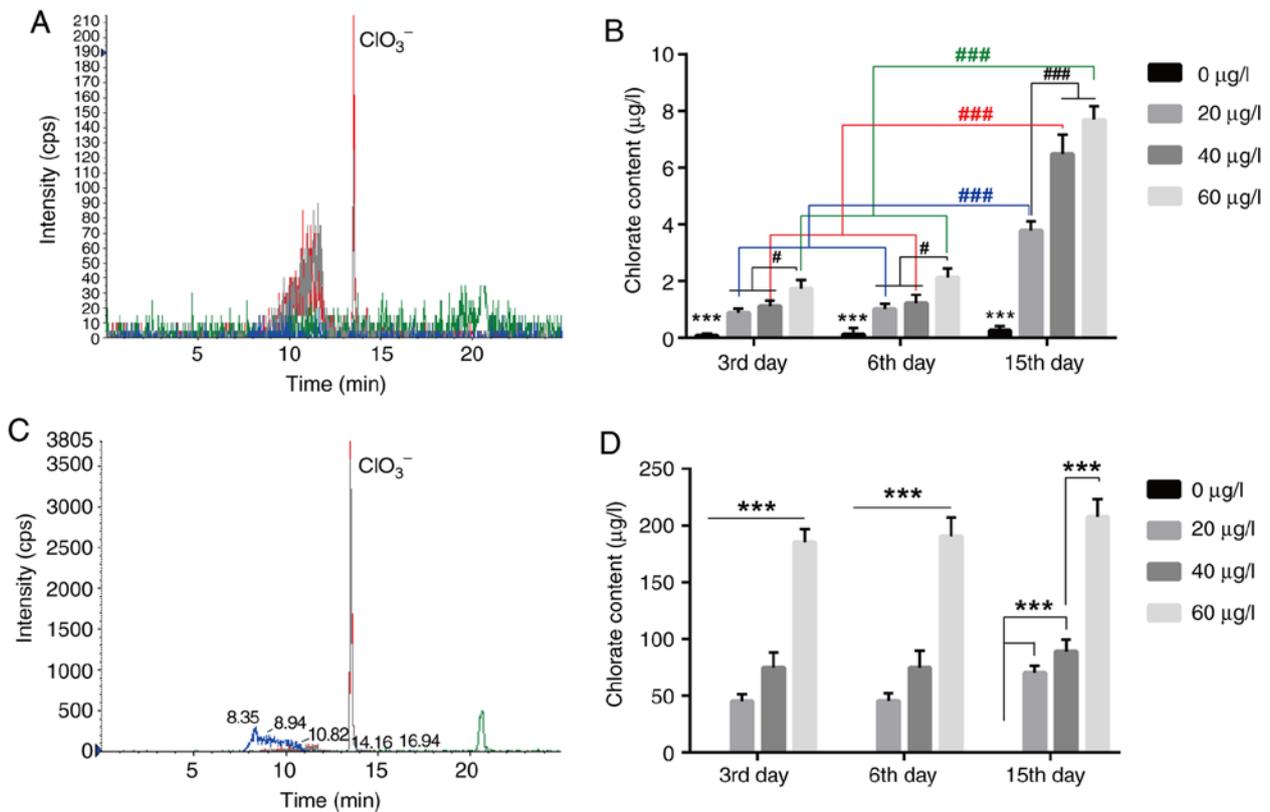


Figure 2. Changes in the chlorate content in ozonated saline on the 3rd, 6 and 15th day. By using IC-MS, the chlorate content was detected on the 3rd, 6 and 15th day in (A and B) the blood bag and (C and D) saline bottle subgroups. The chlorate concentration appeared to increase with time. (A) IC-MS detected chlorate in 40 µg/ml ozonated saline in the blood bag subgroup (peak time, 13.5 min; intensity, 210 cps) on the 3rd day. (B) Chlorate content in the blood bag subgroup with varying concentrations of ozonated saline on the 3rd, 6 and 15th day after mixing. There was no statistically significant difference in the chlorate content between the 3rd day and the 6th day after treating with any concentration of ozone. On the 15th day, the chlorate content increased significantly compared with that on the 3rd and 6th days in all treatment groups. (C) Chlorate was detected by IC-MS on the 3rd day in 40 µg/ml ozonated saline in the saline bottle subgroup (peak time, 13.5 min; intensity, 3,805 cps). (D) Chlorate content in the saline bottle subgroup with varying concentrations of ozonated saline on the 3rd, 6 and 15th day after mixing. On the 15th day, the chlorate content increased significantly compared with that on the 3rd day and the 6th day in the 20 µg/ml ozone-saline solution. There was no significant difference between the 3rd day, the 6th day and the 15th day in the 40 and 60 µg/ml ozone-saline solution. However, the chlorate content in ozonated saline was elevated with the increase of the ozone concentration. Values are expressed as the mean ± standard deviation (n=3 for each group). Statistical analyses were performed by repeated-measures two-way analysis of variance, followed by Tukey's multiple-comparisons tests. \*\*\*P<0.001 compared with the 3rd day and the 6th day; #P<0.05, ###P<0.001. IC-MS, ion chromatography-mass spectrometry; cps, counts per second.

study has reported the toxic dose of intravenously infused chlorate, although the World Health Organization Guidelines for drinking-water quality stipulated that the maximum permitted concentration of chlorate in drinking water is 0.7 mg/l (43). One study observed the effect of intravenous or oral sodium chlorate administration on the fecal shedding of *Escherichia coli* in sheep. Sodium chlorate (150 mg/kg) was infused over 3 h or less (a volume of 0.9% physiological saline containing 150 mg/kg sodium chlorate in a total volume of 250 ml), whilst the control group was administered the same dose of sodium chlorate orally. The content of NaClO<sub>3</sub> in the serum of sheep was measured at 4, 8, 16, 24 and 36 h after administration. The highest concentration of NaClO<sub>3</sub> in the serum was observed at 4 h of infusion (194.1±28.4 µg/l). The same dose (150 mg/kg NaClO<sub>3</sub>) was given orally, which also indicated a peak serum level after 4 h, reaching 138.9±13.2 µg/l (44). According to the results of the present study, the chlorate content in 100 ml ozonized saline was between 0.90±0.14 µg/l and 207.6±15.63 µg/l; this was much lower than the aforementioned toxic dose of chlorate and the maximum limit of the drinking-water standard setting after

metabolism in the serum. However, since the chlorate in the ozone-saline solution is administered intravenously, its toxicity requires careful re-evaluation.

On the 3rd, 6 and 15th day, no chlorite was detected in any of the groups. It is speculated that the ozonated saline did not form any chlorite or the chlorite that had been formed within 3 days continued to be oxidized by ozone, turning into chlorate. On the 3rd, 6 and 15th day, no perchlorate was detected in the two groups. It appears that ozone mixed with normal saline does not produce perchlorate within the observed timeframe (15 days) in the present study. Since hypochlorite is formed instantaneously and decomposes rapidly, no hypochlorite was detected in the present study.

In order to simulate the ozone and saline reaction scenarios in the clinic, the present study used two ozone-saline containers that are commonly used in clinical settings: Saline bottles (polypropylene) and ozone-resistant blood transfusion bags [medical polyvinyl chloride, di(2-ethyl) hexyl phthalate plasticized]. The results suggested that under the same conditions, the chlorate content in ozonated saline in the blood bag made of ozone-resistant material was much lower than that in the saline bottle.

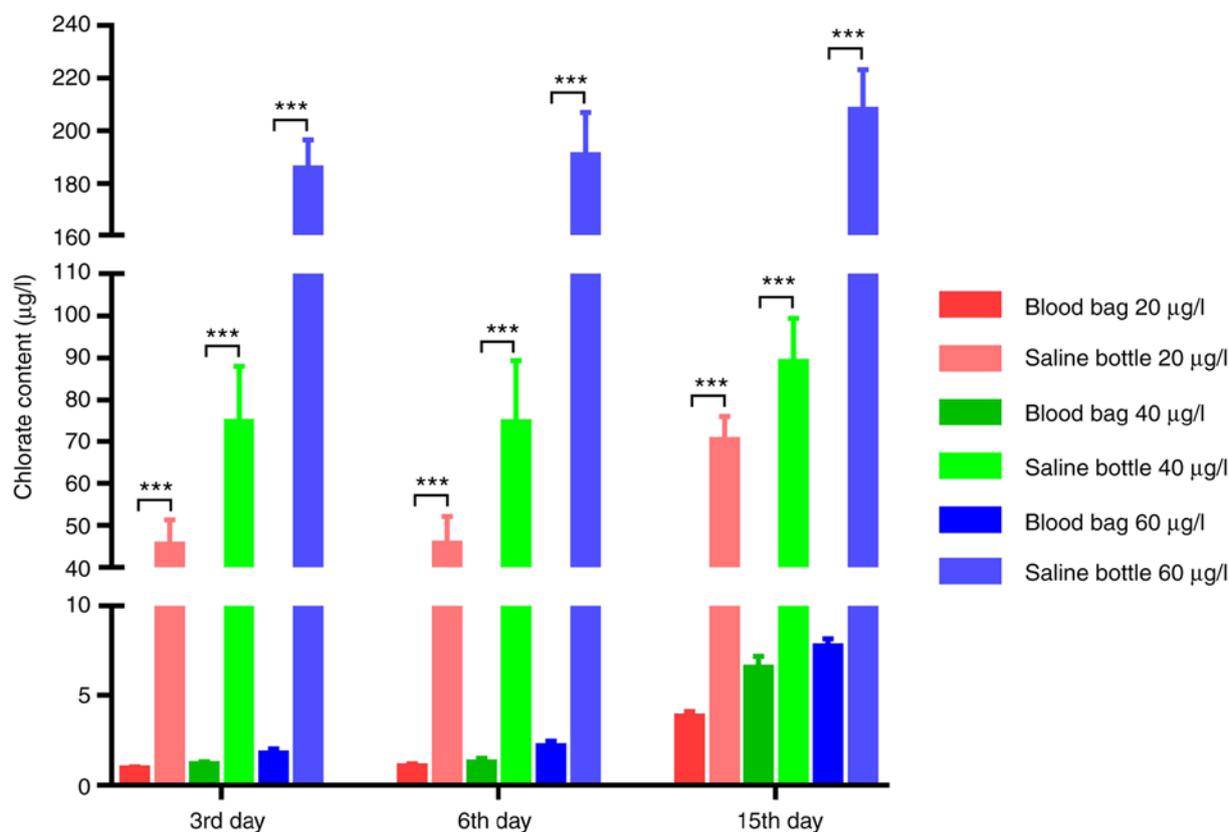


Figure 3. Reaction containers affected the chlorate content in the ozonated saline group. The chlorate content was detected using ion chromatography-mass spectrometry. The chlorate content of the ozonated saline in the ozone-resistant blood bag subgroup was significantly lower than that in the saline bottle subgroup at the same time-point with the same ozone concentration. \*\*\* $P < 0.001$  compared with the blood bag group at the same time-point and with the same ozone concentration. Values are expressed as the mean  $\pm$  standard deviation ( $n=3$  for each group). Statistical analyses consisted of repeated-measures two-way analysis of variance tests, followed by Sidak's multiple-comparisons tests.

As the reaction time went on, the chlorate content increased significantly. It may therefore be indicated that the material of the infusion container is able to affect the content of the toxic substances in the solution. The saline bottle material (polypropylene) may be oxidized by ozone, resulting in an increase of the chlorate content. These results suggested that the syringe and reaction container used for ozone therapy should consist of ozone-resistant materials, which would be effective in reducing the production of chlorate or any other toxic substances.

Previous studies indicated that oral administration of chlorate has multiple toxic effects. Ali *et al* (45) reported that a single oral dose of 100-750 mg/kg sodium chlorate in rats caused intestinal DNA damage through the production of ROS. Another study from the same group suggested that the same dose of sodium chlorate caused acute kidney injury in rats by producing an imbalance of redox reactions (46). Furthermore, different doses of sodium chlorate (0.106-1.06 mg/ml) not only induced oxidative stress in human red blood cells *in vitro*, leading to extensive damage to the cell membrane and reducing the anti-oxidant response, but also changed the morphology of red blood cells, increasing osmotic fragility. At the same time, exposure to different concentrations of sodium chlorate (0-10 nM) may lead to dose-dependent hemolysis (47). Chlorate and perchlorate affect the function of the thyroid gland. Following short-term (7 days) exposure to perchlorate (10 mg/l) and chlorate (100 mg/l) in rats, serum T4 was significantly lower than that in the control group (48). The incidence

of thyroid follicular cell hypertrophy in male and female rats administered sodium chlorate was higher and the incidence of thyroid cancer was higher in the 2,000 mg/l chlorate group compared with that in the control group; furthermore, a small amount of islet cell tumors were produced in female rats exposed to sodium chlorate (49). As intravenous infusion therapy with ozonated saline usually requires repeated administrations in the short-term, further basic and clinical research is required to determine whether the accumulation of chlorate is able to affect blood, kidney and thyroid function.

According to Bocci *et al* (41), intravenous infusion of ozonated saline is potentially toxic and therefore, they do not support the systemic administration of ozone in this way. The specific reasons are as follows: i) Ozonated saline may contain toxic substances including  $H_2O_2$ , hypochlorous and hypochlorous acid; ii) ozonation of saline is an unstable process and if it is not injected on time,  $O_3$  will completely decompose within 60 min; iii) during the infusion, the blood flow rate in the vein affects the concentration of  $H_2O_2$ - $O_3$ , suggesting that there may be a variable biological oxidation process. Therefore, the therapy does not meet the therapeutic principles of clinical drugs, namely stability and clearly defined active components (41).

The present study suggests that there may be toxic substances, e.g. chlorate, in ozonated saline, and the toxic dose of these substances in the human body remains to be determined. As such, the outcome of long-term intravenous infusion of ozonized saline remains uncertain.

Of note, the present study has several limitations. The content of chlorite, chlorate and perchlorate in ozonated saline was only measured after 3 days in the present study, and it remains elusive whether any chlorite was present in the ozonated saline at earlier stages. In the clinic, ozonated saline is prepared upon its use. In addition, chlorate and perchlorate are relatively stable; therefore, their concentrations in the first 3 days should be less than that detected on the third day. As such, the current data are instructive for clinical work. Usually, 100 ml of ozonated saline is infused within half an hour during clinical treatment. Bocci *et al* (41) reported that ozone totally decomposed within 60 min and that intravenous infusion of ozone solutions should be completed within one hour; therefore, detection of chlorite, chlorate and perchlorate in ozonated saline as soon as possible would provide results that are more representative of the clinical scenario. However, in the present study, differences between 3, 6 and 15 days observed indicate that there may have still been some ozone present, that may have led to the generation of chlorate beyond several days.

In conclusion, ozonated saline solution contains chlorate and the ozone may react with the polypropylene of the saline bottle to increase the chlorate content in the solution. Although studies have reported that intravenous infusion of ozonated saline is safe and effective, due to the lack of blood chlorate toxicology studies, it remains elusive whether the levels of chlorate detected in the ozonated saline in this present study ( $0.90 \pm 0.14$ - $207.6 \pm 15.63 \mu\text{g/l}$ ) are safe for the solution to be given intravenously. Therefore, clinical intravenous infusion of ozonated saline should be used with caution. Importantly, ozone reacts with various containers and an ozone-resistant container for ozonated solution infusion is recommended.

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#### Availability of data and materials

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

#### Authors' contributions

LM, SW, JY, DZ, YYL, YZ and SC performed the experiments, wrote the manuscript and prepared figures. YL and SC conceived and designed the experiments and provided the reagents, materials and analysis tools. All authors reviewed the data and drafts of the paper.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

#### References

1. Manoto SL, Maepa MJ and Motaung SK: Medical ozone therapy as a potential treatment modality for regeneration of damaged articular cartilage in osteoarthritis. *Saudi J Biol Sci* 25: 672-679, 2018.
2. Baranova IV: The use of the functional state of the joints for the estimation of the effectiveness of the application of oxygen/ozone therapy for the rehabilitative treatment of the patients suffering from knee arthritis. *Vopr Kurortol Fizioter Lech Fiz Kult* 95: 42-48, 2018 (In Russian).
3. Lopes de Jesus CC, Dos Santos FC, de Jesus LMOB, Monteiro I, Sant'Ana M and Trevisani VFM: Comparison between intra-articular ozone and placebo in the treatment of knee osteoarthritis: A randomized, double-blinded, placebo-controlled study. *PLoS One* 12: e0179185, 2017.
4. Raeissadat SA, Tabibian E, Rayegani SM, Rahimi-Dehgolan S and Babaei-Ghazani A: An investigation into the efficacy of intra-articular ozone (O<sub>2</sub>-O<sub>3</sub>) injection in patients with knee osteoarthritis: A systematic review and meta-analy. *J Pain Res* 11: 2537-2550, 2018.
5. Beyaz SG and Sayhan H: Six-month results of cervical intradiscal oxygen-ozone mixture therapy on patients with neck pain: Preliminary findings. *Pain physician* 21: E449-E456, 2018.
6. Elawamy A, Kamel EZ, Hassanien M, Wahba OM and Amin SE: Implication of two different doses of intradiscal ozone-oxygen injection upon the pain alleviation in patients with low back pain: A randomized, single-blind study. *Pain Physician* 21: E25-E31, 2018.
7. Biazzo A, Corriero AS and Confalonieri N: Intramuscular oxygen-ozone therapy in the treatment of low back pain. *Acta Biomed* 89: 41-46, 2018.
8. Costa T, Linhares D, Ribeiro da Silva M and Neves N: Ozone therapy for low back pain. A systematic review. *Acta Reumatol Port* 43: 172-181, 2018.
9. Braidy N, Izadi M, Sureda A, Jonaidi-Jafari N, Banki A, Nabavi SF and Nabavi SM: Therapeutic relevance of ozone therapy in degenerative diseases: Focus on diabetes and spinal pain. *J Cell Physiol* 233: 2705-2714, 2018.
10. Raeissadat SA, Rayegani SM, Sadeghi F and Rahimi-Dehgolan S: Comparison of ozone and lidocaine injection efficacy vs dry needling in myofascial pain syndrome patients. *J Pain Res* 11: 1273-1279, 2018.
11. Tirelli U, Cirrito C, Pavanello M, Piasentin C, Lleshi A and Taibi R: Ozone therapy in 65 patients with fibromyalgia: An effective therapy. *Eur Rev Med Pharmacol Sci* 23: 1786-1788, 2019.
12. Rowen RJ and Robins H: Ozone therapy for complex regional pain syndrome: Review and case report. *Curr Pain Headache Rep* 23: 41, 2019.
13. Lin SY, Zhang SZ, An JX, Qian XY, Gao XY, Wang Y, Zhao WX, Eastwood D, Cope DK and Williams JP: The effect of ultrasound-guided percutaneous ozone injection around cervical dorsal root ganglion in zoster-associated pain: A retrospective study. *J Pain Res* 11: 2179-2188, 2018.
14. Hu B, Zheng J, Liu Q, Yang Y and Zhang Y: The effect and safety of ozone autohemotherapy combined with pharmacological therapy in postherpetic neuralgia. *J Pain Res* 11: 1637-1643, 2018.
15. Di Mauro R, Cantarella G, Bernardini R, Di Rosa M, Barbagallo I, Distefano A, Longhitano L, Vicario N, Nicolosi D, Lazzarino G, *et al*: The Biochemical and pharmacological properties of ozone: The smell of protection in acute and chronic diseases. *Int J Mol Sci* 20: pii: E634, 2019.
16. Clavo B, Santana-Rodriguez N, Gutierrez D, Lopez JC, Suarez G, Lopez L, Robaina F and Bocci V: Long-term improvement in refractory headache following ozone therapy. *J Altern Complement Med* 19: 453-458, 2013.

17. Li LY and Ni JX: Efficacy and safety of ozonated autohemotherapy in patients with hyperuricemia and gout: A phase I pilot study. *Exp Ther Med* 8: 1423-1427, 2014.
18. Akbudak IH, Kucukatay V, Kilic-Erkek O, Ozdemir Y and Bor-Kucukatay M: Investigation of the effects of major ozone autohemotherapy application on erythrocyte deformability and aggregation. *Clin Hemorheol Microcirc* 71: 365-372, 2019.
19. Sancak EB, Türkön H, Çukur S, Erimsah S, Akbas A, Gulpinar MT, Toman H, Sahin H and Uzun M: Major ozonated autohemotherapy preconditioning ameliorates kidney ischemia-reperfusion injury. *Inflammation* 39: 209-217, 2016.
20. Bocci V: Ozonization of blood for the therapy of viral diseases and immunodeficiencies. A hypothesis. *Med Hypotheses* 39: 30-34, 1992.
21. Bocci V, Borrelli E, Travagli V and Zanardi I: The ozone paradox: Ozone is a strong oxidant as well as a medical drug. *Med Res Rev* 29: 646-682, 2009.
22. Bocci V: Autohemotherapy after treatment of blood with ozone. A reappraisal. *J Int Med Res* 22: 131-144, 1994.
23. Tusat M, Mentese A, Demir S, Alver A and Imamoglu M: Medical ozone therapy reduces oxidative stress and testicular damage in an experimental model of testicular torsion in rats. *Int Braz J Urol* 43: 1160-1166, 2017.
24. Al-Saadi H, Potapova I, Rochford ET, Moriarty TF and Messmer P: Ozonated saline shows activity against planktonic and biofilm growing *Staphylococcus aureus* in vitro: A potential irrigant for infected wounds. *Int Wound J* 13: 936-942, 2016.
25. Sagai M and Bocci V: Mechanisms of action involved in ozone therapy: Is healing induced via a mild oxidative stress? *Med Gas Res* 1: 29, 2011.
26. Borrelli E: Mechanism of action of oxygen ozone therapy in the treatment of disc herniation and low back pain. *Acta Neurochir Suppl* 108: 123-125, 2011.
27. Azuma K, Mori T, Kawamoto K, Kuroda K, Tsuka T, Imagawa T, Osaki T, Itoh F, Minami S and Okamoto Y: Anti-inflammatory effects of ozonated water in an experimental mouse model. *Biomed Rep* 2: 671-674, 2014.
28. Lu L, Pan C, Chen L, Hu L, Wang C, Han Y, Yang Y, Cheng Z and Liu WT: AMPK activation by peri-sciatic nerve administration of ozone attenuates CCI-induced neuropathic pain in rats. *J Mol Cell Biol* 9: 132-143, 2017.
29. Yu M, Zhao Y and Zhang X: Gardenoside combined with ozone inhibits the expression of P2X3 and P2X7 purine receptors in rats with sciatic nerve injury. *Mol Med Rep* 17: 7980-7986, 2018.
30. Wang J, Wu M, Lin X, Li Y and Fu Z: Low-concentration oxygen/ozone treatment attenuated radiculitis and mechanical allodynia via PDE2A-cAMP/cGMP- NF- $\kappa$ B/p65 signaling in chronic radiculitis rats. *Pain Res Manag* 2018: 5192814, 2018.
31. Wu MY, Xing CY, Wang JN, Li Y, Lin XW and Fu ZJ: Therapeutic dosage of ozone inhibits autophagy and apoptosis of nerve roots in a chemically induced radiculoneuritis rat model. *Eur Rev Med Pharmacol Sci* 22: 1787-1797, 2018.
32. Zhao X, Li Y, Lin X, Wang J, Zhao X, Xie J, Sun T and Fu Z: Ozone induces autophagy in rat chondrocytes stimulated with IL-1 $\beta$  through the AMPK/mTOR signaling pathway. *J Pain Res* 11: 3003-3017, 2018.
33. Fuccio C, Luongo C, Capodanno P, Giordano C, Scafuro MA, Siniscalco D, Lettieri B, Rossi F, Maione S and Berrino L: A single subcutaneous injection of ozone prevents allodynia and decreases the over-expression of pro-inflammatory caspases in the orbito-frontal cortex of neuropathic mice. *Eur J Pharmacol* 603: 42-49, 2009.
34. Karatieieva S, Muzyka N, Semenenko S, Bakun O and Kozlovskaya I: Ultrastructural changes of wound macrophages under the influence of intravenous ozone therapy in patients with diabetes and inflammatory processes of soft tissues. *Georgian Med News*: 98-101, 2018.
35. Tafil-Klawe M, Woźniak A, Drewa T, Ponikowska I, Drewa J, Drewa G, Włodarczyk K, Olszewska D, Klawe J and Kozłowska R: Ozone therapy and the activity of selected lysosomal enzymes in blood serum of patients with lower limb ischaemia associated with obliterative atheromatosis. *Med Sci Monit* 8: CR520-CR525, 2002.
36. Katiukhin LN: Influence of the course of treatment by injections of ozonized saline on rheological properties of erythrocytes in patients with complex pathology. *Hum Physiol* 42: 672-677, 2016.
37. Volkhovskaya NB, Tkachenko SB and Belopolsky AA: Modulation of phagocytic activity of blood polynuclear leukocytes with ozonized physiological saline. *Bull Exp Biol Med* 146: 559-561, 2008.
38. Qu DD, Peng FJ, Liu L, Yang SL and Guo YB: Effect of ozonized saline on signaling passway of Keap1-Nrf2-ARE in rat hepatocytes. *Zhonghua Gan Zang Bing Za Zhi* 19: 367-371, 2011 (In Chinese).
39. Razumovskii SD, Konstantinova ML, Grinevich TV, Korovina GV and Zaitsev VY: Mechanism and kinetics of the reaction of ozone with sodium chloride in aqueous solutions. *Kinet Catal* 51: 492-496, 2010.
40. Boyarinov GA, Gordetsov AS, Peretyagin SP, Boyarinova LV and Martusevich AK: Chemical transformations in treatment of saline solution with ozone-oxygen gas mixture. *J Health Inequal* 2: 194-199, 2016.
41. Bocci V, Zanardi I, Borrelli E and Travagli V: Reliable and effective oxygen-ozone therapy at a crossroads with ozonated saline infusion and ozone rectal insufflation. *J Pharm Pharmacol* 64: 482-489, 2012.
42. Zhang T, Cui H, Hu D, Cai F, Ma J and Zhu Q: Simultaneous determination of chlorite, chlorate, perchlorate and bromate of seafood processed products by IC-MS. *Food Sci Technol* 42: 326-330, 2017 (In Chinese).
43. WHO. Guidelines Approved by the Guidelines Review Committee. In: Guidelines for drinking-water quality: Fourth edition incorporating the first addendum World Health Organization, Geneva, 2017.
44. Smith DJ, Taylor JB, West M and Herges G: Effect of intravenous or oral sodium chlorate administration on the fecal shedding of *Escherichia coli* in sheep. *J Anim Sci* 91: 5962-5969, 2013.
45. Ali SN, Ansari FA, Arif H and Mahmood R: Sodium chlorate induces DNA damage and DNA-protein cross-linking in rat intestine: A dose dependent study. *Chemosphere* 177: 311-316, 2017.
46. Ali SN, Arif H, Khan AA and Mahmood R: Acute renal toxicity of sodium chlorate: Redox imbalance, enhanced DNA damage, metabolic alterations and inhibition of brush border membrane enzymes in rats. *Environ Toxicol* 33: 1182-1194, 2018.
47. Ali SN, Ahmad MK and Mahmood R: Sodium chlorate, a herbicide and major water disinfectant byproduct, generates reactive oxygen species and induces oxidative damage in human erythrocytes. *Environ Sci Pollut Res Int* 24: 1898-1909, 2017.
48. Khan MA, Fenton SE, Swank AE, Hester SD, Williams A and Wolf DC: A mixture of ammonium perchlorate and sodium chlorate enhances alterations of the pituitary-thyroid axis caused by the individual chemicals in adult male F344 rats. *Toxicol Pathol* 33: 776-783, 2005.
49. National Toxicology Program: Toxicology and carcinogenesis studies of sodium chlorate (Cas No. 7775-09-9) in F344/N rats and B6C3F1 mice (drinking water studies). *Natl Toxicol Program Tech Rep Ser*: 1-255, 2005.



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