Ascorbic Acid Inhibits Liver Cancer Growth and Metastasis in vitro and in vivo, Independent of Stemness Gene Regulation

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Experimental and clinical evidence has indicated that the natural product ascorbic acid (AA) is effective in preventing and treating various types of cancers. However, the effect of AA on liver cancer metastasis has not yet been reported. Cancer stem cells (CSCs) play pivotal roles in cancer metastasis. Here, we demonstrated that AA selectively inhibited the viability of both liver cancer cells and CSCs, reduced the formation of cancer cell colonies and CSC spheres, and inhibited tumor growth in vivo. Additionally, AA prevented liver cancer metastasis in a xenotransplantation model without suppressing stemness gene expression in liver CSCs. Further study indicated that AA increased the concentration of H₂O₂ and induced apoptosis in liver CSCs. Catalase attenuated the inhibitory effects of AA on liver CSC viability. In conclusion, AA inhibited the viability of liver CSCs and the growth and metastasis of liver cancer cells in vitro and in vivo by increasing the production of H₂O₂ and inducing apoptosis. Our findings provide evidence that AA exerts its anti-liver cancer efficacy in vitro and in vivo, in a manner that is independent of stemness gene regulation.

Keywords: ascorbic acid, cancer stem cells, metastasis, stemness genes, H₂O₂, apoptosis

INTRODUCTION

One of the main causes of cancer-related death is distant metastasis that occurs in cancer patients, and cancer stem cells (CSCs) are an important driving force for cancer metastasis. CSCs, also referred to as tumor-initiating cells, have a stronger tumor-forming ability than somatic or non-tumorigenic cancer cells (Ponti et al., 2005; Ma et al., 2007). CSCs play key roles in the development of metastasis in multiple cancers. In colorectal cancer, CD26⁺ CSCs caused distant metastasis when injected into the mouse cecal wall, while the presence of CD26⁻ CSCs in primary tumors can predict distant metastasis in cancer patients (Pang et al., 2010). Also, Lgr5⁺ or CD44v6⁺ CSCs are required for the generation of metastatic tumors (Todaro et al., 2014; De Sousa e Melo et al., 2017). In squamous cell carcinoma of the head and neck, it was observed that BMI1⁺ CSCs regulated the invasive growth and cervical lymph node metastasis in a mouse model (Chen D. et al., 2017). A recent study at the single-cell level in breast cancer has shown that early-stage metastatic cells possess a distinct stem-like gene expression signature (Wylie et al., 2015).

Liver cancer is a heterogeneous disease, and liver CSCs play important roles in the development of this disease. Inhibition of ICAM-1, a marker of hepatocellular CSCs, suppresses tumor formation
and metastasis in mice (Liu et al., 2013). All-trans retinoic acid can effectively induce the differentiation of CSCs, and it also enhances the cytotoxicity of cisplatin and increases the inhibition of hepatocellular carcinoma (HCC) cell migration in vitro and metastasis in vivo in combination with cisplatin (Zhang et al., 2013). All of these studies have demonstrated a key role for CSCs in cancer metastasis and suggested that CSCs are a promising target for developing effective therapeutic agents that can be used to treat metastatic cancer.

The natural product ascorbic acid (AA) is an important water-soluble vitamin and is one of the early unorthodox therapies that has long been used in the field of alternative and complementary medicine for cancer treatment, with profound safety and anecdotal efficacy (Du et al., 2010; Chen et al., 2015). Many clinical and laboratory studies have revealed its effects on cancer prevention and treatment. AA inhibits the growth of prostate, ovarian, and pancreatic cancer cells and neuroblastoma cells. (Maramag et al., 1997; Carosio et al., 2007; Chen et al., 2008; Du et al., 2010; Yun et al., 2015; Schoenfeld et al., 2017). Cameron et al. demonstrated in the 1970s that there was a potential survival benefit for patients who received oral and intravenous administration of AA (Cameron and Pauling, 1976; Cameron and Pauling, 1978). However, two clinical studies performed at the Mayo Clinic have shown no significant difference between oral ascorbate-treated and placebo-treated patients (Moertel and Fleming, 1985; Creagan et al., 1979).

Additional research has shown that oral ingestion of high doses of AA rarely induce a plasma concentration greater than 200 μM, due to the limited absorption and renal excretion. By contrast, both intravenous (i.v.) and intraperitoneal (i.p.) administration of ascorbate result in pharmacologic serum ascorbate concentrations up to 20 mmol/L (Reczek and Chandel, 2015; Verrax and Calderon, 2009). Subsequent studies have shown that high-dose intravenous administration of AA alleviates symptoms and prolongs survival in patients with advanced cancer (Cameron and Pauling, 1976; Cameron and Pauling, 1978; Cameron and Campbell, 1974; Padayatty et al., 2006; Raymond et al., 2016). AA also significantly reduces the metastasis of B16FO melanoma cells injected into mice who were deficient in AA and unable to synthesize it (Cha et al., 2013). However, there have been no reports describing the effects of AA on liver cancer metastasis.

With the participation of transition metals (such as copper and iron), a high dose of AA as an electron donor produces extracellular ascorbate anion and H₂O₂, which play important roles in AA-induced anticancer activity (Chen et al., 2015). H₂O₂, an important reactive oxygen species (ROS), plays numerous roles in cancer cells, where a low concentration of H₂O₂ is involved in various signal transduction and cell functions, and a high concentration of H₂O₂ causes DNA damage and promotes cell apoptosis. Du et al. demonstrated that AA decreases the clonogenic survival of pancreatic cancer cell lines, while treatment of cells with H₂O₂ scavengers can reverse AA’s anticancer activity (Du et al., 2010). Chen et al. reported that AA causes significant cytotoxicity in cancer cells, while glutathione reduces the cytotoxicity by attenuating AA-induced H₂O₂ production (Chen et al., 2005; Chen et al., 2011).

In this study, we investigated the inhibitory effects of AA on liver cancer cells and liver CSCs in vitro and in vivo. We found that AA inhibited the growth and metastasis of liver cancer cells and liver CSCs, although AA also increased the expression levels of stemness genes. Further molecular mechanism studies indicated that the increased concentration of H₂O₂ and the enhanced apoptosis by AA play vital roles in its efficacy against liver cancer.
MATERIALS AND METHODS

Cell Culture
Human liver cancer cell lines Huh7 and Hep3B and normal human liver cell line L02 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Huh7 and Hep3B CSCs were enriched and maintained on poly-HEMA coated plates in serum-free DMEM/Nutrient Mixture F-12 (F-12) medium containing 20 ng/ml epidermal growth factor (EGF) (236-EG-200, R&D Systems), 10 ng/ml fibroblast growth factor (FGF) (233-FB-025, R&D Systems), and 1% penicillin/streptomycin (Pang et al., 2010; Li et al., 2015). For preparing poly-HEMA coated plates, 6-well plates were pre-coated with 1.2% (w/v) poly-HEMA (Re et al., 1994).

Detection of Cell Viability
Cell viability was measured by Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories) according to the user’s manual. The cell viability in each group is expressed as the percentage of untreated control cell viability (Wu et al., 2017).

Flow Cytometric Analysis
To examine the expression of CD133 and CD44, Huh7 and Hep3B stem cells were digested with 0.05% trypsin. Next, $10^6$ cells/100 μl of single cells were resuspended and incubated with PE-labeled CD133...
or CD44 (1:50, Miltenyi Biotec) in the dark for 15 min, washed twice with cold phosphate-buffered saline (PBS), resuspended in 400 μl PBS, and analyzed using flow cytometry (Becton Dickinson FACS Vantage SE, San Jose, CA, United States).

To analyze cell apoptosis, Huh7 stem cells were digested with 0.05% trypsin. Then, 1 × 10^6 single cells were resuspended and mixed with 10 μl Annexin V-fluorescein isothiocyanate (FITC, 130-097-928, Miltenyi Biotec), incubated in darkness for 15 min, washed with 1 ml Annexin V Binding Buffer and resuspended in 500 μl 1× Annexin V Binding Buffer, mixed with propidium iodide (PI) solution, and then analyzed by flow cytometry (Cheng et al., 2017).

**RNA Isolation and Quantitative Real-Time PCR**

Total RNA was isolated using a Tissue RNA Kit (R6311-01, Biomiga). RNA (1 μg) was reverse-transcribed into cDNA using GoScript Reverse Transcriptase (A5001, Promega). Quantitative real-time PCR was completed using the PowerUp SYBR Green Master Mixture (Thermo Fisher) with the StepOne Plus Real-Time PCR System (Thermo Fisher), according to a protocol from a previous study (Wu et al., 2017). Specific primers for CD90 and EPCAM were created according to Luo et al. (2015). Specific primers for CD133, OCT4 (POU5F1), NANOG, SOX2, and beta-actin were created according to Ma et al. (2010).

**Animal Experiments**

All of the mice were maintained in a pathogen-free facility, and all of the animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Naval Medical University, China. For the animal experiments, 6-week-old female nude BABL/c mice were used, and 2 × 10^6 Huh7 or Hep3B cells were subcutaneously inoculated into the nude mice (Ma et al., 2018; Yuan et al., 2015). Three weeks later, PBS (control group) or 4 g/kg AA was injected intraperitoneally twice daily for 26 days. The tumor volume was calculated as total volume = (length × width^2)/2 (Naito et al., 1986). Lung and liver tissues were fixed with 4% polyformaldehyde, and serial sections (four sections per tissue with a 30-μm step) were created and stained with hematoxylin and eosin (HE) (Cheng et al., 2017).

**Western Blot**

Western blot was completed according to a protocol from a previous study (Wu et al., 2017). Briefly, cells or tissues were lysed with Radioimmunoprecipitation Assay (RIPA) Lysis Buffer (P0013C, Beyotime Biotechnology, China) and centrifuged at 13,000 rpm for 15 min. The supernatant was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated overnight with anti-NANOG (1:500, ab109250, Abcam), anti-SOX2 (1:500, ab92494, Abcam, UK), anti-ALDH1A1 (1:1,000, ab52492, Abcam), or anti-β-actin (1:1,000, 3700S, Cell Signaling Technology) primary antibodies, washed with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) three times, incubated with secondary antibody (926-32210, 1:20,000 for β-actin and 926-32211, 1:5,000 for others, LI-COR, Biosciences), and analyzed with the Odyssey Infrared Imaging System (LI-COR, Biosciences).

**Detection of H$_2$O$_2$**

The H$_2$O$_2$ concentration was measured using a H$_2$O$_2$ Assay Kit (S0038, Beyotime Biotechnology, China) according to the user’s manual. Simply, 1 × 10^6 cells were lysed in 200 μl lysis buffer and...
centrifuged for 5 min at 12,000 rpm. Every 50 μl of the supernatant was mixed with 100 μl of H2O2 detection reagent and incubated for 30 min at room temperature. Absorbance was determined at 560 nm using an Epoch Microplate Spectrophotometer (BioTek). For catalase experiments, catalase was added prior to AA treatment.

Sphere Formation Assay and Colony Formation Assay
For the sphere formation experiment, cells were digested into single cells with trypsin. Then, 100 cells/well were plated into a 96-well ultra-low attachment plate and cultured for 2 weeks in serum-free DMEM/F-12 medium containing 20 ng/ml EGF, 10 ng/ml FGF, and AA (0, 0.5, or 1 mM). The number of spheres was counted and photographed.

For the colony formation experiment, 1,000 cells/well were plated into 6-well plates. The colonies were cultured in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin, and AA (0, 0.5, or 1 mM). The colonies were then stained with 1% crystal violet.

Statistical Analysis
Statistical analysis was performed using unpaired t tests when comparing two different groups or one-way ANOVA with Tukey’s multiple comparison tests. IC50 values were calculated using Prism software (GraphPad, San Diego, CA, USA) by nonlinear regression to dose-response curves, and expressed as mean and 95% confidence intervals (CI). The data are expressed as the mean ± SEM. p < 0.05 was considered statistically significant.

RESULTS
AA Selectively Inhibited the Viability of Liver Cancer Cells and Liver CSCs in vitro
Two human liver cancer cell lines (Huh7 and Hep3B), the respective CSCs, and a normal human liver cell line LO2 were treated with AA at the concentrations of 0, 0.5, or 1 mM, which are easily achievable clinically by intravenous infusion (Chen et al., 2008) (Hoffer et al., 2008). The results showed that AA inhibited the viabilities of liver cancer cells and liver CSCs in a concentration-dependent manner (Figures 1A–D). AA at the concentration of 1 mM decreased the viabilities of Huh7 and Hep3B cells to 12.15 and 5.77%, respectively (Figures 1A,B). For Huh7 and Hep3B CSCs, the viabilities were decreased to 52.37 and 33.04%, respectively, at 1 mM concentration of AA (Figures 1C,D). The IC50 values of AA for Huh7, Hep3B, and Huh7 CSCs and Hep3B CSCs were 0.67, 0.32,
1.21, and 0.52 mM, respectively (Figure 1E). However, AA did not display significant inhibitory effects on the viability of L02 cells at 0.5 mM or 1 mM concentrations (Figure 1F). Together, these data indicated that AA was responsible for selective inhibitory effects on the viabilities of liver cancer cells and liver CSCs.

**AA Inhibits Sphere Formation and Colony Formation in Liver Cancer Cells**

We further examined the effects of AA on sphere formation and colony formation. As shown in Figure 2A, AA treatment reduced the volume of spheres formed by Huh7 cells. The number of spheres larger than 50 μm in diameter was markedly decreased in a concentration-dependent manner in AA-treated Huh7 cells (Figure 2B). Twenty-two spheres were formed for every 100 cells in the control group, whereas only two spheres were formed for every 100 cells in the group treated with 1 mM AA. Similar results were obtained for Hep3B cells (Figures 2C,D). As shown in Figure 2E, AA treatment also markedly decreased colony formation in a concentration-dependent manner in Huh7 and Hep3B cell lines. Collectively, our data showed that AA reduced sphere formation and colony formation by liver cancer cells, indicating the inhibitory effects of AA on self-renewal and tumorigenicity of liver cancer cells.

**AA Inhibited Liver Tumor Growth in vivo**

We determined the effects of AA on tumor growth in mice bearing Huh7 and Hep3B xenografts. As mentioned above, AA concentrations in human plasma and cells were tightly controlled. With the oral ingestion of high doses of vitamin C, even at 100 times the recommended dietary allowance, the plasma concentration rarely exceeds 200 μM. Both i.v. and i.p. administration of ascorbate induced pharmacologic serum ascorbate concentrations up to 20 mmol/L. To obtain a pharmacologic serum ascorbate concentration, the i.p. administration method was selected. Compared with the PBS control group, AA treatment significantly suppressed the growth of Huh7 and Hep3B xenograft tumors in vivo (Figures 3A,B) without significantly changing the animal’s body weight (Figures 3C,D).

**AA Prevents Tumor Metastasis in vivo**

As shown in Figures 4A,B, AA-treated mice developed fewer metastatic lung tumors as compared to the control group. The number of metastatic lung tumors in AA-treated mice was 0.90 ± 0.40 (n = 5), and that in the control mice was 6.25 ± 2.27 (n = 5) (Figure 4C). The area ratio of metastatic lung tumors in AA-treated mice was 0.29 ± 0.17 (n = 5), and that in control mice was 14.61 ± 6.91 (n = 5) (Figure 4D). The metastatic tumors in the livers of either the control or AA groups were small (Figures 4E,F). In the control group, 5 of 5 mice developed metastatic lung tumors, whereas 3 of 5 mice exhibited metastatic lung tumors in the AA-treated group (Figure 4G). Additionally, in the control group, 4 of 5 mice developed metastatic liver tumors, while in the AA-treated group, 1 of 5 mice developed metastatic liver tumors (Figure 4H). In summary, our data demonstrated that AA
treatment reduced liver and lung metastasis of liver cancer cells inoculated subcutaneously into nude mice.

**AA Upregulated the Expression of Stemness Genes in Liver Cancer Cells and Tumors**

We investigated the effects of AA on the expression of stemness genes. Flow cytometric analysis showed that AA treatment increased CD133+ cells and CD44+ cells in both Huh7- and Hep3B-derived stem cells (Figures 5A,B). CD133 antigen was identified as a CSC marker in various cancer types, including liver cancer. CD44, a transmembrane glycoprotein, is also considered as an important liver CSC marker (Zhu et al., 2010; Yang et al., 2008). For Huh7 CSCs, AA at 1 mM increased CD133+ cells and CD44+ cells from 2.90 to 14.70%–4.29 and 24.19%, respectively (Figures 5A,B). For Hep3B CSCs, CD133+ cells and CD44+ cells were increased by AA from 20.40 to 0.75%–24.22 and 4.51%, respectively (Figures 5A,B). Western blot analysis showed that the protein levels of embryonic stem cell markers NANOG and SOX2 as well as liver CSC marker ALDH1A1 were increased after treatment with AA in Huh7- and Hep3B-derived stem cells (Figures 5C,D).

We also examined the effects of AA on the expression of stemness genes in liver tumors in vivo. Consistent with the in vitro results, the mRNA expression levels of NANOG, OCT4, SOX2, EPCAM, CD133, and CD90 were upregulated in the AA-treated tumors (Figure 6A). Also, the protein level of NANOG was increased in the AA-treated group as compared with that of the control group (Figures 6B,C). Collectively, our data showed that AA upregulated the expression of stemness genes in liver cancer cells in vitro and in vivo.

**AA Enhanced the Production of H2O2 and Promoted the Apoptosis of Liver CSCs**

It was reported that H2O2 plays an important role in AA’s anticancer activity (Lennicke et al., 2015; Chaiswing et al., 2018). To determine the role of H2O2 in the inhibitory effect of AA on liver CSCs, we first evaluated the concentrations of H2O2 in Huh7-derived CSCs with or without AA treatment. As shown in Figure 7A, AA treatment increased the concentration of H2O2 in Huh7-derived CSCs. Furthermore, AA increased the protein levels of cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase-7 (Figure 7B) and promoted cell apoptosis (Figures 7C,D).

Catalase, as a specific H2O2 scavenger, converts the ROS H2O2 to water and oxygen and thereby mitigates the cytotoxic effects of H2O2. We also found that the addition of catalase reversed the effects of AA on the production of H2O2 and the cleavage of PARP and caspase-7 (Figures 7E,F). More importantly, the addition of catalase reduced the inhibitory effects of AA on...
FIGURE 7 | AA reduces liver CSC viability via increasing the production of H2O2 and induction of cell apoptosis. (A) The content of H2O2 in Huh7 stem cells treated with different concentrations of AA. (B) The protein levels of cleaved PARP and caspase 7 in Huh7 stem cells treated with different concentrations of AA. (C, D) Apoptosis of Huh7 stem cells treated with different concentrations of AA. (E) The content of H2O2 in Huh7 stem cells treated with different concentrations of AA in the presence of 100 μg/ml catalase (Cat: catalase, Sigma - Aldrich). (F) The protein levels of cleaved PARP and caspase 7 in Huh7 stem cells treated with different concentrations of AA in the presence of 100 μg/ml catalase. (G) Viabilities of Huh7 stem cells treated with different concentrations of AA in the presence of 100 μg/ml catalase. * p < 0.05.
liver CSC viability (Figure 7G), which was consistent with previous reports describing the dependence of AA’s cytotoxicity on the generation of H$_2$O$_2$ (Du et al., 2010; Chen et al., 2015; Chen et al., 2005). In conclusion, our data indicate that AA exerts its inhibitory effects on liver CSCs through the production of H$_2$O$_2$ and the promotion of cell apoptosis.

**DISCUSSION**

Various factors lead to an increased risk of liver cancer. Among these factors, it has been reported that alcoholic liver disease is the most common cause of HCC, accounting for approximately 30% of all HCC cases (Morgan et al., 2004). Liver cancer is one of the common causes of cancer-related death. Metastasis and recurrence are the main causes of primary liver cancer-associated mortality. Liver CSCs, possessing a higher migration ability and tumorigenicity, are closely related to metastasis and recurrence of liver cancer. Liver CSCs are considered an important target for liver cancer therapy. For example, WYC-209, a synthetic retinoid, inhibited the proliferation of malignant murine melanoma tumor-repopulating cells and abrogated 87.5% of lung metastases of melanoma tumor-repopulating cells (Chen et al., 2018).

It was reported that AA inhibited the growth of various types of cancer, including colorectal cancer cells, neuroblastoma cells, and ovarian cancer cells. However, its effect on liver cancer metastasis has not yet been reported. Consistent with a previous study (Lv et al., 2018), we found that AA inhibited the viability of liver cancer cells without significantly inhibiting the viability of L02 cells, which are normal human hepatocytes. Furthermore, AA significantly attenuated the viability of liver CSCs and reduced the colony formation ability and sphere formation ability of liver cancer cells in vitro, indicating the inhibition by AA on self-renewal and tumorigenicity of liver cancer cells. Because CSCs are involved in important functions in cancer metastasis and AA shows inhibitory effects on liver CSCs, we further examined the effects of AA on liver cancer metastasis. As expected, AA inhibited the metastasis of liver cancer cells to the lung and liver in a subcutaneous xenotransplantation model.

Stemness genes play vital roles in regulating cancer metastasis. In most cases, stemness genes promote cancer metastasis (Lv et al., 2017; Baccelli et al., 2013; Tang et al., 2012; Celià-Terrassa and Kang, 2016). Sox2, a transcription factor involved in the regulation of embryonic development, functions as a novel regulator of cell invasion, migration, and metastasis in several cancer types (Feng and Lu, 2017; Weina and Utikal, 2014). However, it was recently reported that REX1, an embryonic stem cell marker, inhibits liver cancer metastasis, indicating the complex functions of stemness genes in the process of cancer metastasis (Luk et al., 2019). AA regulates the expression of stemness genes, and in human embryonic stem cells, AA caused specific DNA demethylation of 1,847 genes (including the important stem cell genes) (Chung et al., 2010) and also inhibited retinoic acid-induced differentiation of embryonic stem cells (Wu et al., 2014). Furthermore, AA alleviated cell aging and increased the production of induced pluripotent stem cells in mice and human cells (Esteban et al., 2010; Wang et al., 2011).

In conclusion, AA inhibited the viability of liver CSCs and prevented liver cancer metastasis without reducing the expression of stemness genes in liver cancer cells. The inhibitory effects of AA on liver CSCs can result from the production of H$_2$O$_2$ and promotion of cell apoptosis. Our findings provide evidence that supports AA as an effective therapeutic agent for liver cancer metastasis and suggest that additional effects other than inhibition of stemness genes may be considered during later evaluation of the effects of AA on CSCs and cancer metastasis.
DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on the Ethics of Animal Experiments of the Naval Medical University.

REFERENCES

Baccelli, I., Schneweiss, A., Riethdorf, S., Stenzinger, A., Schillert, A., Vogel, V., et al. (2013). Identification of a Population of Blood Circulating Tumor Cells from Breast Cancer Patients that Initiates Metastasis in a Xenograft Assay. Nat. Biotechnol. 31, 539–544. doi:10.1038/nbt.2576

Cameron, E., and Campbell, A. (1974). The Orthomolecular Treatment of Cancer II. Clinical Trial of High-Dose Ascorbic Acid Supplements in Advanced Human Cancer. Chem. Biol. Interact. 9, 285–315. doi:10.1016/0009-2797(74)90019-2

Cameron, E., and Pauling, L. (1976). Supplemental Ascorbate in the Supportive Treatment of Cancer: Prolongation of Survival Times in Terminal Human Cancer. Proc. Natl. Acad. Sci. 73, 3685–3689. doi:10.1073/pnas.73.10.3685

Cameron, E., and Pauling, L. (1978). Supplemental Ascorbate in the Supportive Treatment of Cancer: Reevaluation of Prolongation of Survival Times in Terminal Human Cancer*. Proc. Natl. Acad. Sci. 75, 4538–4542. doi:10.1073/pnas.75.11.4538

Carosio, R., Zuccari, G., Orienti, I., Mangraviti, S., and Montaldo, P. G. (2007). Pharmacologic Doses of Ascorbate Act as a Prooxidant and Decrease Growth of Aggressive Tumor Xenografts in Mice. Proc. Natl. Acad. Sci. 105, 11105–11109. doi:10.1073/pnas.0804226105

Chen, Q., Espey, M. G., Sun, A. Y., Pooput, C., Kirk, K. L., Krishna, M. C., et al. (2008). Ascorbate Radical and Hydrogen Peroxide in Extracellular Fluid In Vivo. Proc. Natl. Acad. Sci. U. S. A. 104, 8749–8754. doi:10.1073/pnas.0702854104

Cheng, Z., Yuan, X., Qu, Y., Li, X., Wu, G., Li, C., et al. (2017). Bruceine D Inhibits Hepatocellular Carcinoma Growth by Targeting β-catenin/jagged1 Pathways. Cancer Lett. 403, 195–205. doi:10.1016/j.canlet.2017.06.014

Hoffer, L. J., Levine, M., Assouline, S., Melnychuk, D., Padayatty, S. J., Rosadiuk, K., et al. (2008). Phase I Clinical Trial of i.V. Ascorbic Acid in Advanced Malignancy. Ann. Oncol. 19, 1969–1974. doi:10.1093/annonc/mdn377

Lennicke, C., Rahn, J., Lichtenfels, R., Wessjohann, L. A., and Seliger, B. (2015). Hydrogen Peroxide - Production, Fate and Role in Redox Signaling of Tumor Cells. Cell Commun. Signal. 13, 1–19. doi:10.1186/s12935-015-0188-6

Liu, S., Ni, N., Xu, Y., Xiao, Y., Cheng, K., Hu, J., et al. (2013). Expression of Intercellular Adhesion Molecule 1 by Hepatocellular Carcinoma Stem Cells and Circulating Tumor Cells. Gastroenterology 144, 1031–1041. doi:10.1053/j.gastro.2013.01.046

AUTHOR CONTRIBUTIONS

The work was supported by National Natural Science Foundation of China (81903510), National Major Project of China (2019ZX09201004-003-010).

FUNDING

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JW, JZ, LF, YL, and HWZ designed and conducted experiments, and wrote the manuscript. XK helped study design and interpretation of data. CL, HJ organized, conceived, and supervised the study. All authors read and approved the manuscript.
