# Cold atmospheric plasma-activated Ringer's solution inhibits the proliferation of osteosarcoma cells through the mitochondrial apoptosis pathway

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**Abstract.** The present study aimed to investigate the effects of cold atmospheric plasma (CAP)-activated Ringer's solution on osteosarcoma cell lines MG63 and U2OS, and to identify the molecular mechanism underlying these effects. CAP-activated Ringer's solution was used to treat osteosarcoma cell lines MG63 and U2OS for 30 min. Cell viability was measured using the MTT method. The apoptosis rate was detected using Annexin-V and propidium iodide. The expression levels of cytochrome *c*, caspase-3 and polyADP ribose polymerase (PARP) in MG63 cells were analyzed via western blotting. The change in mitochondrial membrane potential was detected via the JC-1 dye method and verified by the level of reactive oxygen species (ROS). CAP-activated Ringer's solution inhibited the proliferation of MG63 and U2OS cells in a dose- and time-dependent manner. Furthermore, CAP-activated Ringer's solution induced the apoptosis of MG63 cells, increased the intracellular ROS level, decreased the mitochondrial membrane potential level, and induced the release of cytochrome *c*. CAP-activated Ringer's solution inhibits osteosarcoma cell proliferation through intracellular ROS-mediated mitochondrial apoptosis.

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*Abbreviations:* CAP, cold atmospheric plasma; OS, osteosarcoma; ROS, reactive oxygen species; Ringer, lactated Ringer's solution

*Key words:* cold atmospheric plasma, osteosarcoma, mitochondrial, reactive oxygen species, apoptosis

## Introduction

Osteosarcoma (OS) is one of the most common primary malignant bone tumors. It most commonly occurs in the metaphysis of long bones in children and adolescents. Approximately 10-20% of patients have metastasized disease at initial diagnosis (1,2). OS is highly invasive and leads to progressive bone destruction. Currently, the main clinical treatment of patients with OS is still surgery-based, supplemented by chemotherapy (1) or radiotherapy (2). Although the treatment for OS has improved over the past few decades, the 5-year overall survival rate remains extremely low, at ~60% (3). Intraoperative contamination of radical surgery, microlesions in the peritumoral tissue that are unable to be completely removed, and microresidual tumors are the primary causes of local recurrence in patients with OS, and the prognosis of these patients is often poor (4,5). Therefore, new treatment methods are urgently required in order to control the intraoperative tumor contamination and residuals, decrease the recurrence rate and improve the survival rate of patients.

Cold atmospheric plasma (CAP) is the fourth basic material state after solid, liquid and gas. Plasma is a partially ionized gas generated by a focused discharge (6). It contains a variety of physical and chemical components, including electric field, ions, photons, free radicals and other unknown active substances (7). Conventional plasma can only be applied to industry under high temperatures of over 10,000˚C. The temperature range of CAP is 20-50˚C, and then, the molecular structure and cellular integrity can be maintained (8). Reports have demonstrated that CAP can be used in wound healing (9), sterilization (10) and food preservation (11). The antitumor capabilities of CAP have become a popular topic for research, including restoring the sensitivity of chemotherapy-resistant cancer cells (12). Although the direct application of CAP has proven to be sensitive for a number of different types of cancer, the poor penetration of CAP limits its applications in cancer treatments in the clinical practice, particularly for cancer metastasis. Recently, CAP-activated media were demonstrated to have sufficient antitumor effects toward a number

of cell types (13), including HeLa (14), glioblastoma (15), breast cancer (16), ovarian cancer (17) and pancreatic cancer cells (18). There are also reports on the selective antitumor effects of buffered saline (19) and Ringer's solution (20). These findings suggest that CAP treatment is more than a drug therapy. According to previous studies, ROS production may be the main mechanism of CAP-induced apoptosis (21-23). However, the specific description of this mechanism remains unclear. Furthermore, there are reports that different cell lines reveal different responses to CAP treatment.

The present study used Ringer's solution as the mediator of CAP treatment as its composition is simple and easy to obtain. CAP-activated Ringer's solution was prepared and used to treat human OS cells. MTT assay, apoptosis detection [Annexin-V/propidium iodide (PI)], ROS level determination, JC-1 assay and western blot analysis were performed to determine the effects of CAP-activated Ringer's solution on OS cells. The aim of the present study was to analyze the effects of CAP-activated Ringer's solution on human OS cell lines MG63 and U2OS, and to further characterize its cellular effects and potential molecular mechanisms. These results represent an important advancement in the clinical applications of plasma-activated solutions.

## Materials and methods

*Cell culture*. The OS cell lines MG63 and U2OS and the human osteoblast hFOB1.19 cell line were obtained from the Shanghai Institute of Life Sciences, Chinese Academy of Sciences. OS cells were cultured in high glucose Dulbecco's modified Eagle medium (DMEM; HyClone; pH 7.0-7.4) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin, and 100 $\mu$g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). All cells were incubated in a humidified cell incubator (Thermo Fisher Scientific, Inc.) at 37˚C with 5% $CO_2$. The adherent OS cells were trypsinized, collected by centrifugation, resuspended in fresh medium and passaged at a ratio of 1:4.

*Preparation of plasma-activated solution*. The plasma device used in the present study was an air-dependent instrument designed and developed by the Institute of Plasma Physics, Chinese Academy of Sciences. The schematic graph and image are presented in Fig. 1. The air plasma discharge electrode consists of six copper rods with a diameter of 1 mm. Other articles have reported similar types of configuration (24,25). In the present study, the six electrodes were driven by high voltage direct current (DC) without the requirement for any external gas supply. Ringer's solution acted as a grounded electrode. The input voltage was 10 kV, the discharge current was 5 mA, and the ballast resistor R that limited the discharge current was 30 mΩ. Therefore, the gas temperature of the air plasma generated was controlled at 20-30˚C. In the present study, 3 ml Ringer's solution (lactated Ringer's solution) was placed in a 35-mm Petri dish with a distance from the electrode of 1.0 cm, and was treated with a CAP device for 60, 120, 180, 240 or 300 sec.

*Treatment of cells with plasma-activated solution*. MG63, U2OS and hFOB1.19 cells were incubated with CAP-activated Ringer's solution for 30 min, washed twice with phosphate-buffered saline (PBS), and cultured for 24 h with fresh medium. For some cells, ROS and JC-1 detection was immediately performed following treatment with CAP-activated Ringer's solution.

*Cell viability assay*. The effect of CAP-activated Ringer's solution on cell viability was determined using an MTT assay (Sigma Aldrich; Merck KGaA) according to the manufacturer's protocol. Cells were trypsinized with trypsin/ethylenediaminetetraacetic acid (trypsin/EDTA) (HyClone), seeded into 96-well plates at a density of 10,000 cells/well in triplicate, and cultured for 24 h. CAP-activated Ringer's solution was used in the experimental groups, and untreated Ringer's solution functioned as the control group. Cells were incubated with either CAP-activated Ringer's solution or untreated Ringer's solution for 30 min, washed with PBS, and cultured with fresh medium for 24 h Then, 10 $\mu$l MTT solution (5 mg/ml) was added to each well and incubated for 4 h. Next, 100 $\mu$l DMSO was added to each well and thoroughly mixed on a shaker to dissolve the purple-violet crystal. Finally, the optical density (OD) value of the purple-violet crystal at 570 nm was measured using the EnSpire Multimode Plate Reader R (PerkinElmer). In addition, cell viability was calculated as Experimental group OD value/Control group OD value x100%.

*Apoptosis determination*. Apoptosis induced by CAP-activated Ringer's solution was measured using Annexin V-FITC (BB-4101-50T) according to the manufacturer's protocol. The MG63 cells were treated with CAP-activated Ringer's solution for 30 min and cultured for 24 h. OS cells were digested with trypsin without EDTA and washed twice with PBS. A total of $1x10^5$ cells were resuspended in the binding buffer, and 5 $\mu$l Annexin V-FITC was added to the cell suspension and incubated for 15 min at 4˚C in the dark. The cells were then mixed with 10 $\mu$l PI and incubated for another 5 min at 4˚C in the dark. Finally, cells were detected by flow cytometry (BD Biosciences) and analyzed for apoptosis using FlowJo7.6.5 software (Tree Star Inc.).

*Determination of mitochondrial membrane potential ($\Delta\Psi m$)*. The $\Delta\Psi$m was determined using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocya-nineiodide; Beyotime Biotechnology; C2006). According to the manufacturer's protocol, the cells were incubated with CAP-activated Ringer's solution for 30 min, washed twice with PBS, and resuspended in 0.5 ml cell culture medium. Then, 0.5 ml JC-1 staining solution was added and inverted several times to mix well. Next, the cells were incubated at 37˚C for 20 min and centrifuged at 600 x g for 3-4 min at 4˚C to form a pellet. The cells were washed twice with JC-1 staining buffer (1X) and resuspended in an appropriate amount of JC-1 staining buffer (1X). The fluorescence intensities of the fluorescent dye excited at 490 and 590 nm were measured by flow cytometry (BD Biosciences), and the $\Delta\Psi$m was reflected by the JC-1 polymer/monomer ratio or the absorbance of the JC-1 polymer.

*Western blot analysis*. The expression of apoptotic proteins at 0, 2, 4 and 8 h after MG63 cells were treated with CAP-activated Ringer's solution was analyzed via western blotting. Cells

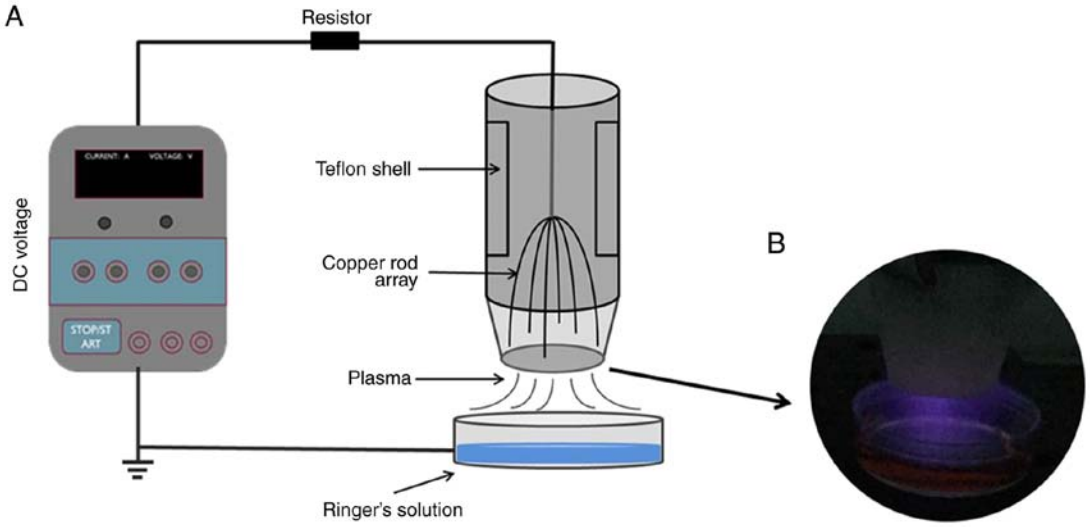


Figure 1. Schematic graph and image of the cold atmospheric plasma equipment used in the present study. (A) Schematic of the cold atmospheric plasma device schematic. (B) Image of the plasma jet.

were incubated with CAP-activated Ringer's solution for 30 min, and adherent and floating cells were collected. Mitochondria and cytoplasmic proteins were extracted using a mitochondrial extraction kit (Solarbio) according to the manufacturer's protocol. Protein quantification was performed using a BCA assay, and 40 $\mu$g of protein samples were separated via SDS-PAGE (10% gel) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with Tris-buffered saline (TBST) buffer and 5% (M/V) skimmed milk powder, and incubated with caspase-3 (dilution 1:500; cat. no. WL03339), cleaved caspase-3 (dilution 1:1,000; cat. no. WL01857), PARP (dilution 1:500; cat. no. WL01578), cytochrome *c* (Cyt c; dilution 1:500; cat. no. WL01571) and β-actin (dilution 1:1,000; cat. no. WL01845) primary antibody at 4˚C overnight (all antibodies were purchased from Wanleibio Co., Ltd.). After washing, the blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (dilution 1:5,000; cat. no. WLA023; Wanleibio Co., Ltd.) for 45 min at room temperature. After washing with TBST, the blot was exposed in a dark room using an ECL+ chemiluminescence kit. The film was scanned, and the OD of the target bands was analyzed using a gel image processing system (Gel-Pro Analyzer software; *Tanon* Science & Technology Co., Ltd.).

*Intracellular reactive oxygen species (ROS) detection*. The ROS level changes in OS cells treated with CAP-activated Ringer's solution were detected using 2',7'-dichlorofluorescein diacetate (DCFH-DA). OS cells were seeded at a density of $4x10^5$ cells/well in 6-well plates in triplicate and cultured for 24 h. Immediately after treatment of cells with CAP-activated Ringer's solution, 10 $\mu$M DCFH-DA was added and incubated for 30 min at 37˚C in the dark with 5% $CO_2$. The intracellular ROS level was measured using an EnSpire multimode plate reader (PerkinElmer) with 488 nm excitation wavelength and 525 nm emission filter.

*Statistical analysis*. The data are presented as the mean ± standard deviation of at least three independent experiments. Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, Inc.). Differences were assessed by two-sample t-test or one-way ANOVA. LSD post hoc and Dunnett's post hoc tests were used where appropriate. $P \leq 0.05$ and $P \leq 0.01$ were considered to indicate a statistically significant difference.

## Results

*CAP-activated Ringer's solution affects cell morphology*. The present study observed that treatment with CAP-activated Ringer's solution altered the morphology of OS cells. Compared with the control cells, the cells demonstrated shrinkage following treatment with Ringer's solution that was exposed to CAP for 180 sec, and a small amount of cells became suspended in the cell culture medium. When the exposure time reached 300 sec, the spindle cells rounded and shriveled, and more cells were suspended in the medium (Fig. 2).

*CAP-activated Ringer's solution decreases cell viability*. OS cells treated with CAP-activated Ringer's solution were assayed using the MTT method after 24 h. CAP-activated Ringer's solution treatment inhibited the proliferation of MG63 and U2OS OS cells in a dose-dependent manner (Fig. 3A). When the exposure time was extended to 240 sec, the viability of MG63 and U2OS cells treated with CAP-activated Ringer's solution was significantly decreased to 40.04 and 35.00%, respectively. When the exposure time was extended to 300 sec, the inhibition rates were >70%. However, the human osteoblast hFOB1.19 cells treated with CAP-activated Ringer's solution did not exhibit significant inhibition of growth, but instead displayed promotion of growth under the exposure time of <120 sec. The inhibition effect was still detectable 48 and 72 h after treatment with CAP-activated Ringer's solution (Fig. 3B).

*CAP-activated Ringer's solution induces apoptosis*. The Annexin V-FITC and PI staining confirmed that when the exposure time to CAP reached 180 sec, the apoptotic rate of OS cells was significantly increased (Fig. 4). When the

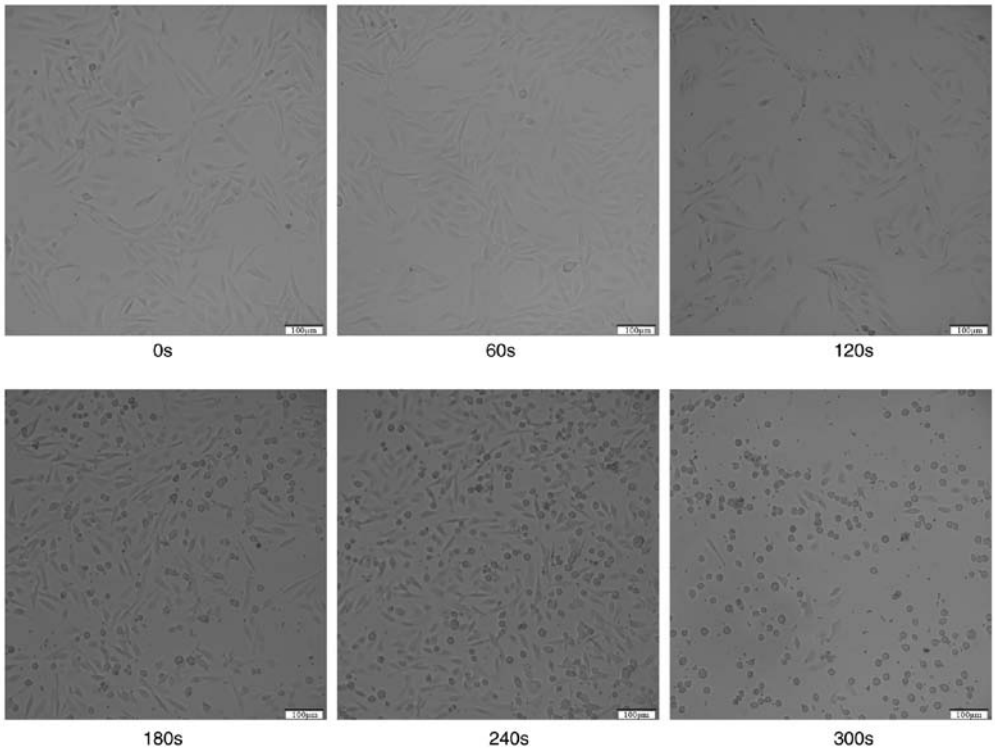


Figure 2. Treatment with CAP-activated Ringer's solution altered the morphology of the OS cells. MG63 cells were incubated with CAP-activated Ringer's solution for 30 min, and the images were captured with an optical microscope. The time listed under every image represents the exposure time for CAP-activated Ringer's solution. CAP, cold atmospheric plasma; OS, osteosarcoma.

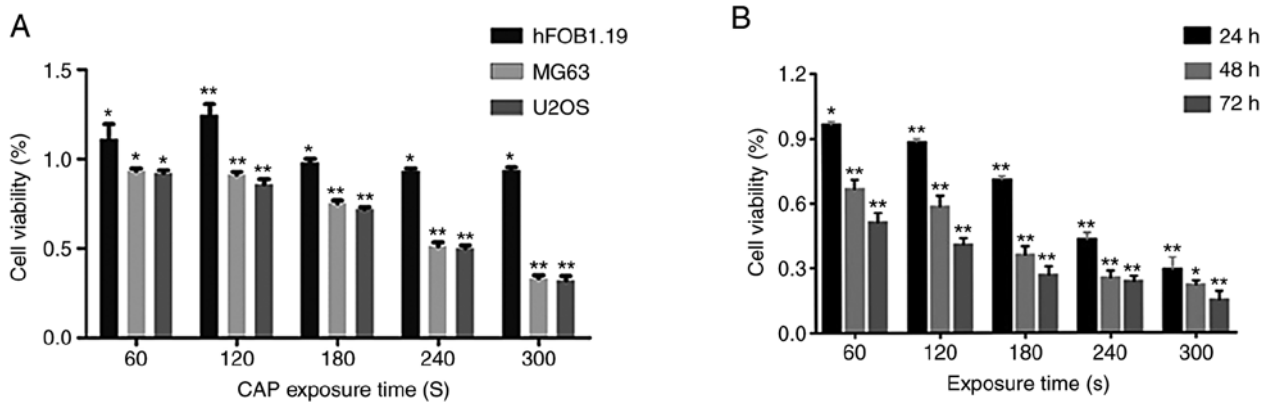


Figure 3. Cell viability of the cells treated with CAP-activated Ringer's solution. (A) CAP-activated Ringer's solution was used to treat OS cell lines MG63 and U2OS and the human osteoblast hFOB1.19 cell line, and the cell viability in the three cell groups was measured using an MTT assay after 24 h of standard culture. (B) The cell viability of MG63 cells at 24, 48 and 72 h after receiving CAP-activated Ringer's solution treatment for the indicated times as determined by MTT assay. The times listed under the histogram represent the exposure time for CAP-activated Ringer's solution. The data are presented as the mean ± standard deviation of three independent experiments. $^{*}$P<0.05; $^{**}$P<0.01. CAP, cold atmospheric plasma; OS, osteosarcoma.

exposure time reached 300 sec, ~84% of the cells were in an apoptotic state. The expression of caspase-3 and PARP in MG63 cells was assessed via western blotting (Fig. 5). The results revealed that at 0, 2, 4 and 8 h after the 30-min incubation with CAP-activated Ringer's solution (CAP exposure time of 180 sec), the expression levels of caspase-3 and PARP were decreased in a time-dependent manner (Fig. 5B and D), and the level of cleaved caspase-3 was significantly increased in a time-dependent manner (Fig. 5C) (P<0.05). These findings indicate that CAP-activated Ringer's solution induces apoptosis in MG63 cells via the caspase-3-dependent pathway.

*CAP-activated Ringer's solution reduces cell mitochondrial membrane potential*. With the elongation of exposure time of Ringer's solution to CAP, the content of JC-1 polymers in the mitochondria of cells of the experimental groups was significantly decreased compared with that of the control group (Fig. 6B), and the JC-1 polymer/monomer ratio was also significantly decreased (Fig. 6A), which indicates a decrease in mitochondrial membrane potential (P<0.01).

*CAP-activated Ringer's solution induces cytochrome c (Cyt c) release*. OS cells treated with CAP-activated Ringer's solution

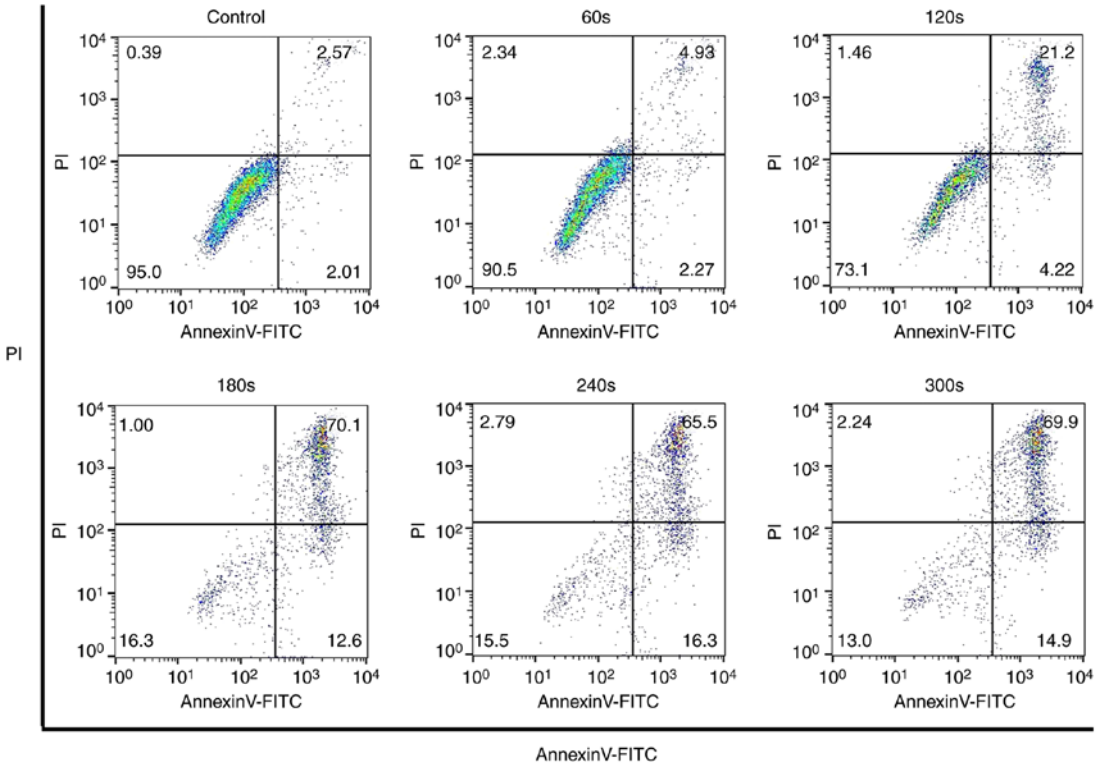


Figure 4. CAP-activated Ringer's solution induces cell apoptosis in a dose-dependent manner. After receiving CAP-activated Ringer's solution treatment, cell staining was performed using Annexin V-FITC/PI; the early and late apoptosis rates of the treated MG63 cells were measured via flow cytometry. Flow cytometry demonstrating apoptosis in cells treated with CAP-activated Ringer's solution. In each of the small images, the lower left quadrant represents living cells, while the lower right quadrant and upper right quadrant represent early and late apoptotic cells, respectively. The time listed at the top of every image represents the exposure time for CAP-activated Ringer's solution. CAP, cold atmospheric plasma; OS, osteosarcoma.

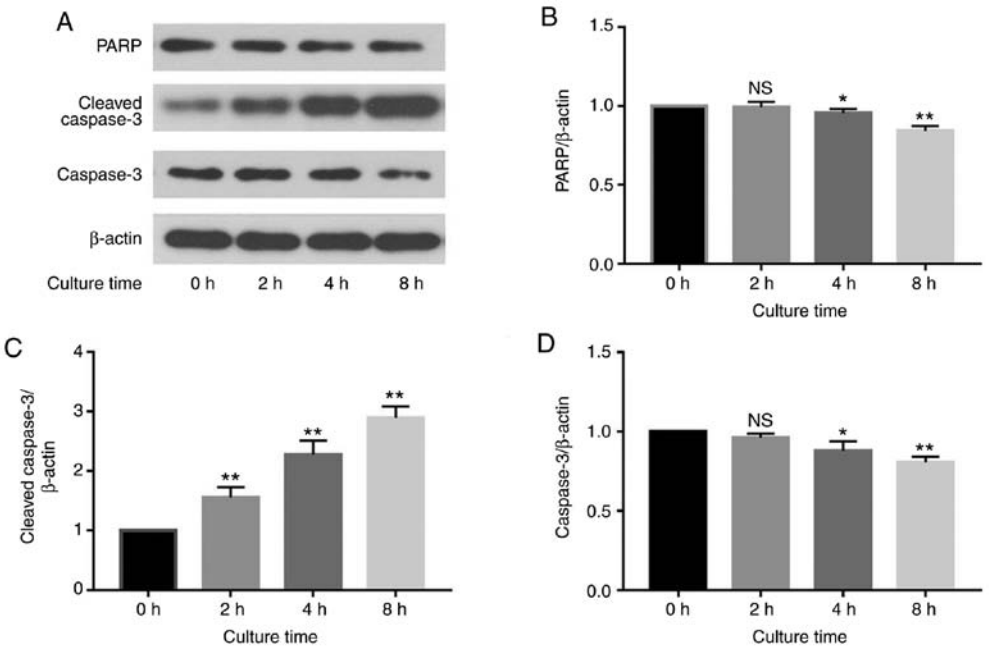


Figure 5. CAP-activated Ringer's solution promotes expression of apoptotic proteins. (A) After 2, 4 and 8 h of treatment with CAP-activated Ringer's solution, the expression of caspase-3, cleaved caspase-3 and PARP in OS MG63 cells was analyzed via western blotting. (B) Changes in the ratio of PARP/β-actin in MG63 cells after CAP-activated Ringer's solution treatment. (C and D) Changes in the ratio of cleaved-caspase-3/β-actin and caspase-3/β-actin after CAP-activated Ringer's solution treatment. $^{*}$P<0.05; $^{**}$P<0.01; NS, not significant. Data are represented as the mean ± SD of three independent experiments. CAP, cold atmospheric plasma; OS, osteosarcoma; PARP, poly(ADP-ribose) polymerase.

with an exposure time of 180 sec showed decreased Cyt c expression in the mitochondria, but significantly increased Cyt c levels in the cytoplasm (P<0.01) (Fig. 7B and C), The total cytochrome *c* content did not change significantly, suggesting

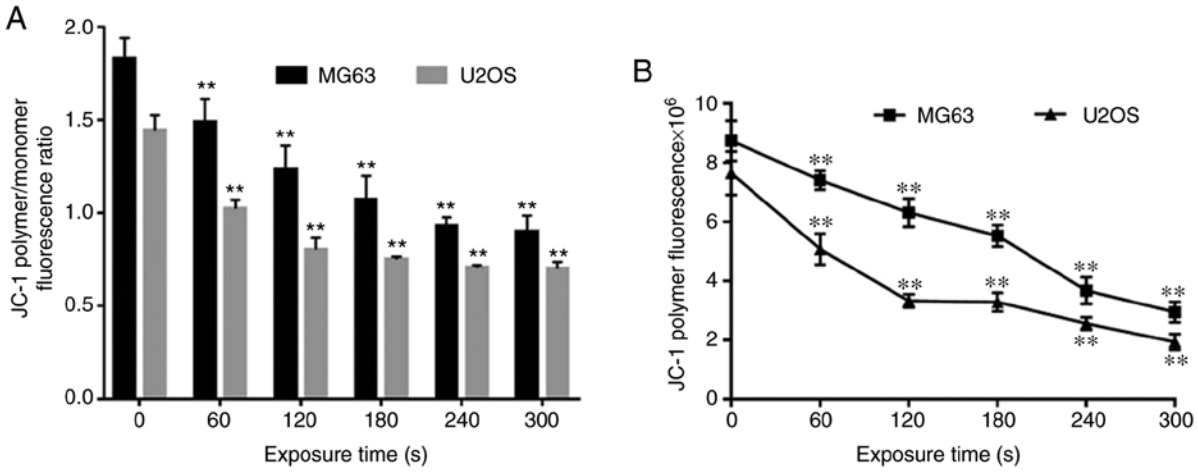


Figure 6. CAP-activated Ringer's solution decreases cell mitochondrial membrane potential. (A) After receiving CAP-activated Ringer's solution treatment, cell staining was performed using JC-1. Changes in the ratio of the JC-1 polymer/monomer in OS MG63 cells after a 2-h treatment with CAP-activated Ringer's solution. (B) Changes in JC-1 polymers in MG63 cells after a 2-h treatment with CAP-activated Ringer's solution. The times on the x-axes represent the exposure times for CAP-activated Ringer's solution. $^{**}$P<0.01. Data are presented as the mean ± standard deviation of three independent experiments. CAP, cold atmospheric plasma; OS, osteosarcoma.

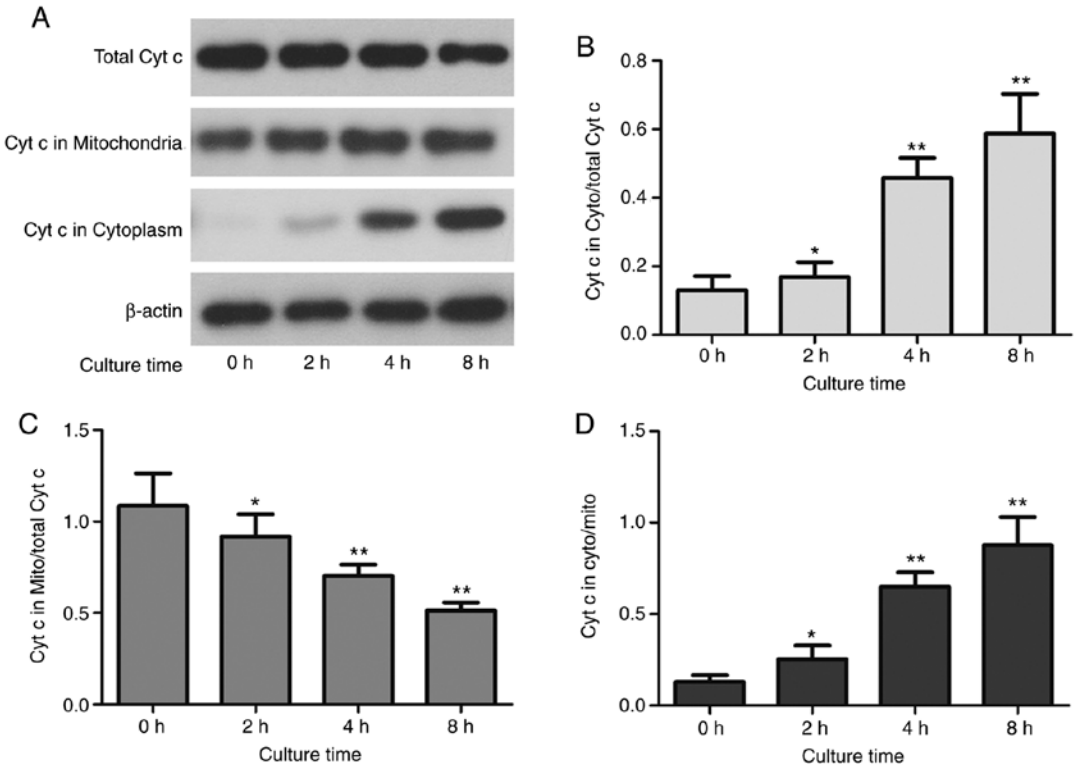


Figure 7. CAP-activated Ringer's solution induces Cyt c release. (A) Following CAP-activated Ringer's solution treatment, the expression of mitochondrial, cytoplasmic and total Cyt c in OS MG63 cells were analyzed by via western blotting. (B) Changes in the ratio of Cyt c in cytoplasm (Cyto)/total Cyt c in MG63 cells after receiving CAP-activated Ringer's solution treatment. (C) Changes in the ratio of Cyt c in the mitrochondia (Mito)/total Cyt c in MG63 cells after receiving CAP-activated Ringer's solution treatment. (D) Changes in the ratio of Cyt c in Cyto/Mito after 2, 4, and 8 h treatment with CAP-activated Ringer's solution. $^{*}$P<0.05; $^{**}$P<0.01. Data are presented as the mean ± standard deviation of three independent experiments. CAP, cold atmospheric plasma; OS, osteosarcoma; Cyt c, cytochrome *c*.

that Cyt c is released from mitochondria into the cytosol in MG63 cells treated with CAP-activated Ringer's solution (Fig. 7A). Furthermore, the level of Cyt c in each component was altered in a time-dependent manner (Fig. 7B-D).

*CAP-activated Ringer's solution causes production of intracellular ROS*. The present study detected a significant increase in intracellular ROS upon the exposure of Ringer's solution to CAP (P<0.01). The ROS level reached its maximum when the exposure time was 300 sec, which was >3 times that without exposure. At 2 h after treatment with CAP-activated Ringer's solution, the intracellular ROS content was significantly decreased compared with the level detected immediately after treatment (Fig. 8).

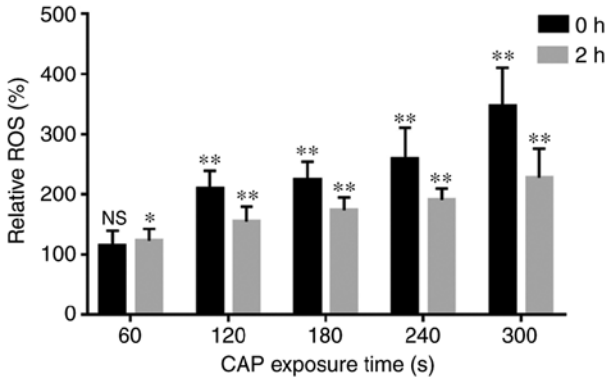


Figure 8. CAP-activated Ringer's solution causes production of intracellular ROS. Following CAP-activated Ringer's solution treatment, the cells were stained with DCFH-DA, and fluorescence was measured using a microplate reader to reflect changes in the levels of ROS in OS MG63 cells. After incubation with CAP-activated Ringer's solution for 30 min, ROS levels in MG63 cells after 0 and 2 h were detected. The ROS level in MG63 cells increased following co-culture with CAP-activated Ringer's solution. However, 2 h after treatment with CAP-activated Ringer's solution, intracellular ROS levels were decreased compared with levels detected immediately after incubation. The times listed on the x-axes represent the exposure time for CAP-activated Ringer's solution. $^{*}$P<0.05; $^{**}$P<0.01; NS, not significant. Data are presented as the mean ± standard deviation of three independent experiments. CAP, cold atmospheric plasma; OS, osteosarcoma; ROS, reactive oxygen species.

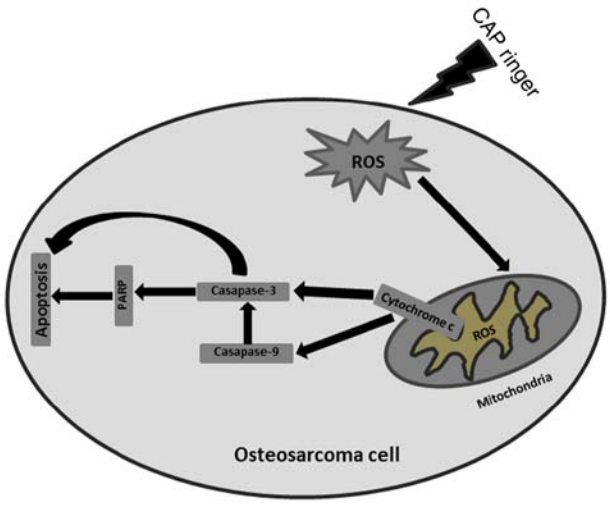


Figure 9. Schematic illustration of the present study. Cold atmospheric plasma (CAP)-activated Ringer's solution inhibits osteosarcoma cell growth, and increases apoptosis, potentially through the mitochondrial apoptosis pathway. ROS, reactive oxygen species; PARP, poly(ADP-ribose) polymerase.

## Discussion

In recent years, techniques for limb salvage surgery have greatly advanced with the improvement of osteosarcoma (OS) treatments (26). However, the accompanying tumor contamination of the surgical area and the microresidual lesions in the peritumoral tissue have become a problem for the orthopedist. Negative microscopic observation results of the margin of the tumor do not guarantee maximum tumor resection (27). The same situation is also present in benign tumors that are prone to recurrence, such as giant cell tumor of bone and osteoblastoma. Akihiko *et al* performed a long-term follow-up of 461 patients with OS, and the results revealed that the 5-year survival rate of 45 patients with recurrence was 30% and the 10-year survival rate was only 13% (28). Therefore, the residual tumor cells in surgeries that are invisible to the naked eye are expected to be destroyed through plasma-activated solution, achieving controlled tumor recurrence. In the meantime, studies have confirmed that cold atmospheric plasma (CAP)-activated solution has little effect on normal cells (19,29). Schuster *et al* found that CAP can increase the frequency of tumor surface response and the rate of apoptosis in clinical studies of CAP in the treatment of human head and neck squamous cell carcinoma (30). Both *in vivo* and *in vitro* experiments have demonstrated that CAP inhibition of a variety of tumor cells is exerted through apoptosis (15,31-34). The determination of apoptosis is usually conducted by a combination of multiple methods. In the present study, the inhibition of proliferation by CAP-activated Ringer's solution was first tested on OS cells using an MTT assay, and the results revealed that the cell proliferation was significantly inhibited when OS cells were treated with activated Ringer's solution, with the inhibition rate reaching 70%. It was also revealed that plasma inhibition of cell proliferation was significantly time- and dose-dependent. Activated Ringer's solution has no obvious inhibitory effect on the growth of human osteoblasts, and so it was also observed that plasma had a selective inhibitory effect on tumor cells. The present study demonstrated that CAP-activated Ringer's solution exhibited a significant inhibitory effect on OS cell viability. In order to prove that this inhibitory effect leads to OS cell death induced by CAP-activated Ringer's solution through programmed apoptosis, the present study examined the double staining of Annexin V/PI on cells via flow cytometry, and revealed that the cell numbers in the early and late stages of apoptosis caused by plasma treatment were higher than those in the no-treatment control group, indicating that plasma treatment can induce apoptosis of OS cells. This apoptosis also demonstrated a significant time- and dose-dependence.

Mitochondria are considered the most important sites of apoptosis during the apoptotic process in a number of different types of cells (35). Decreased mitochondrial membrane potential ($\Delta\Psi$m) is an early marker of apoptosis (36,37). When the mitochondrial membrane of normal live cells is relatively intact, the membrane potential is high, and JC-1 exists in the form of polymers in the mitochondria. When cells undergo apoptosis, the mitochondrial membrane is damaged, the mitochondrial membrane potential is decreased, and JC-1 is present as a monomer (38,39). Therefore, the present study used JC-1 to analyze $\Delta\Psi$m. The results of the present study also revealed that as the plasma exposure time increased, the mitochondrial membrane potential gradually decreased.

There is a large number of apoptosis-inducing factors in mitochondria and on the mitochondrial membrane, such as cytochrome *c* (Cyt c) and caspase precursors. Changes in mitochondrial membrane permeability and mitochondrial membrane potential result in the release of Cyt c from the mitochondria into the cytoplasm, which activates caspase-3 and thereby activates downstream polyADP ribose polymerase (PARP) to amplify apoptotic signals. PARP is an important apoptotic executive protein that directly leads to apoptosis (35,40). Further confirming that the CAP-activated Ringer's solution induces the apoptosis signaling pathway, the western blotting results

of the present study revealed a decrease in mitochondrial Cyt c expression, an increase in cytosolic Cyt c expression, and time-dependent expression of caspase-3 and PARP.

Reactive oxygen species (ROS) are a class of oxygen-containing substances produced by aerobic cells during aerobic metabolism that have extremely high biological activities. ROS play an important role in the proliferation and apoptosis of cells. A certain amount of ROS in the cells is necessary for the maintenance of normal physiological condition of the cells (41). However, high concentrations of ROS can directly induce changes in mitochondrial membrane permeability and even mitochondrial damage, cause damage to intracellular proteins, lipids and DNA molecules, and lead to cell death (42). Studies have demonstrated that ROS are an important factor in CAP-induced apoptosis; however, the stability of ROS is poor (43). The present study used DCFH-DA as a fluorescent probe to measure intracellular ROS levels. The results revealed that as the plasma exposure time increased, the intracellular ROS level gradually increased, indicating that ROS are involved in plasma-induced apoptosis. When the exposure time reached 300 sec, the ROS level reached the maximum. As the post-treatment time was prolonged, the level of ROS was decreased, which may be due to the instability of intracellular ROS or the antioxidant capacity of the cells *per se* that prevents further decrease of ROS. However, 48 and 72 h after CAP-activated Ringer's solution treatment, OS cells still demonstrated certain inhibitory effects, which were stronger than those at 24 h, indicating that ROS may not be the only factor inhibiting the growth of MG63 cells. The activated Ringer's solution may contain substances that are more stable and can continuously inhibit the proliferation of OS cells.

Tanaka *et al* detected little ROS in glioblastoma cells treated with plasma-activated Ringer's solution, while detecting a large amount of $H_2O_2$ in the plasma-activated Ringer's solution, and suggested that $H_2O_2$ is the primary factor that induces apoptosis in plasma-activated Ringer's solution (20). However, in that study, cells were treated with diluted PAL (plasma-activated lactated Ringer's solution) solution, which may result in the active ingredients in diluted plasma-activated Ringer's solution not reaching the threshold to induce massive ROS production. Alternatively, this different result may be due to the different plasma devices and different cell types. The composition of Ringer's solution is simple (NaCl, 6.0 g/l; KCl, 0.3 g/l; $CaCl_2$, 0.2 g/l; and L-sodium lactate, 3.1 g/l). Tanaka *et al* used CAP to separately activate a double concentrated solution of each component and demonstrated that L-lactate exhibited an antitumor effect on U251SP cells (20). However, that study failed to analyze the association between more than two components. In the future, it will be necessary to conduct an in-depth study on the interaction between the components of activated Ringer's solution, to confirm the feasibility of activated Ringer's solution in clinical applications, and to determine the storage methods and conditions.

The present study verified that CAP-activated Ringer's solution has a significant selective inhibition of OS cells, and intracellular ROS was an important factor during this process. The potential mechanism may be that the increase in intracellular ROS content leads to changes in mitochondrial membrane permeability and mitochondrial membrane potential, which lead to the release of Cyt c into the cytoplasm. The release of Cyt c further activates a series of downstream apoptotic responses to induce apoptosis, indicating that activated Ringer's solution activates the mitochondrial pathway of apoptosis (Fig. 9).

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**Availability of data and materials**

The data used to support the findings of the present study are available from the corresponding author upon request.

**Authors' contributions**

YW and SX conceived and designed the experiments. YW performed the experiments. CM, YQ, SC, HW and XY collected the data and performed the statistical analysis. YW with the help of GZ and YH wrote the manuscript and revised it critically for important intellectual content. CY and CC provided the plasma equipment and guided the experiments. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

All experiments conducted in the present study were approved by the Ethics Committee of The First Affiliated Hospital, Anhui Medical University (Anhui, China).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors have declared that they have no competing interests.

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