

Pharmacological Ascorbate Induces Osteosarcoma Cells Death via H₂O₂ Mediated-Oxidative Stress and Enhances the Efficacy of Cisplatin in Osteosarcoma

Liangfu Zhou

Northwestern Polytechnical University

Xixi Zhao

Northwestern Polytechnical University

Shenghang Wang

Northwestern Polytechnical University

Huanhuan Lv

Northwestern Polytechnical University

Atik Badshah Shaikh

Southern University of Science and Technology

peng Shang (✉ shangpeng@nwpu.edu.cn)

Southern University of Science and Technology Hospital <https://orcid.org/0000-0001-5418-6240>

Research

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Abstract

Background:

Finding emerging strategies for new use of the “old drug-vitamin C” on cancer treatment-combined with chemotherapy drugs.

Methods:

This study investigates the mechanisms that pharmacological ascorbate (vitamin C, AA) mediated toxicity in osteosarcoma cells and the effects of pharmacological ascorbate combined with cisplatin (DDP) on osteosarcoma cells in vitro and in vivo.

Results:

Pharmacological ascorbate not only promoted the production of hydrogen peroxide (H_2O_2) to kill osteosarcoma cells, but also improved its lethality to tumor cells by reducing the activity of catalase (CAT). DNA damage was the primary reason leading to osteosarcoma cell death, and depletion of nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP), activated apoptosis-inducing factor (AIF) all contribute to ascorbate-induced toxicity. The combination of pharmacological ascorbate with cisplatin showed synergistic effect on osteosarcoma cells in vitro. Pharmacological ascorbate enhanced the efficacy of cisplatin on inhibiting osteosarcoma tumor growth in orthotopic intra-tibial mouse model.

Conclusion:

These results suggest that pharmacological ascorbate causes DNA damage and induces osteosarcoma cells death via H_2O_2 mediated-oxidative stress. The combination of pharmacological ascorbate and cisplatin has synergistic effects on osteosarcoma cells and orthotopic intra-tibial mouse model. Pharmacological ascorbate could be a safe and effective adjuvant agent against osteosarcoma tumor.

Background

Osteosarcoma derives from primitive bone-forming mesenchymal cells and it is the most common primary malignant bone tumor in childhood and adolescence aged 10–20 years [1]. Surgical treatment combined with chemotherapy is currently the most effective treatment strategy, and has greatly improved the survival rates of patients. However, survival rates continue to be unsatisfactory in the metastatic and relapse setting [2]. Pharmacological methotrexate, doxorubicin, cisplatin, and ifosfamide are four based drugs for chemotherapy regimens [3]. Long-term use of chemotherapy agents has a toxic effect on the main organs, therefore the novel, effective and safe therapeutic approaches are also urgently needed.

Pharmacological ascorbate was firstly proposed as a potential anti-cancer agent in the 1970s by Linus Pauling [4, 5]. However, two followed randomized clinical trials conducted by the Mayo Clinic failed to

demonstrate any effects on cancer therapy with pharmacological oral administration [6, 7]. Later pharmacokinetic studies found that blood levels of ascorbate are tightly controlled by oral use, and intravenous administration can produce high plasma concentrations [8]. This study provided a possible explanation for the discrepancy between the findings of earlier trials. Additional clinical trials showed that patients show good tolerability to pharmacological intravenous ascorbate [9]. Recent researches also suggested that pharmacological intravenous ascorbate could be applied to cancer treatment alone or in combination with the chemotherapies or radiation therapy [10–12].

Chen et al. reported that the anti-cancer effect of pharmacological ascorbate is dependent on the action of ascorbate as a pro-drug for hydrogen peroxide (H_2O_2) generation which involves in the catalytic metals [13]. H_2O_2 and catalytic metals promote the production of reactive oxygen species (ROS) through the Fenton reaction which causes oxidative stress and induces cancer cell death [14, 15]. Although the cytotoxic effect of ascorbate has been demonstrated on many cancer cell lines, its clearly molecular mechanism has not been identified yet [16]. Many previous studies have showed oxidative stress, DNA damage and ATP depletion play critical roles in pharmacological ascorbate-induced cancer cell death [14, 17, 18]. Apoptosis, autophagy and other forms of cell death have been proposed as potential cell death mechanisms [19–21].

In this study, we investigated the mechanisms that pharmacological ascorbate mediated toxicity in osteosarcoma cells. Pharmacological ascorbate mediated toxicity and DNA damage in osteosarcoma cells was found to be dependent upon H_2O_2 mediated oxidative stress, and apoptosis-inducing factor activation was involved in this process. Furthermore, synergistic effects of pharmacological ascorbate combined with cisplatin were also observed in vitro and in vivo.

Materials And Methods

Reagents and cell culture

Ascorbate acid, Cisplatin, N-acetyl-L-cysteine (NAC), bovine catalase (CAT), Deferoxamine (DFO), Chloroquine (CQ) and Ferrostatin-1 were purchased from Sigma-Aldrich. Ascorbate solution was prepared as 1 M stock solution in tri-distilled water. Then the solution was adjusted the pH to 7.0 with NaOH and filtered through 0.22 μ m syringe filter. The solution was divided into several parts into tubes, stored at $-80^\circ C$ and used within 2–3 weeks. Olaparib and PD 151746 were purchased from MedChemExpress. Z-VAD-FMK and Necrostatin-1 were purchased from Beyotime Biotechnology. 3-Methyladenine (3-MA) was purchased from Selleck. Disodium adenosine triphosphate was purchased from Solarbio Science & Technology.

Murine osteosarcoma cell line K7M2, human osteosarcoma cell lines MG63, U2OS and MNNG/HOS were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All osteosarcoma cells were maintained in DMEM (Gibco, USA) supplemented with 10% FBS (Biological Industries, Israel), 100 U/mL penicillin and 100 μ g/mL streptomycin with 5% CO_2 at $37^\circ C$.

Cell viability and colony formation assay

Cells in 96-well plates (5×10^3 per well) were treated with ascorbate (0.1–20 mM) for 1 h, washed with PBS, and incubated for an additional 24 h. The dose of single cell is 0, 20, 100 and 200 pmol/ per cell under the concentration of ascorbate 0, 1, 5, 10 mM. Cell viability assay was performed with the Cell Counting Kit 8 (Beyotime Biotechnology, China).

Colony formation assay was carried out as described previously [22]. Cells were plated and treated with ascorbate for 1 h, then trypsinized and seeded in 24-well plates at a density of 150 cells per well. The cells were further cultured for 10–14 days. Colonies were fixed in 4% paraformaldehyde and dyed with 0.2% crystal violet.

Intracellular H_2O_2 levels and catalase activity detection

Cells were seeded in 6-well plates and incubated for 24 hours in incubator, then the cells were treated with ascorbate and washed with Phosphate Buffer Saline (PBS). Cells were lysed on ice, and centrifuged at 12,000 g for 20 min at 4 °C. The supernatants were collected for detecting H_2O_2 . Intracellular H_2O_2 levels were measured with Hydrogen Peroxide Assay Kit (Beyotime Biotechnology, China) as our previous report [23].

Cells were treated with ascorbate, then washed with PBS and lysed and collected for measuring catalase activity. Catalase activity in cells was measured by Catalase Assay Kit (Beyotime Biotechnology, China) according to the manufacturer's instructions. Catalase activity was then calculated from the consumed hydrogen peroxide. The values were normalized by BCA protein quantification assay.

Detection of reactive oxygen species

Cells were treated with ascorbate for 1 h, washed and incubated with ascorbate-free medium for another 12 h for ROS analysis. After treatment with ascorbate, the cells were trypsinized and washed with PBS three times before being stained with serum-free medium containing 10 μ M 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min at 37 °C in the dark. Then, the cells were washed with PBS three times and resuspended in PBS. The samples were analyzed by flow cytometry (Becton Dickinson, USA).

Measurement of cellular ATP and NAD^+ levels

After treated with ascorbate, cells were washed with PBS and lysed by lysis buffer. The lysates were centrifuged at 12,000 g for 5 min at 4 °C.

The supernatants were collected for the ATP assay with ATP Assay Kit (Beyotime Biotechnology, China). Chemiluminescence mode of microplate reader and black 96-well plates were used for measuring ATP, and cellular ATP content was calculated by ATP standard curve line.

Determination of the total amount of NAD⁺/NADH and the amount of NADH in the supernatants by using NAD⁺/NADH Assay Kit with WST-8 (Beyotime Biotechnology, China). The amount of NAD⁺ was derived by subtracting NADH from total NAD⁺/NADH. All values were normalized by BCA protein quantification assay.

Western blot analysis

Cells (3×10^5 /well) were seeded in 6-well plates and treated with different doses of ascorbate for the indicated times. Subsequently, cells were lysed on ice, and then centrifugation at 12,000 g for 20 min at 4 °C. The lysates were collected and determined using the BCA method and denatured by boiling in loading buffer, and then subjected to Western blot analysis. Blots were enhanced with electrochemiluminescence (ECL) and scanned with a T5200 Multi chemiluminescence system (Tanon, China). The antibodies used for immunoblotting include anti- β -actin (Proteintech Group, USA), anti-H2AX (Cell Signaling Technology, USA), anti-Bcl-2 (Abcam, USA), anti-pADPr (Santa Cruz Biotechnology, USA), anti- γ -H2AX, anti-BAX anti-AIF (Beyotime Biotechnology, China).

Orthotopic intra-tibial mouse model of osteosarcoma

Male 4–6 weeks BALB/c mice were purchased from the SPF Biotechnology Co., Ltd (Beijing, China). All experimental procedures were approved by the Ethics Committee of the Northwestern Polytechnical University. The mice were housed five per cage and kept for 2 weeks under SPF conditions before being used for any experiments. 2×10^6 K7M2 osteosarcoma cells in 20 μ l serum-free DMEM were injected into the left tibial diaphysis with sterile syringe of 1 ml. Once the tumors were established, mice were divided into four groups based on tumor size to ensure the average volume of tumor is consistent in each group. The mice were randomized into the following 4 groups: (1) Ascorbate (AA) group, mice were injected with ascorbate via intraperitoneal injection (4 g /kg daily, IP); (2) DDP group, mice were injected with DDP (5 mg/kg once per week, IP) in 0.9% saline solution; (3) AA + DDP combinational group, both AA and DDP were administered, AA (4 g /kg daily, IP), DDP (5 mg/kg once per week, IP); (4) Control group, mice were injected with equivalent dose of saline (IP). Tumors were measured every other day with Vernier calipers (volume = length \times width² \times 0.5), and mouse weight were also recorded every other day. After 3 weeks, mice were euthanized and sacrificed. Tumors were dissected out, weighed, and stained with hematoxylin and eosin (H&E) and immunohistochemistry (IHC). The major organs were also analyzed by H&E staining.

Statistical analysis

The statistical analysis of the difference between two groups was performed using Student's *t*-test. All statistical analyses were performed using GraphPad Prism 7 Software. The data were presented as the mean \pm standard deviation (SD), *P*-value < 0.05 was considered significant. * represents significant differences (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001), n.s. represents no significant differences (*p* > 0.05).

Results

Pharmacological ascorbate decreases cell viability and inhibits cell proliferation

Previous studies have shown that pharmacological ascorbate presents cytotoxicity and selective sensitivity effects in many cancer cell lines [16]. Therefore, we first detected the effects of pharmacological ascorbate on cell viability in osteosarcoma cell lines. The results showed that pharmacological ascorbate decreases osteosarcoma cell viability in a dose-dependent manner of four osteosarcoma cell lines (Fig. 1A). Then, we performed the colony formation assays to determine the cytotoxic effects of pharmacological ascorbate on cell proliferation. As shown in Fig. 1B, pharmacological ascorbate treatment significantly inhibited cell proliferation in osteosarcoma cell lines. Our results have demonstrated here that pharmacological ascorbate can effectively kill osteosarcoma cells in a dose-dependent manner.

Pharmacological ascorbate improves intracellular H_2O_2 levels and reduces catalase activity

Chen et al. suggested that pharmacological ascorbate act as a pro-oxidant for H_2O_2 generation in cancer cells [13]. In subsequent researches, the pro-oxidative effects of ascorbate have also been confirmed in many cancer cell lines [11, 17]. Here, we detected intracellular H_2O_2 levels after pharmacological ascorbate treatment. As expected, pharmacological ascorbate treatment significantly improved intracellular H_2O_2 levels in four osteosarcoma cell lines, and the intracellular H_2O_2 levels also gradually rose with extension of the treatment time (Fig. 2A). Doskey et al. reported that tumor cells have decreased ability to metabolize H_2O_2 , and the catalase activity may affect tumor cells on ascorbate treatment [22]. We detected the changes of catalase activity in four osteosarcoma cell lines after ascorbate treatment (Fig. 2D). With the increasing treating time, we found that pharmacological ascorbate can reduce catalase activity significantly (Fig. 2B). These results demonstrated that pharmacological ascorbate not only promotes the production of H_2O_2 to kill tumor cells, but also improves its lethality to tumor cells by reducing the activity of catalase. Previous studies have shown that the free radicals generated during the oxidation of ascorbate and semidehydroascorbate are all responsible for the inhibition of catalase [24, 25]. Moreover, we tested the effect of catalase on ascorbate, and excessive exogenous bovine catalase could completely abolish ascorbate-induced toxicity in osteosarcoma cells (Fig. 2C). Our results indicated that the role of H_2O_2 is crucial in the process of ascorbate-induced cell death.

Pharmacological ascorbate-mediated oxidative stress breaks the redox balance and leads to DNA damage

Considering that pharmacological ascorbate treatment improves intracellular H_2O_2 levels and H_2O_2 reacts with intracellular labile iron to generate more reactive hydroxyl radicals ($HO\cdot$) by Fenton reaction [26], we measured the cellular ROS levels in osteosarcoma cells treated with ascorbate by DCFH-DA. As

expected, ascorbate treatment significantly increased cellular ROS levels in osteosarcoma cells, and this effect was reversed by bovine catalase (Fig. 3A). Labile iron promotes the production of hydroxyl radicals, and the cytotoxic effect of hydroxyl radicals is stronger than H_2O_2 [23]. As shown in Fig. 3B, both iron chelator DFO and ROS scavenger NAC could eliminate pharmacological ascorbate-induced toxicity in osteosarcoma cells (Fig. 3B). Excessive ROS can break DNA double-strand and cause cell death [27, 28]. To observe the DNA damage induced by excessive ROS, we used transmission electron microscopy (TEM) to determine morphological alterations. As displayed in Fig. 3C, we observed that obvious disorganized chromatin condensation, vacuolation of the cytoplasm and breakdown of the plasma membrane. We also observed that swelling of mitochondria and disappearance of mitochondria cristae. TEM revealed ascorbate-induced cell death presents necrosis morphological features rather than apoptosis. Additionally, to confirm ascorbate treatment caused DNA double-strand damage in osteosarcoma cells, we detected markers of DNA double-strand damage. As shown in Fig. 3D, the expression of phosphorylation of histone 2AX (γ -H2AX) was significantly increased in cells after ascorbate treatment. While, both catalase and ROS scavenger NAC could prevent the phosphorylation of H2AX caused by ascorbate (Fig. 3E). Next, we tested the ability of five small molecule cell death inhibitors to prevent ascorbate-induced cell death in osteosarcoma cells (Fig. 3F). we found that ascorbate-induced death was not consistently modulated by inhibitors of pan-caspase (Z-VAD-FMK), RIPK1 (necrostatin-1), lysosomal autophagy (3-MA, CQ), lipid ROS (ferrostatin-1) compounds known to inhibit forms of apoptosis, necrosis, autophagic and ferroptosis cell death. The results indicated that ascorbate-induced osteosarcoma cell death is independent of these forms of cell death. Collectively, our data indicated that pharmacological ascorbate breaks cellular redox balance, leads to DNA damage and induces osteosarcoma cell death.

DNA damage depletes intracellular ATP and NAD^+ and activates apoptosis-inducing factor

Poly (ADP-ribose) polymerase (PARP) is an important nuclear enzyme that responds to DNA damage and is required for DNA repair [29]. Activated PARP format Poly (ADP-ribose) (PAR) for DNA damage repair, and the changes in PAR levels reflect the activation of PARP. Therefore, we detected the PAR polymer formation in ascorbate-treat cells. The results showed that the levels of PAR are significantly increased (Fig. 3D). During DNA damage repair process, NAD^+ , as a substrate, is cleaved into ADP-ribose and nicotinamide to format PAR, then cellular NAD^+ levels are restored by recycling nicotinamide with two ATP molecules [30]. Next, we measured the effect of ascorbate on cellular NAD^+ and ATP levels in osteosarcoma cells. As shown in Fig. 4A and 4B, ascorbate treatment for 1 h significantly decreased cellular NAD^+ and ATP levels. Also, both CAT and NAC could restore NAD^+ and ATP levels, indicating that ascorbate induced osteosarcoma cell death probably affected by NAD^+ and ATP depletion during damage repair. In order to further verify the role of activated PARP in osteosarcoma cells, we used olaparib, a PARP inhibitor, and ascorbate to co-treat osteosarcoma cells, and then detected cell viability (Fig. 4C). Interestingly, ascorbate-induced cytotoxicity could be effectively reversed by olaparib in MG63

cells, and olaparib had no effect on ascorbate-induced cytotoxicity in the other three osteosarcoma cell lines. Ma et al. have reported that exogenous ATP can protect neuroblastoma cells from ascorbate-induced cytotoxicity [17]. However, exogenous ATP could inhibit ascorbate-induced cytotoxicity in MG63 cells, and had no effect on the other three cell lines (Fig. 4D). Even more strangely, 5 mM exogenous ATP could kill K7M2 cells directly. Some studies have shown that the activation of P2 × 7 receptor, one of ATP receptors, by high concentrations of ATP can trigger cell death [31, 32]. The effect of exogenous ATP on ascorbate-induced cytotoxicity needs further research. In summary, oxidative stress-mediated DNA damage and NAD⁺ and ATP depletion all contribute to ascorbate-induced cell death.

Although olaparib prevents DNA damage repair by inhibiting PARP, it can partially rescue cell viability in MG63 cells. Yun et al. have reported that NAD⁺ depletion contributes to ascorbate-induced cytotoxicity, and olaparib can rescue cell viability in KRAS and BRAF mutant colorectal cancer cells after ascorbate treatment [33]. However, Ma et al. reported that olaparib preserve cellular NAD⁺ and ATP levels, but cannot prevent ascorbate-induced cell death [17]. Previous studies showed that once excessive PARP activation, the formatted PAR can also act as a pro-death signaling molecule[34, 35]. PAR interacts with the mitochondrial outer surface and induces apoptosis-inducing factor (AIF) release [36]. Then, AIF is cleaved into a soluble form (tAIF) by calpains, and Bax regulates tAIF release from mitochondria [37]. To investigate the role of PAR in ascorbate-induced cell death, we tested the expression of the related proteins. As shown in Fig. 4E, the expression of Bax and the ratio of Bax/Bcl-2 were significantly increased in cells after ascorbate treatment. Moreover, the ratio of tAIF/AIF also significantly increased in cells after ascorbate treatment. The results indicated that ascorbate treatment provokes mitochondrial outer membrane permeability and promotes tAIF release from mitochondria. Considering that calpains regulates tAIF release from mitochondria, we also tested the effect of PD 151746, a calpain inhibitor, on ascorbate-induced cytotoxicity. However, PD 151746 cannot inhibit ascorbate-induced cytotoxicity (Fig. 4F). In summary, we believe that DNA damage is the primary reason leading to osteosarcoma cell death, and NAD⁺ and ATP depletion enhances ascorbate-induced cytotoxicity. DNA damage promotes excessive activation of PARP leading to activation of AIF and release of tAIF, and tAIF induced nuclear condensation also contributes to ascorbate-induced cytotoxicity (Fig. 4G).

Pharmacological ascorbate synergizes with cisplatin in osteosarcoma cell lines

As a first-line chemotherapy for osteosarcoma treatment, cisplatin reacts with DNA to form intra and interstrand crosslinks, which induces DNA damage leading to cell death [38]. Our data have shown that DNA damage is the primary reason in ascorbate-induced cell death in osteosarcoma cells. First, we tested the effect of cisplatin on osteosarcoma cells. As shown in Fig. 5A and 5B, the osteosarcoma cell viability could be effectively inhibited by cisplatin. In addition, the IC₅₀ of K7M2 cells was lower than the other three cell lines. To investigate the effect of ascorbate in combination with cisplatin. As shown in Fig. 5C, the combination of cisplatin and ascorbate significantly suppressed cell viability compared with cisplatin alone in the four osteosarcoma cell lines. We also used Chou-Talalay analysis to determine if ascorbate synergized with cisplatin in osteosarcoma cells, and the combination index (CI) less than 1 indicated a

synergistic effect between cisplatin with ascorbate [39]. As displayed in Fig. 5C, ascorbate and cisplatin synergistically induced cytotoxicity in K7M2, MNNG/HOS and U2OS cells, but we did not observe synergistical effects of two agents in MG63 cells. To further examined the synergy of ascorbate and cisplatin, we detected the expression of γ -H2AX. The combination treatment resulted in a significant increase in γ -H2AX expression compared to cisplatin or ascorbate alone in the three cell lines (Fig. 5D). Given that lower dose of cisplatin was used in combination with ascorbate to treat osteosarcoma cells, our results suggested pharmacological ascorbate can reduce the use of chemotherapeutic agents in vitro.

The combination of pharmacological ascorbate and cisplatin suppresses osteosarcoma tumor growth in orthotopic intra-tibial mouse model

Based on the synergistic effects of ascorbate and cisplatin treatment on osteosarcoma cells in vitro, we tested the efficiency of ascorbate and cisplatin in orthotopic intra-tibial mouse model of osteosarcoma. We found that both pharmacological ascorbate and cisplatin could suppress tumor growth compared with saline group, and the tumor volume and weight were significantly reduced. Although cisplatin alone was better than ascorbate, the combination of two agents was more effective compared to either ascorbate or cisplatin alone (Fig. 6A-C). Then, we tested the tumor tissues from mouse model by H&E staining and IHC analysis to further validate the inhibitory effects of on tumor growth. As shown in Fig. 6D and 6E, drugs treatment induced varying grades of necrosis and the percentage of Ki-67 positive cells was remarkably decreased. In addition, ascorbate alone had no effect on body weight, cisplatin alone and the combination of two agents reduced the body weight compared with saline group (Fig. 6F). Despite the body weight was reduced, we did not observe obvious toxicity in the liver, kidney and spleen in drugs treatment groups (Fig. 6G). Taken together, our results indicated that the treatment with ascorbate alone or in combination with cisplatin significantly suppressed osteosarcoma tumor growth in orthotopic intra-tibial mouse model. All of our findings suggested that pharmacological ascorbate maybe a safe and effective adjuvant agent against osteosarcoma tumor.

Discussion

Osteosarcoma is the most common primary malignant tumor of bone in children and young adults. Before the 1970s, amputation surgery was the main treatment for osteosarcoma. Subsequently, surgery combined with adjuvant systemic chemotherapy significantly improved outcome for patients. However, the treatment and outcome have not changed since the 1980s [40]. Currently, surgery and systemic chemotherapy is still the standard care for osteosarcoma patients. The use of chemotherapy agents has a toxic effect on the main organs, such as cardiotoxic effects of doxorubicin, nephrotoxic effects of methotrexate and cisplatin [3]. Therefore, it is necessary to study safe and effective adjuvant therapeutic agents for patients.

Although the effect of pharmacological ascorbate on cancer treatment is full of controversy, with the deepening of researches, the toxicity effect of ascorbate has been confirmed in a variety of cancer cell lines [12]. Different cancer cells are selective sensitive to ascorbate, and the clearly molecular mechanism

has not been identified yet. Some studies showed that the expression of sodium-dependent vitamin C transporter 2 (SVCT-2) and glucose transporter 1 (GLUT1) in cancer cells, which transport vitamin C and dehydroascorbate (DHA) into the cells, respectively, affected the sensitivities of cancer cells to ascorbate [14, 33]. The abilities of cancer cells to metabolize H_2O_2 might also cause different sensitivities to ascorbate [22]. Our data revealed that ascorbate not only promotes the production of H_2O_2 to kill osteosarcoma cells, but also improves its lethality to tumor cells by reducing the activity of catalase. A recent study reported that the $O_2^{\cdot-}$ and H_2O_2 were able to disrupt cellular iron metabolism, thereby selectively sensitizing cancer cells to ascorbate [11]. In our recent research, similar conclusions were obtained, the basal levels of intracellular labile iron and effects of ascorbate on ferritin expression are related to the sensitivity of human osteosarcoma cell lines to ascorbate [23].

Intracellular labile iron reacts with H_2O_2 to generate more reactive hydroxyl radicals ($HO\cdot$) by Fenton reaction, and leads to DNA damage. TEM and the expression of γ -H2AX have verified that ascorbate-induced significant DNA damage in osteosarcoma cells, which was consistent with results in neuroblastoma cells [17]. Although several forms of cell death have been proposed as potential ascorbate-induced cell death mechanisms, our data showed several small molecule cell death inhibitors cannot prevent ascorbate-induced cell death in osteosarcoma cells. These four osteosarcoma cell lines have different sensitivities to ascorbate, and the factors that affect their death are not the same. During DNA damage repair process, PARP is activated and NAD^+ and ATP are consumed. Over-activated PARP produces a large amount of PAR which in turn activates AIF, then tAIF induces nuclear condensation [36]. Xia et al. also showed that AIF was activated by ascorbate in multiple myeloma cells [41]. We found that NAD^+ and ATP depletion and tAIF also contributes to ascorbate-induced cytotoxicity. Exogenous ATP and PARP inhibitor olaparib can inhibit ascorbate-induced cytotoxicity in MG63 cells, but have no effect on the other three cell lines. Perhaps multiple factors involved in the death of MG63 cells that make it more sensitive to ascorbate compared with the other three cell lines.

Pharmacokinetic studies found that plasma concentrations of ascorbate are tightly controlled by oral use due to the limitation of intestinal absorption, and intravenous injection can produce high plasma concentrations [8]. Therefore, high plasma concentrations of ascorbate in vivo are sufficient to kill tumor cells. Subsequently, many preclinical and clinical studies have shown that intravenous administration of ascorbate can be used as a safe and effective adjuvant to combine with chemotherapies or radiation therapy for cancer treatment [9, 42–45]. Furthermore, ascorbate could also sensitize cancer cells to chemotherapy and protect normal tissues [10, 46]. Our data showed that ascorbate and cisplatin synergistically induce cytotoxicity in different osteosarcoma cell lines, and ascorbate can reduce the use of chemotherapeutic agents in vitro. In vivo experiments, intravenous administration of ascorbate could enhance the efficacy of cisplatin in orthotopic mouse model of osteosarcoma. While, clinical trials will be needed to evaluate the safety and efficacy of ascorbate in osteosarcoma tumor therapy.

Conclusions

In this study, we found that pharmacological ascorbate promotes the production of H₂O₂ and reduces the activity of catalase in osteosarcoma cells. H₂O₂-mediated oxidative stress leads to DNA damage, which induced cell to death. In addition, depletion NAD⁺ and ATP and activation of PARP both contributed to ascorbate-mediated cytotoxicity. We also observed the synergistic effects of pharmacological ascorbate combined with cisplatin in osteosarcoma cells and orthotopic intra-tibial mouse model. In summary, our findings suggested that pharmacological ascorbate could be a safe and effective adjuvant agent against osteosarcoma tumor.

Abbreviations

AA: Pharmacological ascorbate; AIF: Apoptosis-inducing factor; ATP: Adenosine triphosphate; CAT: Bovine catalase; CI: Combination index; CQ: Chloroquine; DCFH-DA: 2,7-dichlorodihydrofluorescein diacetate; DFO: Deferoxamine; DDP: Cisplatin; H₂O₂: Hydrogen peroxide; NAC: N-acetylcysteine; NAD⁺: nicotinamide-adenine dinucleotide; NADH: reduced form of nicotinamide-adenine dinucleotide; PARP: Poly (ADP-ribose) polymerase; PAR: Poly (ADP-ribose); PBS: Phosphate Buffer Saline; ROS: Reactive oxygen species; TEM: Transmission electron microscopy; 3-MA: 3-Methyladenine

Declarations

Ethics approval and consent to participate

All animal studies were approved by the Ethics Committee of the Northwestern Polytechnical University.

Consent for publication

Not applicable.

Availability of data and material

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

LFZ: Conceptualization, Methodology, Visualization, Writing - original draft, Writing - review & editing, Funding acquisition.

XXZ: Investigation, Visualization, Software, Writing - review & editing.

SHW: Methodology, Data curation, Visualization.

HHL: Project administration, Validation.

ABS: Methodology, Formal analysis

PS: Funding acquisition, Supervision, Project administration, Resources.

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Author details

¹ School of Life Sciences, Northwestern Polytechnical University, Xi'an, Shaanxi, 710072, China

² Research & Development Institute of Northwestern Polytechnical University in Shenzhen, Shenzhen, Guangdong, 518057, China

³ Key Laboratory for Space Bioscience and Biotechnology, Institute of Special Environment Biophysics, Northwestern Polytechnical University, Xi'an, Shaanxi, 710072, China

⁴ International Orthopedic Centre, Southern University of Science and Technology Hospital, Shenzhen, Guangdong, 518055, China

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Figures

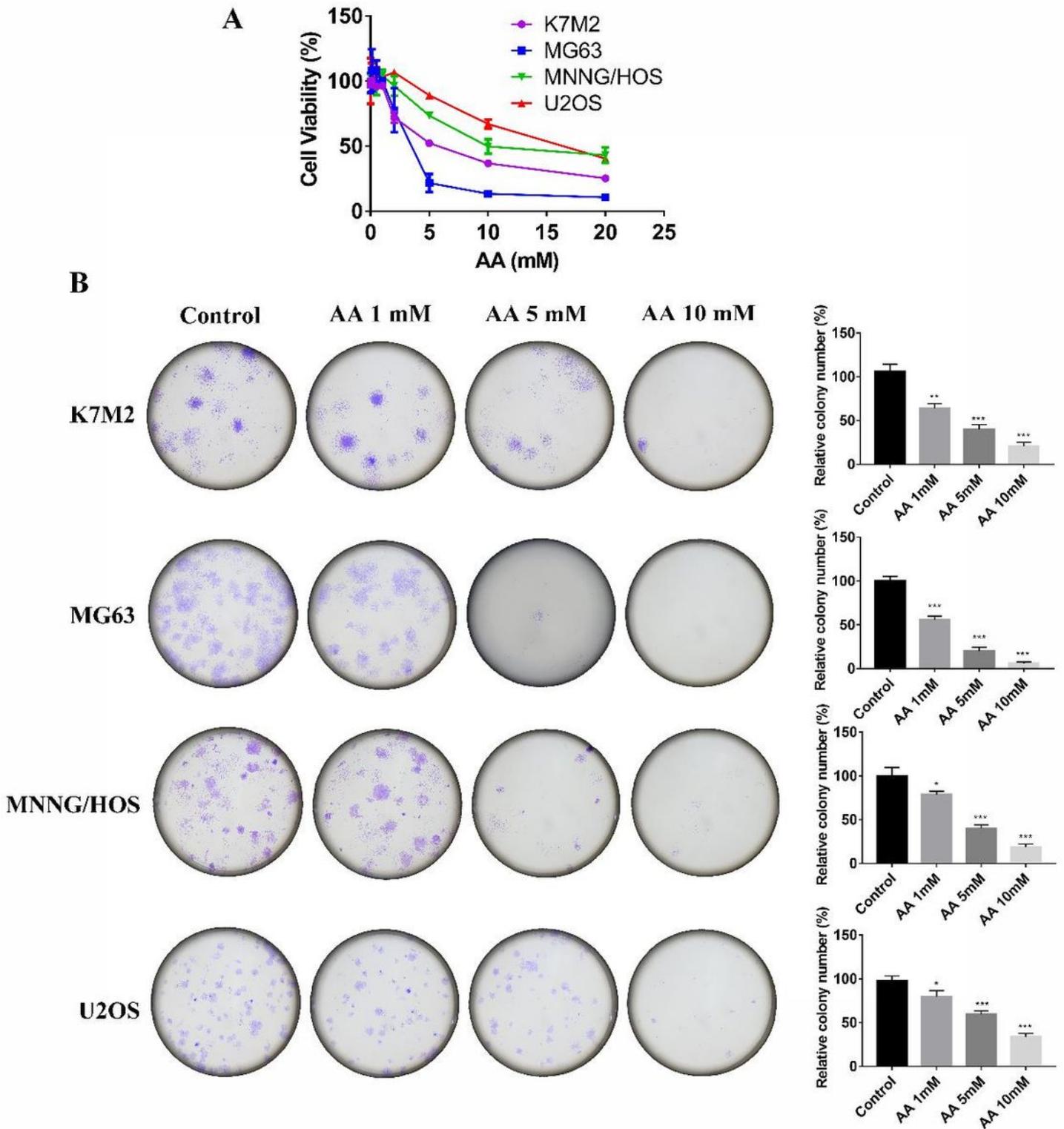


Figure 1

Effects of pharmacological ascorbate on osteosarcoma cells. (A) Cells treated with ascorbate (AA, 0-20 mM) 1 h, washed with PBS, cell viability was determined after 24 h; (B) Cell proliferation of was analyzed by colony formation assay after ascorbate treatment.

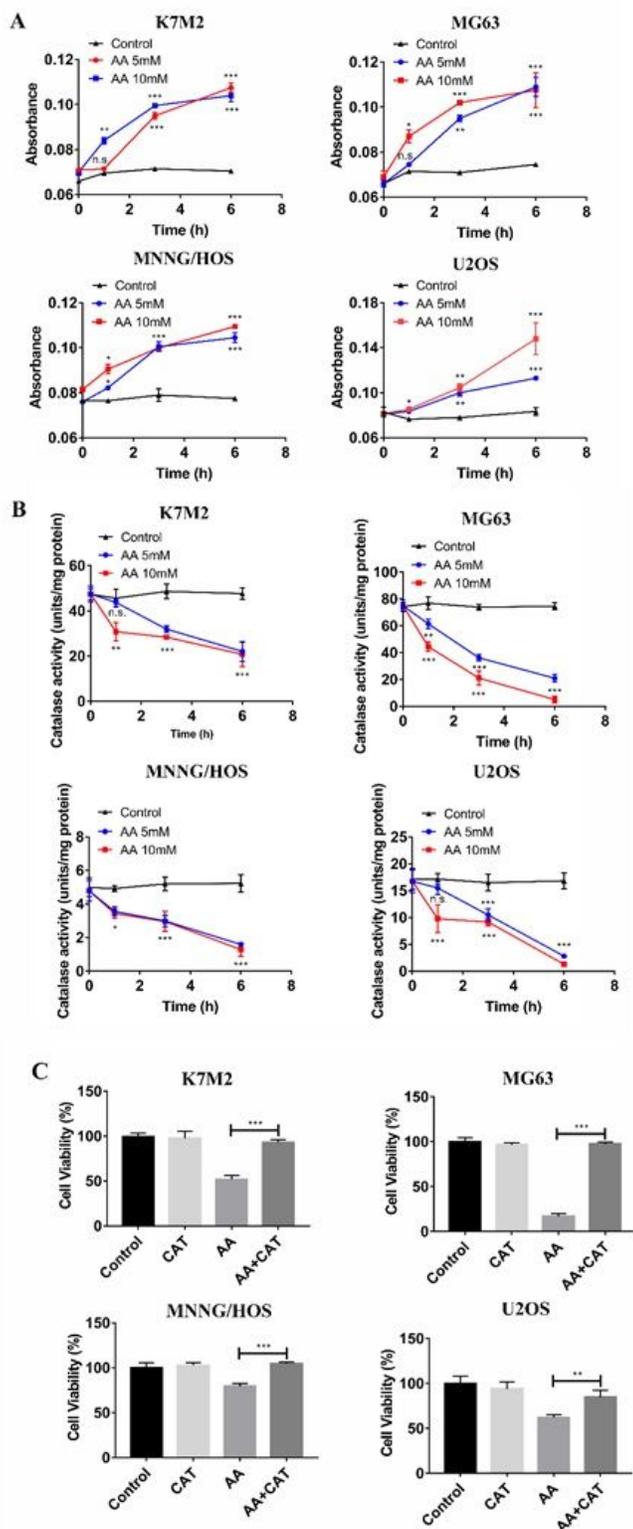


Figure 2

Effects of pharmacological ascorbate on cellular H₂O₂ levels and catalase activity. (A-B) Cells treated with ascorbate (5, 10 mM) for 0, 1, 3, 6 h, washed with PBS, then collected cell lysates for detection of cellular H₂O₂ levels and catalase (CAT) activity; (C) Cells treated with ascorbate 1 h in the presence of 200-500 units/mL bovine catalase, washed with PBS, cultured for an additional 24 h, detected cell viability.

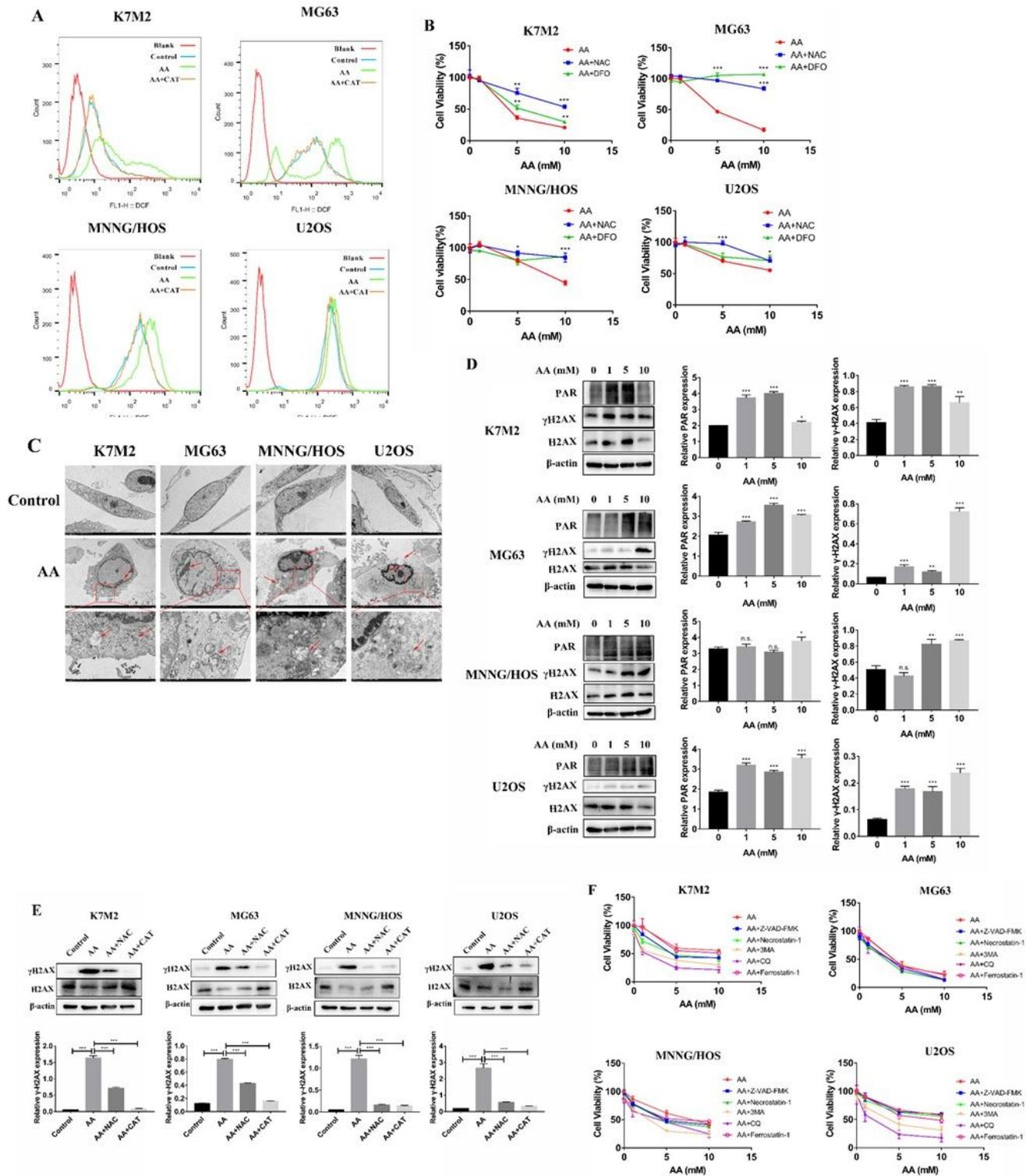


Figure 3

Pharmacological ascorbate-mediated oxidative stress increases cellular ROS levels and damages DNA. (A) Cells treated with ascorbate 1 h in the presence of 200-500 units/mL bovine catalase, washed with PBS, cultured for an additional 12 h. Then, flow cytometry measurement of cellular ROS levels through DCFH-DA staining; (B) Cells treated with 2 mM NAC and ascorbate 1 h, washed with PBS, cell viability was determined after 24 h; Cells pre-incubated with 200 μ M DFO 3 h, washed with PBS, treated with

ascorbate 1 h, washed with PBS, cell viability was determined after 24 h; (C) MG63 and K7M2 cells treated with 5 mM ascorbate 1 h and MNNG/HOS and U2OS cells treated with 10 mM ascorbate 1 h, washed with PBS, cultured for 24 h, fixed and collected cells, detected with TEM; (D) Detected PAR, γ -H2AX and H2AX expression after treated with ascorbate by western blot assay (left panel); Statistical analyses of PAR and γ -H2AX in the four cell lines (right panel); (E) Detected γ -H2AX and H2AX expression after treated with ascorbate and CAT or NAC by western blot assay; Statistical analyses of PAR and γ -H2AX in the four cell lines; (F) Cells pre-incubated with 20 μ M Z-VAD-FMK, 20 μ M Necrostatin-1, 1mM 3-MA, 5 μ M CQ, 10 μ M Ferrostatin-1 for 2h, then inhibitors and ascorbate co-treated cells 1 h, washed with PBS, cultured for 24 h in inhibitor-containing medium, detected cell viability.

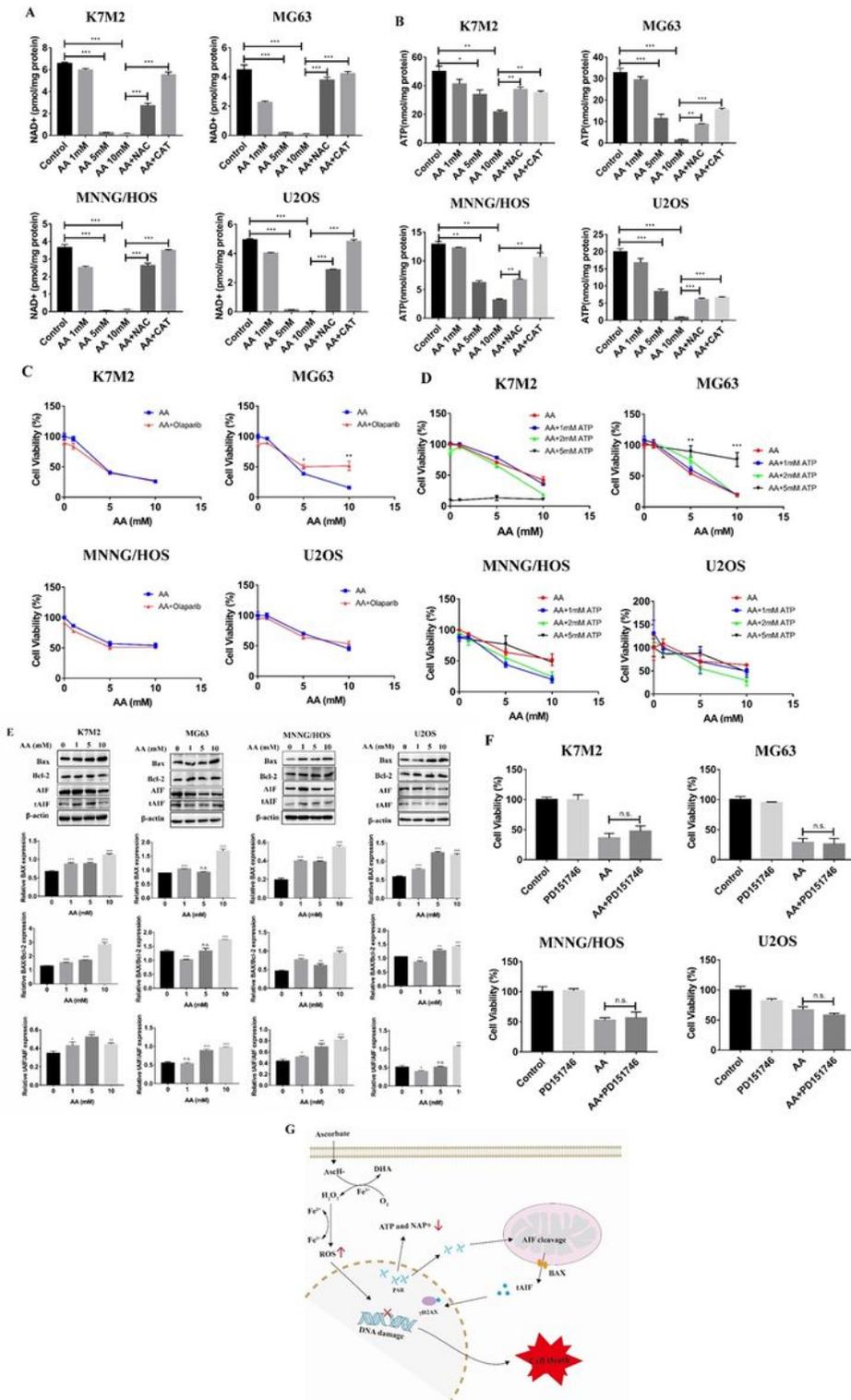


Figure 4

DNA damage depletes cellular ATP and NAD⁺ and activates AIF. (A-B) Cells treated with ascorbate and 2 mM NAC or 200-500 units/mL CAT 1 h, washed with PBS, then collected cell lysates for detection of cellular NAD⁺ and ATP levels ; (C) Cells pre-incubated with 20 μM PARP inhibitor olaparib for 2h, then olaparib and ascorbate co-treated cells 1 h, washed with PBS, cultured for 24 h in olaparib-containing medium, detected cell viability; (D) Cells treated with ascorbate and different concentrations of ATP 1 h,

washed with PBS, cell viability was determined after 24 h; (E) Detected BAX, Bcl-2, AIF and tAIF expression after treated with ascorbate by western blot assay (left panel); Statistical analyses of BAX, BAX/Bcl-2 and tAIF/AIF in the four cell lines (right panel); (F) Cells treated with 10 mM ascorbate and PD 151746 calpain inhibitor 1 h, washed with PBS, cell viability was determined after 24 h; (G) Proposed model of pharmacological ascorbate killed osteosarcoma cells.

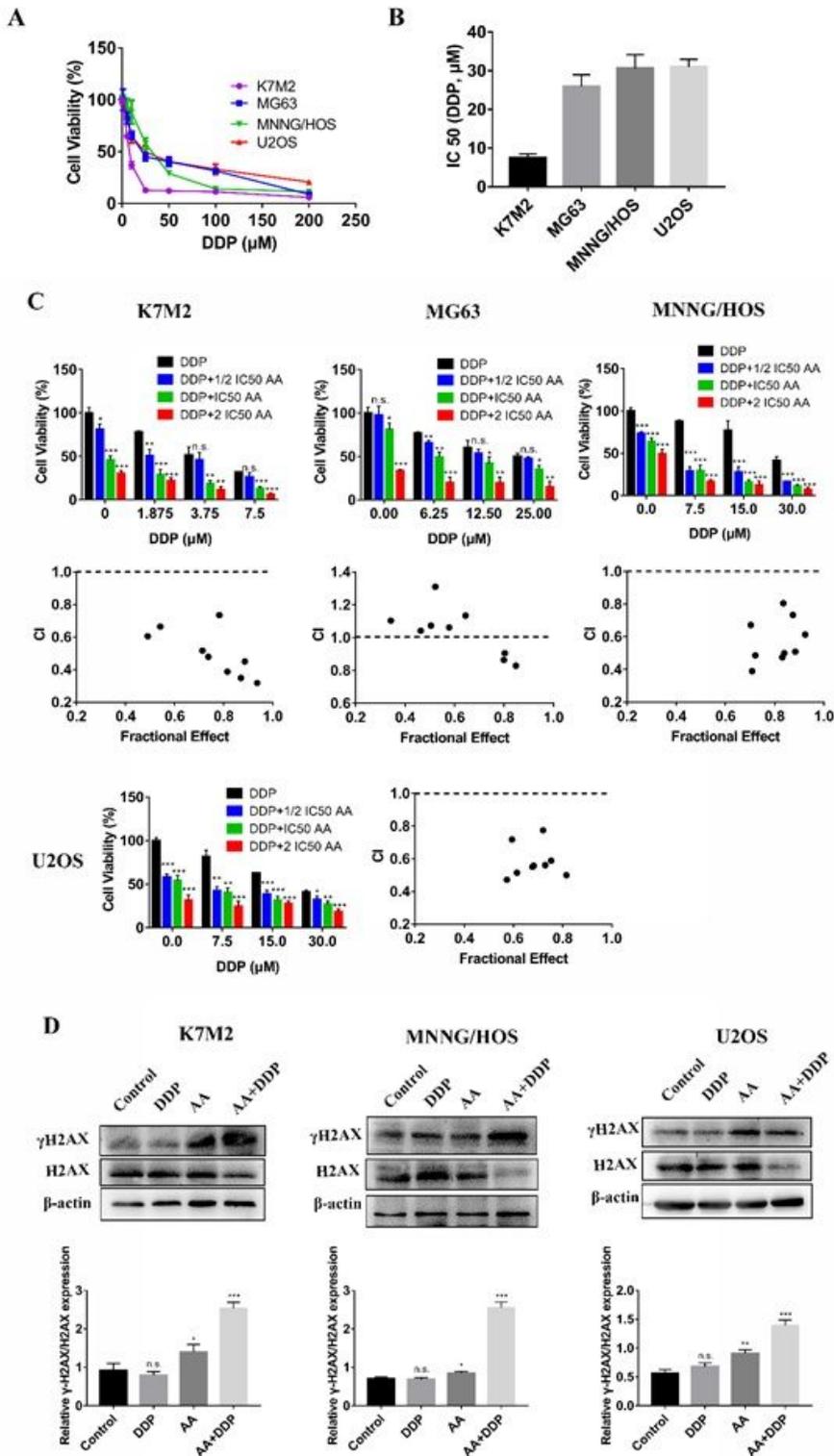


Figure 5

Effects of pharmacological ascorbate and cisplatin (DDP) on osteosarcoma cells. (A) Cells treated with various concentrations of cisplatin (0-200 μM) 24 h, then detected cell viability; (B) IC 50 values of cisplatin in osteosarcoma cell lines; (C) Cells treated with ascorbate 1 h, washed with PBS, then treated with cisplatin 24 h, detected cell viability (left panel); The combination index (CI) of ascorbate and cisplatin was calculated for the osteosarcoma cell lines (left panel), CI analysis was performed using CalcuSyn 2.0 (Biosoft, USA); (D) Cells were treated with ascorbate (1 h) and cisplatin (24 h) alone or in combination(ascorbate 1 h and cisplatin 24 h), the expression of $\gamma\text{-H2AX}$ and H2AX expression were detected by western blot assay.

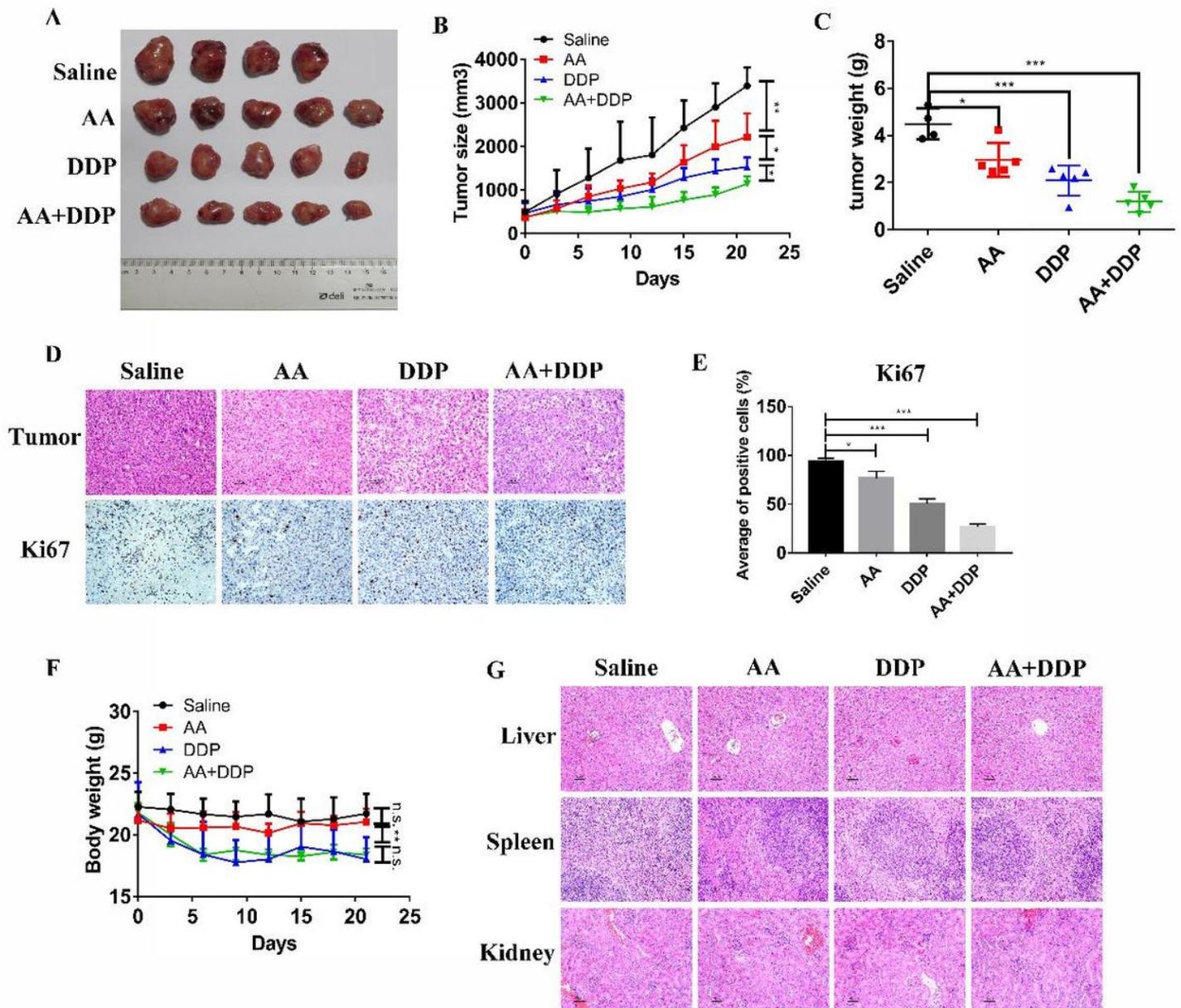


Figure 6

Combination of ascorbate and cisplatin in orthotopic intra-tibial mouse model of osteosarcoma. After treatment, the mice were sacrificed, major organs and tumors were dissected and collected; (A)

Photographs of dissected tumors for each group; (B) The volumes of tumors for each group at the indicated time points; (C) Tumors were dissected and collected, then tumors' weight was recorded; (D) Tumors were sectioned and stained with H&E and Ki67, and representative H&E and IHC images were presented; (E) Statistical analyses of Ki67 expression in each group; (F) Body weight of each group at the indicated time points; (G) Major organs (liver, spleen, kidney) were sectioned and stained with H&E.