An Acad Bras Cienc (2022) 94(Suppl. 4): e20211400 DOI 10.1590/0001-3765202220211400
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

BIOMEDICAL SCIENCES

Oroxin A ameliorates the oleic acid-induced A549 cell injury through the suppression of pyroptosis and degradation of alveolar surfactant

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Abstract: The destruction of the pulmonary epithelial barrier in acute respiratory distress syndrome is caused by the damage of the alveolar epithelial cells. Oroxin A is an effective flavonoid component derived from the medicinal plant Oroxylum indicum (L.) Kurz. In this study, the oleic acid (OA)-induced A549 cell injury model was established in vitro to explore the protective mechanism of Oroxin A. The experiment was divided into the following groups: control, OA and OA + Oroxin A group. The OA-induced A549 cell injury was dose (time)-dependent and was detected by the CCK-8 assay. The protein and mRNA expression levels associated with pyroptosis are detected by Western blotting and RT-qPCR. After Oroxin A treatment, the levels of IL-1β, IL-18 and LDH released were significantly lower than the OA group. In terms of pyroptosis, Oroxin A can inhibit the expression of pyroptosis-related protein and mRNA. Significantly, the surfactant protein C (SPC) level in the OA + Oroxin A group was significantly higher than that in the OA group. The treatment with Oroxin A alleviates the OA-induced injury in the A549 cells, and its mechanism may be related to the inhibition of A549 cells pyroptosis and prevention of the degradation of the SPC.

Key words: Oroxin A, oleic acid, surfactant protein C, pyroptosis, ARDS.

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a severe syndrome commonly seen in the intensive care unit (ICU). With the clinical application of the prone position ventilation and extracorporeal membrane oxygenation (ECMO), the mortality rate of patients with ARDS has decreased, but it is still up to 40% (Huppert et al. 2019). Oleic acid (OA) is an unsaturated fatty acid, which not only destroys the alveolar epithelial barrier by activating thrombin activity, endothelin and oxidative stress response but also has direct toxic effects on the alveolar epithelial type II cells (AEC II) and pulmonary vascular endothelial cells (Sylvester et al. 2013). The OA-induced lung injury model has been widely used, and its acute symptoms and pathological changes closely mimic the human ARDS (Matute-Bello et al. 2008). However, the exact mechanism of OA-induced lung injury is still unclear, and the related *in vitro* mechanism studies are rarely reported. Oroxin A is an effective flavonoid component derived from the traditional Chinese herbal medicinal plant *Oroxylum indicum* (L.) *Kurz*, which is used in the treatment of cough, pharyngitis, whooping cough, bronchitis and other diseases in traditional Chinese medicine (Moirangthem et al. 2013, Tran et al. 2015). Some studies have shown that *Oroxylum indicum* (L.) *Kurz* and its chemical components, such as flavonoid glycosides and cyclohexanol, have anti-inflammatory

and anti-cancer properties (Dinda et al. 2015). Interestingly, a study has shown that Oroxin A can inhibit the hemolytic activity of a-hemolysin in *Staphylococcus aureus*, which may be helpful for the development of antibacterial drugs against *Staphylococcus aureus* in the future. Recent studies have shown that pyroptosis is widely observed during respiratory diseases, and inhibition of pyroptosis can reduce the degree of lung injury and inflammation (Li et al. 2019, Wang et al. 2019). In this study, the A549 human lung adenocarcinoma cell line was selected, which has the same phenotype and characteristics as the AEC II (Bein et al. 2013), to establish a lung injury model induced by OA *in vitro* and explore the protective mechanism of Oroxin A in this process.

MATERIALS AND METHODS

Cell culture and experimental grouping

The human lung adenocarcinoma epithelial cells A549 (were purchased from the American Type Culture Collection) represented the AEC II. The cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM) (Solarbio, China) containing 10% fetal bovine serum (Gibco, USA) in a 5% CO_2 incubator (Thermo, USA) at 37°C and the logarithmic phase cells were selected for the experimental treatment. The experiment was divided into three groups, with specific intervention methods as follows: Control group; OA (300 mM) group; OA (300 mM) + Oroxin A (10 mM) group.

Chemicals

OA was purchased from Sigma-Aldrich (St. Louis, USA), and was prepared as follows: It was compatible with anhydrous ethanol and NaOH solutions and was configured as a solution with a final concentration of 0.2 mM, which was diluted to the desired concentration with DMEM before the experiment. Oroxin A was purchased from Selleck (Houston, USA) and dissolved in dimethyl sulfoxide (DMSO) before use.

Cell viability assay

The A549 cells were inoculated in the 96-well plate with $1^{*}10^{4}$ cells/well. After adherent growth, OA was used to intervene the cells with an increasing concentration gradient (0, 1, 10, 100, 300 and 500 mM) for 24 h and time gradient (0, 12, 24, 36 and 48 h) at 300 mM to select the best experimental conditions, and then the optimal intervention concentration of Oroxin A was determined as 10 μM . The cell viability of each group was detected by CCK-8 assay (Biosharp, China) according to the manufacturer's protocol. The absorbance was measured at 450 nm with a microplate reader (Thermo, USA). The experiment was repeated three times for each group.

Lactate dehydrogenase (LDH) and enzyme-linked immunosorbent assay (ELISA) assay

OA (300 mM) and Oroxin A (10 mM) were added to the culture medium according to the corresponding concentration for 24 hours. Subsequently, the cell culture medium of each group was collected and the levels of LDH were determined by the LDH assay kit (Solarbio, China) and the levels of IL-1β and IL-18 were detected by the ELISA kit (Enzyme-linked Biotech, China) according to the manufacturer's protocol. The experiment was repeated three times for each group.

Western blotting (WB)

The A549 cells in each group were washed with pre-cooled phosphate-buffered saline (PBS), and the collected cells were lysed with the radioimmunoprecipitation assay (RIPA) lysate at 4°C for 30 min and centrifuged at 11000 g for 15 min. The supernatant was taken, and

the protein concentration was quantified in strict accordance with the instructions of the Bicinchoninic Acid (BCA) protein quantitative kit. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membrane incubation (overnight, 4°C) was performed using anti-caspase1 (dilution 1:1000, Proteintech, Wuhan, China), anti-NLRP3 (dilution 1:1000, Abcam, Cambridge, MA, USA), anti-GSDMD (dilution 1:1000, Proteintech, Wuhan, China), anti-ASC (dilution 1:1000, Abcam, Cambridge, USA) and anti-b-actin (dilution 1:1000, Proteintech, Wuhan, China). After scanning the images, Image J software was used to measure the gray values of each strip, and the relative expression level of the target protein was expressed by the ratio of the gray values of the target protein and β-actin.

Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR)

After the experiment, the cells were collected from each group and lysed with Trizol (Takara, Shiga, Japan) to extract the total RNA. After the determination of the concentration and purity of RNA samples, reverse transcription was done to achieve total cDNA. The primers corresponding to the target genes were designed and synthesized, and their sequences are shown in Table I. *β-actin* was taken as the reference gene, and the instructions of the kit were strictly followed. Finally, the QuantStudio 6 & 7 PCR System software (Thermo, MA, USA) was used for data processing and analysis, and 2^{-DDCt} was used to express the relative mRNA expression.

Statistics

The data is presented as mean ± standard error of the mean (SEM). Continuous variables with a normal distribution were analyzed using Students *t*-test, or by analysis of variance (*ANOVA*). For abnormal data distribution, a nonparametric test, such as the Mann-Whitney

Table I .Primer sequences of the target genes.

Target gene	Primer sequence
spc	Forward: 5'-AAGAGATCCCTCTCCCAGCA-3'
	Reverse: 5'-TGGGGTTTGCCGCCATC-3'
nlrp3	Forward: 5′-GTGTGGATCTTTGCTGCGAT-3′
	Reverse: 5′ACAAATGGAGATGCGGGAGA-3′
asc	Forward: 5'-CAGAGTACAGCCAGAACAGGACAC-3'
	Reverse: 5'-GTGGTCTCTGCACGAACTGCC-3'
caspase1	Forward: 5′-ACAAGGCACGGGACCTATG-3′
	Reverse: 5′-TCCCAGTCAGTCCTGGAAATG-3′
gsdmd	Forward: 5′-CCAGCATGGAAGCCTTAGAG-3′
	Reverse: 5′-CAGAGTCGAGCACCAGACAC-3′
β-actin	Forward: 5-CACCATTGGCAATGAGCGGTTC–3'
	Reverse: 5-AGGTCTTTGCGGATGTCCACGT–3'

U test was used. Statistical significance was defined as a two-tailed *p*-value of <0.05. For statistical analysis, GraphPad Prism 7.0 was used.

RESULTS

Effects of Oroxin A on the cell viability of the A549 cells induced by OA

After 24 h of the OA treatment, the cell viability of the A549 cells was inhibited to varying degrees. With the increase of OA concentration, the cell survival rate decreased gradually in a dose-dependent manner (Figure 1a). Then, the A549 cells were stimulated with 300 mM of OA, and the cell viability decreased in a time-dependent manner with the extension of culture time (Figure 1b). According to the above results, OA concentration of 300 mM was selected and cultured for 24 h as the conditions for subsequent experiments. According to the above results, we selected 300 mM OA and cultured it for 24 h as Oroxin A toxicity screening. As shown in Figure 1c, when Oroxin A was exposed to the A549 cells at the concentration of 0-100 mM, the cell viability increased significantly. Therefore, 10 mM concentration was selected for the follow-up experiments in this study.

Oroxin A inhibited the release of IL-1β, IL-18 and LDH in the OA-induced A549 cells

As shown in Figure 2a-c, compared with the control group, the levels of IL-1β, IL-18 and LDH in the cell culture medium of the OA group were significantly increased (p<0.01, p<0.001, p<0.001, respectively). Compared with the OA group, the levels of IL-1β and IL-18 and LDH in the OA + Oroxin A group were significantly lower (p<0.01, p<0.001, p<0.001, respectively).

Oroxin A can prevent the degradation of the alveolar surfactant in the OA-induced A549 cells

The SPC is an alveolar surfactant that reduces the alveolar surface tension and is uniquely expressed in the AEC II (Kalina et al. 1992). In this study, when compared with the control group, the protein and mRNA levels of SPC in the OA group were significantly decreased (p<0.01, Figure 3a-c), while the SPC expression in the OA + Oroxin A group was significantly increased compared with the OA group after Oroxin A intervention (p<0.05, Figure 3a-c).

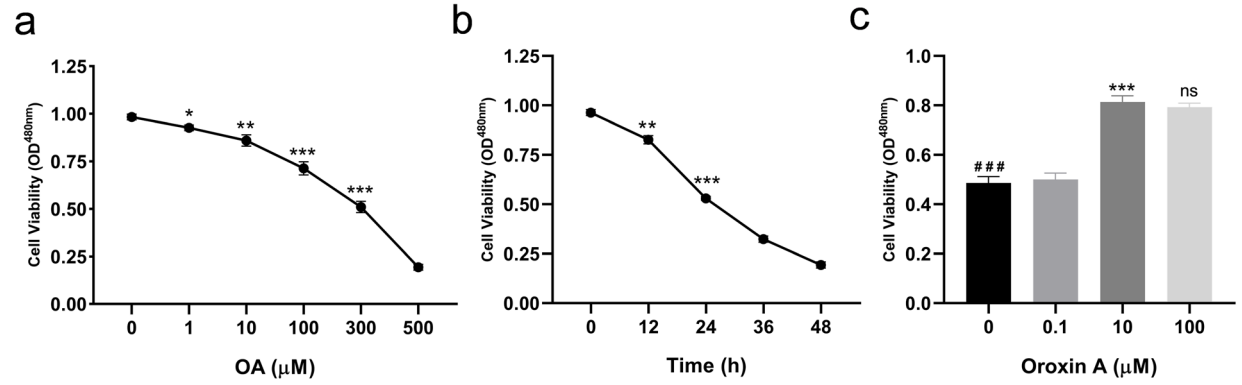

Figure 1. The effects of OA on the A549 cell viability at different concentrations and at different times of stimulation. The effect of OA on the A549 cell viability was dose-dependent and time-dependent, corresponding to 24 h and 300 μM, respectively (a, b). Oroxin A can protect the A549 cells stimulated by OA at a concentration of 10 μM (c). All data were expressed as mean ± SEM. Statistical significance was analyzed by Student's *t* test. $^{*}p$<0.05, $^{**}p$<0.01, $^{***}p$<0.001 compared with 0 group, $^{###}p$<0.001 compared with 100 group, ns means p>0.05 compared with 10 group, n=3 in each group.

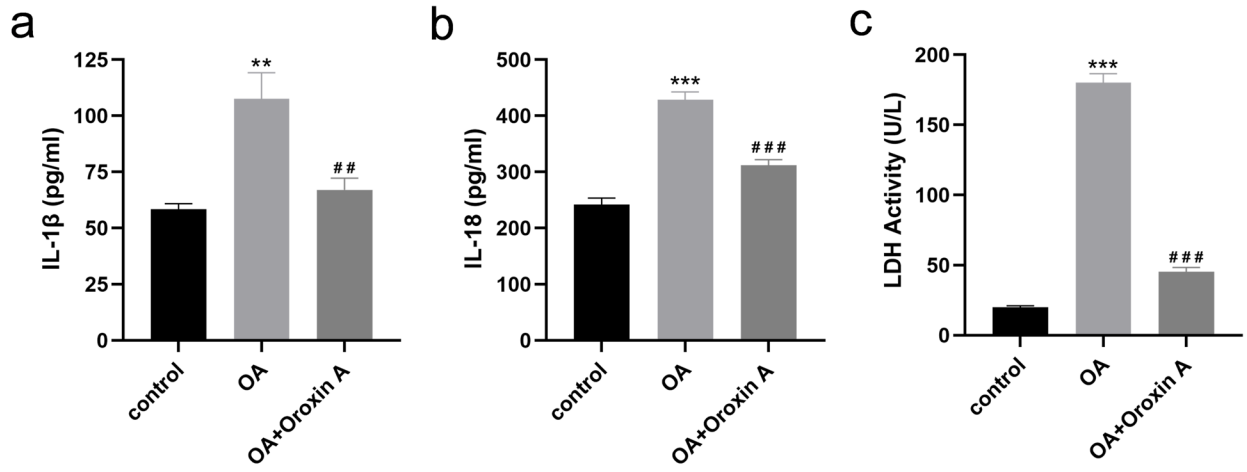

Figure 2. The levels of IL-1β, IL-18 and LDH in the cell culture medium were determined. The levels of IL-1β, IL-18 and LDH in the OA group were significantly higher than that in the control group but the level of IL-1β, IL-18 and LDH in the OA+Oroxin A group was significantly lower than that in the OA group (a-c). All data were expressed as mean ± SEM. Statistical significance was analyzed by Student's *t* test. $^{**}p<0.01$, $^{***}p<0.001$ compared with control group, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with OA group, n=3 in each group.

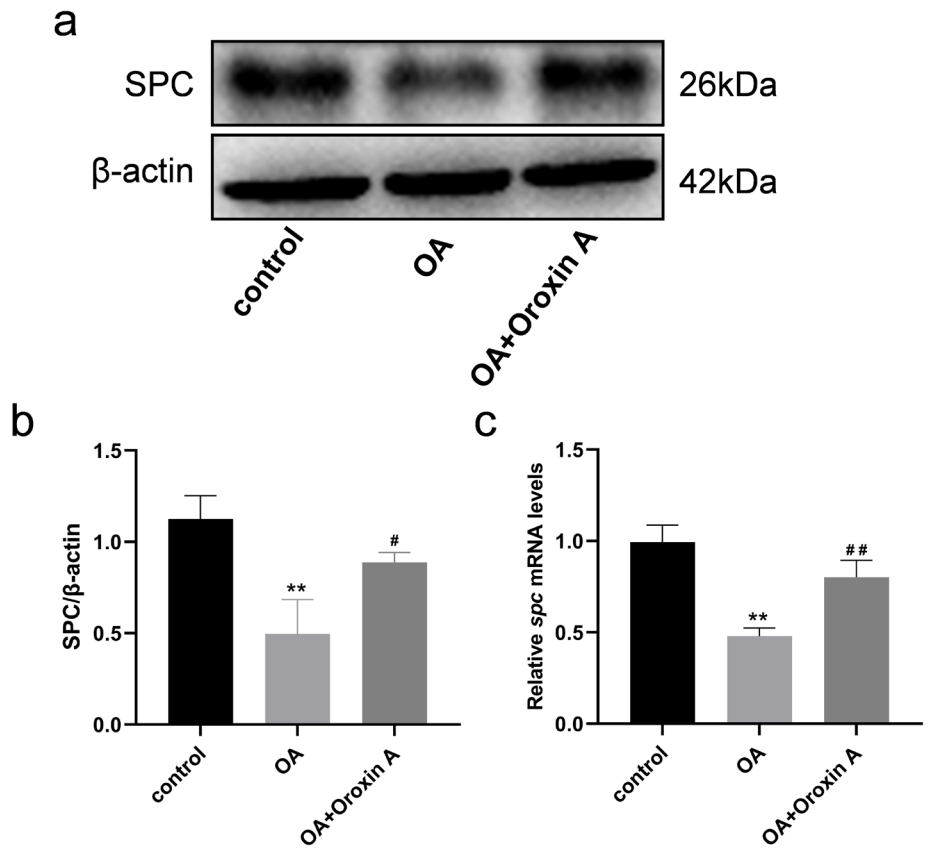

Figure 3. The effect of Oroxin A on the expression of SPC in OA-induced A549 cell injury. The level of SPC in the OA group was significantly lower than that in the control group, but the level of SPC in the OA+Oroxin A group was significantly higher than that in the OA group (a-c). All data were expressed as mean ± SEM. Statistical significance was analyzed by Student's *t* test. $^{**}p<0.01$ compared with control group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with OA group, n=3 in each group.

Oroxin A inhibited the expression of the pyroptosis-related proteins and mRNA in the OA-induced A549 cells

As shown in Figures 4a, b and 5a-d, compared with the control group, the protein and mRNA expression levels of NLRP3, ASC, Caspase1 and GSDMD in the OA group were increased ($p<0.05$), and compared with the OA group, the protein and mRNA expression levels of NLRP3, ASC, Caspase1 and GSDMD decreased in the OA + Oroxin A group ($p<0.05$).

DISCUSSION

Although the treatment of ARDS has improved in recent years, the fatality rate is still very high, ranging from 27 to 45% (Ranieri et al. 2012). The A549 cells were human lung adenocarcinoma epithelial cells that can proliferate indefinitely *in vitro* and has characteristics similar to the AEC II, so it became the first-choice cells for constructing the *in vitro* cell model of acute lung injury (ALI) (Pavlicek et al. 2015, Zhang et al. 2016). OA (18:1 n-9) was a type of unsaturated fatty acid found in plants and animals. It is also the most common and abundant fatty acid in a normal healthy individual body (Ameer et al. 2014, Gonçalves-de-Albuquerque et al. 2015). The exposure of OA to the cells can lead to apoptosis or necrosis (Rockenfeller et al. 2010). Studies have shown that OA affects cells through mechanisms involving the cell-surface receptors or nuclear receptors (Hara et al. 2011). The intravenous injection of OA into the animal mimics human ARDS, producing pathological changes similar to those of human ARDS, with the greatest advantage of being reproducible (Gonçalves-de-Albuquerque et al. 2015). Although OA-induced ARDS has been widely reported in animals, the effect of OA on lung cells *in vitro* has been rarely reported. In this study, OA was used to induce the A549 cell injury *in vitro* to simulate lung injury. The results showed that OA had concentration-dependent

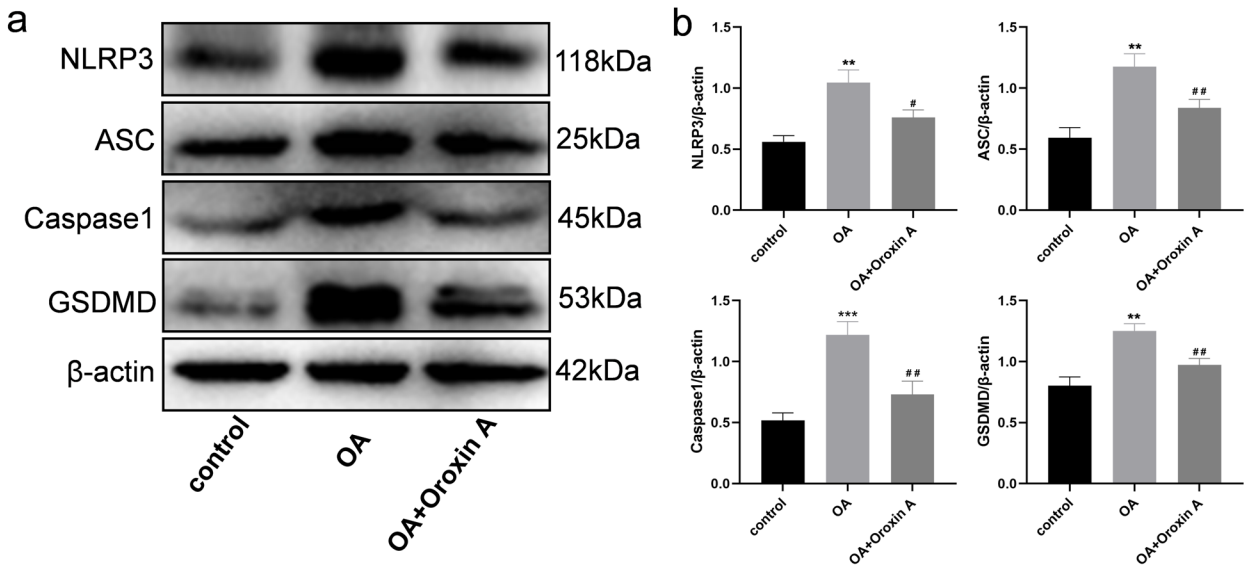

Figure 4. The effects of Oroxin A on pyroptosis-related protein expression in the OA-induced A549 cell injury. The level of NLRP3, ASC, Caspase-1 and GSDMD in the OA group was significantly higher than that in the control group, but the level of NLRP3, ASC, Caspase-1 and GSDMD in the OA+Oroxin A group was significantly lower than that in the OA group (a, b). All data were expressed as mean ± SEM. Statistical significance was analyzed by Student's *t* test. $^{**}p<0.01$, $^{***}p<0.001$ compared with control group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with OA group, n=3 in each group.

and time-dependent effects on the cell viability of the A549 cells and could prevent the degradation of SPC and maintain the stability of cell membrane.

Oroxylum indicum (L.) *Kurz* (Bignoniaceae) was a traditional Chinese herb that has been widely used to treat respiratory infections and gastrointestinal diseases (Sun et al. 2018). Oroxin A, the main ingredient of *Oroxylum indicum* (L.) *Kurz*, has been shown to be effective in diabetes intervention, anti-bacterial and anti-cancer medications with low toxicity (He et al. 2016, Qiu et al. 2013, Sun et al. 2018). Maintaining the AECII function is critical in ALI, and this has been confirmed in several studies (Modelska et al. 1999, Pittet et al. 1997). The SPC is a pulmonary surfactant protein secreted by AECII that reduces the alveolar surface tension, and thus, reduces lung injury (Vaporidi et al. 2005). Individuals who lack SPC tend to develop progressive interstitial lung disease (Meyer 2014). The A549 cells can produce SPC during normal culture and have been widely used in several studies to evaluate surfactant production (Provost et al. 2000, Vaporidi et al. 2005). According to the results, OA-induced A549 cell injury can significantly reduce the expression level of SPC. Interestingly, SPC degradation was significantly reduced after Oroxin A intervention. *In vitro* experiments showed that Oroxin A significantly inhibited the SPC degradation of A549 cells induced by OA. This interesting phenomenon suggests that whether Oroxin A can prevent the degradation of SPC in ARDS rats can be studied in the future. This provides a new treatment strategy for ARDS, a refractory disease.

Pyroptosis is a caspase-1/caspase-11-dependent programmed cell death that was distinct from caspase-3-mediated apoptosis, which activates a number of inflammatory

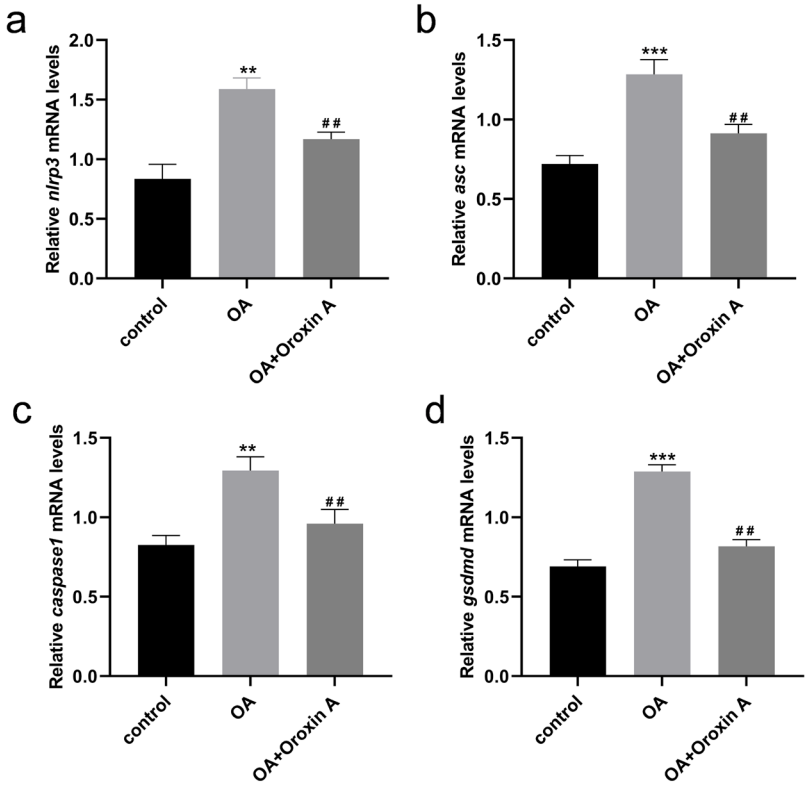

Figure 5. The effects of Oroxin A on pyroptosis-related mRNA expression in OA-induced A549 cell injury. The level of *nlrp3, asc, caspase-1* and *gsdmd* in the OA group was significantly higher than that in the control group, but the level of *nlrp3, asc, caspase-1* and *gsdmd* in OA+Oroxin A group was significantly lower than that in the OA group (a-d). All data were expressed as mean ± SEM. Statistical significance was analyzed by Student's *t* test. $^{**}p<0.01$, $^{***}p<0.001$ compared with control group, $^{\#\#}p<0.01$ compared with OA group, n=3 in each group.

factors such as IL-1β and IL-18. The activated inflammatory factors are released from the extracellular membrane through the pore channels and further trigger the inflammatory responses (Aglietti et al. 2016). Studies have shown that pyroptosis can not only promote lung injury but also inhibit lung cancer cell proliferation (Pinkerton et al. 2017). In this study, it was found that the A549 cells stimulated by OA can release a large amount of IL-1β and IL-18, and the release of these inflammatory factors was significantly inhibited after Oroxin A treatment. This anti-inflammatory effect of Oroxin A was verified for the first time, which provided a certain experimental basis for its application in ARDS model *in vivo*.

LDH is the terminal enzyme of the glycolysis pathway, which normally exists in the cytoplasm and can spill out when the membrane is damaged or has increased permeability. Therefore, the level of LDH in the supernatant of the cell culture medium can reflect the degree of membrane damage, and the change of membrane permeability is a common reaction when toxic substances act on the membrane, as it can show the degree of damage of the cell (Zavala et al. 2016). The results of this study showed that the level of LDH in the cell culture supernatant of the A549 cells stimulated by OA for 24 h was higher than that of the control group, indicating that OA can affect the integrity of cell membrane structure and cause damage to the cells, while Oroxin A can reverse this process.

The classic pyroptosis pathway depends on the formation and activation of the typical inflammasome, which was a polymeric protein complex composed of the Nod-like receptors (NLRs) family proteins (NLRP1, NLRCP3 and NLRC4), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and pro-caspase-1 (Cerqueira et al. 2015). In the process of pyroptosis, Gasdermin D (GSDMD) was the effector protein, which is cleaved by the caspase to GSDMD-N, and then binds specifically to eukaryotic or prokaryotic cell membranes to promote the formation of membrane pores, resulting in cell swelling and pyroptosis (Dong et al. 2018). Interestingly, one study showed that OA protects the membrane against palmitic acid-induced hepatocyte injury by inhibiting endoplasmic reticulum (ER) stress and pyroptosis (Zeng et al. 2020). The results showed that compared with the control group, the protein level and mRNA expressions of NLRP3, ASC and caspase-1 of the A549 cells in the OA group were increased, indicating that OA can activate the cell pyroptosis pathway. However, after treatment with Oroxin A, the expression levels of pyroptosis-related proteins and mRNAs in the OA group decreased significantly, suggesting that Oroxin A protects the A549 cells by inhibiting pyroptosis.

In general, this study established an OA-induced A549 cell injury model to simulate ARDS *in vitro* and explored the protective effect of Oroxin A on the A549 cells by inhibiting the pyroptosis pathway and preventing the degradation of SPC. This has laid the theoretical foundation for the application of Oroxin A in ARDS treatment.

Acknowledgments

This work was supported by the Cuiying Scientific and Technological Innovation Program of Lanzhou University Second Hospital (CY2019-BJ07); the Natural Science Foundation of Gansu Province, China (20JR10RA745, 20JR5RA318); Scientific research project of higher education in Gansu Province (2020B-049); Basic and Clinical Cooperative Research Promotion Program of Anhui Medical University (2020xkjT046) and Open Research fund of Key Laboratory of Anesthesiology and Perioperative Medicine of Anhui Higher Education Institutes of Anhui Medical University (2020mzkf003).

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How to cite

HUANG J, CHEN C, XIE J, ZHAI K, WEI S, CHENG X & ZHANG R. 2022. Oroxin A ameliorates the oleic acid-induced A549 cell injury through the suppression of pyroptosis and degradation of alveolar surfactant. An Acad Bras Cienc 94: e20211400. DOI 10.1590/0001-3765202220211400.

Manuscript received on October 19, 2021;
accepted for publication on May 10, 2022

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