

Alfalfa saponins plays protective roles in oxidative stress-induced apoptotic cells

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Abstract

Background: As is known, alfalfa saponin can be used as a feed additive in the pig's diet. And the addition of alfalfa saponin to the pig's diet could improve animal antioxidant capacity. However, the mechanism by which alfalfa saponins exerts their antioxidant effects has not been studied. To address this issue, H₂O₂-induced rat intestinal epithelial cell was used to explore the protective mechanism of alfalfa saponins in this study. Results: Alfalfa saponin could rescue the cell proliferation activity, elevate the amount of antioxidant enzymes and downregulate the release of MDA and LDH in H₂O₂-induced cells. The results indicated that the antioxidant activity of alfalfa saponin was achieved by restoring GSH homeostasis. Further results demonstrated that alfalfa saponin could inhibit cell apoptosis through activating MAPK signaling pathway. Conclusions: The mechanism by which alfalfa saponins exerts their antioxidant effects was elucidated. Therefore, alfalfa saponin could function as cellular oxidative damage inhibitor, green feed additive or potential drug candidate, providing new strategy for inhibiting cell apoptosis induced by oxidative stress in monogastric animals.

Introduction

Reactive oxygen species (ROS) are normally generated in metabolic processes in all organisms[1, 2]. In general, a small amount of free radicals are constantly produced, which are also reduced or removed in time. But when this balance is disturbed by excessive ROS, oxidative stress would occur. Oxidative stress could result in cell damage, which is often reflected by decreased amount of antioxidant enzymes, increased amount of malondialdehyde (MDA) or lactate dehydrogenase (LDH), and even cell apoptosis[3–5]. At present, early weaning of piglets is one of the main technical means to improve the breeding environment of sows and elevate the utilization efficiency of pigsty. However, due to the physiological developmental characteristics of piglets and the combined effects of environmental, nutritional and psychological factors, oxidative stress is a common state of weaned piglets and one of the major stresses that cause economic losses in piglet production[6, 7].

In recent decades, the growing evidence of the presence of compounds with antioxidant properties in the plant extracts have been studied to reduce or prevent the extent of oxidative stress in animals. *Melissa officinalis* in Brazil showed effective antioxidant effects on prevention of various neurological diseases associated with oxidative stress in rat[8]. And the flower extract of *Etligeria elatior* has powerful antioxidant effects against lead-induced oxidative stress[9]. Furthermore, increasing attention has been paid to saponins on defending against oxidative stress. Notoginsenoside R1 could rescue cell damage in neurons by inhibiting reactive oxygen species and modulating MAPK activation[10]. Ginsenoside Rg1 plays protective roles in hydrogen peroxide-induced injury in human neuroblastoma cells[11]. Alfalfa saponins, extract of Alfalfa (*Medicago sativa*), are naturally bioactive compounds, which consist of a sugar moiety glycosidically linked to a hydrophobic aglycone (sapogenin), mainly pentacyclic triterpenoid[12]. Our previous study has reported that alfalfa saponins was found to be a potential source of natural antioxidants due to their marked antioxidant activity[13]. However, the mechanism of anti-

oxidation of alfalfa saponins in monogastric animals, especially in the intestinal epithelial cells in vitro, has not been investigated.

To explore the antioxidant effects of alfalfa saponins on monogastric animal epithelial cells, rat intestinal epithelial cells (IEC-6 cell line) were used as experimental materials, and H₂O₂ was exploited as a stressor to establish the oxidative stress model. In order to reveal the protective effects of alfalfa saponins on rat intestinal epithelial cells in oxidative stress, the influence of alfalfa saponins on the cell proliferation activity and anti-oxidation of IEC-6 were explored. Furthermore, the mechanism of alfalfa saponin on IEC-6 cell apoptosis under oxidative stress was investigated, providing a new method for improving the antioxidant stress resistance of intestinal epithelial cells.

Materials And Methods

Cell culture

Rat intestinal epithelial cells (IEC-6 cell line) were purchased from BeNa Culture Collection, Beijing, China. IEC-6 cells were cultured in RPMI-1640 (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂.

The establishment of oxidative stress model

To select the optimal concentration of H₂O₂, cells (1.2×10^4 /well) were plated onto a 96-well plate. After culture for 24 h, the cells treated with 0, 75, 100, 150, 200 and 300 μmol/L H₂O₂, respectively. After treated with H₂O₂ for 24 h, the cell medium was discarded. Cell proliferation activity was monitored using MTT Solution Cell Proliferation Assay Kit (Solarbio, Beijing, China) according to the manufacturer's protocol. The cell proliferation rate (absorbance at 490 nm) was examined in an iMARK™ microplate reader (Bio-Rad, USA).

To select the optimal time of H₂O₂, cells (1.2×10^4 /well) were plated onto a 96-well plate. After culture for 24 h, the cells treated with 150 μmol/L H₂O₂ for 0, 3, 6, 12, 24 and 48 h, respectively. After treated with H₂O₂ for 24 h, the cell medium was discarded. Cell proliferation activity was monitored as described above.

The determination of alfalfa saponins concentration in IEC-6 cells

To explore the appropriate concentration of alfalfa saponins in IEC-6 cells, cells (1.2×10^4 /well) were plated onto a 96-well plate. After culture for 24 h, the cells treated with 0, 25, 50, 100, 200, 400 and 800 μg/mL alfalfa saponins, respectively. After treated with alfalfa saponins for 24 h, the cell

proliferation activity was monitored using MTT Solution Cell Proliferation Assay Kit (Solarbio, Beijing, China) according to the manufacturer's protocol.

Cell proliferation assay

Cells (1.2×10^4 /well) were plated onto a 96-well plate. After culture for 24 h, the cells were dealt with different treatments. The control group (negative control) was without any treatment. The cells in groups of 25 $\mu\text{g}/\text{mL}$ alfalfa saponins and 40 $\mu\text{g}/\text{mL}$ alfalfa saponins were treated with 25 and 40 $\mu\text{g}/\text{mL}$ alfalfa saponins, respectively. The cells treated with 150 $\mu\text{mol}/\text{L}$ H_2O_2 was named as H_2O_2 while cells pre-incubated with 25 or 40 $\mu\text{g}/\text{mL}$ alfalfa saponins and subsequently induced by H_2O_2 were separated into groups of 25 $\mu\text{g}/\text{mL}$ alfalfa saponins + H_2O_2 or 40 $\mu\text{g}/\text{mL}$ alfalfa saponins + H_2O_2 . All groups were treated for 24 h, and then, the cell proliferation activity was monitored using MTT Solution Cell Proliferation Assay Kit (Solarbio, Beijing, China) according to the manufacturer's protocol.

Detection of cellular antioxidant enzymes, MDA and GSSG/GSH

Cells (3×10^5 /well) were plated onto a 6-well plate. After culture for 24 h, the cells were dealt with different treatments as described above. 24 h later, the cells were collected and lysed with a sonicator to prepare a cell suspension. After centrifuge for 10 min at 1000 rpm/min, the cell supernatant were examined by T-AOC, GSH-PX, CAT, MDA and GSH/GSSG kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

LDH detection assay

Cells (1.2×10^4 /well) were plated onto a 96-well plate. After culture for 24 h, the cells were dealt with different treatments as described above. After cells were treated for 24 h, 120 μL cell supernatant in different groups was collected and plated onto a new 96-well plate to detect the amount of LDH using LDH Cytotoxicity Assay Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol.

Annexin V assay of apoptosis

Apoptosis assay of IEC-6 cells by use of annexin V (Invitrogen) was performed according to the manufacturer's protocol. Briefly, cells ($5 \cdot 10^4$ /well) were plated onto a 24-well plate. After culture for 24 h, the cells were dealt with different treatments as described above. 24 h later, cells were washed twice in cold PBS. The cells were resuspended in 195 μL Annexin V-FITC binding buffer, followed by the addition

of 5 μ L Annexin V-FITC and 10 μ L propidium iodide (PI). The cells were incubated for 20 min in the dark, and then analyzed by fluorescent microscope.

Western blot analysis

Western blot analysis was performed by SDS-PAGE. Before proteins transferred to a nitrocellulose membrane (Bio-Rad, USA), the membrane was blocked with 5% milk at 4 °C overnight. The membrane was incubated with a primary antibody for 2 h and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma-Aldrich, USA) for 2 h at room temperature. Proteins were detected using Western Lightning™ Plus-ECL Oxidizing Reagent Plus (Perkin Elmer, USA). The antibody of Bcl-2 (26 KD), Caspase-3 (32/17 KD), Caspase-3 (32/17 KD), Caspase-9 (46 KD), Bax (21 KD) were purchased from PROTEINTECH GROUP, USA. The antibody of p38 (43 KD), p-p38 (thr180/tyr182, 43 KD), ERK (44/42 KD), p-ERK (thr 202/tyr 204, 44/42 KD), JNK (45/53 KD), p-JNK (thr183/tyr185, 46/54 KD) were purchased from Cell Signaling Technology, USA. The antibody of GAPDH (37 KD) was purchased from TransGen Biotech, Beijing, China.

Statistical analysis

The mean and standard deviations for triplicate assays were calculated by one-way analysis of variance (ANOVA). The statistical significance between different treatments was determined using Student's t test.

Results

Establishment of oxidative stress model

To establish an oxidative stress model, the appropriate time and concentration for H₂O₂ to act on the cells was first determined. To investigate the appropriate time for H₂O₂ to treat cells, the cell proliferation rate was analyzed after IEC-6 cells were treated with 150 μ mol/L H₂O₂ for different time course (0, 3, 6, 12, 24 and 48 h). The result demonstrated that the cell proliferation rate showed no significant difference in groups of cells without H₂O₂ treatment ($P \geq 0.05$, Fig. 1A). But the survival rate of IEC-6 cells significantly reduced when compared with that in cells treated with same time course (0, 3, 6, 12, 24 and 48 h), reducing to 81%, 71%, 62%, 51% and 33%, respectively ($P \leq 0.01$, Fig. 1A). As the survival rate of IEC-6 cells was 51% when cells treated for 24 h, 24 h was selected as the appropriate time for H₂O₂ to act on the cells of this experiment. To determine the appropriate concentration for H₂O₂ to stimulate oxidative stress, 6 concentration gradients (0, 75, 100, 150, 200 and 300 μ mol/L) were set to treat IEC-6 cells. The cell survival rate decreased significantly after cells were treated 75, 100, 150, 200 and 300 μ mol/L for 24 h, reducing to 76%, 61%, 53%, 41% and 39%, respectively (Fig. 1B). From the above results, the median lethal concentration of H₂O₂ on IEC-6 cells was 150 μ mol/L. Therefore, 150 μ mol/L H₂O₂ was selected for oxidative stress research in subsequent experiments. According to the results obtained, the oxidative stress model was established by exploiting 150 μ mol/L H₂O₂ treatment for 24 h.

The effects of alfalfa saponins on proliferation rate of IEC-6 cells

To explore the influence of alfalfa saponins on proliferation rate of IEC-6 cells, the survival rate of IEC-6 cells was examined. Different concentration gradients (0, 25, 50, 100, 200, 400, 800 $\mu\text{g}/\text{mL}$) were set to treat IEC-6 cells for 24 h. The result indicated that there was no significant difference in cell viability when cells were treated with 0-400 $\mu\text{g}/\text{mL}$ alfalfa saponins, although the cell viability decreased with increasing concentration ($P > 0.05$, Fig. 2A). However, when IEC-6 cells were treated with 800 $\mu\text{g}/\text{mL}$ alfalfa saponins, the cell activity significantly reduced ($P < 0.01$, Fig. 2A). To ensure that alfalfa saponins were not toxic to IEC-6 cells, the concentration of alfalfa saponins was less than 50 $\mu\text{g}/\text{mL}$ in the subsequent experiments. Thus, the low concentration of alfalfa saponins was 25 $\mu\text{g}/\text{mL}$ and the high concentration of alfalfa saponins was 40 $\mu\text{g}/\text{mL}$ saponins. In order to investigate whether alfalfa saponins could resist the cellular stress caused by H_2O_2 , the cells were pre-incubated with low and high concentration (25 and 40 $\mu\text{g}/\text{mL}$) of alfalfa saponins for 24 h. The alfalfa saponins treated groups showed higher cell viability when compared with control group but without significant difference ($P > 0.05$, Fig. 2B). However, 25 and 40 $\mu\text{g}/\text{mL}$ alfalfa saponins could obviously rescue the cell viability in oxidative stress model. The survival rate of IEC-6 cells increased by 40% and 67%, respectively ($P < 0.01$, Fig. 2B). Thus, the concentration of 25 and 40 $\mu\text{g}/\text{mL}$ alfalfa saponins were used in subsequent experiment.

The anti-oxidant activity of alfalfa saponins in oxidative damage cells

There is a stable dynamic balance of oxidation/reduction in animal. In general, a small amount of free radicals are constantly produced, which are also reduced or removed in time. But when this balance is disturbed by H_2O_2 , a large number of free radicals accumulate in the body, exceeding the capacity of antioxidant defense system, and resulting in cell oxidative damage. To explore the role of alfalfa saponins in cell oxidative damage, the expression level of T-AOC, CAT and GSH-PX were examined. The results demonstrated that alfalfa saponins of 25 and 40 $\mu\text{g}/\text{mL}$ could gradually elevate the amount of T-AOC in cells without H_2O_2 treatment but there was no significance in these two groups of different concentration ($P > 0.05$, Fig. 3A). In oxidative stress model, the cells suffered significant decrease of expression level of T-AOC ($P < 0.05$, Fig. 3A); alfalfa saponins of 25 and 40 $\mu\text{g}/\text{mL}$ could rescue expression level of T-AOC in oxidative stress model but without significance compared with cells with H_2O_2 treatment ($P > 0.05$, Fig. 3A). However, the the enzyme activity of CAT gradually increased ($P > 0.05$) while the enzyme activity of GSH-PX could be significantly increased ($P < 0.05$) by alfalfa saponins of 25 and 40 $\mu\text{g}/\text{mL}$ in cells without H_2O_2 treatment compared with control group (cells without treatment). In oxidative stress model, the the enzyme activity of CAT and GSH-PX decreased significantly but could be rescued by pre-incubation of cells with alfalfa saponins of 25 and 40 $\mu\text{g}/\text{mL}$ (Fig. 3A).

MDA is one kind of lipid peroxide metabolites which is produced through the non-enzymatic system when oxygen free radicals attack the membrane of polyunsaturated fatty acids (PUFA). Therefore, the amount of MDA indirectly reflects the degree of cell damage. Compared with negative control group, the amount of MDA significantly decreased when cells were treated with alfalfa saponin of 25 and 40 $\mu\text{g}/\text{mL}$ ($P < 0.05$). However, the amount of MDA elevated obviously in oxidative stress model while the cells pre-

incubated with alfalfa saponin of 25 and 40 $\mu\text{g}/\text{mL}$ showed less amount of MDA, indicating that alfalfa saponin could protect cells from peroxide (Fig. 3B). Large amount of free radical accumulation can cause lipid peroxidation of polyunsaturated fatty acids in intestinal epithelial cells, resulting in damage of cell membrane and increasing its permeability. LDH is a stable cytosolic enzyme that can be rapidly released into the extracellular plasma once the cell membrane is damaged. Therefore, LDH is often used as one of the indicators of degree of cell damage. In order to investigate the role of alfalfa saponins in protecting cells from damage caused by H_2O_2 , the amount of released LDH was analyzed. Consistent with the above result (Fig. 3B), alfalfa saponins of 25 and 40 $\mu\text{g}/\text{mL}$ had little effects on the release of LDH compared with that of cells without treatment ($P > 0.05$, Fig. 3C). However, H_2O_2 treated cells showed much more release of LDH. The further investigation demonstrated that the pre-incubation of cells with alfalfa saponins with 25 and 40 $\mu\text{g}/\text{mL}$ can significantly reduce the LDH release of H_2O_2 treated cells, reducing by 45% and 56%, suggesting that alfalfa saponin reduced the LDH release dose-dependently ($P < 0.01$, Fig. 3C). Taken together, alfalfa saponins could protect cells from peroxide, reducing cell damage caused by H_2O_2 .

The anti-oxidative effects of alfalfa saponins by restoring GSH homeostasis

To explore the regulatory mechanism of alfalfa saponins on oxidative damage cells, the amount of GSH and GSSG were detected. Compared with the negative control group, the intracellular GSSG concentration and GSSG/GSH ratio in H_2O_2 group were significantly increased ($P < 0.01$, Fig. 4). After 25 and 40 $\mu\text{g}/\text{mL}$ alfalfa saponins pre-treatment, the GSSG content and GSSG/GSH ratio were gradually decreased compared with that in H_2O_2 group, indicating that the antioxidative effects of alfalfa saponins on oxidative damage cells are achieved by restoring GSH homeostasis.

The downstream pathway of H_2O_2 -induced cell apoptosis by alfalfa saponins

To explore the mechanism of alfalfa saponins on H_2O_2 -induced oxidative damage, the cell apoptosis rate was firstly investigated by fluorescence microscope. The result demonstrated that cells treated with alfalfa saponins with 25 and 40 $\mu\text{g}/\text{mL}$ showed no significant difference compared with negative control while cells treated with H_2O_2 presented higher apoptosis rate (Fig. 5A). However, the pre-incubation of cells with alfalfa saponins with 25 and 40 $\mu\text{g}/\text{mL}$ could significantly reduce the cell apoptosis rate (Fig. 5A). It was suggested that the antioxidant effect of alfalfa saponins on H_2O_2 induced oxidative stress in IEC-6 cells may be achieved by inhibiting cell apoptosis. In order to further determine the regulatory mechanism of alfalfa saponins on H_2O_2 -induced IEC-6 cell apoptosis, the expression profile of Caspase-3, Caspase-9, Bax and Bcl-2 were determined by western blot. The results showed that there was no difference of Pro-caspase-3 expression in different groups. However, both 25 and 40 $\mu\text{g}/\text{mL}$ alfalfa saponins reduced the expression of Cleaved caspase-3, caspase-9 and Bax in cells, and increased the expression of Bcl-2 gene. The addition of 40 $\mu\text{g}/\text{mL}$ alfalfa saponins was more effective. After treatment with H_2O_2 , Cleaved caspase-3, caspase-9 and Bax expression levels were significantly increased, and Bcl-2 gene expression was significantly decreased. 25 and 40 $\mu\text{g}/\text{mL}$ alfalfa saponins pre-treatment in

oxidative stress model reduced intracellular Cleaved caspase-3, caspase-9 and Bax, and increased bcl-2 gene expression, suggesting that the mechanism of alfalfa saponins against H₂O₂-induced IEC-6 cell apoptosis may be achieved by up-regulating Bcl-2 and down-regulating the expression of Cleaved caspase-3, caspase-9 and Bax, thereby inhibiting mitochondrial apoptosis (Fig. 5B).

In order to study the signaling pathway of alfalfa saponins on the anti-oxidation protection of IEC-6 cells, the expression of three signaling pathway proteins of MAPK was detected, including ERK1/2, JNK and p38. There was no significant difference in total protein expression of ERK1/2, JNK and p38 (P > 0.05). Compared with the negative control group, the phosphorylation levels of ERK1/2, p38 and JNK in H₂O₂-treated cells significantly increased (P < 0.01), while the phosphorylation levels of 3 proteins in 25 and 40 µg/mL alfalfa saponins pre-incubated groups were effectively reduced (P < 0.01, Fig. 5C). The results showed that alfalfa saponins could inhibit the activation of MAPK signaling pathway in IEC-6 cells induced by H₂O₂, thus protecting IEC-6 cells.

In conclusion (Fig. 5D), alfalfa saponins pre-treatment could achieve antioxidative effects by restoring GSH homeostasis and inhibit mitochondrial apoptosis by MAPK signaling pathway, thereby further alleviating oxidative damage of cells.

Discussion

Alfalfa saponins enhanced antioxidant system

Studies have reported that H₂O₂ can be used to induce cells to establish an oxidative stress model, which is widely applied in the modeling of oxidative stress in intestinal epithelial cells[14, 15]. In general, the content of free radical maintains a dynamic balance in cells. Once this balance is destroyed, it will cause oxidative damage of cells, leading to the content changes of the substrates related to redox. T-AOC can be used to indicate the total antioxidant capacity of organism[16]. GSH-PX is one kind of important antioxidant enzyme[17]. CAT is widely distributed in living organisms, which can promote the decomposition of H₂O₂ and protect cells from free radical damage[18]. MDA is one kind of lipid peroxide metabolites which is produced through the non-enzymatic system when oxygen free radicals attack the membrane of polyunsaturated fatty acids (PUFA)[19]. LDH is a stable cytosolic enzyme that can be rapidly released into the extracellular plasm once the cell membrane is damaged[16]. Therefore, the amount of MDA and LDH indirectly reflects the degree of cell damage[19]. At present, researchers have proved that most saponins have certain antioxidant capacity[10, 11]. In this study, the survival rate of cells, the activities of T-AOC, GSH-PX and CAT were significantly improved by pre-incubating with alfalfa saponins. Moreover, the content of MDA and LDH were significantly lower in oxidative stress model pre-incubating with alfalfa saponins, indicating that alfalfa saponins could increase the antioxidant enzyme activity and can enhance the ability of clearing free radicals.

The antioxidative effects of alfalfa saponins are achieved by restoring GSH homeostasis

The oxidative cytotoxic agent, H₂O₂, typically considered as a cause of oxidative stress always induces cell apoptosis[20, 21]. GSH plays an important role in maintaining the redox homeostasis of organism[22]. Once the cells are damaged by oxidation, GSH participates in the antioxidant reaction to be oxidized to GSSG, resulting in an increase in the ratio of GSSG/GSH. The result of this study demonstrated that the antioxidative effects of alfalfa saponins on oxidative damage in IEC-6 cells may be achieved by increasing the amount of GSH synthesis and reducing the ratio of GSSG/GSH to restore the mechanism of GSH homeostasis.

Alfalfa saponins can inhibit cell apoptosis through MAPK signaling pathway in oxidative stress model

Apoptosis is one of the important outcomes of oxidative damage in cells. Caspase-3 is an executor of cell apoptosis and is directly involved in apoptotic events. Activated Caspase-3 (Cleaved Caspase-3, 17 kD) is activated when cells undergo apoptosis[23]. In addition, anti-apoptotic genes (Bcl-2, Bcl-xL) and pro-apoptotic genes (Bax, Bad, Bid, Bnip3) in the Bcl-2 family are also involved in the regulation of apoptosis[24]. The results of this study showed that alfalfa saponins pre-treatment reduced expression of intracellular Cleaved caspase-3, Caspase-9 and Bax, and increased Bcl-2 expression in oxidative stress model, suggesting that the mechanism of alfalfa saponin against H₂O₂-induced IEC-6 cell apoptosis may be through up-regulation of Bcl-2 and down-regulation of Cleaved caspase-3, Caspase-9 and Bax gene expression. The MAPKs signal transduction pathway is involved in a series of physiological and biochemical reactions (such as cell proliferation, differentiation, metabolism, transformation and apoptosis). MAPKs are mainly composed of three subfamily proteins, ERK1/2, p38 and JNK. Zhou Y *et al.* reported that ERK1/2, JNK and p38 MAPK could be significantly activated when oxidative damage occurs in intestinal epithelial cells[25]. Moreover, the activation of these kinases will further promote the increase expression of proteoglycans, leading to the occurrence of apoptosis[26]. The results of this study found that the degree of phosphorylation of the three proteins was significantly reduced by the addition of alfalfa saponin in oxidative stress model. It is indicated that alfalfa saponins can prevent oxidative damage by inhibiting H₂O₂-induced activation of cell apoptosis, thereby protecting cells.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YHS and YLC designed, conducted and wrote the manuscript. BSL, XS, ZDL, YYC, GZG and HL helped to do the experiment and analyzed the data. DFL, CZW and XYZ analyzed and wrote part of the data.

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Figures

Fig.1

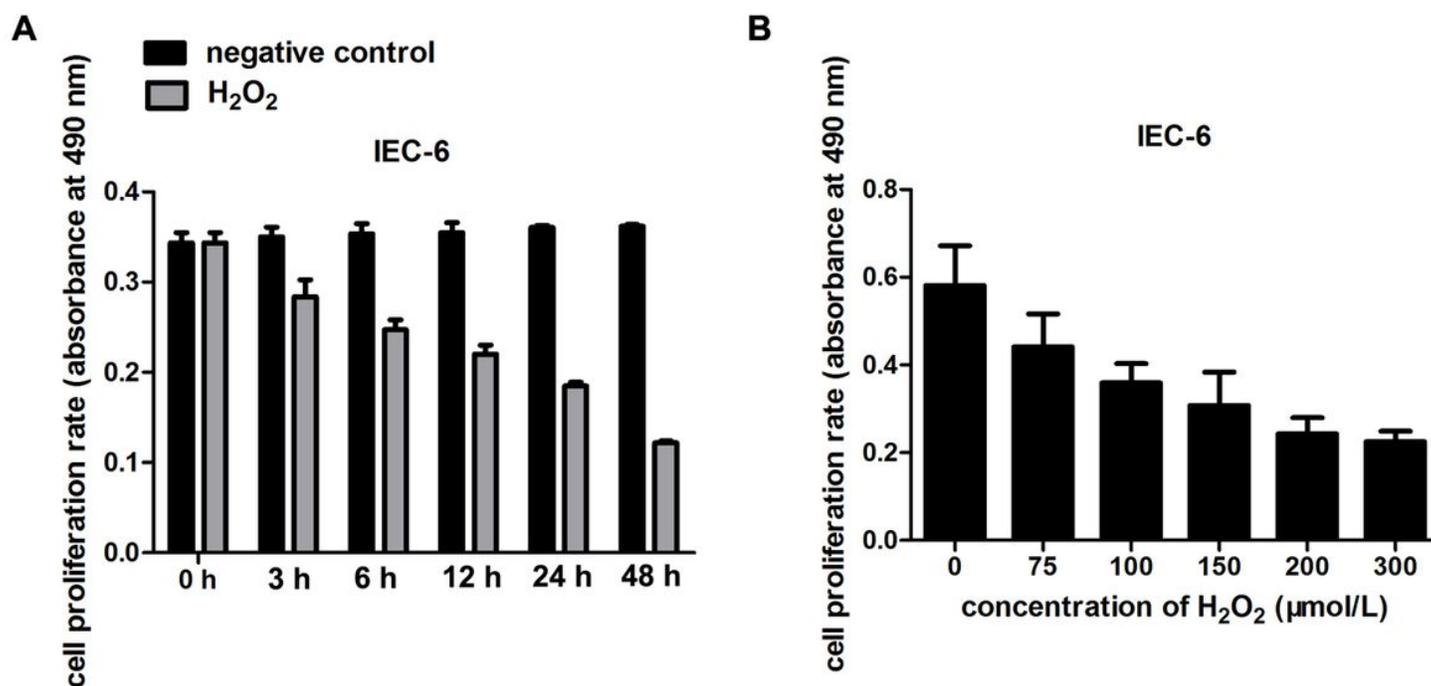


Figure 1

Effects of different H₂O₂ concentrations and treatment time on the activity of IEC-6 cells. (A) Time-dependent effect of H₂O₂ on cell viability in IEC-6 cells. (B) Effects of different concentrations of H₂O₂ on proliferation activity of IEC-6 cells.

Fig.2

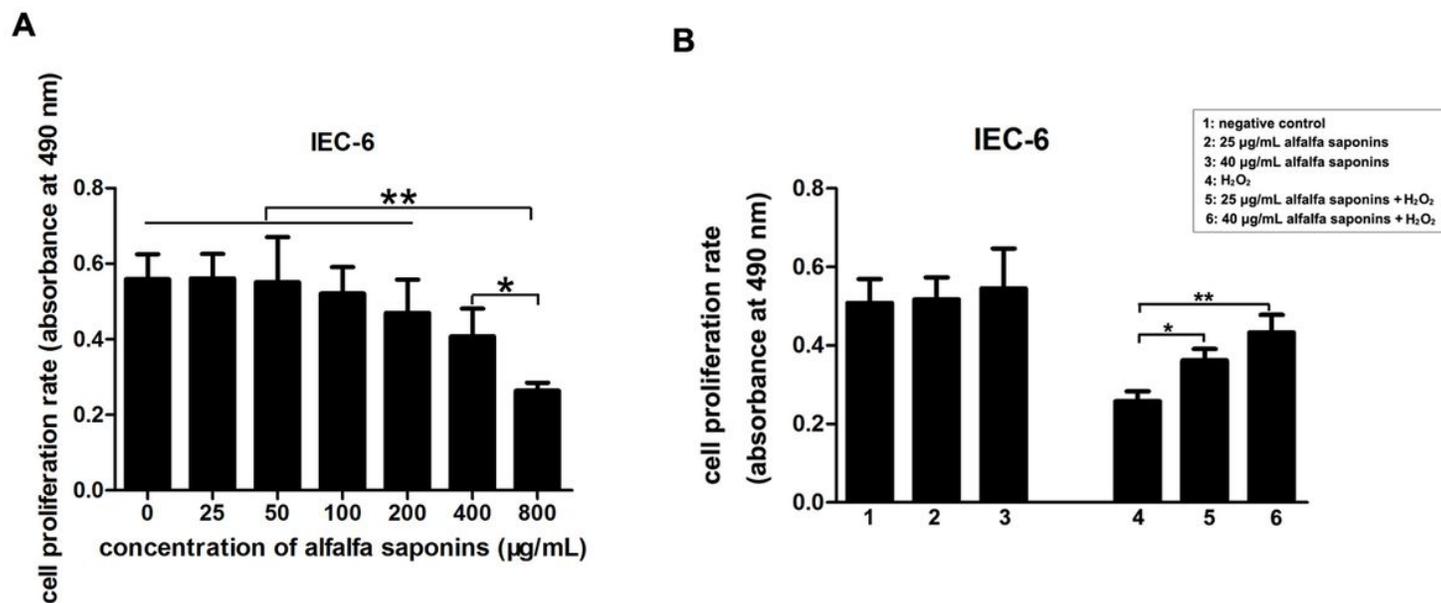
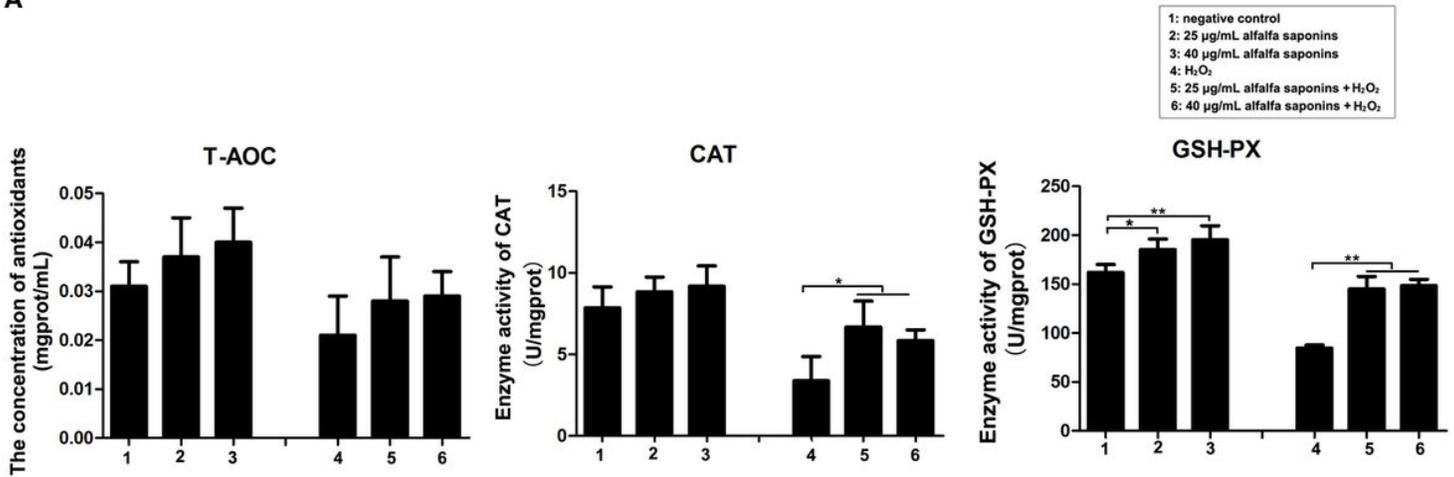


Figure 2

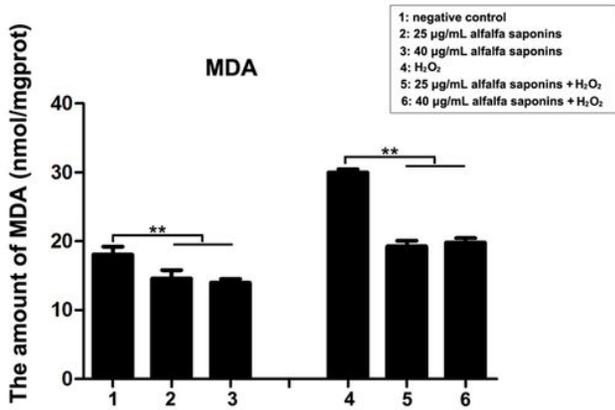
Effects of alfalfa saponins on proliferation activity of IEC-6 cells. (A) Effects of alfalfa saponins on proliferation activity of IEC-6 cells. (B) Influence of alfalfa saponins on IEC-6 cells activity in oxidation stress model. In all panels, statistically significant differences between treatments were represented with asterisks (*, $p < 0.05$; **, $p < 0.01$).

Fig.3

A



B



C

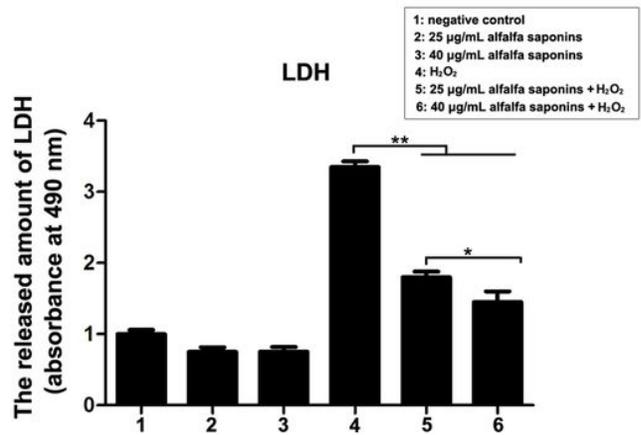


Figure 3

Effects of alfalfa saponins on oxidative stress. (A) Detection of antioxidation enzyme (T-AOC, CAT and GSH-PX) in cells with different treatments. (B) Examination of MDA in IEC-6 cells. (C) Effects of alfalfa saponins against LDH release in H₂O₂ treated cells. In all panels, statistically significant differences between treatments were represented with asterisks (*, $p < 0.05$; **, $p < 0.01$).

Fig.4

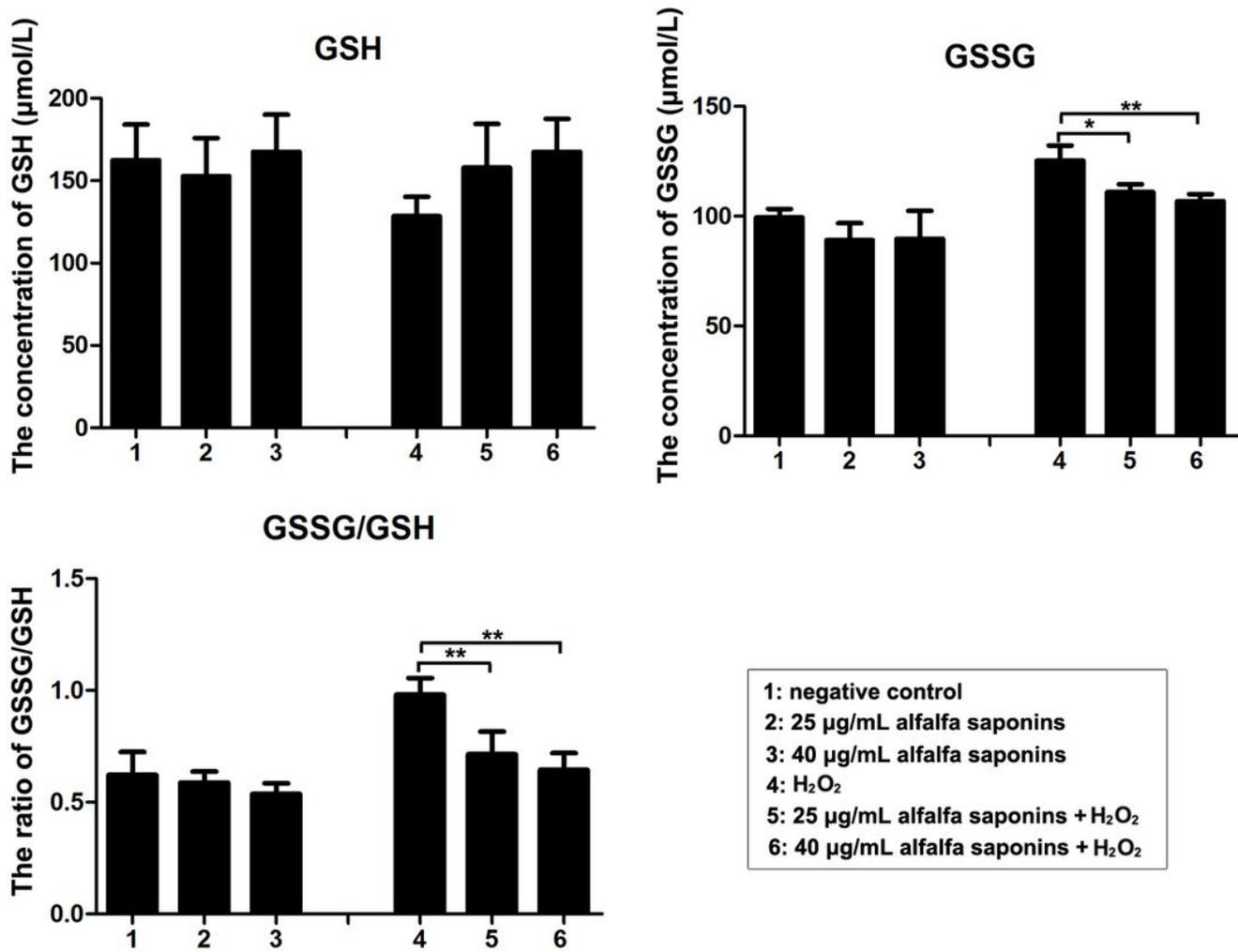


Figure 4

Detection of GSH and GSSG in IEC-6 cells and the ratio of GSSG/GSH was calculated. In all panels, statistically significant differences between treatments were represented with asterisks (*, $p < 0.05$; **, $p < 0.01$).

Fig.5

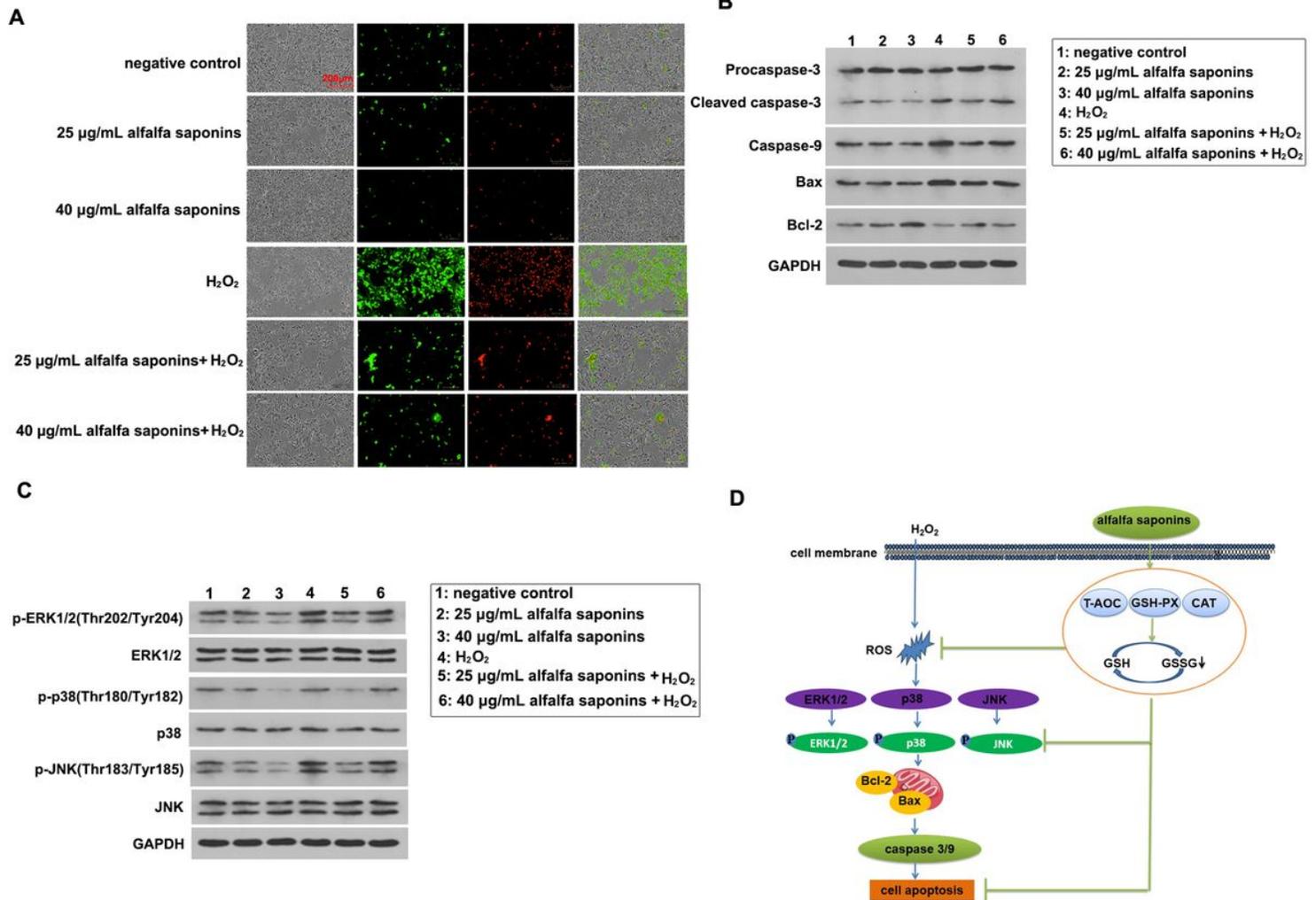


Figure 5

Cell apoptosis analysis of IEC-6 cells. (A) The cells were stained with Annexin V-FITC/PI. Apoptosis rate of IEC-6 cells with different treatments was observed by fluorescence microscope. The green fluorescent stained by Annexin V-FITC presented early apoptotic cells; the red fluorescent stained by PI presented necrotic cells and apoptotic cells in the middle and late stage; cells presented the yellow colour indicated the cells could be stained by Annexin V-FITC and PI. (B) The detection of expression level of apoptotic-related proteins by western blot analysis. (C) Effects of alfalfa saponins on MAPKs pathway in IEC-6 cells. (D) Model for the role of alfalfa saponins in oxidative stress cells.