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# Protective effect of *Ganoderma atrum* polysaccharide on acrolein-induced macrophage injury via autophagy-dependent apoptosis pathway

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ABSTRACT

The study aimed to investigate the protective effect and underlying mechanism of *Ganoderma atrum* (*G. atrum*) polysaccharide (PSG-1) on macrophage injury induced by acrolein. The results showed that PSG-1 restored the cell viability damaged by acrolein. In addition, PSG-1 significantly reduced the acrolein-induced occurrence of apoptosis via increase of Bcl-2 expression, mitochondrial membrane potential (MMP), decrease of ROS, cytochrome *c* (Cyt-C), caspase-3, caspase-9. Moreover, the overexpressions of autophagy-related proteins (LC3, Beclin-1, Atg7 and Atg5) were suppressed by PSG-1, which demonstrated that PSG-1 inhibited autophagy in acrolein treated macrophage. Beside, PSG-1 significantly elevated the expression level of p-mTOR, suggested that PSG-1 mediated autophagy through mTOR pathway. Furthermore, inhibitor of autophagy could inhibit apoptosis in acrolein-induced macrophage, suggesting that autophagy may be involved in the regulation of apoptosis. In summary, the present study demonstrated that PSG-1 protected acrolein-induced macrophage injury via autophagy-dependent apoptosis.

## 1. Introduction

As one of the most commonly used chemotherapeutic drugs, cyclophosphamide (CY) is still widely used in clinical treatment. However, long-term use of high dose of CY had harmful side effects such as immunosuppression, myelosuppression and leukopenia (Zhou et al., 2018). Researches showed that the toxic side effects of CY on normal organisms are mainly caused by acrolein (Kachel and Martin, 1994; Tong et al., 2016), which is one of the two main metabolites of CY (Jeelani et al., 2017). In addition, acrolein is also one of the main products of cigarettes, and widely found in cooked foods and the environment (Stevens and Maier, 2010), it is a common environmental toxin with reproductive toxicity, neurotoxicity and carcinogenicity (Dwivedi et al., 2018; Feng et al., 2006; Jeelani et al., 2018; Wu et al., 2018). To alleviate the side effects of acrolein and protecting the human body from food hazards, it is meaningful to find an active substance that can alleviate or antagonize the side effects of acrolein.

Programmed cell death (PCD) serves as a major mechanism for the precise regulation of cell numbers and as a defense mechanism to remove unwanted and potentially dangerous cells (Jorgensen et al., 2017). Apoptosis is a type of PCD that is characterized by cell membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Su et al., 2015). There are two main signal transduction pathways involved in the apoptosis, including the extrinsic and the intrinsic pathways (Cho et al., 2018). In the indigenous pathway, also known as mitochondrial pathway, is mainly induced by stress, ultraviolet light, ionizing radiation, arsenic, etc. In the early stage, mitochondrial permeability changes, Δψm decreases, cytochrome *c* (Cyt-C) release, caspase-9 activation and caspase cascade reaction are triggered (Adams and Suzanne, 2007).

In addition, a non-apoptotic form of PCD has been described and classified as autophagic cell death (Song et al., 2017). Autophagy is a generic term for the degradation of cellular components in lysosomes (Taichi et al., 2006). As far as we know now, autophagy can be divided into three types: macroautophagy, microautophagy and chaperone-mediated autophagy. Compared with the other two types of autophagy, macroautophagy is considered as the main type of autophagy, which has been widely studied and plays an important physiological role in human health (Mizushima and Komatsu, 2011). Therefore, herein we refer to macroautophagy simply as autophagy. The process of autophagy has been extensively reviewed elsewhere. In short, double-

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membrane vesicles form in the cytoplasm and encapsulate the entire organelle and a large number of cytoplasm. Then the autophagosome fuses to the lysosome, where the contents are degraded and recycled. (Edinger and Thompson, 2004), and in this process, some genes such as Atg, LC3, Beclin-1 play a very significant role (He and Klionsky, 2009). Alterations in the autophagy machinery may lead to diverse pathological conditions, such as neurodegeneration, ageing, and cancer (Su et al., 2015).

Recent research indicated that biopolymers, especially polysaccharides and polysaccharide-derived polymers, have great potential in the areas of materials, food and health care (Persin et al., 2011). Polysaccharides control biological functions, regulate cytokine networks, and new understanding of the structure and function of natural polysaccharides will open up new avenues for the application of polysaccharide engineering (Tzianabos, 2000). *Ganoderma atrum* (*G.atrum*) is a popular medicinal mushroom that has been used in Asian countries for the past two thousand years. The long-term use of *G.atrum* is considered to have the functions of maintaining human health and prolonging life (Yu et al., 2014). Recently, in our laboratory one major water-soluble polysaccharide (PSG-1) was extracted and purified from *G. atrum,* which compose of glucose, mannose, galactose and galacturonic acid in a molar ratio of 4.91:1:1.28:0.71, with an average molecular weight of approximately 1013 kDa (Zhang et al., 2012), and exerted various functions such as antitumor (Chen et al., 2008), hypoglycemic (Zhu et al., 2013), cardiovascular protection (Wen-Juan et al., 2010), immunomodulatory (Yu et al., 2015). However, the information is rare about the effect of PSG-1 on the harmful activity caused by acrolein. Therefore, this study aimed to investigate the protective effect of PSG-1 on acrolein-induced macrophage injury, and provide a theoretical basis for reducing the clinical side effects of cyclophosphamide.

## 2. Materials and methods

### 2.1. Materials and reagents

Acrolein was purchased from Shandong Xiya Chemical Industry Co., Ltd. (Shandong, China). DMEM high glucose medium was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China), and the fetal bovine serum (FBS) was provided from Biological Industries (Karmiel, Israel). Cell Counting Kit-8 Assay Kit (CCK-8) was from Dongren Chemical Technology Co., Ltd. (Shanghai, China). Annexin V-FITC apoptosis detection kits, 2′7′-dichlorofluorescein diacetate (DCFH-DA) and the JC-1 assay kit, cell lysis buffer for Western and IP, Enhanced BCA Protein Assay Kit and Beyo ECL Star Assay Kit were all purchased from Beyotime Biotechnology (Shanghai, China). Antibodies to Cyt-C, Bcl-2, caspase-3, caspase-9, LC3, Beciln-1, Atg7, Atg5, p-mTOR were purchased from CST (Massachusetts, America), and Anti-β-actin and the horseradish peroxidase linked secondary antibody were from ZSGB Biotechnology (Beijing, China). 3-Methyladenine (3-MA) was purchased MedChemExpress (State of New Jersey, USA).

### 2.2. Cell culture

RAW264.7 cells were cultured in high glucose DMEM medium supplemented with 10% FBS at 37 °C in a 5% $CO_2$ humidified incubator. According to the growth of the cells, change the liquid once every other day and pass for 2–3 days.

### 2.3. Cell viability

Cells were seeded in 96-well plates at a density of $5 \times 10^4$ cells per well for 4 h. After being treated with different concentrations of acrolein for the indicated time periods, the medium containing 10% CCK-8 was added to the cells, and it was placed in an incubator for further 1–4 h. Finally, the optical absorbance was read at 450 nm using a Microplate Reader.

### 2.4. Apoptosis assay

Wash the cells twice with PBS, then collected 1–5 × $10^5$ cells by centrifugation at 2000 rpm for 5 min; 500 μL of Binding Buffer was added to suspend the cells; add 5 μL of Annexin V-FITC and mix, add 5 μL of PI solutions and mix. Then the cells were incubated for 5–15 min in the dark, and the flow cytometry was observed and detected within 1 h.

### 2.5. Detection of ROS

DCFH-DA was added to the cells at a concentration of 10 μmol, and the cells were cultured in a 37 °C incubator for 20 min. Collect cells after washing three times in the dark, and cells illuminated with green excitation light were measured with flow cytometry.

### 2.6. Detection of mitochondrial membrane potential (MMP)

The cells were resuspended with 500 μL of cell culture medium after collected, then being added 500 μL JC-1 staining solution and mixed thoroughly. The cells were cultured in a 37 °C incubator for 20 min. After staining, the cells were washed twice with JC-1 staining buffer and analyzed by flow cytometry.

### 2.7. Western-blot analysis

Cells ($3 \times 10^6$/well) were plated in 6-well plate, after treatment with acrolein and PSG-1, cells were harvested and resuspended in ice-cold cell lysis buffer, and the homogenate was centrifuged at 10,000 g for 10 min at 4 °C. The total supernatant protein concentration was measured using BCA protein assay kit. The loading quantity of protein samples were 20–40 μg. SDS–PAGE (5%–12%) electrophoresis and electrotransfers were performed routinely. The antibodies and dilutions used were: LC3B, Beclin-1, Atg7, Atg5, Bcl-2, caspase-3, caspase-9, Cyt-C, β-actin, (1:1000). Secondary anti-rabbit IgG (1:10000) and anti-mouse IgG (1:10000) antibodies were used to tag the proteins, and a chemiluminescence (ECL) kit was used for detection.

### 2.8. Pathway inhibitor assay

Acrolein-induced cells were pretreated with autophagy inhibitor 3-MA for 1 h, cell lysates were extracted, and the expression of autophagy and apoptosis proteins were detected by western-blot method to investigate the interaction between autophagy and apoptotic pathway.

### 2.9. Statistical analysis

Data were presented as mean ± standard deviation and analyzed using Graphpad prism 6.01 statistical software (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA). Results were performed using a one-way analysis of variance. There was a significant difference of data between different groups, when p-value was below 0.05 and 0.01.

## 3. Results

### 3.1. PSG-1 elevated cell viability exposed to acrolein in a dose-dependent manner

According to the curve of macrophage viability determined by CCK-8 analysis (Fig. 1A), acrolein inhibited the proliferation of macrophage, and its inhibition is dose-dependent. Cell viability decreased as the concentration of acrolein increased. Moreover, when acrolein concentration was 25 μM, its inhibitory ability to cells was about 50%.

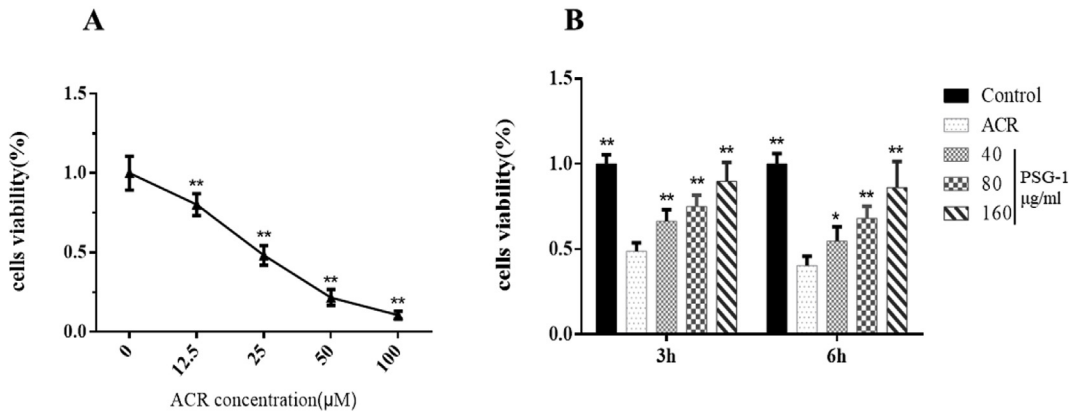


**Fig. 1.** PSG-1 elevated cell viability exposed to acrolein in a dose-dependent manner. Cell viability was measured with CCK-8 assay. (A) Effect of acrolein on macrophage viability. (B) Effect of PSG-1 on acrolein-induced macrophage viability. *P < 0.05, **P < 0.01 compared to acrolein group.

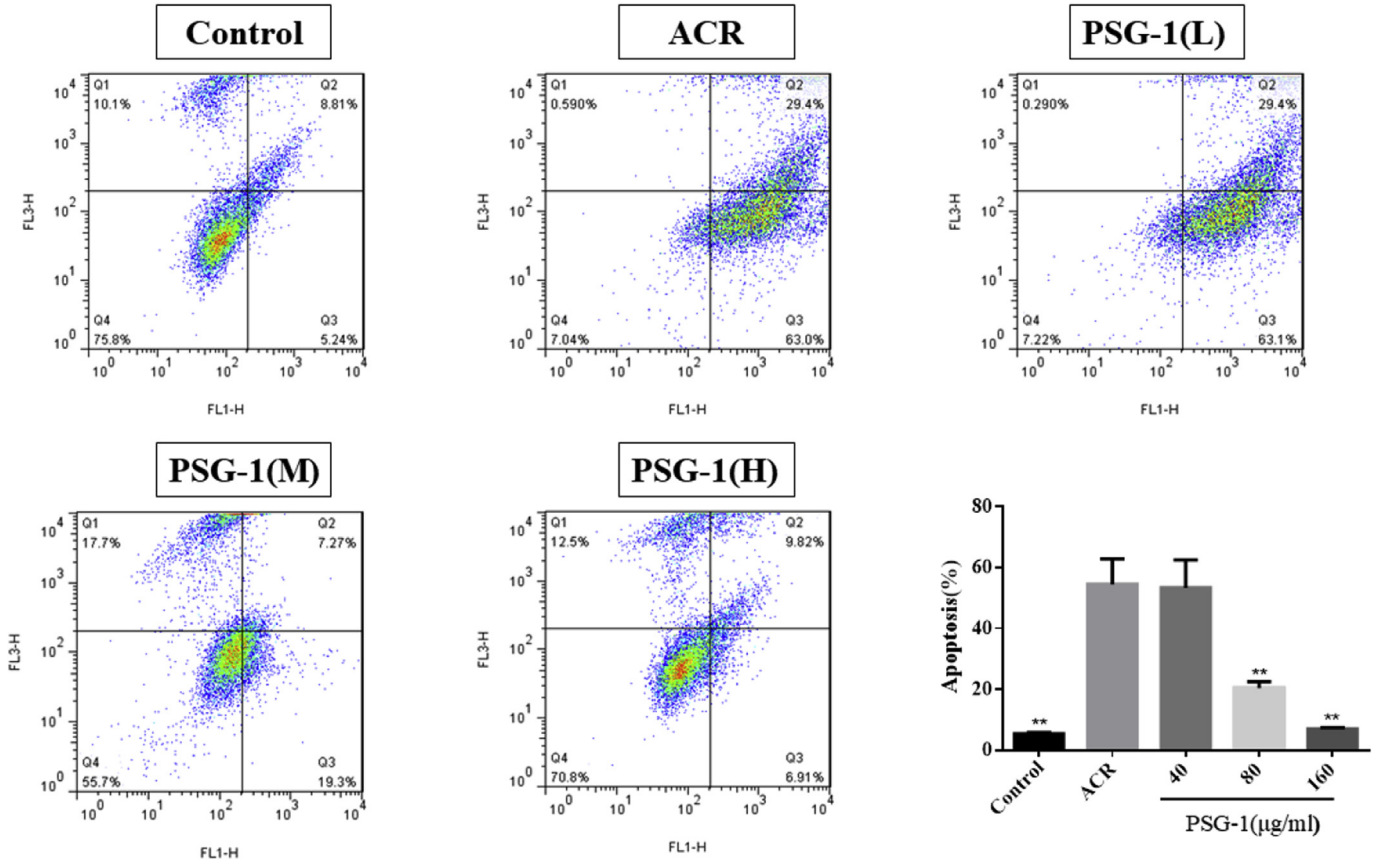


**Fig. 2.** PSG-1 attenuated acrolein-induced apoptosis. Apoptosis measured by flow cytometry with AnnexinV-FITC and PI. *P < 0.05, **P < 0.01 compared to acrolein group.

Therefore, in the following experiments, a concentration of 25 μM was selected as the optimal dose of acrolein. 24 h was taken as the optimal action time (data not shown).

Fig. 1B showed the effects of different concentrations of PSG-1 at 3 h and 6 h on acrolein-induced cell viability decline. Fig. 1B displayed that at the time of 3 h, the toxicity of acrolein on macrophage was obviously prevented by PSG-1 treatment, and the inhibition was dose-dependent, especially at a dose of 160 μg/mL, it can almost restore cell viability to normal levels. Besides, it also can be seen from Fig. 1B that there is a dose-dependent positive correlation between PSG-1 concentration and viability of macrophage exposed to acrolein at the time of 6 h. Since there was no significant difference in the effect on the cell viability of 3 h and 6 h, we chose 3 h as the optimal action time of PSG-1.

### 3.2. PSG-1 reduced acrolein-induced apoptosis

Apoptosis was measured by Annexin V and PI double staining. Fig. 2 exhibited a significant increase of apoptosis in acrolein-induced cells compared with the vehicle treated cells. However, when cells were pretreated with different concentrations of PSG-1 for 3 h and then subjected to acrolein treatment, the number of apoptotic cells decreased. The inhibitory effect of PSG-1 on acrolein-induced macrophage apoptosis was revealed.

### 3.3. PSG-1 reduced acrolein-induced ROS elevation

ROS were detected by fluorescence probe DCFH-DA. As can be seen

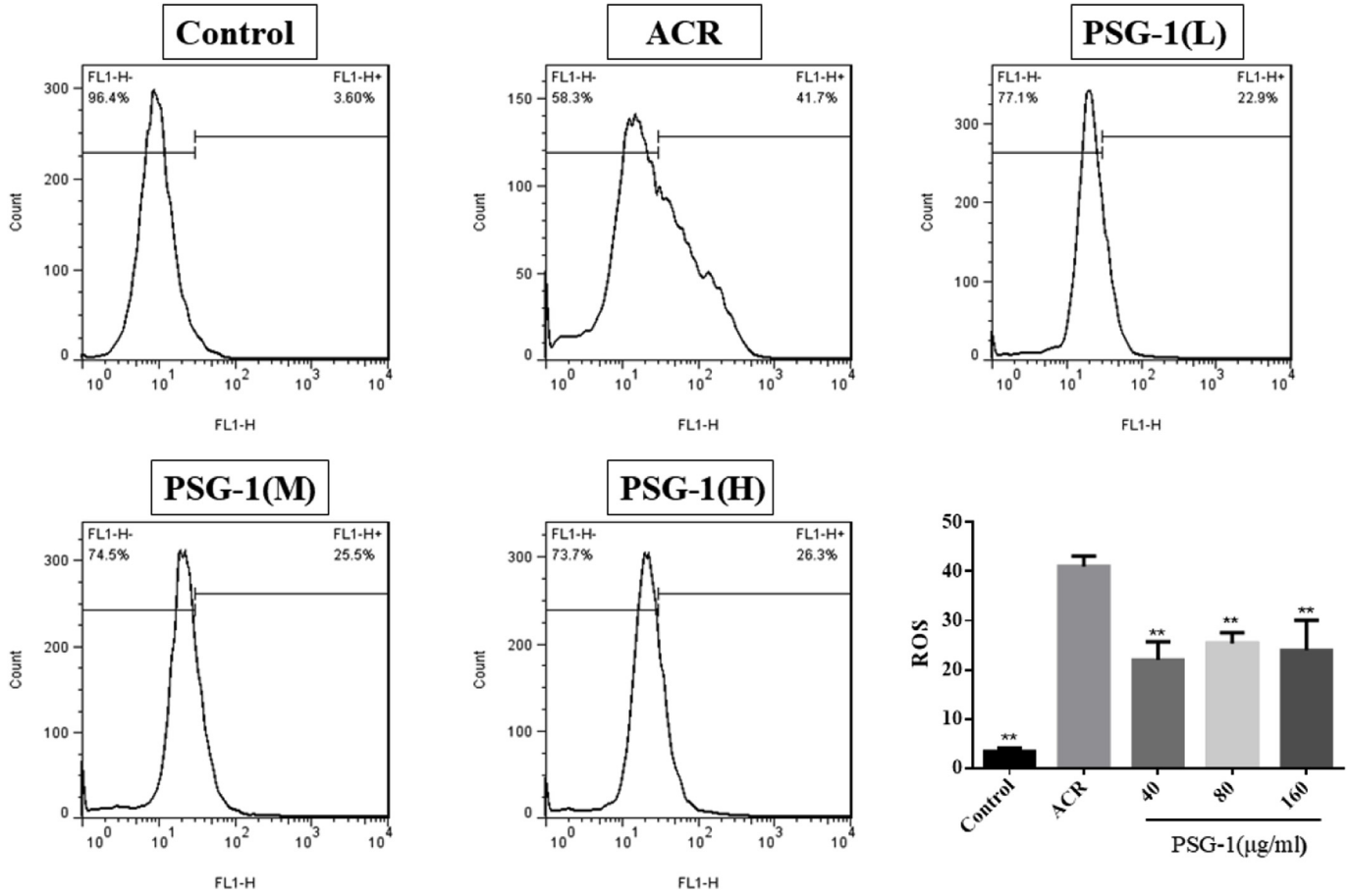


**Fig. 3.** PSG-1 attenuated acrolein-induced apoptosis. ROS content was measured by flow cytometry with DCFH-DA. *P < 0.05, **P < 0.01 compared to acrolein group.

from Fig. 3, the ROS content in cells induced by acrolein was significantly higher than that in the control group. However, after treatment with different concentrations of PSG-1 and acrolein, the ROS content of cells was significantly lower than the acrolein group, indicated that PSG-1 can reduce acrolein-induced macrophage ROS increase.

### 3.4. Effects of PSG-1 on acrolein-induced MMP in macrophage

The change of mitochondrial membrane potential was detected by JC-1 fluorescent probe. As shown in Fig. 4, the amount of JC-1 aggregates decreased significantly in acrolein-induced cells, while the amount of JC-1 monomer increased significantly, indicated that mitochondrial permeability is damaged. However, the loss of MMP was significantly blocked by PSG-1 pretreatment.

### 3.5. PSG-1 regulated the expression of apoptosis-related proteins in acrolein-induced macrophage

As shown in Fig. 5, it can be seen that acrolein significantly reduced the expression of anti-apoptotic protein Bcl-2, and significantly promoted the expression of three positive correlated proteins Cyt-C, caspase-3 and caspase-9, suggesting that acrolein induced apoptosis. However, the PSG-1 treatment group successfully inhibited acrolein-induced apoptosis, which was manifested by the increase of Bcl-2 protein content and the decrease of Cyt-C, caspase-3 and caspase-9 protein, especially at a dose of 160 μg/mL, the protein expression level of almost consistent with the normal group.

### 3.6. PSG-1 weakened acrolein induced autophagy

Fig. 6 revealed that the content of Beclin 1 in acrolein induced cells was significantly higher than that in vehicle treated cells, and the degree of LC3I transform into LC3II in the acrolein treatment group was significantly enhanced compared with control group. By contrast, the expression of Beclin 1 and the ratio of LC3II/I in the PSG-1 treatment group were significantly lower than those in the acrolein treatment group. Moreover, the PSG-1 treatment group also successfully inhibited the overexpression of two other autophagy-related proteins Atg5 and Atg7 caused by acrolein.

### 3.7. PSG-1 activated the mTOR pathway inhibited by acrolein

The expression of the m-TOR pathway-associated protein was also examined. As can be seen from Fig. 7, the expression of p-mTOR in acrolein-induced cells was significantly impaired as compared with the control group. And when the cells were pretreated with different concentrations of PSG-1, the expression level of p-mTOR was significantly elevated, and the effect was in dose-dependent.

### 3.8. The effect of autophagy inhibitor on the apoptosis

You can see in Fig. 8 that treatment of cells with autophagy inhibitor significantly reduced the increase in autophagy-associated protein LC3II induced by acrolein. In addition, to investigate the effect of autophagy inhibitor on the apoptosis, the expression of apoptosis-related protein Cyt-C was measured by western-blot, the result proved that autophagy inhibitor could reduce the overexpression of apoptosis-

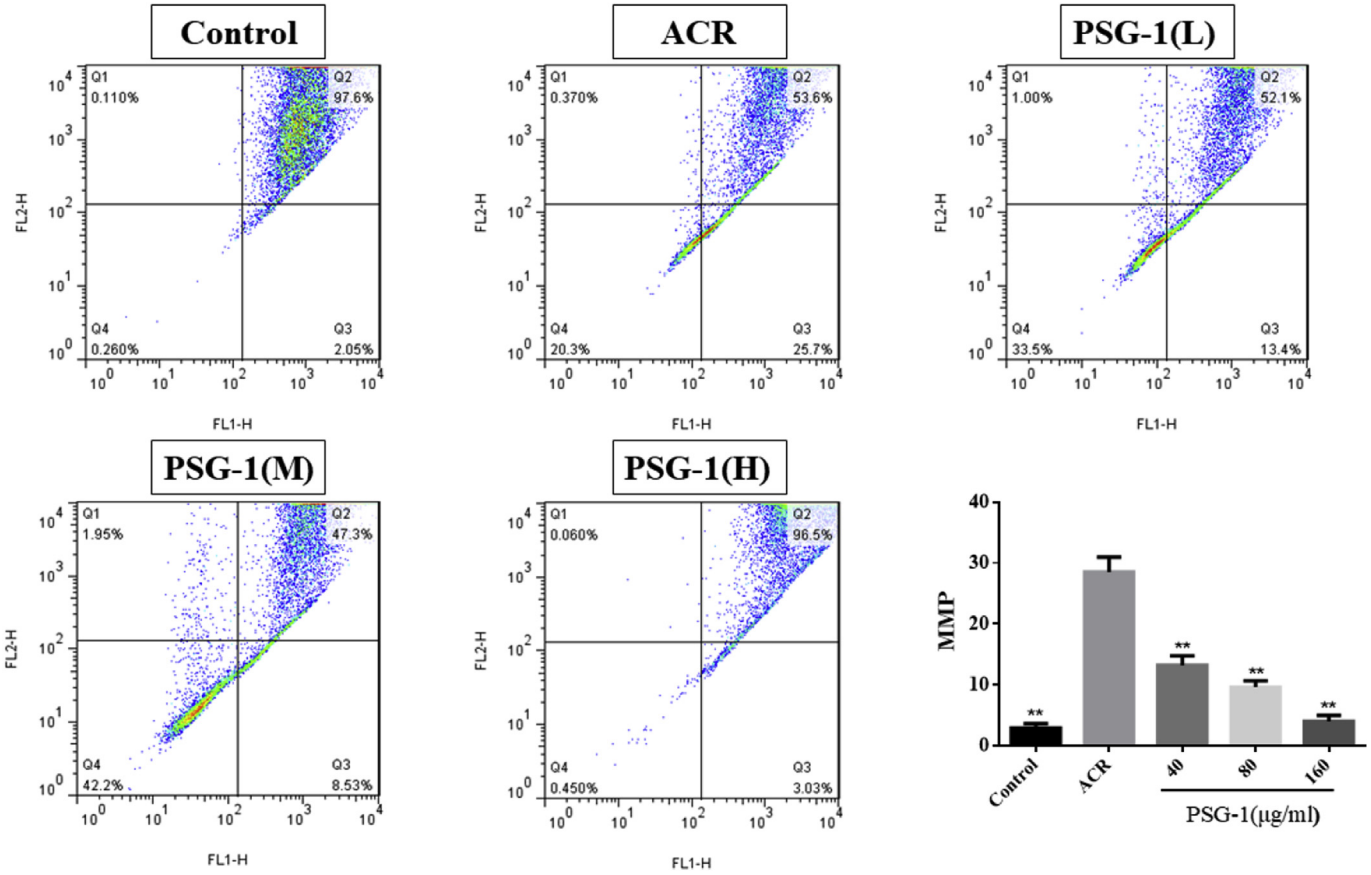


**Fig. 4.** PSG-1 reduced the decrease of MMP induced by acrolein. Flow cytometric analysis of MMP as determined by JC-1staining. FL1-H, green; FL2-H, red. *P < 0.05, **P < 0.01 compared to acrolein group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

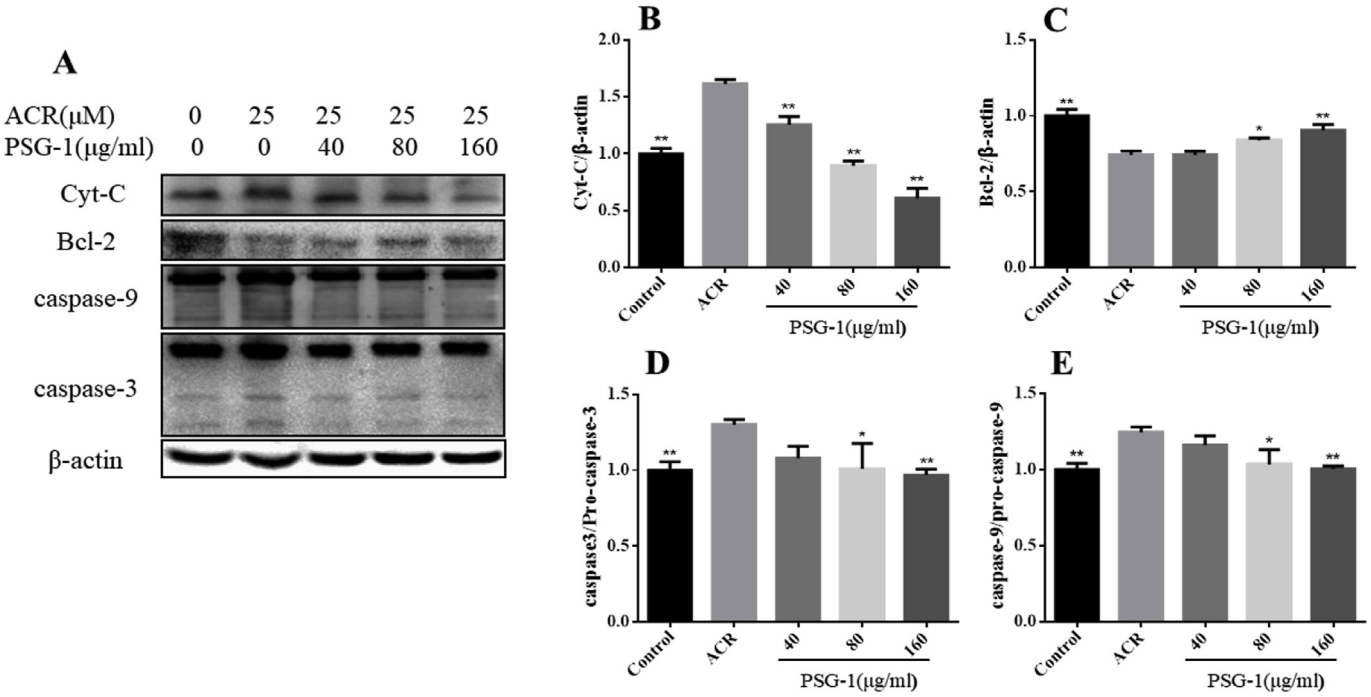


**Fig. 5.** PSG-1 regulated the expression of apoptosis-related proteins in acrolein-induced macrophage. Apoptosis-associated proteins, caspase-9, caspase-3, Cyt-C, Bcl-2 were detected by western-blot. (A) The protein levels of caspase-9, caspase-3, Cyt-C, Bcl-2. (B–E) The relative intensities of caspase-9, caspase-3, Cyt-C, Bcl-2. *P < 0.05, **P < 0.01 compared to acrolein group.

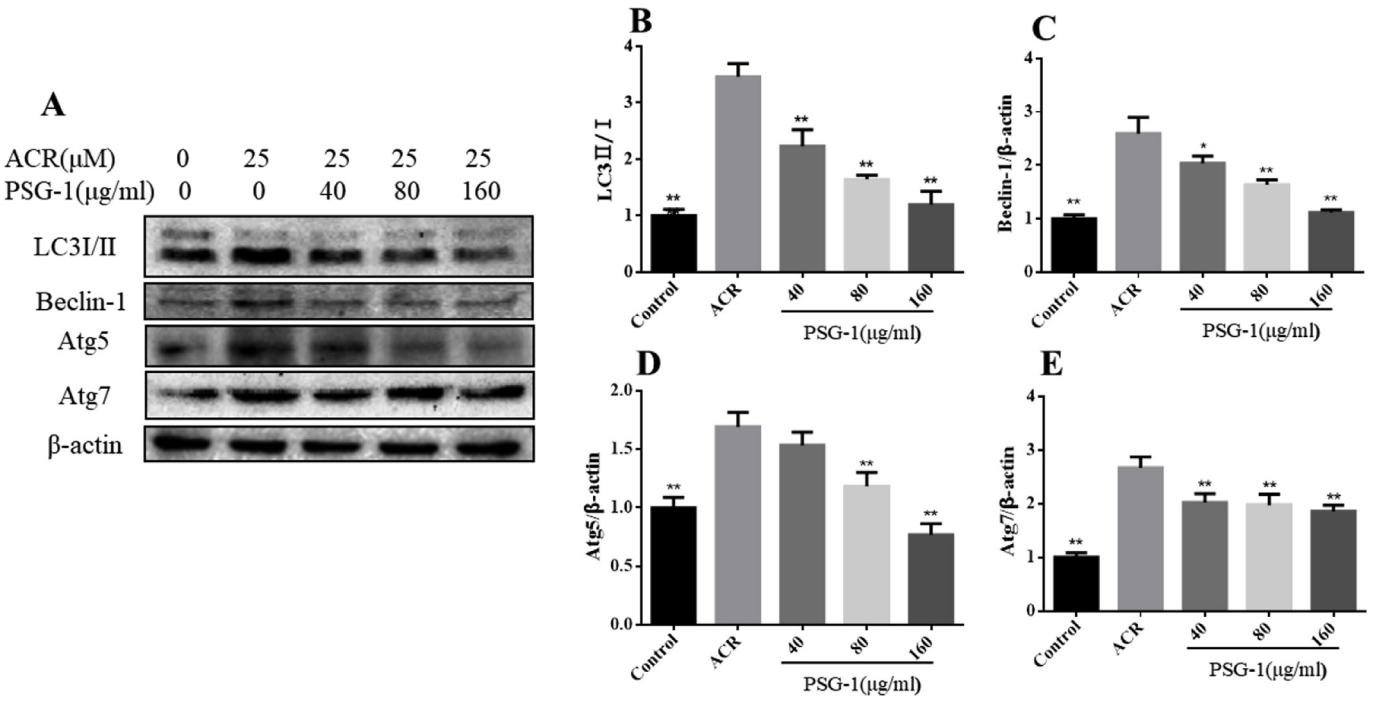


**Fig. 6.** PSG-1 inhibited autophagy through inhibiting the expression of related proteins. Autophagy-associated proteins, Beclin-1, LC3, Atg5, Atg7 were detected by western-blot. (A)The protein levels of Beclin-1, LC3, Atg5, Atg7. (B–E) The relative intensities of Beclin-1, LC3, Atg5, Atg7. *P < 0.05, **P < 0.01 compared to acrolein group.

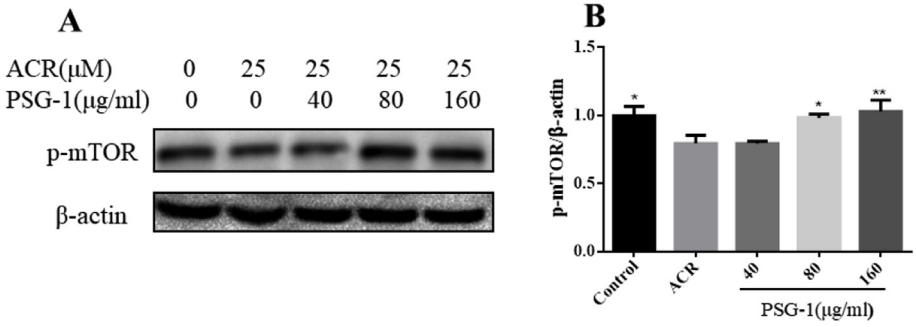


**Fig. 7.** PSG-1 activated the mTOR pathway inhibited by acrolein. MTOR-associated protein, p-mTOR was detected by western-blot. (A) The protein level of P-mTOR. (B) The relative intensities of p-m-TOR. *P < 0.05, **P < 0.01 compared to acrolein group.

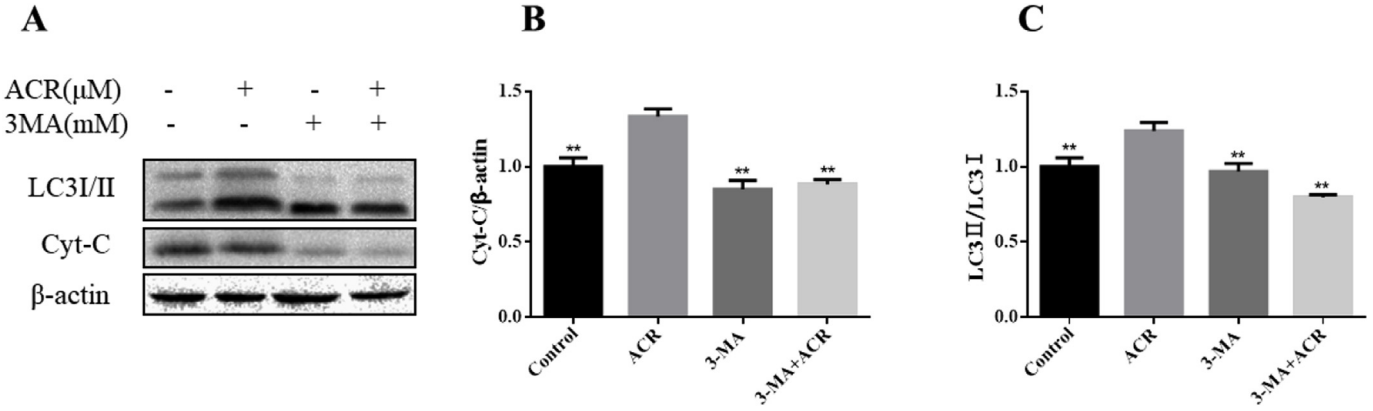


**Fig. 8.** Autophagy inhibitor attenuated apoptosis. LC3, Cyt-C were detected by western-blot. (A)The protein levels of LC3, Cyt-C. (B–C) The relative intensities of LC3 and Cyt-C. *P < 0.05, **P < 0.01 compared to acrolein group.

related protein Cyt-C induced by acrolein.

## 4. Discussion

Macrophage has long been considered to be important immune effector cells (Mosser and Edwards, 2008), and it exists in almost all tissues of the body. Macrophage plays a broad role in the maintenance of tissue homeostasis, through the clearance of senescent cells as well as the remodeling and repairing of tissues after inflammation (Siamon and Taylor, 2005). In this study, the results showed that acrolein with a concentration of 25 μM caused injury to the viability of macrophage, confirming that acrolein induced macrophage damage. However, the results indicated that PSG-1 treatment could significantly protect cell injury by acrolein.

As part of the cellular mechanism, apoptosis is a very important phenomenon that occurs under a range of physiological and pathological conditions (Hengartner, 2000). At pathophysiological concentrations, acrolein causes apoptosis in hepatocytes and lung cells (Mohammad et al., 2012; Park et al., 2018). To explore whether apoptosis plays a role in current research, the change of apoptosis rate in macrophage was first detected. The results showed that the rate of apoptosis in acrolein-induced cells was significantly higher than in the normal group. However, the number of apoptotic cells was reduced with PSG-1 pretreatment, suggesting that PSG-1 has a protective effect on acrolein-induced macrophage apoptosis.

To further determine the specific pathway of apoptosis, the relevant indicators of apoptosis were detected. In the apoptotic pathway, ROS acts as a second messenger to exert its pro-apoptotic effect (Liu et al., 2008). The results indicated that acrolein treatment increased ROS generation in macrophage, and the increase was significantly reduced by PSG-1. Mitochondria are the main site of ROS production, and the permeability of mitochondrial membrane is closely related to apoptosis (Li et al., 2015), so flow cytometry was used to detect the change of MMP. The results showed that acrolein treatment could significantly decrease the MMP, however, PSG-1 treatment could increase the MMP to a certain extent. Therefore, it can be preliminarily speculated that acrolein induced apoptosis through mitochondrial pathway.

To further elucidate the detail pathway involved in the apoptosis, we detected the expression of apoptosis-related proteins. Release of Cyt-C from mitochondria to cytosol is acentral step in the initiation of mitochondrial apoptotic pathway (Zhang et al., 2017), so the increase of Cyt-C is regarded as a sign of apoptosis. Consistent with the expected results, the expression of Cyt-C in acrolein-induced cells was significantly higher than that in the control group. Bcl-2 was the first inhibitor of apoptosis to be discovered (Adams and Cory, 2018), so the experiment also detected the expression of Bcl-2. The expression of Bcl-2 in acrolein-induced cells was significantly decreased, and PSG-1 treatment restored its expression. Caspase-9 is the main caspase of the endogenous pathway of apoptosis (Efferth et al., 2007), and caspase-3 is the main caspase in the apoptotic mechanism (Kuo et al., 2018), so the expressions of these two proteins were also detected. As shown in Fig. 5, PSG-1 inhibited the overexpression of cleaved-caspase-3 and cleaved-caspase-9 induced by acrolein. The above data indicated that acrolein could increase ROS generation, Cyt-C release, expression of cleaved-caspase-3 and cleaved-caspase-9, decrease Bcl-2 protein expression and MMP, thereby induce cells apoptosis via the mitochondrial pathway. However, PSG-1 treatment markedly alleviated these changes, suggesting that PSG-1 could prevent macrophage from acrolein-induced apoptosis.

It is well known that autophagy also plays an important role in regulating PCD (Li et al., 2018). To determine whether autophagy was involved in the protection of PSG-1 against acrolein-induced macrophage injury, autophagy was assessed using western-blot. LC3 is a molecular marker of autophagy (Cheng et al., 2018), so we first tested the changes of LC3 protein levels in macrophage, results showed that the ratio of LC3II/LC3I in acrolein-treated cells increased, demonstrating the occurrence of autophagy. Beclin1 is the mammalian orthologue of yeast Atg6, has a central role in autophagy (Kang et al., 2011), the increased expression of Beclin1 was observed after acrolein treatment in Fig. 6. Moreover, the expression of the other two positively related proteins Atg5 (Yuya et al., 2009) and Atg7 (Hara et al., 2006) were also detected, and the results showed that both of these two proteins were increased in acrolein-induced cells. The above results indicated that acrolein induced the occurrence of autophagy in macrophage, which could be significantly inhibited by PSG-1 treatment. Mammalian target of rapamycin (mTOR) is a key regulator of autophagy initiation, and activated mTOR can inhibit the occurrence of autophagy (Joungmok et al., 2011). Given the importance of mTOR in autophagy regulation, we also investigated whether mTOR was involved in acrolein-induced autophagy. The results showed a dramatic decline of content of p-mTOR in acrolein treated cells, and PSG-1 treatment successfully promoted its expression, which proved that PSG-1 could inhibit acrolein-induced autophagy of macrophage by promoting mTOR pathway. Therefore, we inferred that PSG-1 protected acrolein-induced cell injury via mTOR-mediated autophagy.

There is a complex interaction between autophagy and apoptosis (Roos et al., 2015). For example, SGK1 inhibition induced autophagy dependent apoptosis via the mTOR-Foxo3a(Liu et al., 2017) and Sepia esculenta ink protected mouse Leydig cells by inhibiting acrolein-induced autophagy and apoptosis (Gu et al., 2017). In the present study, pathway inhibitor assay was used to investigate the interaction between acrolein-induced autophagy and apoptosis. It was found that in autophagy inhibitor-treated cells, the expression of autophagy-related proteins was decreased. Moreover, the expression of key apoptotic proteins (Cyt-C) was inhibited by autophagy inhibitor, indicating that the occurrence of apoptosis may be regulated by autophagy in acrolein-induced macrophage.

## 5. Conclusion

In conclusion, PSG-1 treatment could protect cell injury induced by acrolein in macrophage, in which autophagy-dependent apoptosis pathway played an essential role. Thus, PSG-1 may have potential as a novel agent to alleviate side-effect in the clinics.

## Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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