

The anti-ovarian carcinoma activity of L-amino Acid Oxidase from *Crotalus adamanteus* venom in vivo and in vitro

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Abstract

The anti-tumor potential of animal toxins has fully attracted the attention of researchers. Snake venoms is a complex mixture of different components and has revealed high toxicity on normal and tumoral tissues or cells. The snake venom L-Amino-acid oxidase (svLAAO) has grown up to be a critical research target in molecular biology sciences and medicine sciences since widespread presence and various biological roles, including antitumor application. We found that *Crotalus adamanteus* (*C. adamanteus*) venom LAAO significantly decreased the viability of ovarian cancer cells and caused morphological changes preceded cell death. Cell experiments confirmed that *C. adamanteus* venom LAAO caused alterations of intrinsic or extrinsic apoptosis pathway-related genes in ovarian cancer cells. Animal experiments and histological analysis also proved that *C. adamanteus* venom LAAO could effectively inhibit the damage of ovarian cancer to tissues. The major apoptosis induction of *C. adamanteus* venom LAAO on ovarian cancer cells can be blocked by catalase, suggesting that the cytotoxicity of *C. adamanteus* venom LAAO on ovarian cancer cells was mainly mediated by H₂O₂. Our preliminary results revealed that *C. adamanteus* venom LAAO may induce apoptosis of ovarian cancer cells through the death receptor pathway and mitochondrial pathway. It is inferred that *C. adamanteus* venom LAAO will be some advantages in New Drug Research and Development of antitumor drugs in the future. Nevertheless, extra studies on the pharmacological actions and molecular mechanism of svLAAO in anti-cancer are necessary in order to better promote its application.

Introduction

Ovarian cancer is one of the most lethal gynecological diseases worldwide, which is characterized by high heterogeneity and rapid progression [1]. Most ovarian cancer patients are usually diagnosed too late to cure because of the lack of specific early symptoms and effective early detection strategies, which prevents early discovery and treatment [2]. Epithelial ovarian cancer (EOC) is the most common type, accounting for 90% of all cases [3], which is a devastating disease and the tumor cells have disseminated beyond the ovaries and pelvic organs when diagnosed. Although many ovarian cancer patients have experienced successful initial therapy, the overall survival rate has only modestly improved of patients with EOC, which remains a deadly disease due to extensive peritoneal dissemination, chemotherapy resistance, immune escape and immune counterattack of tumor cells [4]. The traditional therapeutic methods and currently available medications often have difficulty in curbing the recurrence and metastasis of ovarian cancer. Thus, new and highly effective natural bioactive therapeutic agents targeting carcinogenesis, survival, growth, apoptosis and transformation of ovarian cancer cells may be useful strategies to control the cancer progression and reduce the side effects of radiochemotherapy or the chance of complications.

LAAO is widely distributed in snake venom, and svLAAO can catalyze stereospecific oxidative deamination of L-amino acid to α -keto acid along with the generation of hydrogen peroxide (H₂O₂) [5, 6]. svLAAO has attracted considerable attention due to its numerous clear biological or pharmacological functions, such as hemorrhage, antiparasitic, cytotoxicity and so on [7, 8]. Increasing studies have shown

that svLAAO can inhibit tumor cell proliferation and induce apoptosis of tumor cells [9, 10]. Many authors support the anticancer activity of svLAAO is attributed to its cytotoxicity of H₂O₂[9, 10]. Nonetheless, the exact physiological role and mechanism of svLAAO against tumor are poorly understood. In order to further realize the anti-tumor activity of svLAAO, we consider the use of LAAO from *C. adamanteus* venom as therapeutic agents to investigate its apoptosis-inducing effect on ovarian cancer cells.

Cell damage manifests itself in diverse forms, but the major fate of severely damaged cells is apoptosis or necrosis. Apoptosis is a physiological process and the main character of growth and development of multicellular organic body that plays an important role in diverse biological processes. Substantial evidence indicates that apoptosis is controlled by a diversity of extracellular and intracellular signals, and dysregulation is associated with pathogenesis in human tumors [11, 12]. And the apoptosis of tumor cells usually regulated by the major pathways: the death receptor pathway and mitochondria-mediated pathway [13, 14]. The activated tumor cell membrane bound death receptors or mitochondrial perturbation may lead to the activation of downstream caspases, which may disrupt cytoskeleton, shut down DNA replication and repair, degrade chromosomal DNA, and stepwise disintegrate cell into apoptotic bodies [15, 16]. The key regulators of apoptosis signal pathway include members of Fas/FasL and Bcl-2 protein family. The Fas/FasL signaling pathway plays an important role in tumor inhibition [17]. The cellular Bcl-2 protein family consists of both pro- and anti-apoptotic protein members, which play an important role in regulation of cell suicide by regulating the efflux of apoptogenic proteins from mitochondria [14, 18]. In this paper, we will discuss the anticancer potential of *C. adamanteus* venom LAAO in ovarian cancer.

Materials And Methods

Details on reagents, cell culture, cell viability, cell morphological alteration, cell apoptosis and statistical analysis are provided in supplementary information.

Experimental protocols of molecular experiment

Two ovarian cancer cells were seeded (5.0×10^5 cells per dish) in 6-well microplates and cultured to 70-80% cell fusion. Next the tumor cells were treated with 5 μ g/mL svLAAO and different concentrations of catalase enzyme at the logarithmic growth phase. The sampling scheme of svLAAO and catalase is the same as “AO/PI double fluorescence-staining test” (**See supplementary information**). And the control group was treated with 10 μ L PBS per dish under the identical experimental conditions. At last, the stimulated tumor cells were collected at 24 h for further experimental detection of gene expression and protein expression. This protocol was carried out three times independently.

RNA isolation and qRT-PCR

Total RNA was extracted from CAOV-3 and OVCAR-3 cells with TaKaRa kit (Takara Bio Inc., Japan) depending on the instructions of the manufacturer. 1 μ g of total RNA from each group was used to form cDNA using the HiScript®II Q RT SuperMix for qPCR(+gDNA wiper) (Vazyme, China).The Cham^QTM

Universal SYBR® qPCR Master Mix (Vazyme, China) and Bio-Rad CFX Manager Detection system (Bio-Rad, USA) were used for detecting real-time PCR products from the reverse-transcribed RNA samples. The primers for 11 candidate genes and GAPDH as control were shown in Table 1. The primer sequences of GAPDH and 11 candidate genes all were from <http://pga.mgh.harvard.edu/primerbank/>; The concentrations of all primers were 400 nmol/L. The PCR cycling conditions were performed as follows: 30 sec at 95°C for pre-denaturation; 40 cycle reactions (10 sec at 95°C for Champagne™ Taq DNA Polymerase activation, 30 sec at 60°C for annealing/extension); and the melting steps (65°C to 95°C, increment 0.5°C). The comparative CT method was used to determine the relative quantitation of gene expression for each gene compared with the housekeeping gene GAPDH.

Measurement of changes in apoptosis related protein expression

The treated cells were collected and washed with ice-cold PBS, then were lysed in RIPA buffer (Sigma, USA) plus protease inhibitors (Dingguo Changsheng Biotech Co., Ltd, China) for 30 min. Next, the cell lysates were centrifuged at 12,000g at 4 °C for 5 min and supernatant were denatured with SDS-PAGE Sample Loading Buffer (Beyotime Biotechnology, China) in boiling water for 5 minutes. 40µg amounts of total protein was separated by 10% or 12% SDS-PAGE, electro-transferred to PVDF membrane and then immunoblotted with antibodies of apoptosis related proteins. Fas, Caspase 7, Caspase 8, Fadd, Bid and Cytochrome C (Cyto C) antibodies were taken from Proteintech Group™ (Wuhan, China). Caspase 3 antibody was from OriGene (Rockville, USA) and Caspase 9 antibody was purchased from Ruiying Biological (Suzhou, China). Protein expression levels were standardized with a polyclonal antibody against GAPDH. GAPDH antibody was from Hua Bio (Hangzhou, China) and used for loading control. All blots were scanned and quantified using an electrochemiluminescence (ECL) western blotting detection system with Image Quant software (Bio-Rad Image Lab system, USA).

Construction of animal model for Ovarian cancer's disease

Swiss-Kunming female mice (16 ± 2 g) were purchased from the Laboratory Animal Centre of Army Medical University (Chongqing, China). One-week environmental acclimatization were performed for all experimental animals under standard conditions (12-h light/dark cycle at 22 ± 1°C, relative humidity 40-70%). Experimental groups (3 female mice per group): (1) control group: normal feeding without any treatment; (2) experimental group: 1) subcutaneous (s.c.) injection group: Each female mouse was injected with 3.0×10^5 CAOV3/OVCAR3 cell suspensions under the left foreleg armpit; 2) intraperitoneal (i.p.) injection group: Each female mouse was injected intraperitoneally with 3.0×10^5 CAOV3/OVCAR3 cell suspensions. All female mice were fed for 4 weeks under standard conditions, and then abdominal dissection of each group was performed to observe tumor development in female mice. All animal procedures were conducted in strict accordance with Methods for the Management of Experimental Animals in Chongqing (No. 195).

Histological Analysis

After the successful construction of ovarian cancer mouse model, mice of the experimental group were injected svLAAO every 24 h for 4 times, the dosage was 0.5, 1.0, 1.5 µg/g body weight. The negative control group was received injection of identical volume of normal saline at the same injection site as described above. Next, we dissected the female mice, observed the visceral tissue and took out the gastric tissue. The gastric tissues were immediately fixed in 4% paraformaldehyde, and dehydrated with ethanol of gradient concentration, then cleared in xylene, embedded in paraffin. The paraffin-embedded tissue blocks were sectioned into thin, 2.5-µm slices and fixed on standard glass microscope slides which used for histological study. Finally, the sections were stained with 0.25% (w/v) haematoxylin and eosin (H&E) and photographed under an optical microscope.

Results

Cytotoxic effect assessment of *C. adamanteus* venom LAAO on ovarian cancer cells

The morphological variation of CAOV3/OVCAR3 was observed by inversion digital imaging microscope after exposure to different concentrations of svLAAO for incubation periods of 6, 12, 24 and 36 h. Our studies showed that the cytotoxicity of svLAAO on the two cells was elevated in a dose dependent manner. Especially, at 24 h time point, the morphology of two cells changed obviously in 3, 5 and 7 µg/mL svLAAO treatment groups, and a large number of cells appeared apoptosis or death (Fig. S1). The of CCK-8 analysis indicated that svLAAO significantly reduced the survival rate of cells (Fig. 1 A). The cell viability of CAOV3/OVCAR3 following treatment with 5 µg/mL svLAAO was also measured with the AO/PI staining assay. The results of AO/PI staining demonstrated that, as shown in Fig. 1 B, the cell DNA in control group was uniform yellow, and the morphological structure was normal; In 5 µg/mL svLAAO group, the cell membrane blebbing, the chromatin condensation, the nucleus was cleaved into small spots, showing dense staining of different sizes, and the living cells decreased significantly. In addition, we treated the two kinds of cells with 5 µg/mL svLAAO adding different concentrations of catalase, a H₂O₂ scavenger, the percentage of living cells increased greatly, but a large number of cells still exhibited abnormal morphology (Fig. 1 B).

The activation of the Fas/FasL and Mitochondrial signaling pathway in CAOV3/OVCAR3 cells treated with *C. adamanteus* venom LAAO

To investigate the molecular mechanism of apoptosis induced by svLAAO in ovarian cancer cells, we examined the mRNA and protein expression changes of Fas/FasL and Mitochondrial signaling pathway-related genes in CAOV3/OVCAR3 cells after incubation with 5 µg/mL *C. adamanteus* venom LAAO for 24 h.

(1) The mRNA expression changes of apoptosis related genes

As illustrated in Fig. 2 & 3, the mRNA levels of apoptotic genes were altered in both cells after incubation with 5 µg/mL svLAAO for 24h. In CAOV3 cells, the mRNA levels of Fas (Fig. 2a), Bid (Fig. 2e), Caspase 3 (Fig. 2k) were increased obviously than that in control group; Meanwhile, the mRNA levels of Caspase 8

(Fig. 2c), Caspase 7 (Fig. 2d), Bcl-2 (Fig. 2f), Bcl-xL (Fig. 2g), Caspase 9 (Fig. 2i) and Apaf-1 (Fig. 2j) in 5 µg/mL svLAAO-stimulated group were decreased substantially; However, there was no significant difference in mRNA expression of Fadd (Fig. 2b) and Cyto C (Fig. 2h) compared with the control group. In OVCAR3 cells, the mRNA expressions of Fas (Fig. 3a), Fadd (Fig. 3b), Caspase 8 (Fig. 3c), Bid (Fig. 3e), Cyto C (Fig. 3h), Apaf-1 (Fig. 3j) and Caspase 3 (Fig. 3i) were up-regulated significantly compared to control; Bcl-2 (Fig. 3f), Bcl-xL (Fig. 3g) and Caspase 9 (Fig. 3i) mRNA levels were down-regulated markedly as control; But Caspase 7 (Fig. 3d) mRNA level was no obvious difference from that of control group. These results suggested that the apoptosis-inducing effect of svLAAO on ovarian cancer cells may be related to the type of cells.

(2) The protein expression alterations of apoptosis related genes

The western blot results showed that the protein levels of Fas (Fig. 4A, Fig. S2a; Fig. 4B, Fig. S3a) and Cyto C (Fig. 4A, Fig. S2f; Fig. 4B, Fig. S3f) were raised distinctively both in CAOV3 and OVCAR3 cells as control. The protein levels of Caspase 8 (Fig. 4A, Fig. S2c; Fig. 4B, Fig. S3c) and Caspase 9 (Fig. 4A, Fig. S2g; Fig. 4B, Fig. S3g) were significantly decreased than that in control group. The Caspase 7 (Fig. 4A, Fig. S2d) and Caspase 3 (Fig. 4A, Fig. S3h) protein levels were up-regulated markedly in CAOV3 cells, and down-regulated significantly in OVCAR3 cells (Fig. 4B, Fig. S4d&h) respectively. In CAOV3 cells, lower protein expressions of Fadd (Fig. 4A, Fig. S2b) and Bid (Fig. 4A, Fig. S2e) were detected than their matched controls. However, in OVCAR3 cells, the Fadd protein level (Fig. 4B, Fig. S3b) was not significantly different from the control level, and the Bid protein expression (Fig. 4B, Fig. S3e) was distinctively increased comparing to its control group. These results suggested that, at the transcription level and protein level, there are certain differences in some gene expression changes caused by svLAAO at 24 h time point. Furthermore, our results also hinted that there may be a few differences in apoptosis induction triggered by svLAAO among different ovarian cancer cells.

H₂O₂ produced by svLAAO participated in the regulation of apoptosis

It is important to clarify the toxicity mechanism of svLAAO against ovarian cancer cells, which helps to improve its clinical application. Some researchers believed that most of the svLAAO activities are mediated by H₂O₂, which can induce oxidative stress in cancer cells and result in disruption of normal cell physiology [11, 19]. We incubated CAOV3/OVCAR3 cells with 5 µg/mL svLAAO and different concentrations of catalase for 24 h. As indicated in Figures (Fig. 2 & 3, Fig. 4 A & B, Fig. S2 & S3), to a certain extent, the mRNA and protein expressions of apoptosis-induced genes triggered by 5 µg/mL svLAAO at 24 h time point were regulated by catalase.

Anti-tumor effect of svLAAO in vivo

(1) Tumorigenicity in vivo

The immunocompetent female mice were used to evaluate the infection of tumor cells. The CAOV3/OVCAR3 cell lines were injected either s.c. injection and/or i.p. injection (3.0×10⁵ cell suspensions

containing no additives). Mice injected with ovarian cancer cells were fed under standard conditions for one month, and then, all mice were killed. The peritoneal and thoracic cavities were opened from the midline. There were no visible tumors in the peritoneal or thoracic cavities after s.c. injection and i.p. injection by gross inspection. However, vesicular bulges appeared on the surface of small intestine after injection of ovarian cancer cells in both ways (Fig. S4). And a small amount of ascitic fluid was observed in i.p. injection group as well. Subsequently, we treated the established immunocompetent mice model bearing human with normal saline and different concentrations of LAAO (0.5, 1.0, 1.5 $\mu\text{g/g}$ body weight), respectively. Here, we present only part of the mouse anatomical materials of the svLAAO-treated group (Fig. 5). The results indicated that svLAAO can effectively inhibit the invasion of tumor cells to the small intestine of infected mice (Fig. 5c, e, g, i).

(2) Histological analysis of tumors

Histologically the tumor tissues exhibited high heterogeneity in spatial arrangement compared to normal tissues. The tumor cells were more disorganized and lost their normal arrangement structure, hierarchy or polar orientation. We performed histological examination on the gastric tissues of CAOV3/OVCAR3 cell-infected mice (Fig. 6 & Fig. S5). Our results demonstrated the cells of normal mouse gastric tissue were closely arranged, well-organized, orderly in structure, regular in shape, and the nucleus is clearly visible (Fig. 6a). However, the structure of the gastric tissue was greatly damaged by either subcutaneous or intraperitoneal injection of CAOV3 cells compared to control. The cell arrangement was sparse, the level was unclear, the structure was confused, the cell shape was irregular, and the cell structure was mostly unclear and incomplete in the CAOV3-infected group (negative control group) (Fig. 6b & f). To some extent, the structure of gastric tissue in infected mice was improved after subcutaneous injection of different concentrations of svLAAO (Fig. 6c, d & e); And the structure of the gastric tissues of infected mice was significantly improved by intraperitoneal injection of corresponding concentrations of svLAAO (Fig. 6g, h & i). The change of gastric tissues in infected mice was similar to that seen in CAOV3 cell treatment group after intraperitoneal injection of OVCAR3 cells (Fig. S5).

Discussion

SvLAAO, as a protein toxin, has gained widespread interests in the field of biology and medicine for its apoptosis-inducing effect on tumor cells and non-tumor cells [5, 10]. The *C. adamanteus* venom LAAO is easy to obtain and purify, which has been used in biochemical studies, such as enzymatic, kinetic and mechanistic investigations [20]. Our studies indicated that *C. adamanteus* venom LAAO impaired viability of CAOV3 and OVCAR3 cells in a dose-dependent manner at 24 h time point (Fig. S1, Fig. 1A), and induced chromatin condensation and segregation (Fig. 1B), which were consistent with previous reports of svLAAO-induced apoptosis of tumor cells [9, 19].

Earlier studies showed that alterations in gene and protein expression level of Fas family members or Bcl-2 family members might play a critical role in epithelial ovarian cancer [21]. The Fas/FasL system is recognized as a primary mechanism for the induction of apoptosis in cells and tissues, which may be

involved in the occurrence, development and chemoresistance of ovarian cancer [22, 23]. Fas can initiate the extrinsic apoptotic pathway to induce the cell death signal cascade by autocrine-paracrine, and eventually leads to cell apoptosis [24]. The existing experimental results proved that the low-level expression of Fas may be more favourable to the survival of tumor cells and escape from human immune monitoring [25, 26]. Our results indicated that svLAAO may lead to a higher rate of programmed cell death due to the activation of the Fas/FasL apoptotic pathway by upregulating Fas mRNA and protein levels (Fig. 2a, Fig. 3a, Fig. 4A&B, Fig. S2&3). Once Fas is activated, which could trigger off intracellular chain reaction, leading to continuous activation of genes related to Fas signaling pathway [27, 28]. Fas may serve as a direct link between external ligand and the basal effector machinery of apoptosis through recruitment of the death-inducing signaling complex [11]. On the one hand, the activated executioner caspases, such as caspase-3 and -7, split specific substrates to execute the apoptotic dissolution of the cell [13]. On the other hand, in the mitochondrial pathway, the anti-apoptotic Bcl-2 family members (such as Bcl-2 and Bcl-xL) and pro-apoptotic Bcl-2 family members (such as Bax and Bid) resident in the cytoplasm translocated to mitochondria when intracellular death-inducing signaling triggered [29].

Bcl-2 and Bcl-xL belong to anti-apoptotic homologues, which stabilize in the outer membrane of mitochondria and inhibit the release of Cytochrome C (Cyto C) from mitochondria, play an important role in tumor occurrence and progress by regulating the permeabilization of the outer mitochondrial membrane and prolonging the survival of malignant cells [14]. There had been several studies shown that the Bcl-2 expression was correlated with a survival advantage in ovarian cancer [21]. Our experiments using Quantitative Real-time PCR analysis indicated that *C. adamanteus* venom LAAO may promote apoptosis of ovarian cancer cells by decreasing Bcl-2 and Bcl-xL mRNA expression and increasing Cyto C mRNA level (Fig. 2f &g, Fig. 3 f, g &h). Bid is a member of Bcl-2 family and recognized as one of the most effective inducer of mitochondrial priming in ovarian cancer, which can induce the release of apoptogenic factors during cell death [30, 31]. Activated caspase-8 (an initiator of apoptosis, containing death effector domain) cleaves cytoplasmic Bid to truncated Bid (tBid, a potent inducer of cell apoptosis), which represents the connection between the extrinsic and intrinsic apoptosis pathways, is translocated to the mitochondrial membrane [32]. tBid targets with BCL-2 proteins and has been proposed as a promising molecular target for killing cancer cells, triggers the release of Cyto C from mitochondria to the cytosol [33, 34]. The Cyto C in cytosol triggered the assembly of the Apaf-1/caspase-9(an executor of apoptosis, containing caspase recruitment domain) holoenzyme that forming an "apoptosome", and in turn activated downstream caspase-3/-7, and eventually led to the permeability transition at the inner membrane in response to activation of cell surface death receptors [35, 36]. In our study, we found that *C. adamanteus* venom LAAO activated Fas/FasL and Mitochondrial pathways in vitro. However, the expression trends of some genes were not consistent between mRNA and protein levels (Fig. 2,3&4, Fig. S 2&3), which may be related to the complex transcriptional processes and post-translational modifications of genes.

We established immunocompetent mice model bearing human ovarian cancer to confirm the anti-ovarian carcinoma of *C. adamanteus* venom LAAO in vivo. Although most models of ovarian cancer use mice

with non-functioning immune systems, normal immune mouse model can be used for exploring the role of immune system in the occurrence and progression of disease [37]. The results of gross inspection revealed that ovarian cancer cells have the ability to survive and grow in mice with intact immune systems (Fig. S4). And intestinal symptoms in ovarian cancer-infected mice were eliminated by s.c. and i.p. injection of *C. adamanteus* venom LAAO for 24h (Fig. 5). Histological results were also showed that *C. adamanteus* venom LAAO significantly ameliorated the structural damage to infected-mouse gastric tissues (Fig. 6, Fig. S5). These results indicated that *C. adamanteus* venom LAAO has anti-tumor potential in vivo and in vitro.

Numerous studies have shown that the damage caused by svLAAO to normal cells is usually negligible compared with that of tumor cells [5, 38, 39]. This implies that svLAAO has a good prospect in anti-tumor research and application. Accumulated researches disclosed that the relevant action mechanisms of biological functions of svLAAO likely depends on the oxidative stress arising from the production of H_2O_2 [40]. There's a popular supposition that H_2O_2 plays a critical role in svLAAO-induced cell apoptosis by generating membrane oxidation stress that can lead to disruption of normal cell physiology, of which activity can be inhibited by adding catalase or other H_2O_2 scavengers [41]. Our results, as shown in Fig. 1B, indicated that the number of cell survival increased greatly and the cell morphology also restored to a certain extent when the cells were co-treated with catalase and svLAAO for 24 h, which is consistent with previous studies [7]. In addition, our work indicated that, as shown in Fig. 2a, Fig. 3a, Fig. 4 A&B, Fig. S 2&3, the change of a majority of mRNA and protein levels triggered by 5 $\mu\text{g}/\text{mL}$ svLAAO can be inhibited by catalase. These findings support Mates and Sanchez-Jimenez's view, that is, a plethora of biological effects by H_2O_2 can trigger the alterations in both signal transduction and gene expression, as well as cell death [42]. However, there exists a very considerable difference between svLAAO and exogenous H_2O_2 for cell apoptosis mechanism. The differences are mainly embodied in several aspects: the morphological changes of cells induced by svLAAO and exogenous H_2O_2 were different, and the apoptosis induced by svLAAO can be inhibited or abolished with antioxidant or catalase, but not inhibited the apoptosis of exogenous H_2O_2 [9, 43]. Our observation also found that catalase couldn't completely inhibit the variation in gene expression caused by svLAAO, which further confirmed that there may be other ways for svLAAO to induce apoptosis of tumor cells. Perhaps there are other components in svLAAO or intermediate metabolites that regulate tumor cell apoptosis, which need to be further investigated in depth. Of course, in addition to the well-known cytotoxicity of svLAAO, it is also involved in the regulation of tumor cell cycle processes, which is an important link in regulating tumor cell apoptosis [44, 45]. However, the research in this area is still scarce and more efforts are needed to support the regulation of svLAAO on tumor cell apoptosis through cell cycle pathway.

In conclusion, our studies indicate that *C. adamanteus* venom LAAO exerts anti-tumor effect via regulation of the death receptor and mitochondrial signaling pathway, which is a new material for the study of anti-ovarian cancer mechanism and development of drug. But tumor cell apoptosis or death is a complex process involving the balance of interactions between pro- and anti-apoptotic regulatory

networks, and more research is needed to elucidate the action mechanisms of svLAAO in tumor cell death process.

Declarations

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Conflict of interest We declare that we have no commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Consent for publication All authors agree with publication.

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Table

Table 1 Primers sequences used for quantitative real-time PCR

Gene symbol	Forward/Reverse primers	Primer sequences 5'-3'
GAPDH	F	CTGGGCTACACTGAGCACC
	R	AAGTGGTCGTTGAGGGCAATG
Fas	F	TCTGGTTCTTACGTCTGTTGC
	R	CTGTGCAGTCCCTAGCTTTCC
Fadd	F	GTGGCTGACCTGGTACAAGAG
	R	GGTAGATGCGTCTGAGTTCCAT
Caspase8	F	CGGACTCTCCAAGAGAACAGG
	R	TCAAAGGTCGTGGTCAAAGCC
Caspase7	F	AGTGACAGGTATGGGCGTTC
	R	CGGCATTTGTATGGTCCTCTT
Bid	F	ATGGACCGTAGCATCCCTCC
	R	GTAGGTGCGTAGGTTCTGGT
BcL-2	F	GGTGGGGTCATGTGTGTGG
	R	CGGTTCAAGTACTCAGTCATCC
BcL-xL	F	GAGCTGGTGGTTGACTTTCTC
	R	TCCATCTCCGATTCAGTCCCT
Cyto C	F	CTTTGGGCGGAAGACAGGTC
	R	TTATTGGCGGCTGTGTAAGAG
Caspase9	F	CTGTCTACGGCACAGATGGAT
	R	GGGACTCGTCTTCAGGGGAA
Apaf-1	F	AAGGTGGAGTACCACAGAGG
	R	TCCATGTATGGTGACCCATCC
Caspase3	F	AGAGGGGATCGTTGTAGAAGTC
	R	ACAGTCCAGTTCTGTACCACG

Figures

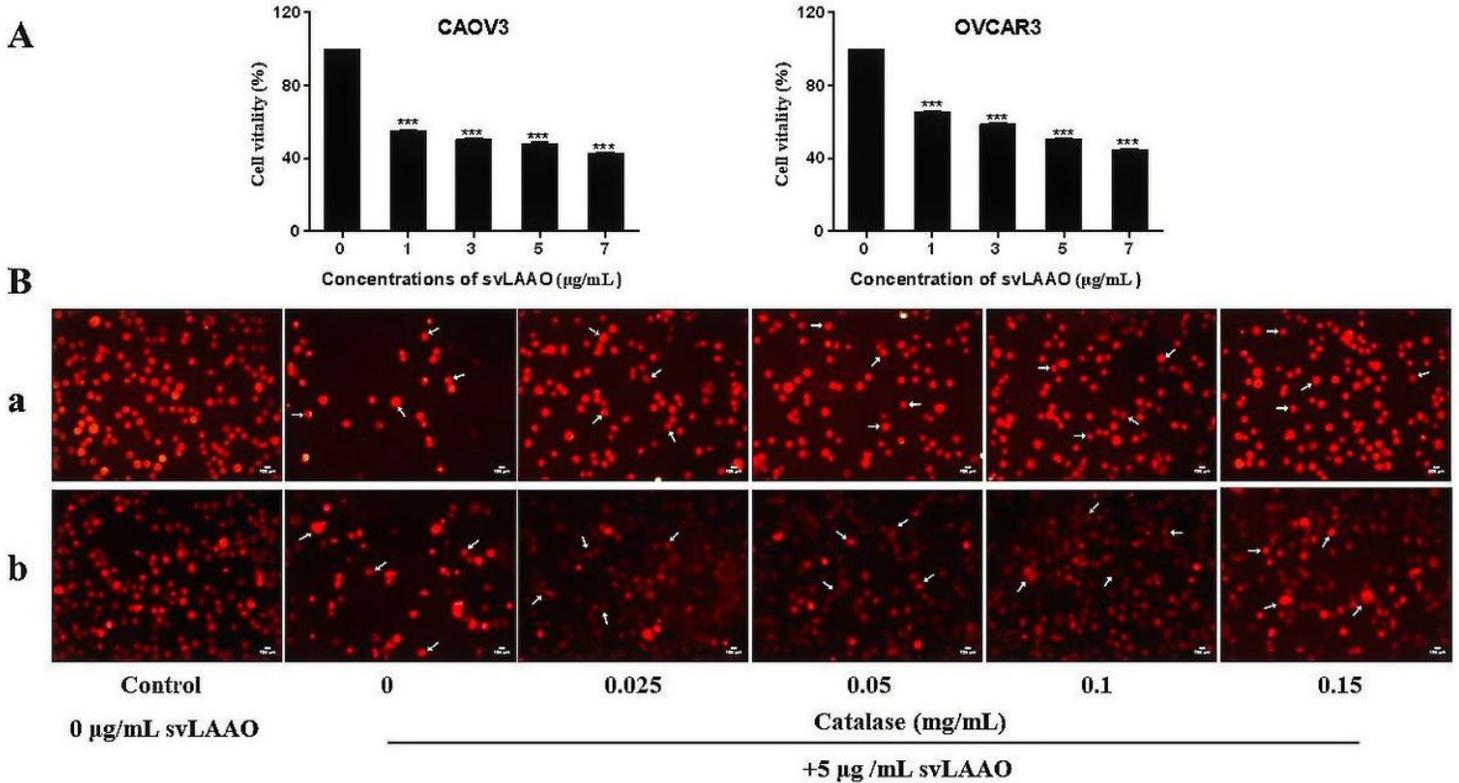


Figure 1

The survival and morphological changes of ovarian cancer cells. A svLAAO cytotoxicity against ovarian cancer cells by CCK-8 assay. The CAOV3 and OVCAR3 cells were incubated with different concentrations of svLAAO for 24 h. Data are representative results of three independent experiments as Mean \pm SD (***) $p < 0.001$). B The structural and morphological alterations of ovarian cancer cells. The CAOV3 (a) and OVCAR3 (b) cells were treated with svLAAO and different concentrations of catalase assessed at 24h time point by AO/PI staining. White arrows indicate apoptotic cells or necrotic cells. Scale bar=100 μ m.

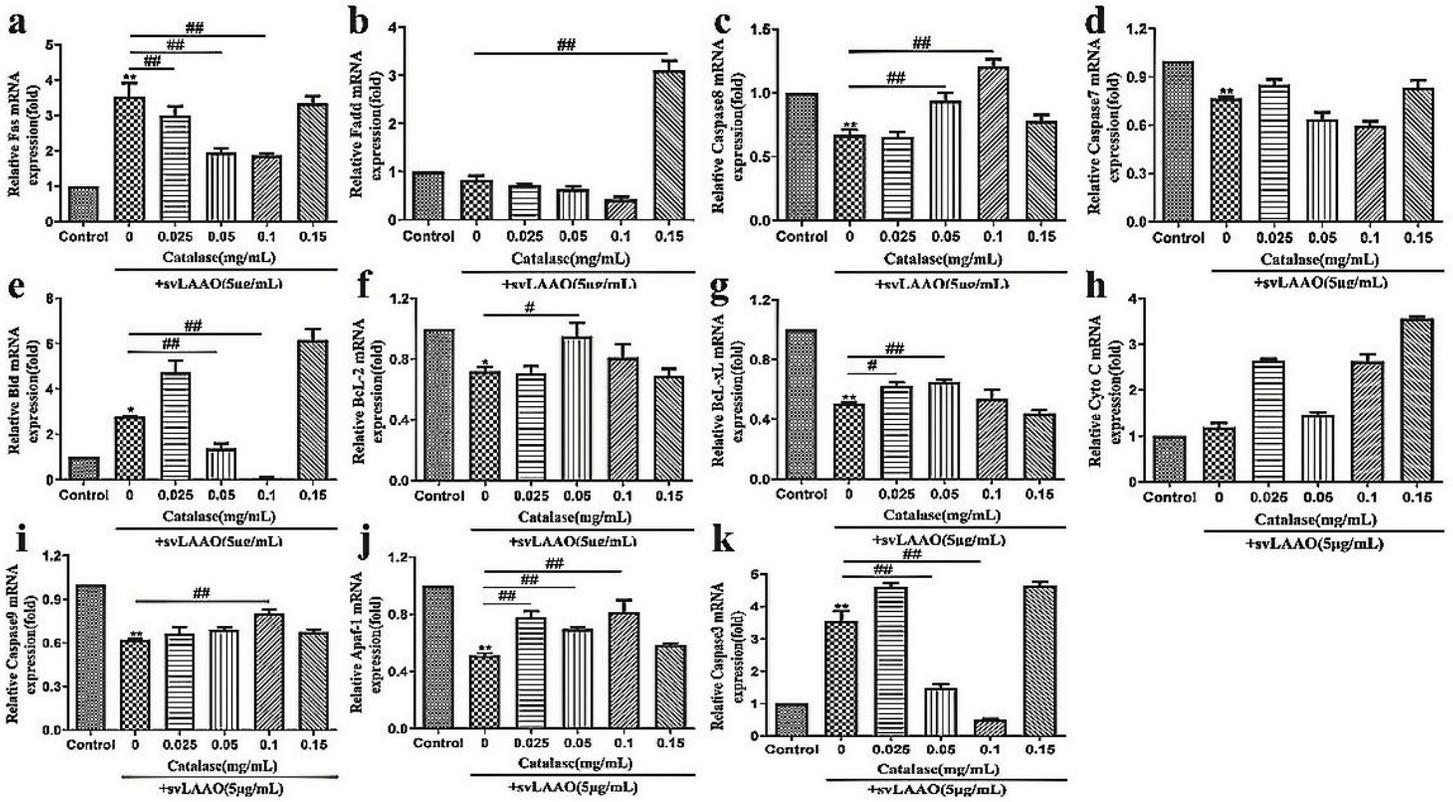


Figure 2

The mRNA expression levels of Fas/FasL and Mitochondrial pathway-related genes were altered in CAOV3 cells. The cells were exposed to 5 µg/mL svLAAO and different concentrations of catalase for 24 h. GAPDH expression served as control. * and ** Statistically significant difference as compared to control group (0 µg/mL svLAAO), n=3, *p < 0.05, **p < 0.01; # and ## Statistically significant difference as compared to 5 µg/mL svLAAO+0 mg/mL catalase group, n=3, #p < 0.05, ##p < 0.01.

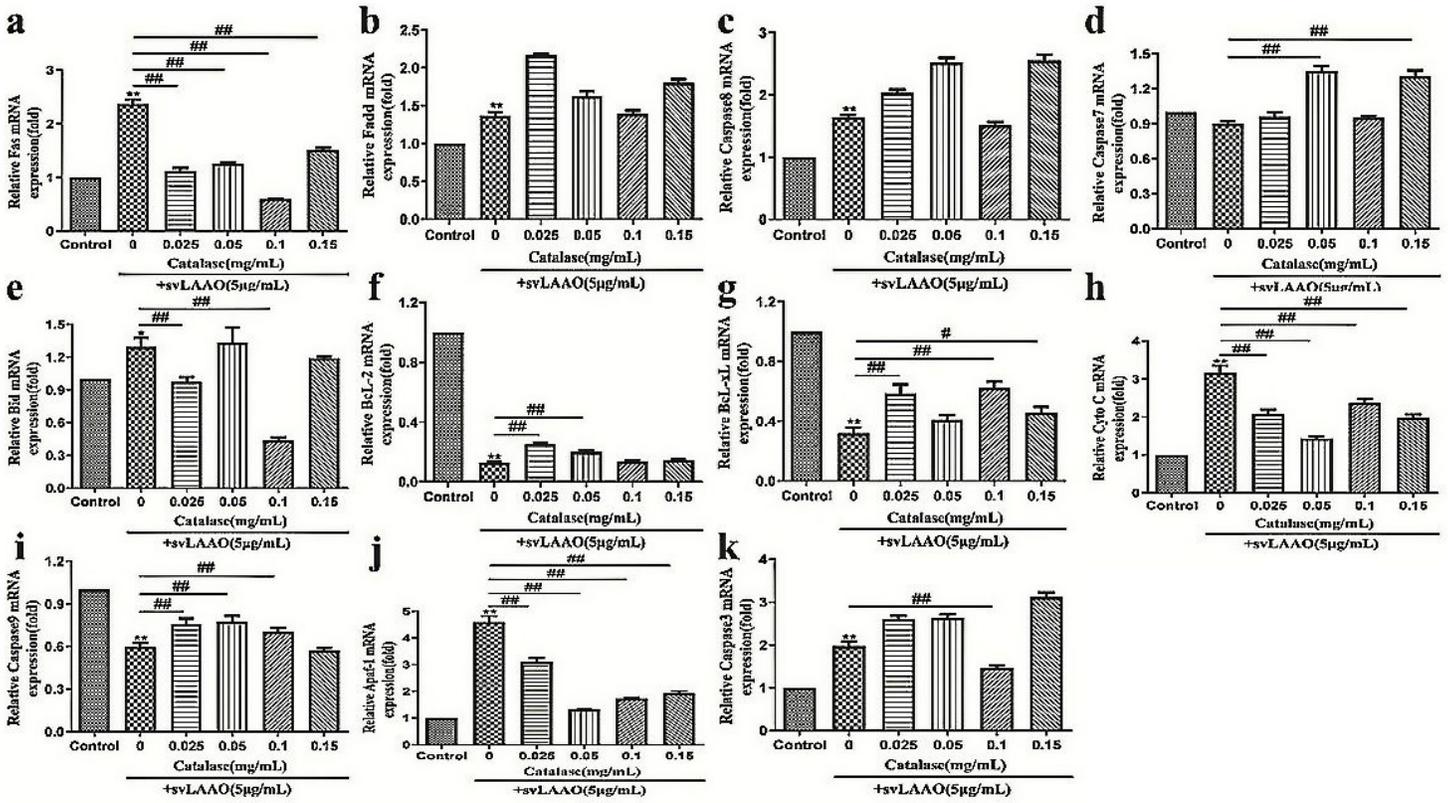


Figure 3

The mRNA expression levels of Fas/FasL and Mitochondrial pathway-related genes were altered in OVCAR3 cells. The cell processing and statistical processing methods are the same as Figure 2.

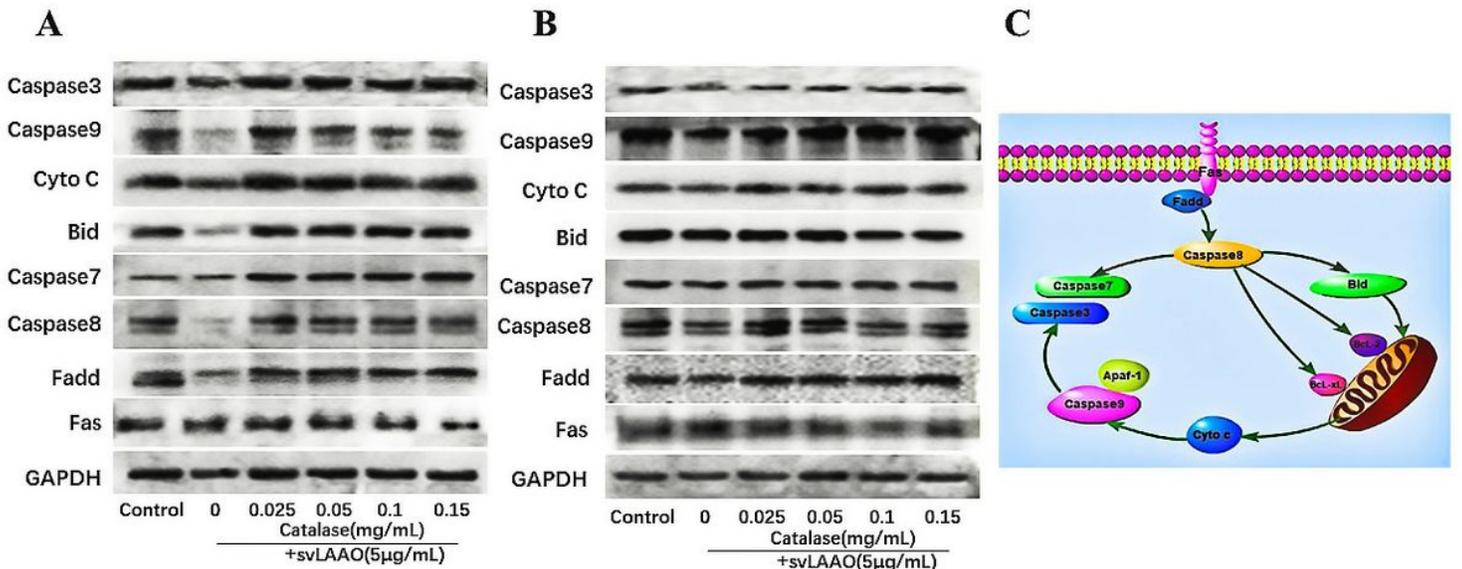


Figure 4

The protein expressions of Fas/FasL and Mitochondrial pathway-related genes in the two cells and the apoptotic cascade signaling in ovarian cancer cells. A The protein expressions of Fas/FasL and Mitochondrial pathway-related genes in CAOV3 cells. B The protein expressions of Fas/FasL and

Mitochondrial pathway-related genes in OVCAR3 cells. The two cells were exposed to 5 $\mu\text{g}/\text{mL}$ svLAAO and different concentrations of catalase for 24 h, and the cell lysates were examined by western blot. GAPDH expression served as control. The results for western blot are one of the three independent experiments. C The schematic diagram of apoptotic cascade signaling in ovarian cancer cells induced by *C. adamanteus* venom LAAO.

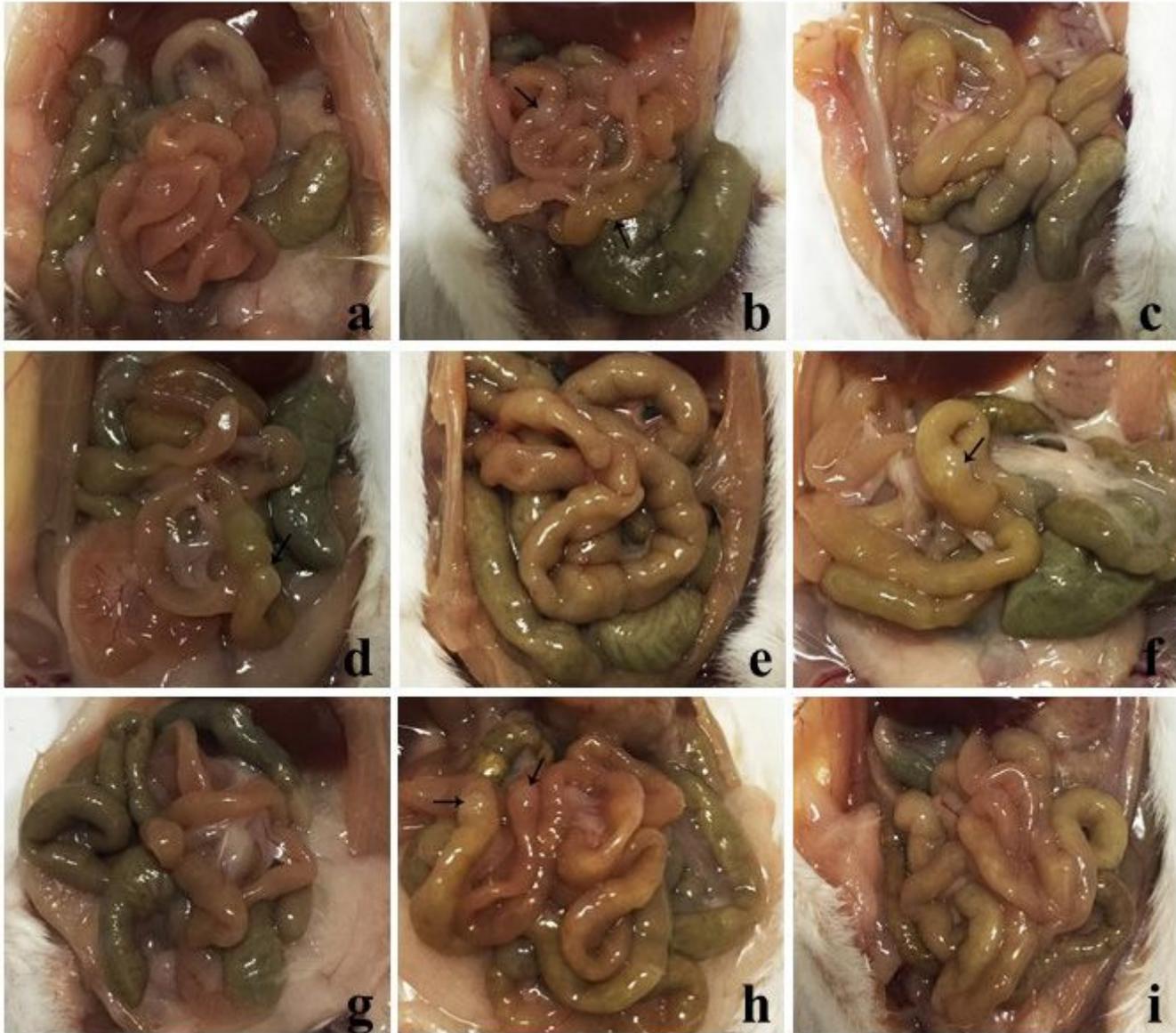


Figure 5

C. adamanteus venom LAAO can effectively inhibit the invasion of tumor cells to the small intestine of infected mice. a blank control. b, d Normal saline was injected by s.c. and i.p. into CAOV3-infected mice, respectively. c, e svLAAO of 1.5 $\mu\text{g}/\text{g}$ body weight was injected by s.c. and i.p. into CAOV3-infected mice, respectively. f, h Normal saline was injected by s.c. and i.p. into OVCAR3-infected mice, respectively. g, i svLAAO of 1.5 $\mu\text{g}/\text{g}$ body weight was injected by s.c. and i.p. into OVCAR3-infected mice, respectively. These samples were collected after s.c. or i.p. injection of svLAAO every 24 h for 4 times. Black arrows indicate vesicular bulges on the surface of small intestine.

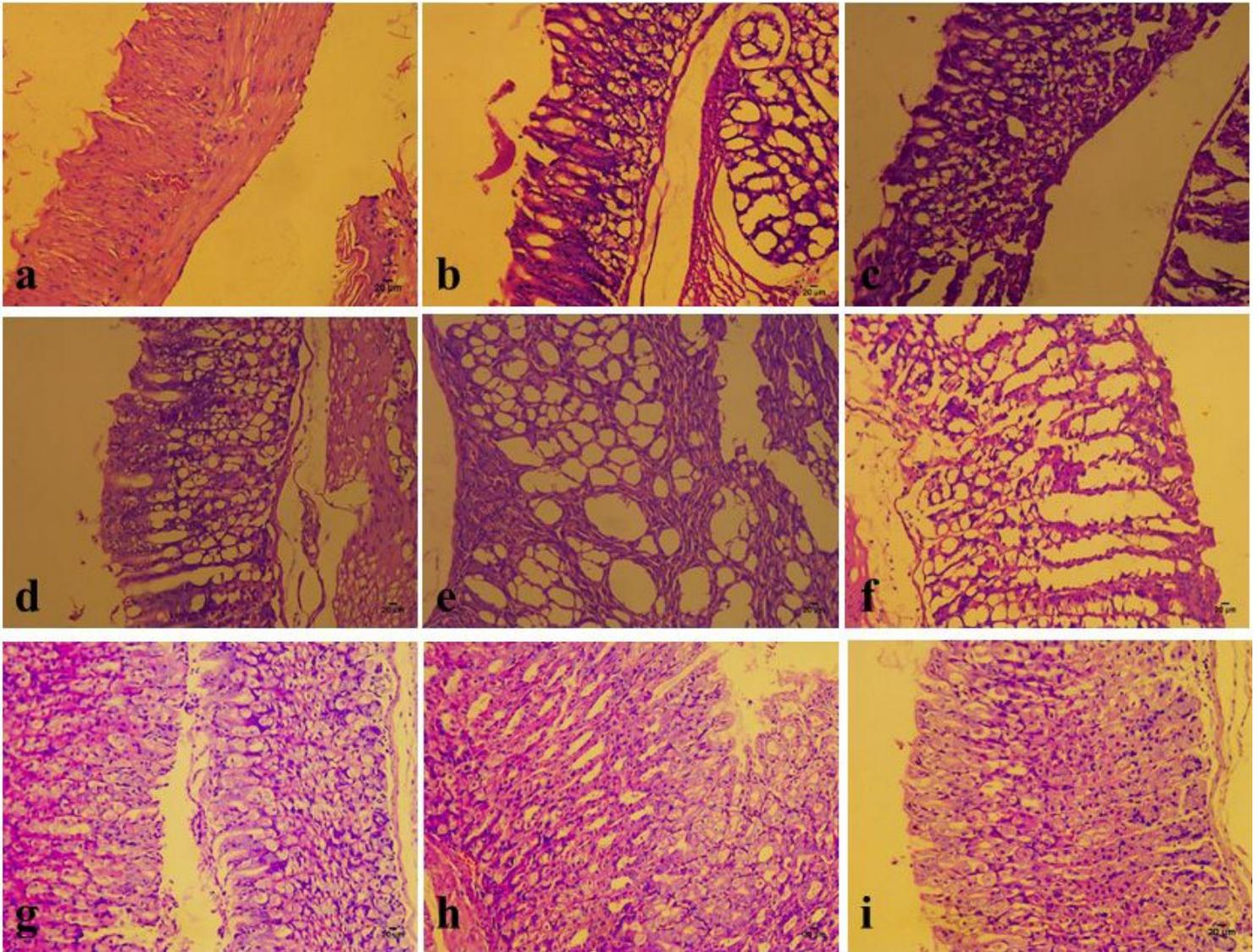


Figure 6

Histological appearance of gastric tissues in CAOV3-infected mice after treatment with different concentrations of *C. adamanteus* venom LAAO. a Normal gastric mucosa. b Gastric tissue of infected-mice by s.c. injection of CAOV3 cell suspension. c, d, e Stomach tissues after treatment with svLAAO of 0.5, 1.0, 1.5 µg/g body weight by s.c. injection, respectively. f Gastric tissue of infected-mice by i.p. injection of CAOV3 cell suspension. g, h, i Stomach tissues after treatment with svLAAO of 0.5, 1.0, 1.5 µg/g body weight by i.p. injection, respectively. These samples were collected after s.c. or i.p. injection of svLAAO every 24 h for 4 times. Scale bar=20 µm.

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