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Original Article

3-Hydroxybenzaldehyde and 4-Hydroxybenzaldehyde enhance survival of mouse astrocytes treated with *Angiostrongylus cantonensis* young adults excretory/secretory products

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ABSTRACT

Background: Human cerebral angiostrongyliasis, induced by *Angiostrongylus cantonensis*, is an emerging disease in many parts of the world. *A. cantonensis* is also an important causative agent of eosinophilic meningitis and eosinophilic meningoencephalitis in humans. 3-Hydroxybenzaldehyde (3-HBA) and 4-Hydroxybenzaldehyde (4-HBA) have been shown to increase intracellular antioxidant activity, vasculoprotective potency, wound healing, and cell migration. However, the function of 3-HBA and 4-HBA in mouse astrocytes in response to *A. cantonensis* young adults excretory-secretory products (ESPs) treatment remains unclear.
Methods: Here, we examined the effect of 3-HBA and 4-HBA by real-time qPCR, western blotting, and cell viability assay in astrocytes after *A. cantonensis* young adults ESPs treatment. The real-time qPCR, western blotting were employed to detect the expression of apoptosis- and Shh pathway-related molecule. The percentage of cell viability was monitored by CCK-8 assay.
Results: We demonstrated that expression of apoptosis-related molecules was increased in response to *A. cantonensis* young adults ESPs treatment. However, the cell viability of astrocytes was elevated by treatment with 3-HBA and 4-HBA. Further investigation found that 3-HBA and 4-HBA activate the Shh signaling pathway and inhibit apoptosis-related molecule expression.
Conclusions: These findings were confirmed using *A. cantonensis* young adults ESPs to activate apoptosis-related pathways in astrocytes. Moreover, 3-HBA and 4-HBA induced a

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protective phenotype through regulation of apoptosis in response to *A. cantonensis* young adults ESPs treatment. Hence, 3-HBA and 4-HBA represent potential therapeutic drugs for the treatment of human angiostrongyliasis.

At a glance of commentary

Scientific background on the subject

Angiostrongylus cantonensis, a zoonotic parasitic nematode, is a major etiologic agent of cerebral angiostrongyliasis in human. Excretory-secretory products (ESPs) are important investigation targets for studying the relationship between hosts and nematodes. These products assist worms in penetrating the blood-brain barrier and avoiding the host immune response.

What this study adds to the field

This study confirmed the expressions of apoptosis-related molecules after treatment with the ESPs of *A. cantonensis* young adults. On the other hands, we determined the function of 3-HBA and 4-HBA in astrocytes. The results should provide useful information for treatment of *A. cantonensis* infection.

The rat lungworm *Angiostrongylus cantonensis*, a foodborne zoonotic parasitic nematode, is an important etiologic agent of cerebral angiostrongyliasis in humans. It can induce eosinophilic meningitis and eosinophilic meningoencephalitis [1,2], particularly in the Pacific islands and Southeast Asia [3–10]. Clinical manifestations of *A. cantonensis* infection in the central nervous system (CNS) have been reported in many studies, including headache, fever, nausea, vomiting, neck pain, visual impairments, and neurological abnormalities [11].

The immune regulatory role of excretory-secretory products (ESPs) is increasingly the most in-depth area of research on parasitic infection pathogenesis. ESPs have resulted in vaccine generation, immunodiagnostic tools, immunomodulatory properties, functional analysis, and signal transduction [12]. During helminth infection, ESPs include a variety of molecules, including proteins, lipids, glycans and nucleic acids, that protect worms against a host immune attack and penetrate the host's defensive barriers [13,14]. Therefore, ESPs represent a valuable target for investigating the relationship between host and parasite.

Some reports have demonstrated that benzaldehyde-related compounds can reduce oxidative stress by inhibiting ROS production after H_2O_2 treatment. In addition, benzaldehyde reduces the inflammatory response by decreasing expression of VCAM-1, ICAM-1, CD40, phospho–NF–κB, phospho-p38 and HIF-1α. Finally, benzaldehyde is also a potential cancer drug because it inhibits cell migration and proliferation [15]. 3-Hydroxybenzaldehyde (3-HBA) and 4-Hydroxybenzaldehyde (4-HBA), benzaldehydes commonly found in nature, have one hydroxyl (OH) group at the meta position of the phenolic ring. This OH group possesses high intracellular antioxidant activity. 3-HBA and 4-HBA are strong free radical inhibitors that exert their effects through activation of nitric oxide synthase (NOS) expression and inhibition of oxidative stress [16]. 3-HBA, a natural active compound isolated from *Gastrodia elata* (Tianma), is a substrate of aldehyde dehydrogenase in humans [17]. Some studies have shown that 3-HBA plays an important role in resistance to oxidative stress, and it has vasculoprotective potency and antibacterial activity against gram-positive and gram-negative strains of bacteria [18,19]. 4-HBA can promote wound healing, cell migration and invasion by increasing Src activity [20]. 4-HBA is also a Chinese herbal medicine that can be used to treat headaches, migraines, and nervous disorders. 4-HBA's protective effects in H_2O_2-induced oxidative stress were demonstrated by activation of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) [21].

Accordingly, 3-HBA and 4-HBA may represent potential drugs for the regulation of ROS generation and apoptosis in response to *A. cantonensis* infection. In our previous studies, we found that *A. cantonensis* young adults ESPs induce oxidative stress, apoptosis, and cytokine secretion in astrocytes [22,23]. In this study, we evaluated the protective function and molecular mechanism of 3-HBA and 4-HBA in mouse astrocytes after *A. cantonensis* young adults ESPs treatment. The results revealed that treatment with 3-HBA and 4-HBA has a protective effect in astrocytes.

Materials and methods

Ethics statement

All procedures involving experimental animals and their care were reviewed and approved by the Chang Gung University Institutional Animal Care and Use Committee (IACUC) and followed the guidelines for Laboratory Animal Facilities and Care (The Council of Agriculture. Executive Yuan, ROC). Animals were housed in plastic cages and provided with food and water ad libitum. Experimental animals were euthanized by anesthesia with isoflurane (1 ml/min).

Animals and infection

A Taiwanese strain of *A. cantonensis* was used in this study. Its life cycle has been maintained in our laboratory through *Biomphalaria glabrata* snails and Sprague–Dawley (SD) rats. SD rats and BALB/c mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). On day 21 post infection, third-stage *A. cantonensis* larvae (L3) were isolated from infected snails by digestion with 0.6% (w/v) pepsin-HCl (pH 2–3) for 1 h. Each rat was inoculated with 50 L^3 individuals by stomach intubation.

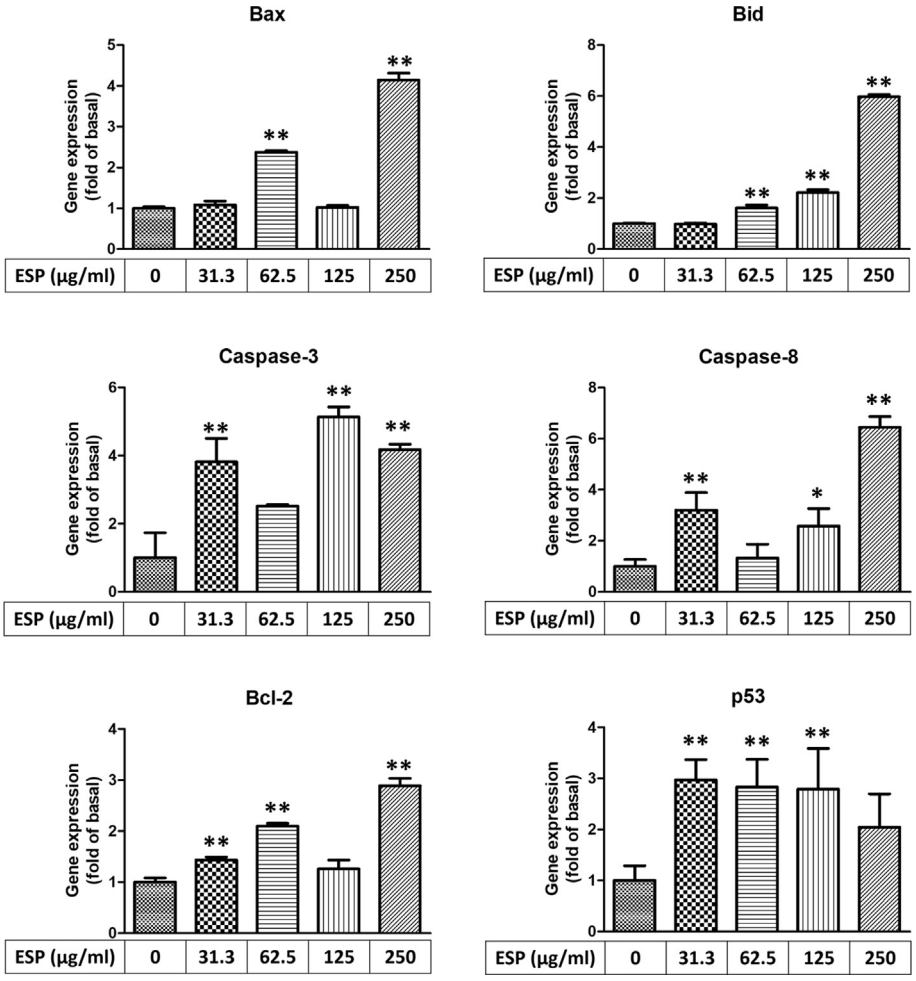

Fig. 1 Excretory/secretory products of *A. cantonensis* young adults larvae stimulate apoptosis-related molecule expression in astrocytes. Cells were treated with 0, 31.3, 62.5, 125 or 250 μg/ml excretory/secretory products (ESPs) from *A. cantonensis* young adults larvae for 12 h. mRNA expression levels of Bax, Bid, Caspase-3, Caspase-8, Bcl-2, and p53 were detected by real-time qPCR. The data are expressed as the mean ± SD from three independent experiments (n = 3). *$p < 0.05$ and **$p < 0.01$ compared to cells treated with 0 μg/ml ESPs of *A. cantonensis* young adults larvae for 12 h.

Preparation of excretory/secretory products

After anaesthetizing with 3% (v/v) isoflurane, living young adults specimens were collected from the brain tissues of infected rats on day 21 post infection [24]. The larvae were carefully removed from the tissue debris using a dissecting microscope. Worms were washed with saline, phosphate-buffered saline (PBS), distilled water and RPMI containing a high concentration of antimycotic solution (200 units/ml penicillin G, 200 μg/ml streptomycin sulfate and 0.5 mg/ml amphotericin B) (Sigma–Aldrich, St. Louis, USA) before incubation in RPMI without fetal bovine serum (FBS) for 24, 48, 72, and 96 h (37 °C; 5% CO_2). *A. cantonensis* young adults excretory/secretory products (ESPs) were concentrated using Amicon Ultra-15 10K centrifugal filter devices (Merck Millipore, Germany). The protein concentration of ESPs was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Concentrated ESPs were used to treat astrocytes, and molecular expression level changes were detected [22].

Mouse astrocyte cultures

The mouse brain astrocytic cell line CRL2535 from the ATCC was employed in this study. Cells were cultured in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) (Corning, USA) with 10% FBS (Gibco, USA), penicillin and streptomycin in poly-L-lysine-coated culture flasks at 37 °C in 5% CO_2. Using GFAP staining, over 95% of cultured cells were identified as astrocytes. Cells were plated onto 10 cm culture dishes and incubated in serum-free DMEM/F-12 for 24 h, followed by pretreatment with drugs for 12 h, and then stimulation with *A. cantonensis* ESPs. Finally, cells were pretreated with 3-HBA or 4-HBA for 12 h and then incubated with 250 μg/ml *A. cantonensis* young adults excretory/secretory products (ESPs) for 12 h.

RNA extraction and real-time qPCR

Total RNA was extracted from astrocytes treated with *A. cantonensis* young adults ESPs at the indicated doses using the GENEzol TriRNA Pure Kit (Geneaid, Taiwan). Each total RNA sample was measured using a spectrophotometer (OD260 nm),

and RNA quality was checked by agarose gel electrophoresis. First-strand cDNA was obtained using the iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, USA) with random hexamers according to the manufacturer's instructions. Real-time qPCR was performed using iQ™ SYBR® Green Supermix (Bio-Rad, USA) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA). GAPDH was used as an internal control. Expression levels were detected with specific primers (Appendix).

SDS-PAGE electrophoresis and western blotting analysis

Proteins from astrocytes were separated by 12% SDS-PAGE after treatment, and samples were analyzed by western blotting. Proteins in the gels were transferred to nitrocellulose membranes using a semidry transfer unit at 0.04 mA for 50 min. The membranes were washed with TBS/T three times and then with a blocking buffer. The membranes were incubated overnight in antibodies against GFAP (Proteintech, USA), Bax (ABclonal, USA), Bid (ABclonal, USA), Caspase-3 (ABclonal, USA), Caspase-8 (ABclonal, USA), Bcl-2 (ABclonal, USA), p53 (ABclonal, USA), and β-actin (Proteintech, USA). The membranes were washed with TBS/T three times and then incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Sigma–Aldrich, USA). Immunoreactive bands were detected using ECL reagents (EMD Millipore, USA) and captured by a ChemiDoc Imaging System (Bio-Rad, USA). ImageJ software analysis was employed to measure the image densitometry of target protein bands.

Cell viability assay

To determine cell survival in response to *A. cantonensis* young adults ESPs treatment, astrocytes (1×10^7 cells/ml) were measured using the CCK-8 assay (Cell Counting Kit-8) (Sigma–Aldrich, USA) at 37 °C in the dark with mild shaking. In the presence of cells, highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction. Cell survival is monitored by measuring formazan dye absorbance at 450 nm using a spectrophotometer (Molecular Devices, USA).

Statistical analysis of data

All the data were analyzed using GraphPad Prism 5 software (GraphPad, USA). The expression levels are shown as the mean ± SD and were analyzed by Student's t-test. p-values < 0.05 and <0.01 were considered statistically significant.

Results

Expression of apoptosis-related molecules in astrocytes treated with A. cantonensis *young adults ESPs*

Our previous studies demonstrated that oxidative stress and apoptosis were induced in astrocytes in response to *A. cantonensis* young adults excretory/secretory product (ESPs) treatment [22]. In this study, we first examined whether *A. cantonensis* ESPs can stimulate the expression of apoptosis-related molecules in astrocytes. Real-time qPCR was used to detect the expression of apoptosis-related genes, including Bax, Bid, Caspase-3, Caspase-8, Bcl-2, and p53. The data revealed that the mRNA expression levels of Bax and Bid were significantly increased in response to treatment with 62.5 μg/ml ESPs and that Caspase-3, Caspase-8, Bcl-2, and p53 levels were significantly elevated after treatment with 31.25 μg/ml ESPs [Fig. 1]. These results demonstrate that *A. cantonensis* young adults ESPs induce apoptosis-related molecule expression in astrocytes.

3-HBA and 4-HBA increase cell viability in astrocytes treated with A. cantonensis *young adults ESPs*

To investigate the protective effects of 3-HBA and 4-HBA in astrocytes with respect to treatment with *A. cantonensis* young adults ESPs, cells were pretreated with different concentration of 3-HBA and 4-HBA (0.1 and 0.5 mM) and then treated with *A. cantonensis* young adults ESPs (250 μg/ml). Cell viability was subsequently measured by the CCK8 assay [Fig. 2]. First, the results showed that ESPs induce cell death in astrocytes. In contrast, cell viability was significantly increased in response to 3-HBA and 4-HBA pretreatment. These results demonstrate that 3-HBA and 4-HBA have protective properties in astrocytes treated with *A. cantonensis* young adults ESPs.

3-HBA and 4-HBA induce astrocyte activation

To determine the effect of 3-HBA and 4-HBA on astrocyte activation after *A. cantonensis* young adults ESPs treatment, astrocytes were pretreated with different concentrations of 3-HBA or 4-HBA (0.1 and 0.5 mM), followed by treatment with 250 μg/ml *A. cantonensis* young adults ESPs. Real-time qPCR

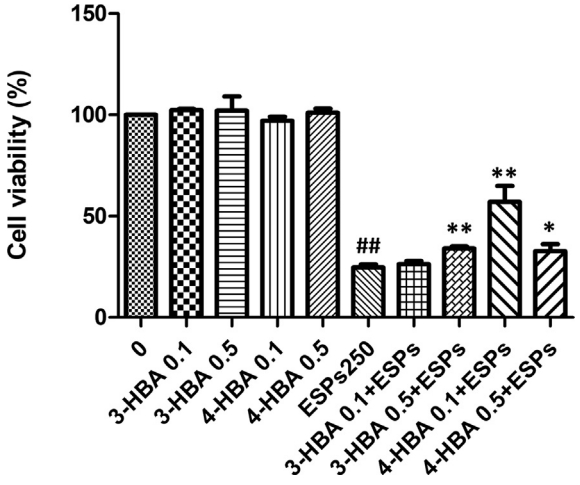

Fig. 2 Protective effects of 3-HBA and 4-HBA in astrocytes treated with *A. cantonensis* young adults ESPs. Cells were pretreated with 3-HBA and 4-HBA (0.1 and 0.5 mM) for 12 h and then incubated with 250 μg/ml *A. cantonensis* young adults excretory/secretory products (ESPs) for 12 h. Astrocyte viability was analyzed by the CCK-8 assay. The data are expressed as the means ± SD from three independent experiments (n = 3). $^{\#\#}p < 0.01$, compared to control. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared to cells exposed to ESPs.

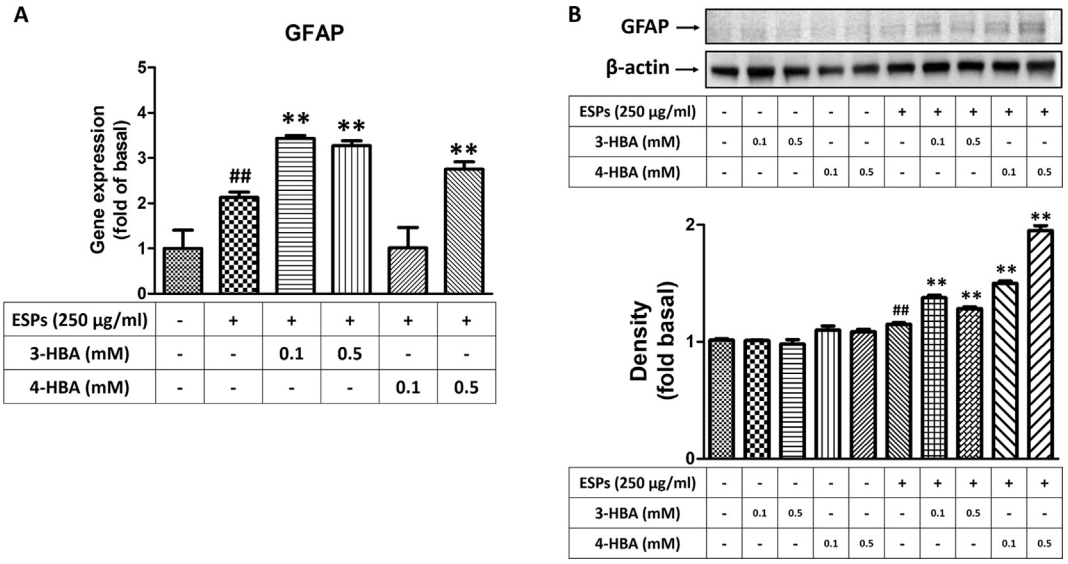

Fig. 3 3-HBA and 4-HBA induce GFAP expression in excretory/secretory product-treated astrocytes. Cells were pretreated with 3-HBA and 4-HBA (0.1 and 0.5 mM) for 12 h and then incubated with 250 μg/ml A. *cantonensis* young adults excretory/secretory products (ESPs) for 12 h. (A) mRNA and (B) protein expression levels of GFAP were detected by real-time qPCR and western blotting. The data are expressed as the means ± SD from three independent experiments (n = 3). $^{\#\#}p < 0.01$, compared to control. $^{**}p < 0.01$, compared to cells exposed to ESPs.

and western blotting were employed to detect gene and protein expression of the astrocyte activation marker GFAP. First, the data showed that the mRNA and protein levels of GFAP were significantly increased after treatment with ESPs. Moreover, the expression of GFAP was also significantly elevated in response to 3-HBA or 4-HBA treatment [Fig. 3]. Taken together, these results suggest that 3-HBA and 4-HBA stimulate astrocyte activation in response to *A. cantonensis* young adults ESPs treatment.

3-HBA and 4-HBA activate the Shh signaling pathway

To determine whether 3-HBA and 4-HBA stimulate Shh signaling pathway activation in ESPs-treated astrocytes, cells were pretreated with different concentrations of 3-HBA or 4-HBA (0.1 and 0.5 mM), followed by treatment with 250 μg/ml A. *cantonensis* young adults ESPs. Real-time qPCR and western blotting were used to detect the gene and protein expression of Shh signaling-related molecules, including Shh, Smo, and Gli. The data indicated that the expression of signaling-related molecules was significantly increased after 3-HBA or 4-HBA treatment [Fig. 4]. These results suggest that 3-HBA and 4-HBA induce the activation of the Shh signaling in astrocytes after treatment with *A. cantonensis* young adults ESPs.

3-HBA and 4-HBA inhibit apoptosis-related molecule expression

In Fig. 1, we demonstrated that *A. cantonensis* young adults ESPs stimulate apoptosis-related molecule expression in astrocytes. Next, we investigated whether 3-HBA and 4-HBA inhibit the expression of apoptosis-related molecules in astrocytes after *A. cantonensis* young adults ESPs treatment. Cells were pretreated with different concentrations of 3-HBA or 4-HBA (0.1 and 0.5 mM), followed by treatment with 250 μg/ml A. *cantonensis* young adults ESPs. Real-time qPCR and western blotting were used to detect gene and protein expression of apoptosis-related molecules, including Bax, Bid, and Bcl-2. The data revealed that the expression of Bax and Bid were significantly decreased, while expression of Bcl-2 was increased in response to 3-HBA or 4-HBA treatment (Fig. 5). These results indicate that 3-HBA or 4-HBA exert potentially protective effects in astrocytes through suppression of apoptosis in response to *A. cantonensis* young adults ESPs treatment.

Discussion

A. cantonensis's complex life cycle has a definitive host (rodent) and an intermediate host (mollusk) [25]. Adult worms live and mate in the pulmonary arteries of rats. Eggs are produced and hatch into first-stage larvae (L1) from female worms in the lung capillaries. L1 larvae then penetrate into the alveolar capillaries and migrate to the throat. Finally, these larvae are released into the environment via rat feces after being swallowing into the gastrointestinal tract. Afterward, L1 individuals may infect the intermediate host by penetrating the skin or through ingestion, where they develop into the infective third-stage larvae (L3). After a human ingests L3 larvae from an intermediate or paratenic host, the larvae can develop into young adults in the CNS. These larvae induce severe host immune responses, mechanical injuries and cell death in human brains [26].

The performance of anthelmintics, such as albendazole and mebendazole, or supportive treatment with corticosteroids, such as dexamethasone, to inhibit inflammatory responses remains controversial. Previous studies have demonstrated

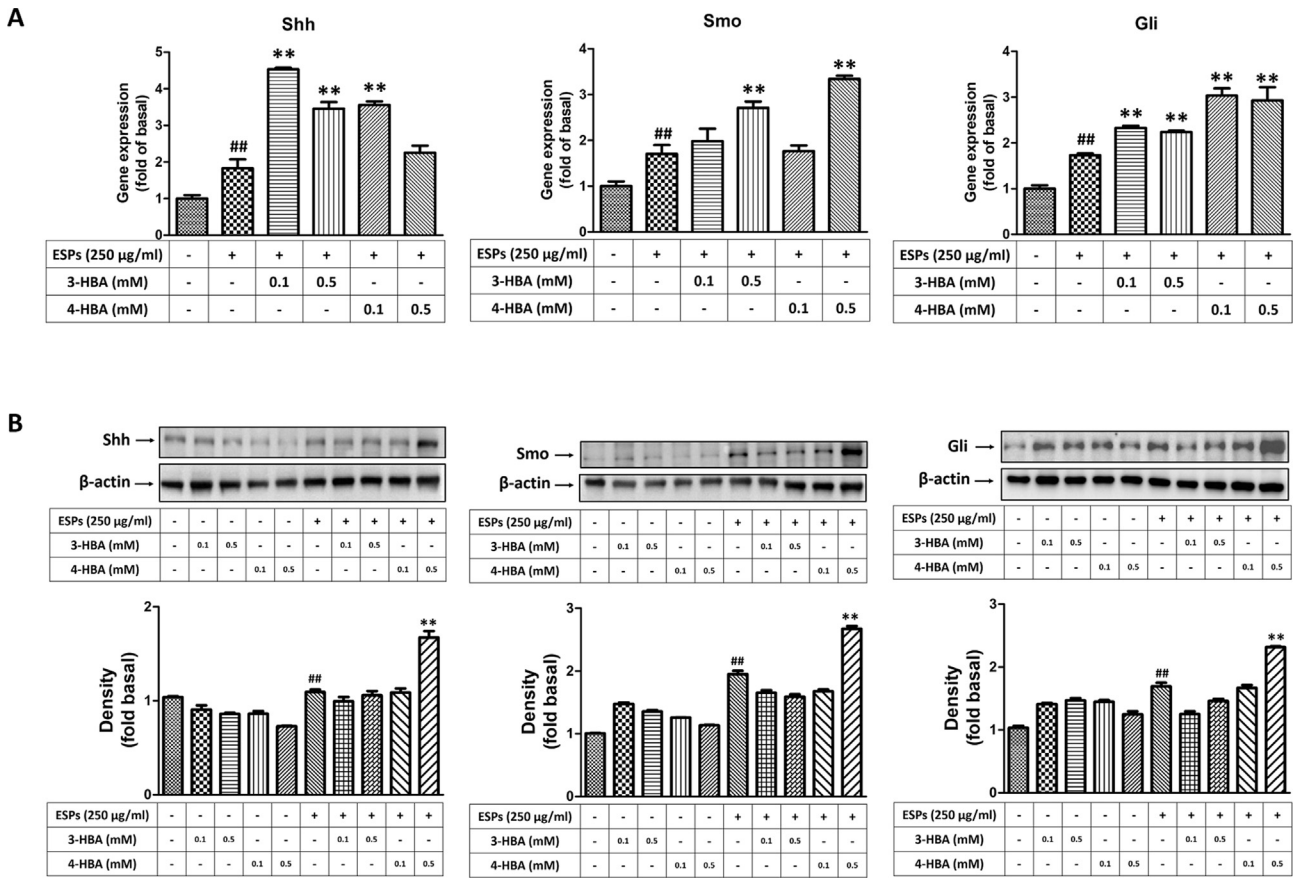

Fig. 4 3-HBA and 4-HBA induce Shh signaling pathway activation in excretory/secretory product-treated astrocytes. Cells were pretreated with 3-HBA and 4-HBA (0.1 and 0.5 mM) for 12 h and then incubated with 250 μg/ml A. *cantonensis* young adults excretory/secretory products (ESPs) for 12 h. (A) mRNA and (B) protein expression levels of Shh, Smo, and Gli were detected by real-time qPCR and western blotting. The data are expressed as the means ± SD from three independent experiments (n = 3). $^{\#\#}p < 0.01$, compared to control. $^{**}p < 0.01$, compared to cells exposed to ESPs.

that albendazole may be an effective drug for the treatment of *A. cantonensis* infection in the CNS [27–32]. Therefore, this drug represents a potential therapeutic strategy for further study. However, our previous study showed that pathological changes in rabbit brains become more severe in response to albendazole treatment, including eosinophilic meningitis, encephalitis, and hydrocephalus [1]. This finding indicates that albendazole is not very appropriate for treating cerebral angiostrongyliasis. In contrast, some reports found that corticosteroids also have treatment efficacy for *A. cantonensis* infection [33,34]. Dexamethasone reduces blood–brain barrier breakdown, neuropathological changes, meningitis and apoptosis in *A. cantonensis* infected brains [35–37]. However, these studies observed only pathological changes in the entire brain after treatment of cerebral angiostrongyliasis. In this study, we focused on the molecular mechanism and signaling transduction in astrocytes in response to 3-HBA and 4-HBA treatment. These results demonstrate that 3-HBA and 4-HBA increase the viability of astrocytes in response to *A. cantonensis* ESPs treatment.

In this study, we demonstrated that *A. cantonensis* young adults ESPs induces cell death and apoptosis-related molecules expression. Excretory-secretory products (ESPs) is an important target for studying on the interaction between host and parasite. In helminths infection, ESPs contains a wide range of molecules (proteins, lipids, glycans, and nucleic acids) that can assist in the penetration of host defensive barriers and avoid the host immune attack [14]. In *A. cantonensis* expressed sequence tags (ESTs) analysis, the putative excretory/secretory proteins were detected. These proteins may play an important role in the lifecycle of *A. cantonensis* [38]. Recent *A. cantonensis* studies have demonstrated that the ESPs can stimulate host immune responses and aid to the penetration of host intestine [23,39,40]. The proteomic results found that the ESPs from *A. cantonensis* contains many proteins, including protein disulfide-isomerase, calreticulin, aspartic protease, heat shock protein 70, aspartyl protease inhibitor, cathepsin B-like cysteine proteinase and hemoglobinase-type cysteine proteinase, and these proteins have been implicated in host infection and immune response [11,41].

This study demonstrated that 3-HBA and 4-HBA activate astrocytes under *A. cantonensis* young adults ESPs treatment. Astrocytes are the most abundant glial cells, and they plays a major role in modulation of neuronal activation and immune responses after pathogen infection via secretion of regulatory proteins or cytokines in the CNS [42–46]. Astrocytes also

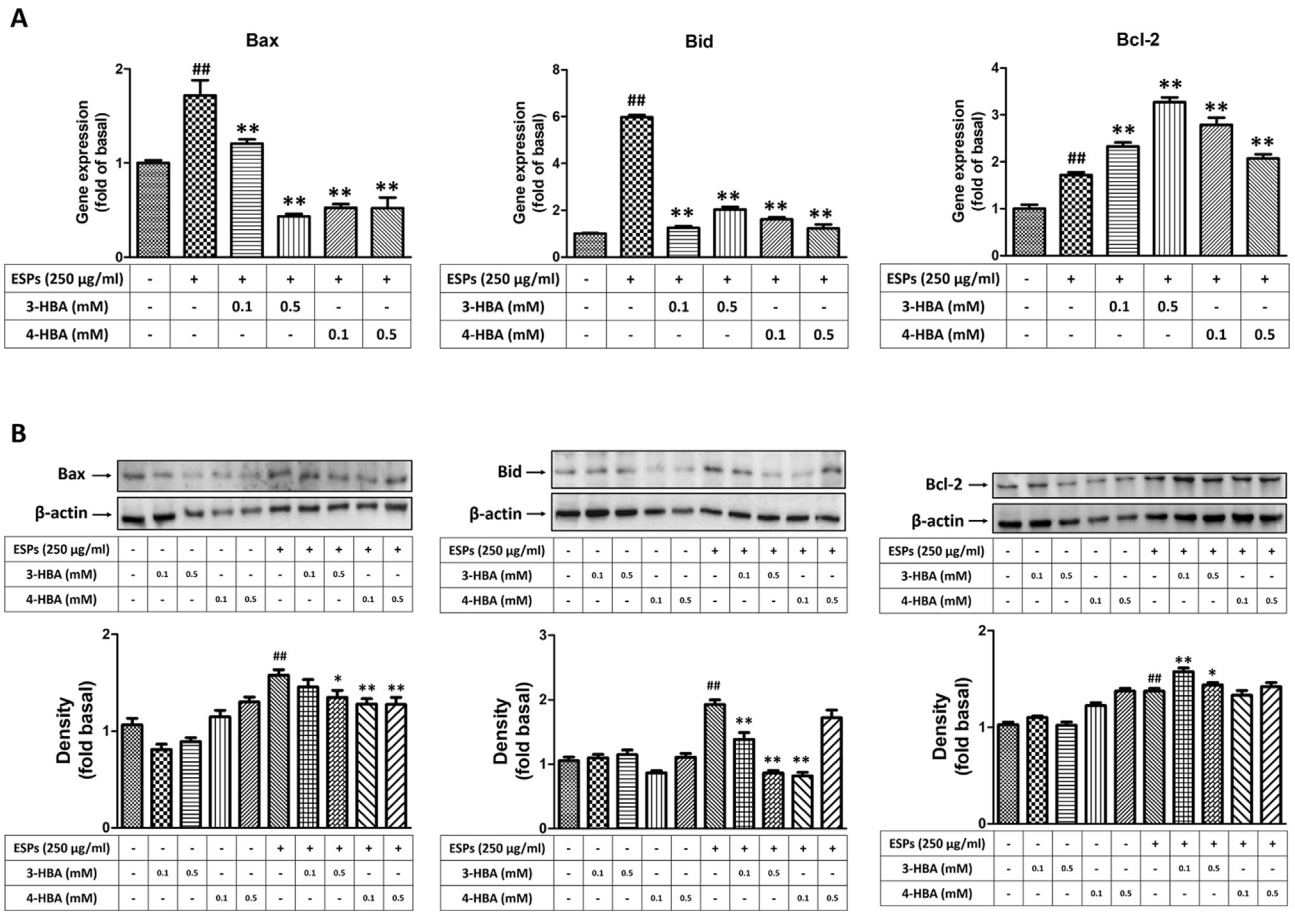

Fig. 5 3-HBA and 4-HBA inhibit apoptosis-related molecule expression in excretory/secretory product-treated astrocytes. Cells were pretreated with 3-HBA and 4-HBA (0.1 and 0.5 mM) for 12 h and then incubated with 250 μg/ml A. *cantonensis* young adults excretory/secretory products (ESPs) for 12 h. The (A) mRNA and (B) protein expression levels of Bax, Bid, and Bcl-2 were detected by real-time qPCR and western blotting. The data are expressed as the means ± SD from three independent experiments (n = 3). $^{\#\#}p < 0.01$, compared to control. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared to cells exposed to ESPs.

maintain homeostasis in the brain [47]. In the CNS, astrocytes regulate molecular transport by formation of the blood–brain barrier (BBB) with endothelial cells (ECs), basal lamina, and pericytes [48,49]. This barrier protects the CNS by separating the blood and brain parenchyma. The BBB allows only very small or hydrophobic molecules to cross into the brain tissue, including O_2, CO_2, hormones and glucose [50]. In pharmacological research, approximately 100% of large molecule drugs and 98% of small molecule drugs are unable to enter the brain tissue through the BBB.

In this study, we found that 3-HBA and 4-HBA stimulate Shh signaling pathway activation in the setting of *A. cantonensis* young adults ESPs treatment. Our previous research showed that expression of Shh, Ptch, and Gli-1 was elevated in response to ESPs treatment [23]. Shh plays an important role in animal development and a variety of tissues' morphogenesis [51,52]. This signaling can trigger other important pathways, such as the AKT and NF-kb pathways. Shh signaling can also influence BBB function and CNS immune responses by regulating entry of immunocytes [53]. Furthermore, Shh can be activated in reactive astrocytes during brain injury, and Shh expression may play a role in promoting cell proliferation [54]. The Shh pathway protects astrocytes and cortical neurons from oxidative stress by activating Bcl-2 and inhibiting Bax [55].

Finally, our previous research found that apoptosis in astrocytes is induced in response to treatment with soluble antigens of *A. cantonensis* by evaluation of apoptosis-related protein expression [56]. We also demonstrated that *A. cantonensis* young adults ESPs stimulate apoptosis in astrocytes [22]. In this study, we demonstrated that expression of apoptosis-related molecules (Bax, Bid, and Bcl-2) is significantly changed after 3-HBA or 4-HBA treatment. These results indicate that in astrocytes, 3-HBA or 4-HBA regulate the expression of apoptosis-related molecules in the setting of *A. cantonensis* young adults ESPs treatment.

Conclusion

In conclusion, we demonstrated that *A. cantonensis* young adults ESPs induce apoptosis in activated astrocytes. 3-HBA or 4-HBA exert protective effects in these astrocytes through regulation of apoptosis in the setting of *A.*

cantonensis young adults ESPs treatment. These drugs may represent useful therapeutic targets for the treatment of human angiostrongyliasis.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2020.11.008.

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