

IL-6/STAT3 signaling pathway-mediated apoptosis induced by medical ozone water in liver cancer: A mechanistic study

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Received April 12, 2025; Accepted November 28, 2025

DOI: 10.3892/br.2026.2167

Abstract. Liver cancer is one of the most common and aggressive malignancies worldwide, with high rates of proliferation, migration and invasion contributing to poor patient outcomes. Ozonated water has potential anti-tumor effects. However, the underlying mechanisms, particularly its interaction with key signaling pathways such as IL-6/STAT3, remain poorly understood. The IL-6/STAT3 pathway serves a critical role in cancer progression, regulating processes such as cell survival and metastasis. To the best of our knowledge, however, the association between ozonated water and this pathway in liver cancer cells has not been thoroughly investigated. Therefore, the objective of the present study was to explore the effects of ozonated water on the proliferation, migration and invasion of HepG2 and Huh-7 liver cancer cells and determine the relationship between ozone and the IL-6/STAT3 signaling pathway, and the potential mechanism of this pathway in HepG2 cells. Proliferation was evaluated using the Cell Counting Kit-8

assay. Wound healing assay was used to evaluate migration ability. Transwell assay with Matrigel was used to evaluate invasion ability. Flow cytometry was used for cell apoptosis analysis. Protein expression was estimated by western blot analysis. Treatment with ozone at a concentration of 20 μg for 24 h inhibited liver cancer cell proliferation, migration and invasion capacities. Flow cytometric analysis revealed a marked increase in apoptosis rate in ozone-treated HepG2 cells (25.15 \pm 0.72 vs. 12.90 \pm 0.83% in control treated with MEM). Western blotting indicated downregulation of IL-6/STAT3 pathway components in ozone-treated HepG2 cells, with IL-6 and phosphorylated (p-)STAT3 expression levels showing the most pronounced reduction. Concurrently, Bcl-2 protein expression significantly decreased in the ozone-treated cells, whereas cleaved caspase-3 exhibited upregulated expression in treated cells. Ozone inhibited the proliferation, migration and invasion of and induced the apoptosis of liver cancer cells. Medical ozone water may induce liver cancer cell apoptosis by downregulating the IL-6/STAT3 signaling pathway, as evidenced by decreased expression of p-STAT3 and downstream anti-apoptotic proteins (Bcl-2) alongside apoptotic induction.

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Abbreviations: TACE, transcatheter arterial chemoembolization; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; HMGB1, high mobility group box 1; TNF- α , tumor necrosis factor- α ; VEGF, vascular endothelial growth factor; ARE, antioxidant response element; IC50, half-maximal inhibitory concentration

Key words: ozone, IL-6, STAT3, apoptosis, liver cancer

Introduction

Globally, liver cancer ranks as the fifth most commonly diagnosed cancer and the third leading cause of cancer-related mortality. In 2021, there were ~906,000 new cases and 830,000 cases of mortality worldwide (1). In China, liver cancer had the second highest mortality rate among all malignant tumors, with an estimated 316500 deaths in 2022, accounting for ~22.42% of all cancer-associated mortality (2-4). Although treatments, such as surgical resection, interventional therapy [including transarterial chemoembolization (TACE), hepatic artery infusion chemotherapy and ablation] and systemic therapy, prolong patient survival, the 5-year survival rate remains between 25 and 39% (5). In addition, the recurrence rate is up to 80% (6).

Medical ozone, which consists of a reactive mixture of oxygen and ozone, has been documented to exhibit diverse therapeutic properties and is widely used for the treatment of conditions such as mucositis, psoriasis, acute pain, neurovascular disease and cancer (7-9). The potential of ozone in oncology was highlighted in 1980: Ozone selectively inhibits the proliferation of lung, breast and uterine tumor cells without affecting normal cells (10). Accumulating evidence has further substantiated its role in cancer therapy (11-13). Zänker and Kroczeck (12) demonstrated that ozone enhanced the efficacy of 5-fluorouracil by reversing chemoresistance in breast cancer cells. In addition, Cannizzaro *et al* (13) showed that ozone induces apoptosis in the neuroblastoma cell line SK-N-DZ via caspase-3 and poly-ADP ribose polymerase pathways, whilst impeding cell cycle progression in SK-N-SH cells by modulating cyclin B1/cyclin-dependent kinase 1 activity. In the context of liver cancer, Li *et al* (14) demonstrated that medical ozone can inhibit proliferation, migration and epithelial-mesenchymal transition in hepatocellular carcinoma (HCC) cells via reactive oxygen species (ROS) accumulation and suppression of the PI3K/AKT/NF- κ B pathway (14). Similarly, Tang *et al* (15) found that ozonated water suppresses HCC cell proliferation, invasion and metastasis by regulating the high mobility group box 1 protein (HMGB1)/NF- κ B/STAT3 pathway. Additionally, ozone induces apoptosis in BEL7402 cells through the mitochondrial pathway (16). Despite its diverse advantages, such as anti-inflammatory effects, pain relief and improving immune function, ozone gas has limited clinical applications due to respiratory toxicity and instability. By contrast, ozonated water is safer and easier to handle. Ma *et al* (17) reported that intratumoral injection of ozonated saline promotes necrosis and suppresses tumor growth, potentially through the IL-6 and TNF- α pathways. Kuroda *et al* (18) demonstrated that local ozone injection dose-dependently induced tumor apoptosis or necrosis without damaging normal tissue. Consistent findings were also reported by Kızıltan *et al* (19), Peirone *et al* (20), and Yıldırım *et al* (21), supporting the translational potential of ozone therapy.

Chronic inflammation serves a key role in tumor progression. Within the tumor microenvironment, key inflammatory mediators, such as NF- κ B and STAT3, can activate genes involved in cell proliferation and angiogenesis, thereby promoting tumor growth and metastasis (22). Notably, NF- κ B perpetuates inflammatory signaling by sustaining the expression of proinflammatory cytokines, including IL-6 and IL-8 (23). This process not only amplifies local inflammation, but also fosters genetic instability and aberrant cell proliferation, establishing a microenvironment that supports tumorigenesis. As a pleiotropic cytokine, IL-6 is highly expressed in numerous types of malignancy, including HCC (24) and colorectal (25) and breast cancer (26). It functions by binding to its specific receptor, IL-6 receptor α , leading to the activation of glycoprotein 130 and subsequent initiation of the JAK/STAT3 signaling pathway (27). Upon activation, phosphorylated (p-)STAT3 translocates into the nucleus and upregulates the expression of target genes, such as Bcl-2, VEGF and MMP2 (28,29). These genes collectively contribute to tumor progression by inhibiting apoptosis whilst promoting proliferation, metastasis and angiogenesis.

Ozone reacts with biological components to induce controlled oxidative stress, leading to the formation of reactive species (30). This process activates the antioxidant defense system. Moderate oxidative stress stimulates the transcription of the antioxidant response element (ARE), promoting the production of several antioxidant enzymes, including glutathione S-transferase, catalase, heme oxygenase-1 and NADPH quinone oxidoreductase 1 (31). These enzymes protect cells from inflammatory damage (32). Concurrently, moderate levels of oxidative stress suppress the NF- κ B signaling pathway, attenuating inflammatory responses and decreasing the levels of proinflammatory factors (such as IL-6) in the microenvironment (33).

Based on the aforementioned findings, it was hypothesized that ozone induces apoptosis in liver cancer cells by decreasing IL-6 levels within the tumor microenvironment, thereby inhibiting the IL-6/STAT3 signaling pathway and suppressing anti-apoptotic factor release. To evaluate this hypothesis, HepG2 and Huh-7 cells were cultured in medical ozone-enriched medium to assess its effects on cell proliferation, migration and invasion. Apoptosis rates were measured using flow cytometry, whilst western blot analysis was performed to determine whether ozone-mediated apoptosis occurs through suppression of the IL-6/STAT3 pathway.

Materials and methods

Cell culture. Human HCC cell lines HepG2 and Huh-7 and the normal hepatic cell line THLE-2 were obtained from Procell Life Science & Technology Co., Ltd. Cell identity was authenticated via short tandem repeat (STR) profiling (performed by the Cell Resource Center of JENNIO Biological Technology and Zhejiang Baidi Biotechnology Co., Ltd.). STR loci matched the American Type Culture Collection reference profile with >98% similarity, confirming no cross-contamination. Mycoplasma contamination was tested monthly using the MycoAlert™ Mycoplasma Detection kit (Lonza Walkersville, Inc) and all cultures were negative. HepG2 cells were cultured in MEM supplemented with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin. Huh-7 cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS and 1% penicillin-streptomycin. THLE-2 cells were cultured in THLE-2 Cell Complete Medium (Wuhan Pusainuo Life Science Co., Ltd.). All three cell lines were cultured at 37°C with 5% CO₂. Cells were divided into experimental and control groups. The experimental group was treated with ozonated medium, while the control group received ozone-free complete medium. The THLE-2 cell line was used to assess the safety of ozonated water on normal hepatocytes.

Preparation of ozonated medium. Complete medium was aliquoted into a sterile 250 ml saline bottle. After removing air from the bottle, it was stored at 4°C. The ozonated medium was freshly prepared 1 h before application using a Medozon compact ozone generator (Humares GmbH). The ozone generator was calibrated monthly using a standard ozone solution. Specifically, an oxygen (95%)/ozone (5%) gas mixture was infused into the pre-chilled medium at a flow rate of 3 l/min for 20 min at room temperature. The final ozonated medium

was stored at 4°C. Ozone concentration was determined using an ozone colorimeter (Huankai Microbial).

Determination of half-maximal inhibitory concentration (IC₅₀). Cells in the logarithmic growth phase were seeded into 96-well plates at 5x10³ cells/well. After 24 h incubation, cells were treated at 37°C with ozonated medium at concentrations of 0, 5, 10, 20, 40 and 60 µg/ml, each with three replicates. Following 0, 24 and 48 h treatment, cell viability was assessed using Cell Counting Kit-8 (CCK-8; Nanjing KeyGen Biotech Co., Ltd.). Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.) to calculate the IC₅₀.

Cell proliferation assay. Cell proliferation was evaluated using the CCK-8 assay. HepG2, Huh-7 and THLE-2 cells were seeded into 96-well plates at 5x10³ cells/well and treated at 37°C with ozonated or control medium for 0, 24, 48 and 72 h. Supernatant was removed and replaced with 100 µl medium containing 10% CCK-8 reagent. Following 1.5 h incubation in the dark, absorbance was measured at 450 nm. Each experimental condition included three replicate wells and a blank control. The experiment was repeated three times.

Wound healing assay. HepG2 and Huh-7 cells were seeded at 37°C into 6-well plates at 4x10⁵ cells/well (three replicates/group). After 24 h, when cells reached 90% confluency, three straight scratches were made in each well using a 20-µl pipette tip. The cells were washed three times with PBS and incubated in serum-free MEM and DMEM. Images were captured at 0 and 72 h using an inverted light microscope (Nikon Corporation), before scratch areas were measured by Image J software (version 1.53k, National Institutes of Health). The assay was performed in triplicate.

Transwell invasion assay. Transwell chambers were pre-coated with 50 µl Matrigel (1 µg/µl) at 37°C for 2 h. HepG2 and Huh-7 cells were serum-starved for 8 h, detached with EDTA-trypsin, washed and resuspended in serum-free MEM and DMEM. In total, 200 µl cell suspension (1x10⁵ cells) was added to the upper chamber and 600 µl complete MEM or DMEM supplemented with 10% FBS, Gibco) was added to the lower chamber. Following 24 h incubation at 37 °C with 5% CO₂, non-invading cells were removed with a cotton swab. Invaded cells were fixed with 20% methanol at room temperature for 15 min, stained with 0.1% crystal violet at room temperature for 20 min and imaged under an inverted light microscope. Each group had three replicates and the experiment was repeated three times.

Apoptosis analysis by flow cytometry. HepG2 cells were seeded into 6-well plates at 5x10⁵ cells/well (three replicates/group) and treated at 37°C with ozonated or control medium for 24 h. Supernatant was collected, cells were washed with PBS, detached using EDTA-free trypsin and centrifuged at 300 x g for 5 min at 4°C. The cell pellet was resuspended in 500 µl loading buffer and stained with Annexin V-FITC and PI (Nanjing KeyGen Biotech Co., Ltd.; cat. no. KGA1012) for 15 min at 37°C in the dark. Samples were analyzed using a FACSCalibur flow cytometer and the data was analyzed by Flow Jo software

(version 10.8.1; both BD Biosciences). Single-stained Annexin V-FITC and PI controls confirmed no signal overlap, and gating excluded debris to ensure accurate apoptotic cell counting. Apoptotic cells were defined as the sum of cells in both the early and late (Annexin V-FITC-positive, PI-positive) apoptotic quadrants. The experiment was repeated three times.

Western blot analysis. HepG2 cells were lysed with RIPA buffer (Shanghai Epizyme Biomedical Technology Co., Ltd.) containing PMSF and phosphatase inhibitors (100:1:1). Following centrifugation at 1,800 x g for 15 min at 4°C, protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology) with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Proteins (20 µg/lane) were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk for 1 h at room temperature and incubated overnight at 4°C with the following primary antibodies: Caspase-3 (1:2,000; cat. no. ET1602-39; Hangzhou HuaAn Biotechnology Co., Ltd.), Bcl-2 (1:2,000; cat. no. ab182858; Abcam), Bax (1:20,000; cat. no. ET1603-34; Hangzhou HuaAn Biotechnology Co., Ltd.), cleaved caspase-3 (1:2,000; cat. no. ET1602-47; Hangzhou HuaAn Biotechnology Co., Ltd.), JAK2 (1:5,000, cat. no. ab108596; Abcam), p-JAK2 (1:2,000; cat. no. ab32101; Abcam), STAT3 (1:2,000, cat. no. ab68153; Abcam), p-STAT3 (1:2,000, cat. no. 05-485; Hangzhou HuaAn Biotechnology Co., Ltd.), IL-6 (1:1,000; cat. no. FNab04282) and GAPDH (1:2,000, cat. no. FNab03342; both Wuhan Fine Biotech, Co., Ltd.). After washing four times with PBST buffer, the membrane was incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:10,000, cat. no. FNSA-0004; Wuhan Fine Biotech, Co., Ltd.) for 1 h at 37°C. Signals were detected using an enhanced chemiluminescence reagent (MilliporeSigma; cat. no. WBKLS0100) and quantified with a chemiluminescence imaging system (ChemiDoc Go; Bio-Rad Laboratories, Inc.). Images were analyzed with Image Lab Touch Software 2.4 (Bio-Rad Laboratories, Inc.). All signals were normalized to GAPDH (loading control) to ensure equal protein loading. A total of three independent experiments was performed.

Statistical analysis. Data from three independent experiments are presented as the mean ± SD. Statistical analyses were conducted using SPSS 20.0 (IBM Corp.). Comparisons between two groups were performed using an unpaired independent samples t-test. The normality of data distribution was assessed using the Shapiro-Wilk test with SPSS software. Non-normally distributed data were analyzed by Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference. Graphs were generated using GraphPad Prism 8.0 (Dotmatics).

Results

Ozone selectively inhibits proliferation of liver cancer cells. Cell proliferation was evaluated using the CCK-8 assay. HepG2, Huh-7 and THLE-2 cells were treated with ozone at 0, 5, 10, 20, 40 and 60 µg/ml for 24, 48 and 72 h. Absorbance was measured at each time point, where the IC₅₀ was determined. Based on comparative, treatment with 20 µg/ml ozone for 0, 24, 48 and 72 h significantly suppressed the proliferation of HepG2 and

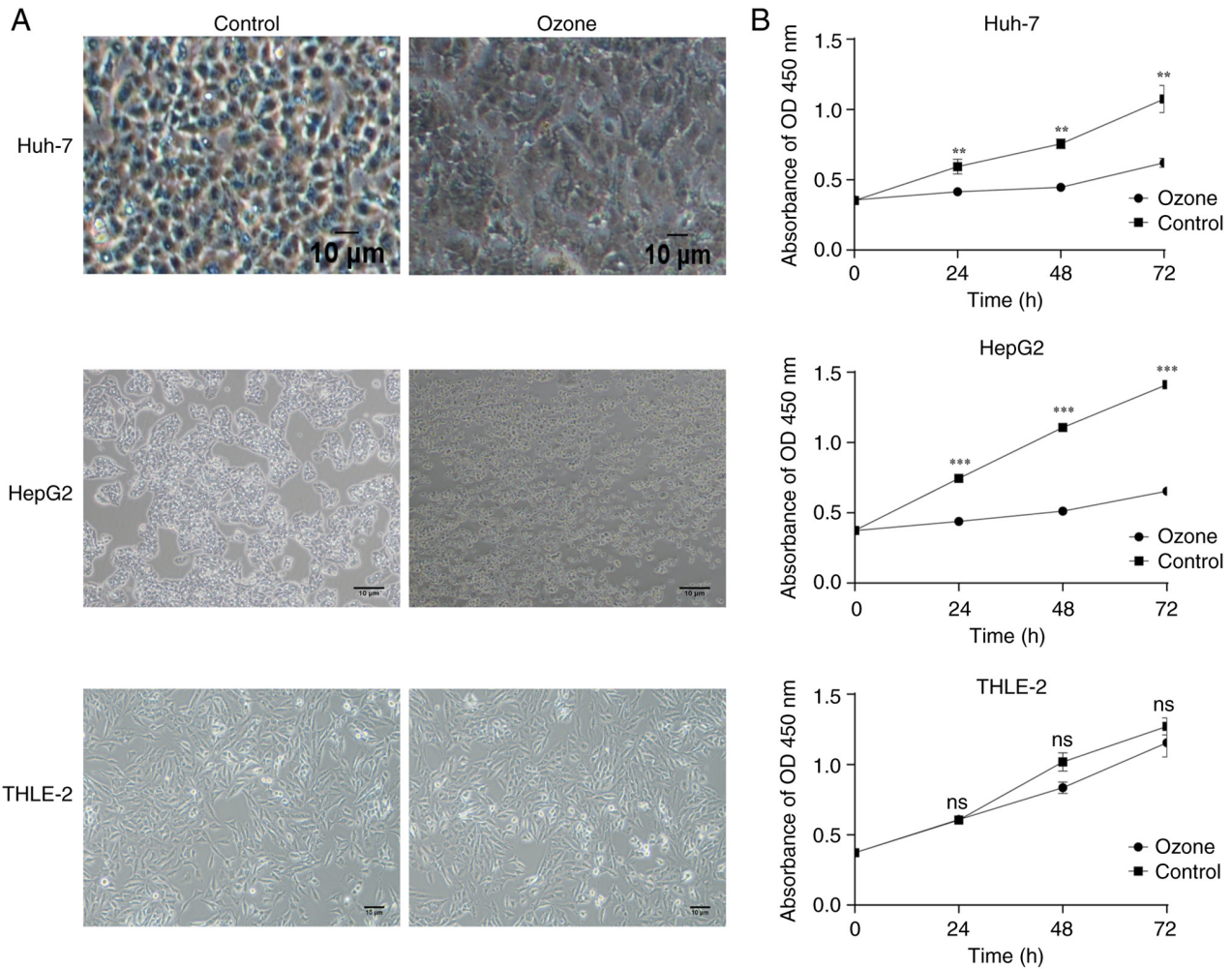


Figure 1. Effect of ozone on cell viability. (A) Morphology of Huh-7, HepG2 and THLE-2 cells. (B) Viability of Huh-7, HepG2 and THLE-2 cells treated with 20 µg/ml ozonated medium. n=3. **P<0.01, ***P<0.001 vs. control. ns, not significant; OD, optical density.

Huh-7 cells in a time-dependent manner, while no significant effect was observed on THLE-2 normal liver cells (Fig. 1). Therefore, 20 µg/ml was selected for subsequent experiments.

Ozone suppresses migration of liver cancer cells. The effect of ozone on cell migration was assessed using a wound healing assay. Ozone-treated HepG2 and Huh-7 cells exhibited significantly decreased migration, with larger remaining scratch areas compared with those in the control group (Fig. 2).

Ozone inhibits invasion of liver cancer cells. A Transwell invasion assay was conducted to evaluate the effect of ozone on the invasive capability of HepG2 and Huh-7 cells. Following 24 h of ozone treatment, the number of invading cells was significantly lower compared with the control group, indicating that ozone effectively inhibited the invasiveness of liver cancer cells (Fig. 3).

Ozone induces apoptosis in HepG2 cells. Flow cytometry was used to analyze apoptosis in HepG2 cells following ozone treatment, which demonstrated a notable increase in apoptotic cells in the treated group (Fig. 4A). The apoptosis rate was significantly higher in the ozone group compared with that in the control, demonstrating an induction of apoptosis (Fig. 4B).

Western blot analysis revealed that ozone treatment significantly upregulated cleaved caspase-3 and downregulated Bcl-2, with a non-significant decrease in Bax expression (Fig. 5). Bcl-2/Bax ratio was decreased in the ozone-treated compared to the control group. These results indicated that ozone promoted apoptosis in HepG2 cells primarily through the intrinsic mitochondrial pathway (Fig. 5).

Ozone suppresses the IL-6/STAT3 signaling pathway. To investigate the molecular mechanism underlying ozone-induced apoptosis, the IL-6/STAT3 pathway was examined using western blotting. Ozone significantly reduced IL-6 expression and decreased the phosphorylation levels of JAK2 and STAT3, while total JAK2 and STAT3 expression levels remained unchanged. These findings suggested that ozone inhibited IL-6/STAT3 signaling, contributing to apoptosis induction in HepG2 cells (Figs. 6 and 7).

Discussion

The present study demonstrated that ozone inhibited the proliferation of HepG2 and Huh-7 cells, while exhibiting no significant cytotoxic effects on the normal hepatocyte line THLE-2. This selective anti-tumor activity aligns with

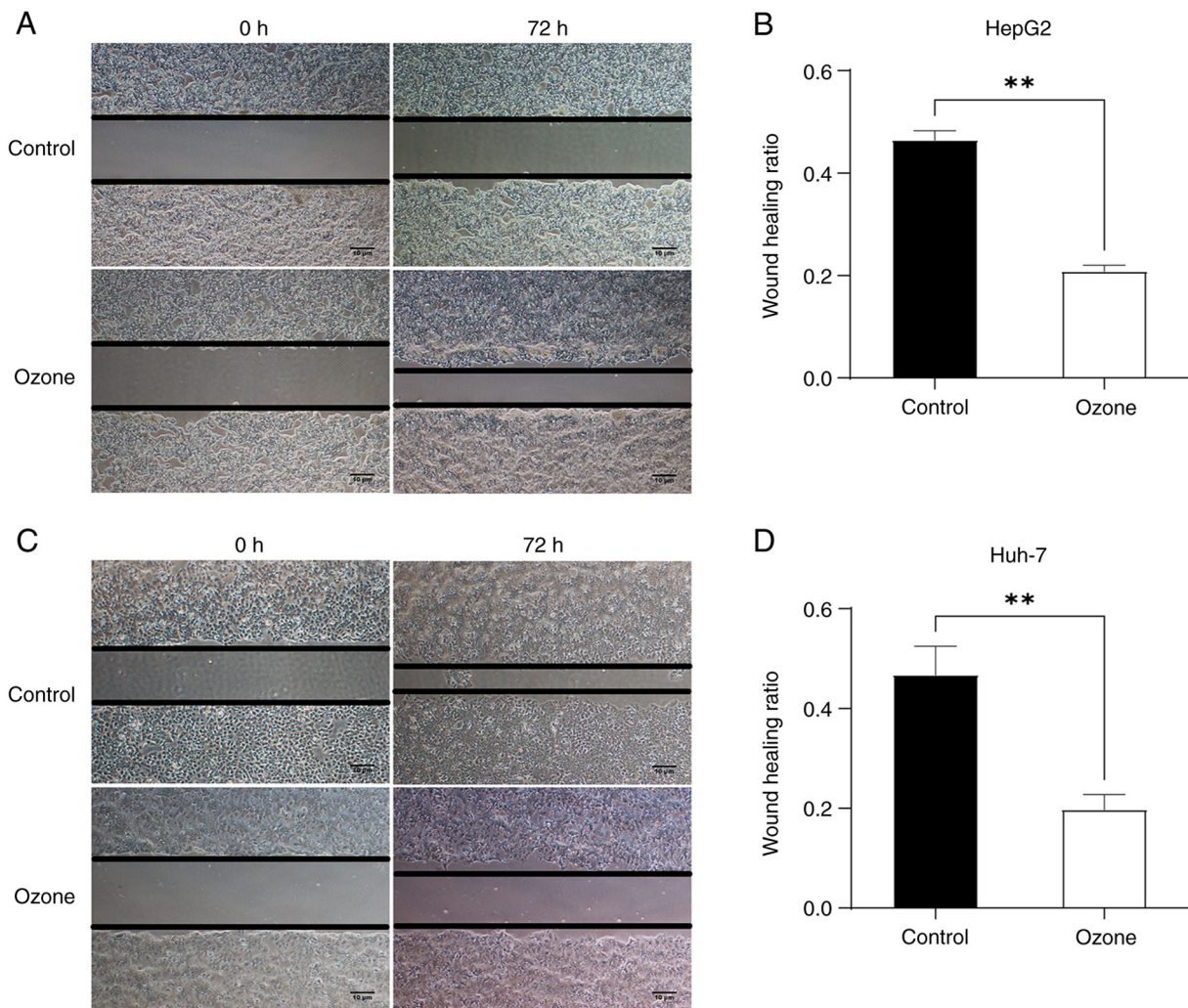


Figure 2. Effect of ozone on migration ability of liver cancer cells. (A) Representative wound healing assay of HepG2 cells. (B) result of the healing ability of the two groups of HepG2 cells. (C) shows representative wound healing assay for two groups of Huh-7 cells; (D) healing ability of the two groups of Huh-7 cells. n=3. **P<0.01.

previous reports (10-14) that also indicate that ozone can effectively eliminate cancer cells while sparing normal cells. Simonetti *et al* (34) reported time- and concentration-dependent cytotoxic effects of ozone on HT-29 colon cancer cells. A study by Schulz *et al* (35) found that intraperitoneal insufflation of an ozone/oxygen mixture at an advanced disease stage significantly improved survival, induced complete tumor regression, and appeared to activate an immune-mediated antitumor response.

In the present study, medical ozone water directly inhibited the IL-6/STAT3 pathway, resulting in dose-dependent decreases in p-STAT3 and Bcl-2, and subsequent apoptosis. Western blot analysis confirmed downregulation of Bcl-2, upregulation of cleaved caspase-3 and a reduced Bcl-2/Bax ratio, indicating mitochondrial apoptotic pathway activation, consistent the pro-apoptotic effects of ozone (36). Additionally, ozone water significantly suppressed migration and invasion in HepG2 and Huh-7 cells, which may decrease metastasis. While Li *et al* (14) and Tang *et al* (15) described ozone-induced suppression via ROS/PI3K/AKT/NF-κB and HMGB1/NF-κB/STAT3 pathways, respectively, the present study provides novel insights into IL-6/STAT3 pathway

involvement. By contrast with Costanzo *et al* (37), which found no effect in HeLa cells, the present results emphasize cell type-dependent responses. The present study demonstrated that ozone water had a minimal impact on normal liver cell (THLE-2) morphology and proliferation, highlighting its translational relevance.

Chronic inflammation serves a role in tumorigenesis by fostering a microenvironment conducive to proliferation, angiogenesis and immune evasion (38). Inflammatory mediators, such as ROS and nitric oxide synthase, induce DNA damage and dysregulate oncogenic signaling (39). On one hand, these substances cause double-strand DNA breaks and induce gene mutations; on the other hand, they promote the activation of proto-oncogenes and inactivation of tumor suppressor genes (40). NF-κB is a central inflammatory transcription factor that can promote sustained IL-6 expression, which inhibits apoptosis and supports tumor survival (41). Ozone exerts anti-inflammatory effects through moderate oxidative stress. This mechanism is initiated when ozone-derived ROS and lipid oxidation products, such as 4-hydroxynonenal, act as signaling molecules (30). These molecules modify critical cysteine residues on the

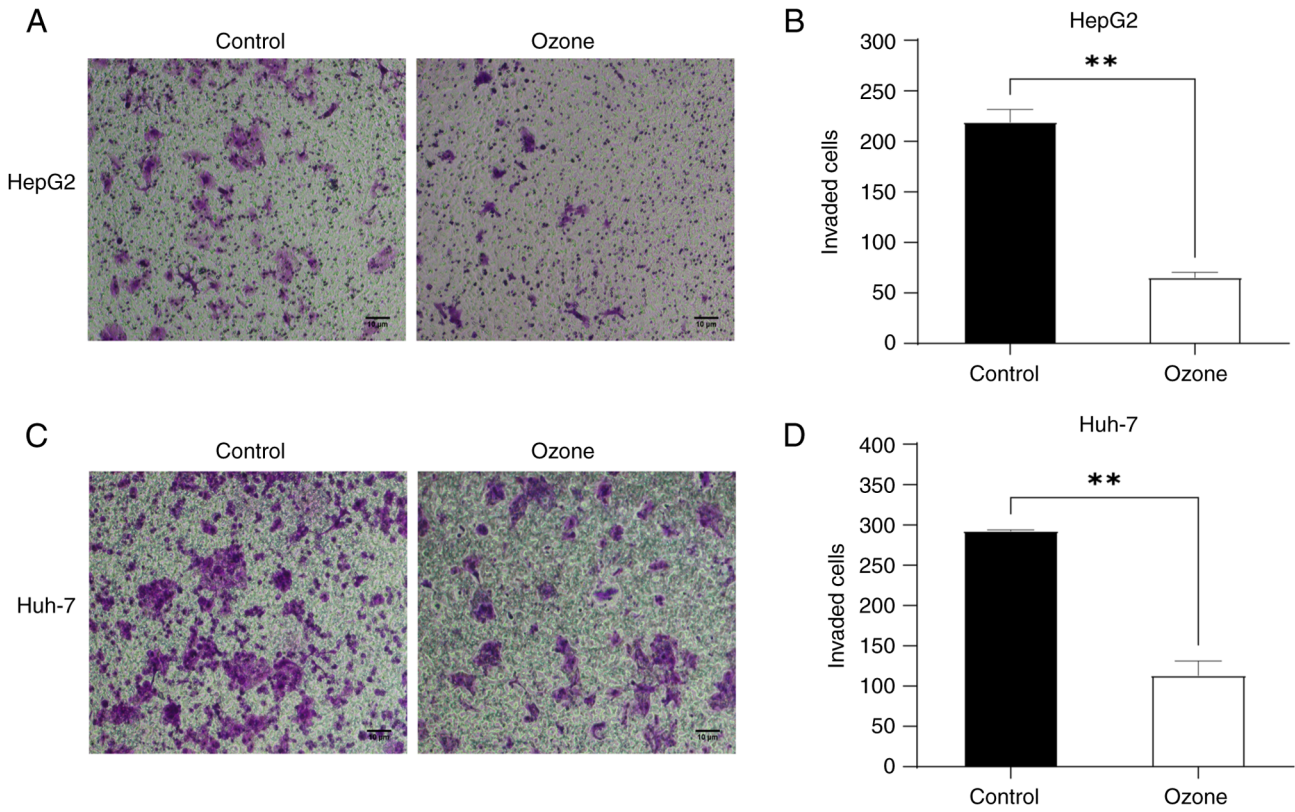


Figure 3. Invasion ability of liver cancer cells. (A) Representative Transwell invasion assay of (B) HepG2 and (C) Huh-7 cells. n=3. **P<0.01.

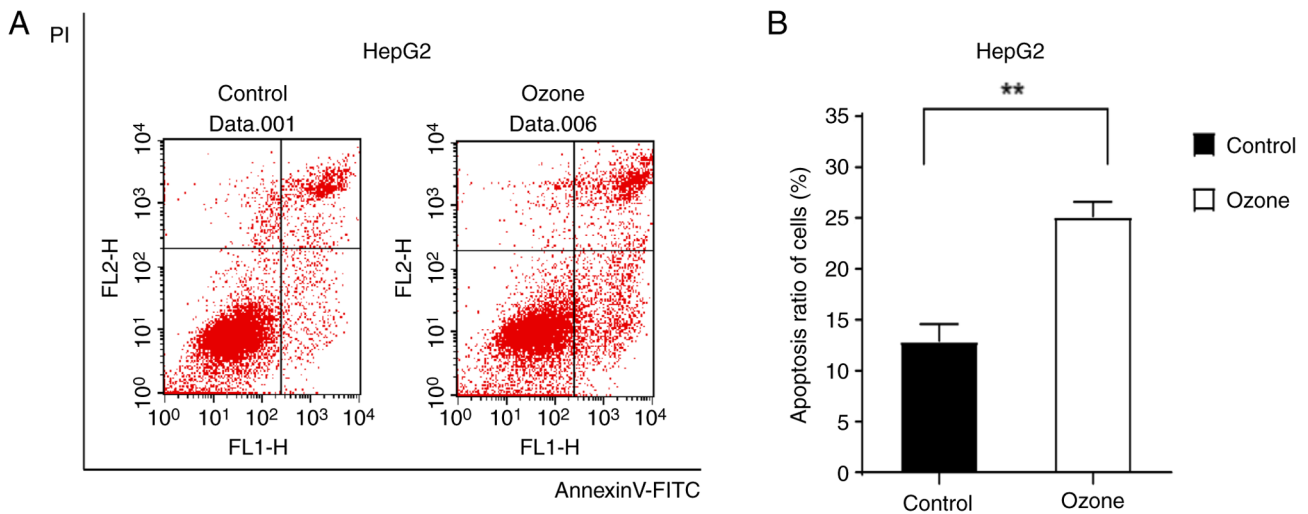


Figure 4. Apoptosis of HepG2 cells *in vitro*. (A) Representative images of cell apoptosis analysis measured by flow cytometry (stained by Annexin V-FITC/PI). (B) Quantitative analysis of apoptotic cells. n=3. **P<0.01.

Keap1 protein, disrupting its association with Nrf2. This leads to Nrf2 stabilization and translocation into the nucleus. Upon binding to the ARE, Nrf2 drives the transcription of cytoprotective enzymes, including heme oxygenase-1, NAD(P)H quinone dehydrogenase 1 and glutamate-cysteine ligase (42,43). The upregulation of this antioxidant repertoire effectively restores cellular redox homeostasis. Consequently, this Nrf2-mediated adaptive response suppresses the NF-κB signaling pathway, a master regulator of IL-6 transcription, thereby inhibiting its activation. The net outcome is a

notable downregulation of IL-6 gene expression and protein synthesis (44,45).

IL-6 is a key cytokine linking inflammation and cancer. It activates the JAK/STAT3 pathway, leading to the transcription of genes involved in proliferation (cyclin D1), invasion (MMP2/3), apoptosis resistance (Bcl-2) and angiogenesis (VEGF) (46,47). Aberrant activation of IL-6/STAT3 signaling is frequently observed in tumors and associated with poor prognosis (48,49). The present results showed that ozone downregulated IL-6 expression

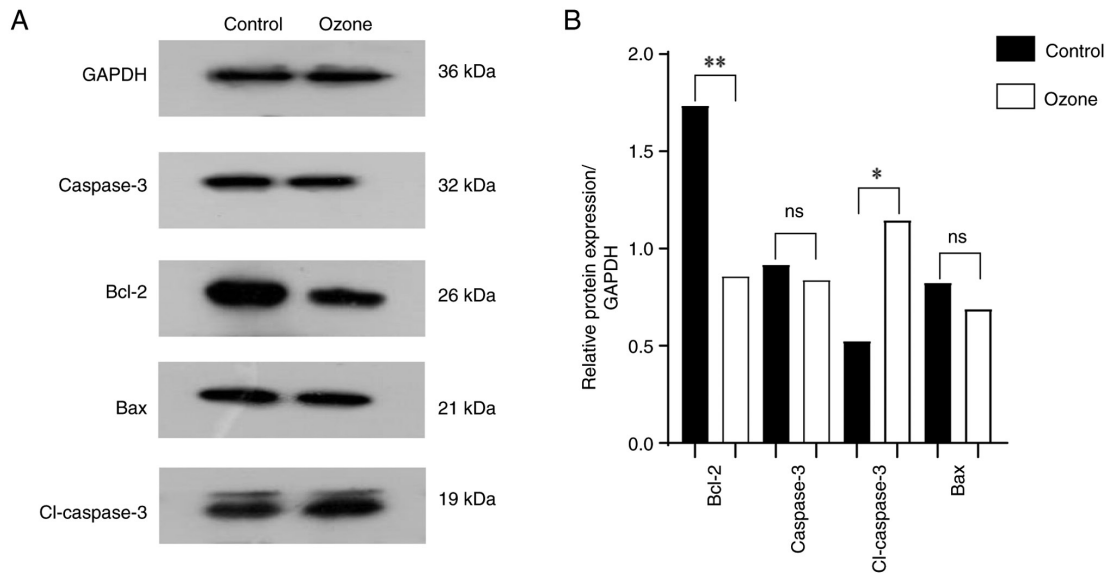


Figure 5. Relative expression of apoptosis-associated protein in HepG2 cells *in vitro*. (A) Representative western blot analysis of (B) apoptosis-associated proteins in HepG2 cells. n=3. *P<0.05, **P<0.01 vs. control. ns, not significant; cl, cleaved.

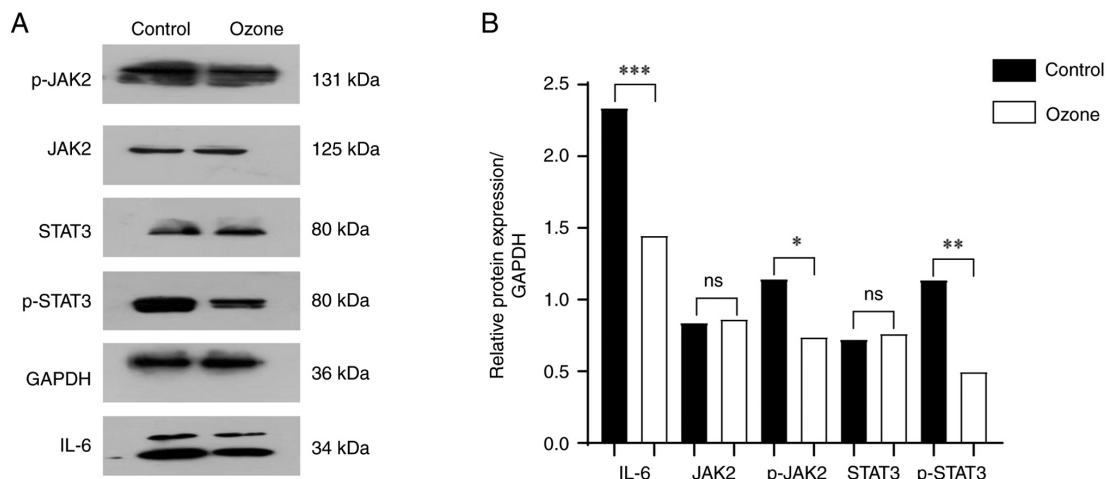


Figure 6. Modulation of IL-6/STAT3 pathway proteins in HepG2 cells *in vitro*. (A) Representative western blot analysis of (B) IL-6/STAT3 pathway proteins n=3. *P<0.05, **P<0.01, ***P<0.001. p-, phosphorylated; ns, not significant.

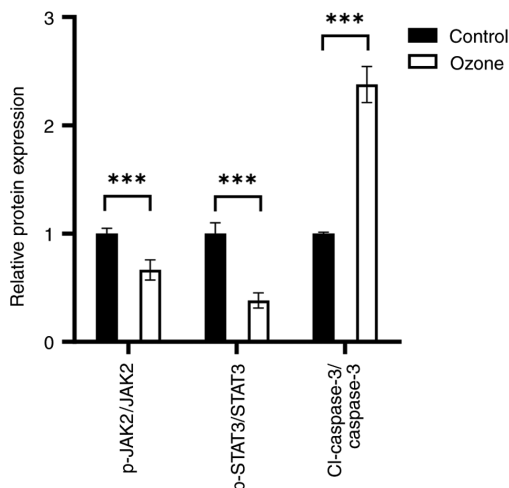


Figure 7. Relative expression of p-JAK2, p-STAT3 and cl-caspase-3 in HepG2 cells. ***P<0.001. p-, phosphorylated; cl, cleaved.

and decreased the phosphorylation of JAK2 and STAT3, thereby inhibiting this oncogenic pathway (50,51). This is consistent with studies across various types of cancer, such as colorectal cancer, myeloma and gallbladder cancer; where STAT3 inhibition suppresses tumor growth and induces apoptosis (48,51).

Ozone is administered in various forms clinically, including as gas (52,53), ozonated water, oil and by autohe-motherapy (54). Its potential use in liver cancer management may include sensitizing tumor cells to chemo- or radiotherapy, decreasing drug resistance and enhancing the efficacy of TACE by increasing drug concentration and decreasing tumor blood supply. In addition, ozone exhibits immunomodulatory properties. Rossmann *et al* (55) observed that ozone treatment increased tumor-infiltrating lymphocytes and confers transferable anti-tumor immunity through peripheral blood mononuclear cells in a VX2 rabbit model, suggesting that ozone may activate systemic anti-tumor immune responses.

In summary, the present study demonstrated that ozone inhibited migration, invasion and proliferation, while promoting apoptosis in liver cancer cells *in vitro*. Ozone suppresses the IL-6/STAT3 signaling pathway, likely through its anti-inflammatory effects. However, limitations should be acknowledged. The present study was conducted only on two liver cancer cell lines (HepG2 and Huh-7), therefore generalizability to other liver cancer subtypes remains unclear. In addition, the impact of ozone on the cell cycle was not investigated. The role of IL-6/STAT3 signaling was also not validated through genetic approaches, such as silencing or overexpression. All experiments were performed *in vitro*, precluding the determination of the efficacy of ozone in *in vivo* settings. Another limitation of the present study is the absence of transcriptome analysis based on next-generation sequencing (NGS). The present study focused on the role of the IL6/STAT3 signaling pathway in ozone water-induced apoptosis of liver cancer cells, but apoptosis regulation typically involves complex interactions among multiple signaling networks (such as MAPK, PI3K/Akt or NF- κ B pathways). The lack of NGS data restricts understanding of the mechanism of action, preventing systematic identification of differentially expressed genes, verification of whether the effects are specifically mediated by IL6/STAT3 or involve broader signaling networks, and discovery of potential synergistic targets. Therefore, future studies should integrate transcriptomics to expand mechanistic insights and animal models are necessary to evaluate the therapeutic potential of ozone *in vivo*. Further research is warranted to explore the effects of ozone on a broader panel of liver cancer models, its influence on cell cycle progression and its efficacy in combination with existing therapy.

Acknowledgements

Not applicable.

Funding

The present study was supported by the school-level youth research project of Chuanbei Medical College (grant no. CBY23-QNA17).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LZ conceived and designed the study and analyzed and interpreted data. SQ, CC, ZZ, NY, DD and JH performed experiments and analyzed data. SQ and CC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

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.

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