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Allicin alleviates acrylamide-induced oxidative stress in BRL-3A cells

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ABSTRACT

Aim: Acrylamide (AA) is a common heat-generated toxicant in some food. Inhibiting its formation with natural antioxidants is of great significance. The current study aims to investigate the alleviative effect and the underlying mechanism of allicin against AA-induced oxidative stress in BRL-3A cells.

Main methods: BRL-3A cells were pretreated with allicin at different concentrations for 2 h, followed by AA treatment. Cell viability was determined by Cell Counting kit-8 (CCK-8). Intracellular reactive oxygen species (ROS) status was measured using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) method. Levels of oxidative stress markers were determined by measuring total superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and 8-hydroxy-desoxyguanosine (8-OHdG) using commercial kits. Expression of the mitogen-activated protein kinase (MAPK) pathway-related proteins was determined by Western blotting.

Key findings: Allicin markedly mitigated oxidative and DNA damage by increasing the activities of SOD and GSH-Px and decreasing the levels of ROS and 8-OHdG. Concomitant with these biochemical parameters, pretreatment with allicin reversed the impact of AA on the expression of p-JNK, p-ERK1/2 and p-p38. Allicin combined with SP600125 (JNK inhibitor) and SB202190 (p38 inhibitor) enhanced cell viability in the presence of AA, as opposed to SCH772984 (ERK inhibitor). Notably, allicin ameliorated the expression of KGF, Gadd45a, c-Fos, Dusp5 and Phospholipase A2, which were related to liver injury.

Significance: Collectively, these findings demonstrate that allicin exerts protective effects against AA-induced oxidative stress by modulating the MAPK signaling pathway in BRL-3A cells.

1. Introduction

Acrylamide (AA) is a common endogenous contaminant in cooked food [1]. Due to its toxicity to humans and animals, much attention has been paid over the past few years. AA is formed mainly via the Maillard reaction, with levels depending on precursors in food materials, food composition, processing conditions and parameters [2]. *In vivo* and *in vitro* research has both indicated that AA leads to neurotoxic, genotoxic and cancerogenic effects [3]. AA was classified as “probably carcinogenic to humans” by International Agency for Research on Cancer (IARC) in 1994 [4], and many studies have focused on its formation mechanism, risk assessment, intake exposure and toxicological mechanism [5].

Allicin is a liposoluble and organosulfur compound obtained from garlic. It is converted from alliin by alliinase when fresh garlic is chopped or crushed. Allicin has various beneficial physiological functions, such as antioxidative, antiviral, neuroprotective, anti-inflammatory and anti-fatty liver properties by its bioactive ingredients [6]. Allicin can scavenge oxygen free radicals, which determines its antioxidant capacity [7]. Studies have revealed that allicin defends cells against oxidative stress by inducing the production of antioxidant products [8]. Besides, allicin can exert protective effects on oxidative stress and inflammatory responses *in vitro* and *in vivo* [9]. Our previous research has proposed that allicin has a significant *in vivo* protective effect on damage mediated by AA, while its mechanism against AA-induced cellular oxidative stress is still vague [10]. Furthermore, we found that the MAPK pathway-related genes (e.g. *Fgf7*, *Dusp5*, *Gadd45a*, *Fos*) in BRL-3A cells were significantly up-regulated and *Pla2g4d* was down-regulated when exposed to 2 mM AA by RNA sequencing and real-time PCR. In such a case, we hypothesized that allicin could exert antioxidant activity against AA-induced oxidative stress via the modulation of cellular defense and the MAPK pathway-related key proteins.

Stimulation of cellular signaling cascades eventually result in manifold alterations in the activity of nuclear transcriptional regulators [11]. The MAPK family is one of the vital pathways of eukaryotic signaling transduction, which closely relates to oxidative stress and various cellular processes, including gene expression, proliferation, differentiation, apoptosis and inflammation [12]. Numerous studies have focused on the activation of the Ras/MAPK signaling pathway in human hepatoma cells, finding that the MAPK pathway is closely related to the

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occurrence and development of liver tumors [13,14]. The MAPK pathway is stimulated by a variety of extracellular signals, especially intracellular oxidative stress [15]. Previous studies have demonstrated that JNK and p38 signaling pathways play an important role in cell apoptosis and death activated by numerous cellular stresses [16,17]. Moreover, the ERK1/2 signaling pathway also exerts crucial effects on oxidative stress and inflammation [18]. Drugs, radiation and environmental pollutants can mediate oxidative stress, which in turn leads to inflammation and apoptosis, and induces the development of liver diseases [19]. Oxidative stress is associated with a wide range of diseases as a result of the production of ROS, the damage of cellular macromolecules and the activation of signaling pathways [20]. Kim et al. found that elevated ROS activated the MAPK pathway in response to glutamate-induced oxidative stress [21].

The mechanism of the MAPK signaling pathway in hepatocellular injury protected by allicin arouses our great interest to examine the protective effects of allicin against oxidative stress in AA-induced BRL-3A cells. In addition, how allicin protects AA-induced BRL-3A cells is to be investigated by evaluating the markers of oxidative damage, antioxidant defense and related signaling pathways.

2. Materials and methods

2.1. Chemicals and reagents

AA (2-propene amide) (CAS 79-06-1, purity > 99.8%), dimethyl sulfoxide (DMSO) and allicin were purchased from Sigma Chemical Co. (St Louis, MO, USA). RPMI 1640 medium, penicillin and streptomycin were obtained from Gibco BRL Co., Ltd. (Grand Island, NY, USA). Mycoplasma-free fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Cell Counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). L-glutamine powder was obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). D-Hanks buffer solution (without Ca^{2+}, Mg^{2+} and phenol red) was purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and commercial kits used for determination of SOD and GSH-Px were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Genomic DNA mini kit was obtained from Thermo Fisher Scientific Inc. (Shanghai, China). 8-OHdG ELISA kit was obtained from Shanghai Yuanye Biotechnology (Shanghai, China). SP600125 (JNK inhibitor), SCH772984 (ERK1/2 inhibitor) and SB202190 (p38 inhibitor) were purchased from Selleck Chemicals (Houston, TX, USA) The primary antibodies against Dusp5, Gadd45a were obtained from Thermo Fisher Scientific Inc. (Shanghai, China) and JNK, ERK1/2, p38, p-JNK, p-ERK1/2, p-p38, Phospholipase A2, KGF, c-Fos, GAPDH were obtained from Abcam (Cambridgeshire, UK).

2.2. Cell culture and treatment

The study employed normal rat liver cells (BRL-3A), purchased from the BeNa Culture Collection (BNCC, China). The cells were incubated in RPMI 1640 medium, supplemented with 10% (v/v) FBS, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) and maintained at 37 °C with 5% (v/v) CO_2 in a humidified incubator.

BRL-3A cells were seeded at a density of 2×10^5 cells per well in a clear 6-well tissue culture plate and incubated for 24 h. Then they were divided into six groups, labeled as the control group, the AA group and four allicin groups. Allicin stock solution of 30 mM was dissolved with DMSO and stored at −20 °C. Prior to incubation with 30 μM allicin, the allicin stock solution was diluted with serum-free RPMI 1640 medium to obtain different concentrations (3.75, 7.5, 15, 30 μM) and filtered through a 0.2 μm membrane. The final DMSO concentration was < 0.1% (v/v). AA was dissolved with distilled deionized water (ddH_2O), diluted to 2 mM in serum-free RPMI 1640 medium and filtered through a 0.2 μm membrane.

For the control group, BRL-3A cells were incubated with serum-free RPMI 1640 medium for 24 h. For the allicin groups, the cells were cultured with different concentrations of allicin (3.75, 7.5, 15, 30 μM) for 2 h. After being cultured with allicin, these cells were treated with 2 mM AA for another 24 h. For the AA groups, BRL-3A cells were incubated with 2 mM AA for 24 h. To evaluate the protective effect of allicin against AA-induced oxidative stress, cells and cell lysate were collected from each group for future analysis.

2.3. Cell viability assay

Cell viability assays were performed using the CCK-8 kit. In brief, the cells were seeded at a density of 1×10^5 cells per well in a clear 96-well microtiter plate and incubated at 37 °C with 5% CO_2 in a humidified incubator for 24 h. The cells were cultured with 100 μL (1, 2, 4, 8, 16, 32 mM) of AA for 24 h. After AA treatment, 10 μL of CCK-8 solution was added to each well, and the 96-well microtiter plate remained at 37 °C for another 2 h. The optical density was measured at 490 nm on a microplate reader (Synergy HT, BioTek, USA).

2.4. Determination of cellular ROS

Fluorescent probe, DCFH-DA was selected to determine the production of cellular ROS. DCFH-DA penetrates the cells and is hydrolyzed to nonfluorescent DCFH by esterases. In the presence of ROS, DCFH is further oxidized to highly green fluorescent DCF. After treatment, cells were incubated with 10 μM DCFH-DA for 30 min in the dark. Then, cells were washed twice with PBS and the fluorescent was detected using a fluorescence microscope (Nikon, Japan).

2.5. Activities of GSH-Px and SOD

Enzymatic activities of GSH-Px and SOD were determined according to the manufacturer's guidelines. After being treated as previously described, the cells were washed with 2 mL phosphate buffer saline (PBS) and lysed in RIPA lysis buffer containing 1% protease inhibitor cocktail on ice. The cell lysate was then centrifuged at 10,010 ×*g* for 10 min to obtain clarified lysate, which was stored at −20 °C until used. The absorbance was recorded at 405 nm for GSH-Px and 450 nm for SOD activities.

2.6. Determination of 8-OHdG

BRL-3A cells were seeded into a 6-well plate at a density of 2×10^5 cells/mL and incubated for 24 h. After treatment, the cell lysate was collected and the DNA of cells was purified using a commercially available extraction kit according to the manufacturer's instructions. The DNA sample of the cultured cells were used to determine the level of 8-OHdG with a competitive ELISA kit. Briefly, 100 μL of HRP-conjugate reagent was added to each well and they were incubated for 1 h at 37 °C. The wells were washed five times with wash solution. Then 50 μL of chromogen solution A and B were added to each well and incubated for 15 min away from light at 37 °C. After that, stop solution was added and the absorbance was recorded at 450 nm within 15 min.

2.7. Protein levels by Western-blotting

BRL-3A cells in the control group, AA groups and allicin groups were washed three times with 4 °C PBS for 1 min and lysed on ice with RIPA lysis buffer containing 1% protease and phosphatase inhibitor cocktails obtained from Beyotime Institute of Biotechnology (Jiangsu, China). The lysates were lysed for 30 min before collection by cell scraper, centrifuged at 10,010 ×*g* for 10 min at 4 °C and stored at −80 °C. To quantify the protein concentrations of each sample, the BCA

protein assay kit was used and the protein samples were heated 10 min at 100 °C to denature. The protein samples were electrophoresed on 10% SDS-PAGE gel at 90 V for 10 min and 120 V for 1 h and then transferred to a polyvinylidene fluoride (PVDF) membrane at 15 V according to protein molecular weight for 30 or 45 min. The membrane was blocked by TBST buffer containing 5% skimmed milk powder for 1 h at room temperature and incubated with corresponding primary antibodies against GAPDH, Phospholipase A2, c-Fos, Gadd45a, Dusp5, KGF, JNK, ERK1/2, p38, p-JNK, p-ERK1/2 and p-p38 (1:10000, 1:500, 1:2000, 1:1000, 1:500, 1:2000, 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:1000 dilutions) overnight at 4 °C. Afterwards, the membranes were washed twice for 10 min with TBST buffer and washed with TBS buffer once , and incubated with HRP-conjugated goat-anti rabbit antiserum (1:10000 dilutions) as the secondary antibody for 2 h at room temperature. The immunoreactive bands were washed the same as before and visualized by reacting with ECL luminescent Kit (Beyotime). The images were detected using a Canon Scan LiDE 100 scanner (Canon) and the immunoreactive bands were analyzed by the Image-J software.

2.8. Statistical analysis

The obtained data were analyzed using GraphPad Prism 6 software. The results are presented as means ± standard deviation. Significant differences between groups are shown by one-way analysis of variance and the results analyzed by software with $p < 0.05$ are regarded as statistically significant.

3. Results

3.1. Appropriate doses of AA and allicin on the viability of BRL-3A cells

To optimize AA and allicin concentrations for further investigation, cells in logarithmic growth phase were collected and incubated with AA at 1, 2, 4, 8, 16, 32 mM for 24 h and viability of BRL-3A cells was determined by CCK-8 assays. The viability was evidently restrained in a dose-dependent manner, down to 73.6%, 68.8% and 23.0% following 2, 4, 8 mM AA treatment, respectively (Fig. 1). Moreover, as AA increased to 32 mM, the cell viability reached an extremely low level, only at 3.2%. In view of IC_{50} of AA according to improved Karber's method, AA at 2 mM is optimal for further study. As present in Fig. 2, treatment with allicin based on our previous experiments at 3.75, 7.5, 15, 30 μM showed no cytotoxic effect on the viability of BRL-3A cells, indicating these doses do not induce cellular damage during incubation and are suitable for experiments.

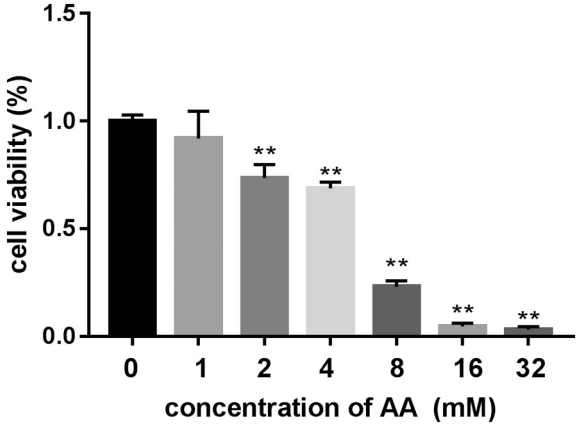

Fig. 1. AA inhibited BRL-3A cell viability. Cells were plated in 96-wells and incubated with various concentrations of AA (0–32 mM) for 24 h. Data are presented as the mean ± SD ($n = 6$). ** $p < 0.01$, versus the control group.

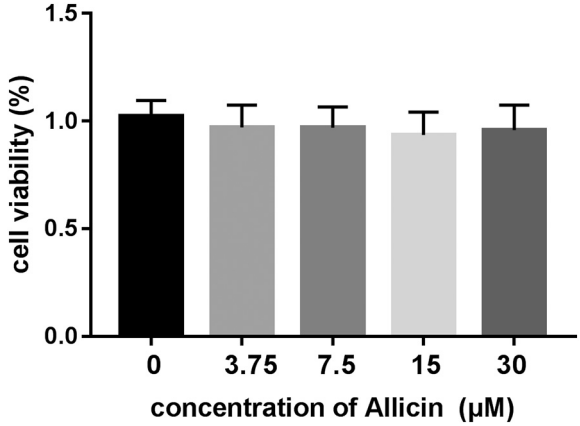

Fig. 2. Allicin had no significant difference in BRL-3A cell viability. Cells were plated in 96-wells and incubated with various concentrations of Allicin (0–30 μM) for 2 h. Data are presented as the mean ± SD (n = 6).

3.2. Protective effect of allicin on oxidative stress and DNA damage induced by AA in BRL-3A cells

The effect of allicin on AA induced oxidative stress damage was evaluated by the level of ROS (Fig. 3) and the activities of SOD and GSH-Px in BRL-3A cells (Fig. 4A, B). Treatment of BRL-3A cells with 2 mM AA exhibited higher fluorescence intensity, which was increased by 149.3% in comparison to the control group. In the presence of allicin, ROS level was remarkably reduced in a dose-dependent manner as compared with the AA group. Activities of SOD and GSH-Px were significantly reduced by 49.9% and 32.9% respectively, compared with the control group. Nevertheless, cells pre-treated with allicin at all concentrations had an enhancement in SOD and GSH-Px activities compared with the AA group. With the increase of AA concentration, SOD and GSH-Px activities increased in a dose-dependent manner. However, both SOD and GSH-Px activities of BRL-3A cells pre-treated with 3.75 μM allicin had no significant difference compared with the AA group. In addition, we found the SOD activity of the group pre-treated with 30 μM allicin increased to 31.67 U/mgprot, which was close to that of the control group. Conclusively, allicin inhibited the decrease of SOD and GSH-Px activities and alleviated the oxidative damage caused by AA.

Compared with the control group, AA caused an increase of 8-OHdG level in DNA sample of cells to 2.14 ng/mL (Fig. 4C). After pretreatment with allicin, 8-OHdG level was notably reduced in comparison with the AA group. For the group pre-treated with 3.75 μM allicin, there was no significant difference compared with the AA group in 8-OHdG level. Nevertheless, cells pre-treated with 15 and 30 μM allicin showed markedly decreased 8-OHdG level by 36.5% and 39.3% respectively compared with the AA group. Hence, this dose-response relationship indicated that allicin could suppress 8-OHdG, thereby mitigating DNA oxidative damage induced by AA.

3.3. Allicin mediated protection via the MAPK signaling pathway in BRL-3A cells

For deeper insight into the effects of allicin against AA-induced oxidative stress on the MAPK signaling pathway, phosphorylated protein levels of JNK, ERK1/2, p38 were determined in BRL-3A cells by Western-blotting (Fig. 5). Immunoblotting results indicated that AA significantly induced the phosphorylation of JNK and p38, while the phosphorylation of ERK1/2 was downregulated notably. JNK and p38 phosphorylation increased by 3.7- and 4.3-fold, respectively, in comparison with that of the control cells and ERK phosphorylation decreased by 0.36-fold. Interestingly, the total protein levels of JNK, ERK1/2 and p38 were not altered remarkably. It was worth nothing

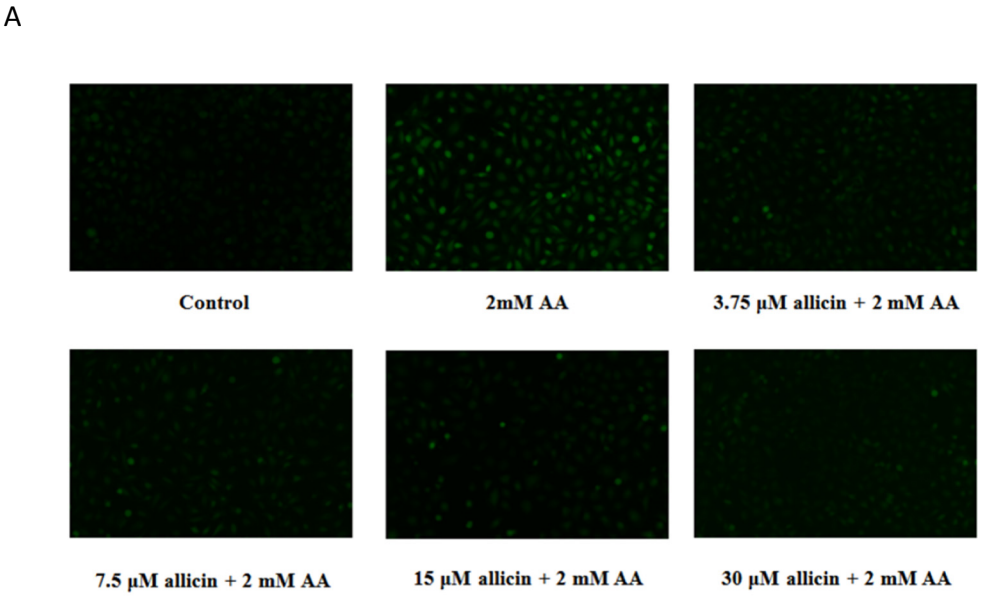

Fig. 3. Allicin inhibited the production of ROS induced by AA. After pretreatment with allicin (0, 3.75, 7.5, 15, 30 μM) for 2 h then treated with AA (2 mM) for 24 h, cells were incubated with 10 μM DCFH-DA for 30 min. (A) Photomicrographs showed the generation of intracellular ROS by fluorescence microscope. (B) The quantitative data of panel (A) and results were expressed as mean DCF fluorescence intensity. Data are presented as the mean ± SD ($n = 3$). $^{**}p < 0.01$, versus the control group; $^{\#\#}p < 0.01$, versus the model group.

that pretreatment with allicin significantly restrained the enhanced phosphorylated levels of JNK and p38 and the level of p-ERK1/2 was increased in the allicin-related dose-dependent manner compared with the AA group.

In addition, involvement of activation of MAPK subfamilies in the protective mechanism was further detected using specific inhibitors. BRL-3A cells were treated with RPMI 1640 medium for 24 h in the absence or presence of SP600125 (JNK inhibitor, 10 μM) or SCH772984 (ERK inhibitor, 2 μM) or SB202190 (p38 inhibitor, 10 μM). As shown in Fig. 6, the JNK, ERK and p38 activation were inhibited by SP600125, SCH772984 and SB202190, respectively. SP600125, SCH772984 and SB202190 had no significant effects on cell viability. Compared with allicin group in the presence of AA, BRL-3A cells treated with allicin combined with SP600125 or SB202190 in the exposure of AA increased cell viability to 82.6% and 84.7% respectively, as opposite to SCH772984 decreased to 59.8%.

3.4. Allicin treatment influences the expression of some MAPK-related proteins induced by AA

Combined with the significantly different genes (e.g. *Fgf7*, *Dusp5*, *Gadd45a*, *Fos*, *Pla2g4d*) obtained from our previous research, some proteins related to liver injury were selected to further investigate the protective mechanism of allicin. The expression of KGF, Gadd45a, c-Fos, Dusp5 and Phospholipase A2 was shown in Fig. 7, involving cell proliferation, oxidative damage, DNA damage, lipid metabolism in the MAPK pathway. Treatment with 2 mM AA significantly increased KGF, Gadd45a, c-Fos and Dusp5 levels, whereas it decreased Phospholipase

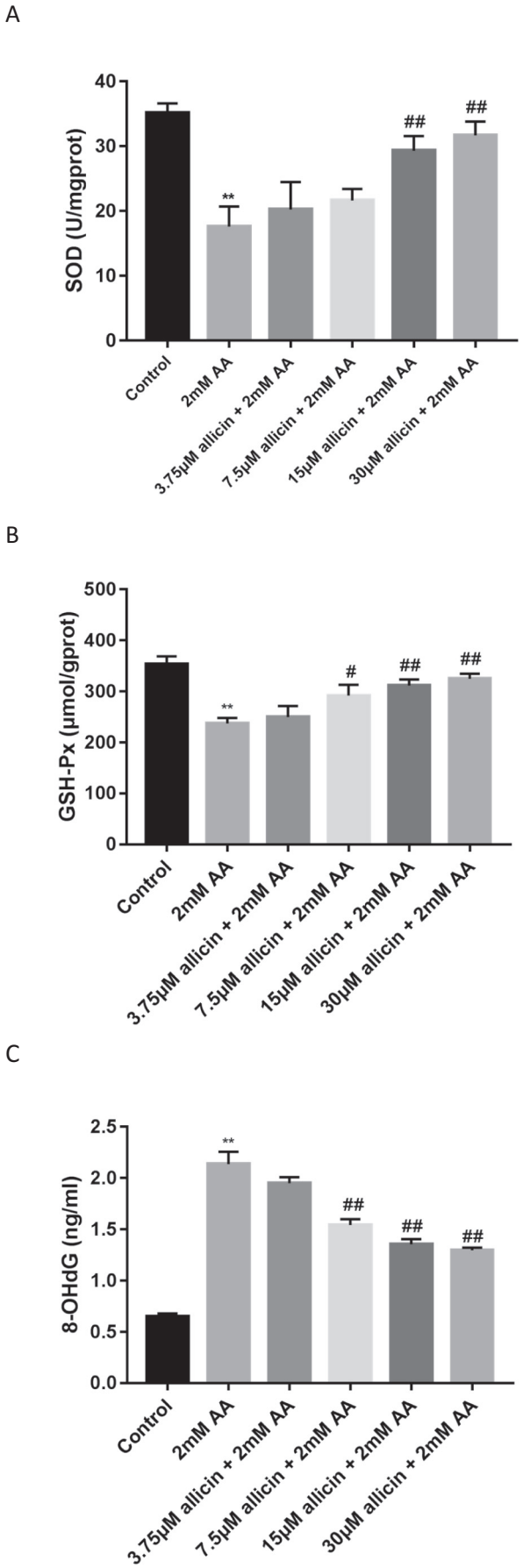

Fig. 4. Effects of allicin on activity of oxidative and DNA damage indicators in AA-treated BRL-3A cells. BRL-3A were pre-treated with allicin (0, 3.75, 7.5, 15, 30 μM) for 2 h then treated with AA (2 mM) for 24 h. Cells were harvested and subjected to assessment of SOD and GSH-Px activities. The DNA sample of cells were subjected to assessment of 8-OHdG level. (A) The activity of SOD in BRL-3A cells. (B) The activity of GSH-Px in BRL-3A cells. (C) The level of 8-OHdG in BRL-3A cells. Data are presented as the mean ± SD ($n = 3$). $^{**}p < 0.01$, versus the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus the model group.

A2 level in comparison with the control group. The expression of KGF, Gadd45a, c-Fos and Dusp5 was increased by 9.3%, 18.9%, 40.4% and 25.6%, respectively, whereas Phospholipase A2 was reduced by 7.6%. Pretreatment with allicin of different concentrations reversed the effect of AA on the expression of these proteins, resulting in markedly decreased expression of KGF and Gadd45a. Among the allicin-treated groups, the expression of KGF and Gadd45a of cells treated with 30 μM allicin was the lowest, 50.3% and 54.0% of the AA group. In particular, for the groups pre-treated with allicin at 3.75, 7.5 and 15 μM, there was no significant difference in the expression of c-Fos or Dusp5. After pretreatment with allicin at 30 μM, Dusp5 level decreased to 34.3% of the AA group. Moreover, Phospholipase A2 level was slightly lower than the AA group in BRL-3A cells pre-treated with allicin at a low dose. With allicin increasing to 30 μM, the expression of Phospholipase A2 was notably enhanced and higher than that of the control and the AA groups.

4. Discussion

AA is a potential toxin in diet, with complicated toxic mechanisms. Its cytotoxicity and genotoxicity are characterized by the destruction of oxidative defense system of cells and the release of ROS [22]. Other studies tend to focus on inhibiting its formation, particularly on phenotypes [23]. In recent years, dietary supplementation of natural antioxidants as well as their potential protection mechanisms at the molecular level has become a hotspot.

Compounds with strong antioxidant properties exert protective effects against oxidative stress [24]. Allicin has drawn particular attention on account of its antioxidant activity, antitumor activity and antibacterial effects [25]. Currently, it is used as drug to alleviate the oxidative stress caused by AA, H_2O_2 and lipopolysaccharide [9,26,27]. Nevertheless, information about the effects of allicin on AA-induced toxicity is very limited. BRL-3A cells have many morphological characteristics of normal human hepatocytes. In such a case, they were selected as an *in vitro* model to investigate the protective effect of allicin against AA-induced oxidative stress, and whether the underlying mechanism is closely dependent on the MAPK signaling pathway as well.

The ameliorative effects of allicin against the cell damage induced by AA were investigated on ROS level and activities of SOD and GSH-Px in BRL-3A cells. Increase in ROS production in AA-induced cells was affected by the destruction of redox homeostasis [28]. ROS level can directly reflect the degree of oxidative stress in cells. We detected the level of ROS in AA-treated BRL-3A cells and discovered a significant increase in ROS level. In this study, it is clearly certified that ROS production was markedly attenuated after BRL-3A cells were pretreated with allicin.

Once excessive cellular ROS is generated, it would result in the reduction of antioxidant enzyme activities to balance the redox state. Antioxidant enzymes can defend cells against oxidative stress caused by free radicals, and are therefore chosen to evaluate oxidative stress in different types of cell lines [29]. The imbalance between oxidation and antioxidant systems may mediate oxidative stress when the body is exposed to various harmful stimulations, which is the common pathophysiological basis of various liver diseases [30]. SOD and GSH-Px act critical roles in protecting several tissue and cellular injuries induced by ROS [31]. Previous studies have revealed that the activities of GSH-Px

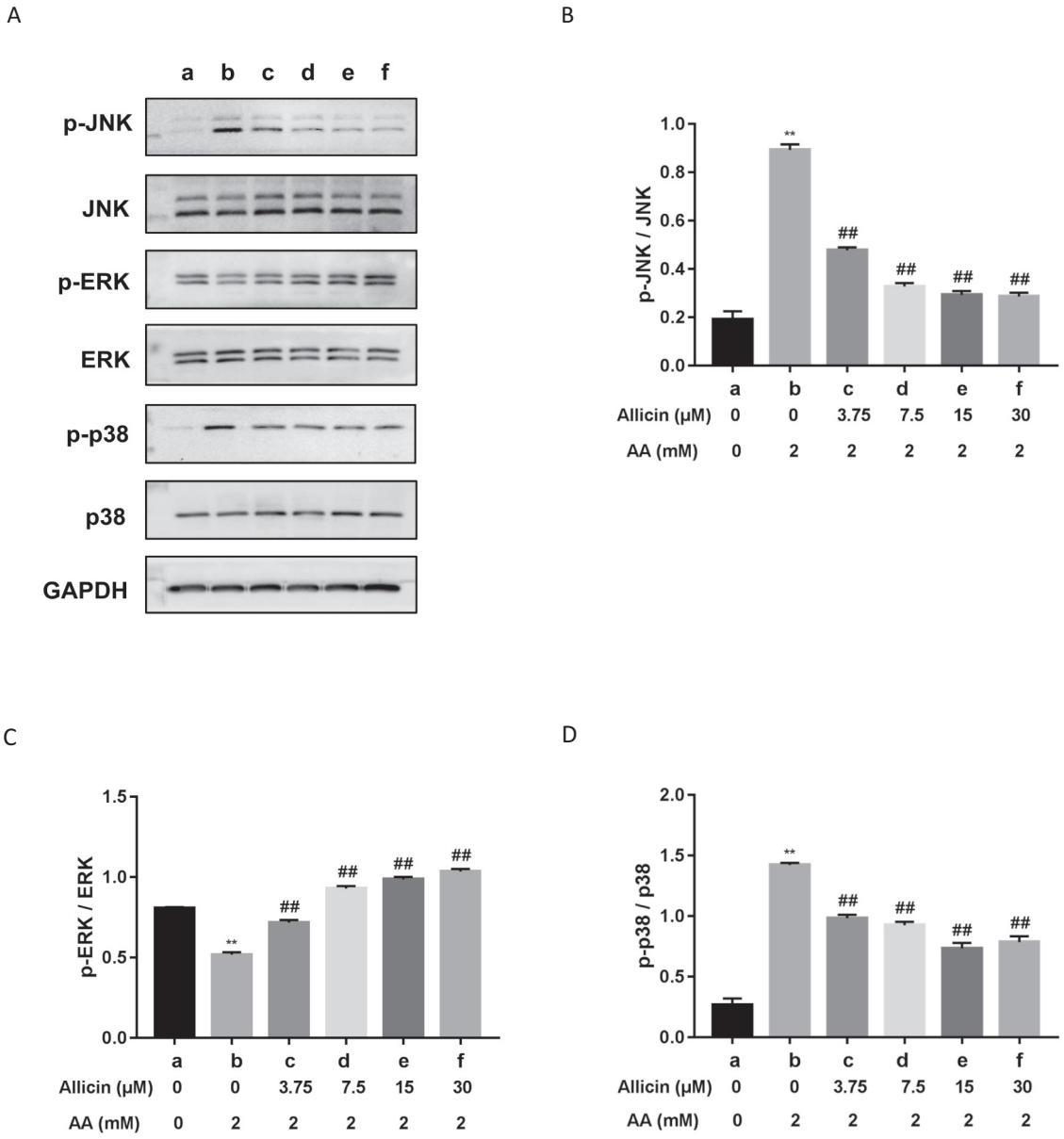

Fig. 5. Effect of allicin on MAPKs phosphorylation. BRL-3A were pre-treated with allicin (0, 3.75, 7.5, 15, 30 μM) for 2 h then treated with AA (2 mM) for 24 h. (A) Representative Western blotting analysis showing the effect of allicin on JNK, ERK and p38 phosphorylation in BRL-3A cells. Immunoblot bands corresponding to p-JNK (B), p-ERK (C) and p-p38 (D) were quantified by densitometric analysis and then normalized to their respective total kinase. Data are presented as the mean ± SD ($n = 3$). $^{**}p < 0.01$, versus the control group; $^{\#\#}p < 0.01$, versus the model group.

and antioxidant enzymes were notably reduced *in vitro* and *in vivo* models treated with AA [32,33]. Allicin could enhance GSH-Px activity and ameliorates hepatotoxicity in ethanol-induced mice [34]. In the present study, we observed a significant reduction in SOD and GSH-Px in BRL-3A cells treated with 2 mM AA, suggesting antioxidant defense system was impaired. Nonetheless, when BRL-3A cells were pre-treated with allicin, these AA-induced cellular events were blocked to a great degree. Our results have found that allicin is capable of recovering the activity of antioxidant enzymes and improving oxidative stress injury in AA-induced BRL-3A cells, as obvious from the variations in SOD and GSH-Px activities.

In addition, we checked the intracellular 8-OHdG level in AA-treated BRL-3A cells. 8-OHdG, whose level reflects the relationship between oxidative stress and DNA damage [35], is featured in the biomarker of oxidative stress caused by exogenous or endogenous factors. Previous studies corroborated that the levels of ROS and 8-OHdG were markedly enhanced in HepG2 cells treated with AA, thereby exerting toxic effects [22]. Similarly, our results are in line with previous results that treatment with AA exhibites evidently increased 8-OHdG level, indicating the redox imbalance in AA-exposed cells. Besides, we have found that pretreatment with allicin is able to reverse this effect in a dose-dependent manner. Overall, allicin pretreatment could efficiently prevent the imbalance of cellular redox status, further supporting its antioxidant role.

Oxidative stress-induced damage implicates profound changes in signaling pathways [36]. Although activation of various signaling pathways is involved in AA-induced oxidative stress, the protective mechanism of allicin on AA-induced cell damage has not been clarified [37]. Based on our previous results, we selected the MAPK pathway to explore the potential molecular mechanisms. The classical MAPK pathway, one of downstream signal transduction pathways of ROS, plays a vital role in gene expression regulation, cell growth, survival and apoptosis [38,39]. Numerous previous investigations have indicated that antioxidants such as astaxanthin and oligonol exert

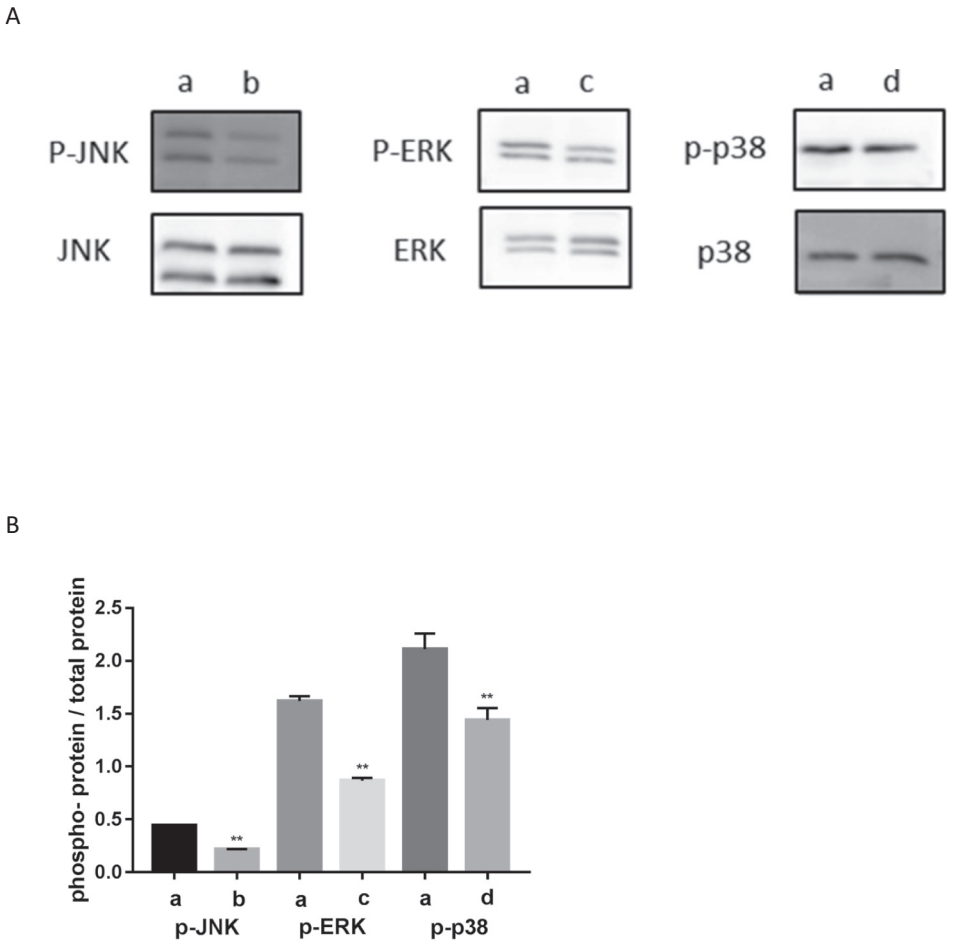

Fig. 6. Effect of SP600125 (JNK inhibitor), SCH772984 (ERK1/2 inhibitor) and SB202190 (p38 inhibitor) on activation of MAPK subfamilies. BRL-3A were treated with RPMI 1640 medium for 24 h in the absence or presence of 10 μM SP600125, 10 μM SCH772984 or 2 μM SB202190 (added 2 h before). (A) Representative Western blotting analysis showing the effect of inhibitors on JNK, ERK and p38 phosphorylation in BRL-3A cells. (B) Immunoblot bands corresponding to p-JNK, p-ERK and p-p38 were quantified by densitometric analysis and then normalized to their respective total kinase. Data are presented as the mean ± SD (n = 3). $^{**}p < 0.01$, versus the control group. (C) Effect of SP600125, SCH772984 and SB202190 on BRL-3A cell viability. BRL-3A cells were pre-treated on 10 μM SP600125 or 10 μM SCH772984 or 2 μM SB202190 or a combination of both allicin and SP600125 or SCH772984 or SB202190 for 2 h before 2 mM AA treatment for 24 h. Data are presented as the mean ± SD (n = 3). $^{**}p < 0.01$, versus the control group; $^{\#\#}p < 0.01$, versus the model group; $^{\&}p < 0.05$, versus the allicin + AA group.

protective effects in responses to liver injury via inhibiting the activation of MAPK cascade [40,41]. The present study showed that allicin downregulated the expression of p-JNK and p-p38, which was significantly upregulated following AA treatment. A recent investigation indicated that addition of 0.6, 1.25, 2.5 and 5 mM AA for 24 h significantly reduced ERK phosphorylation. However, phosphorylation of ERK1/2 was promoted at 0.5–6 h and remarkably reduced at 12 h after exposure to 2.5 mM AA, implying that the MAPK pathway could act as access to rapid response types. In addition, research observed that treatment with various concentrations (25, 50 and 100 μmol/L) of diallyl disulfide, a compound of garlic, dramatically enhanced p-ERK level in human colorectal cancer cells [42]. Allicin elevated ERK1/2 phosphorylation in IL-1β-treated U87MG human glioblastoma cells [43]. As allicin activated the ERK1/2 pathway in the present study, we

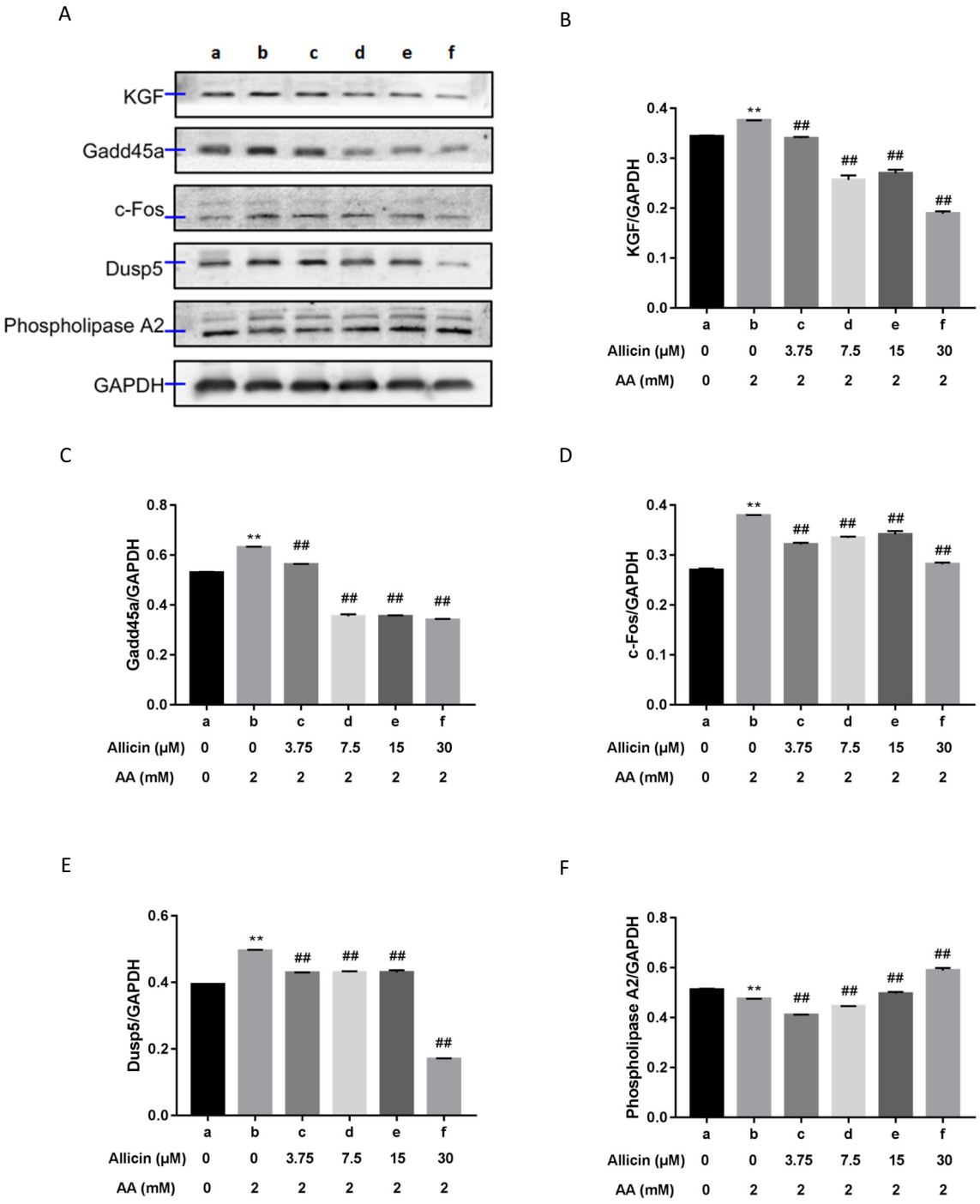

Fig. 7. Effect of allicin on KGF, Gadd45a, c-Fos, Dusp5 and Phospholipase A2 expression. BRL-3A were pre-treated with allicin (0, 3.75, 7.5, 15, 30 μM) for 2 h then treated with AA (2 mM) for 24 h. (A) Representative Western blotting analysis showing the effect of allicin on KGF, Gadd45a, c-Fos, Dusp5 and Phospholipase A2 in BRL-3A cells. Immunoblot bands corresponding to KGF (B), Gadd45a (C), c-Fos (D), Dusp5 (E) and Phospholipase A2 (F) were quantified by densitometric analysis and then normalized to β-actin. Data are presented as the mean ± SD (n = 3). $^{**}p < 0.01$, versus the control group; $^{\#\#}p < 0.01$, versus the model group.

conjecture the JNK and p38 pathways were suppressed and the ERK1/2 pathway was activated in this process. To further corroborate the contributions of MAPK subfamilies in the positive effect of allicin on AA-induced BRL-3A cells, we detected the viability of cells exposed to AA with SP600125, SCH772984 and SB202190, respectively. Notably, BRL-3A cells treated with allicin coculture with SP600125 or SB202190 in the presence of AA displayed a much higher cell viability compared to the group treated with allicin and AA, which is contrary to the effect of SCH772984. These specific inhibitory tests indicated that allicin attenuated AA-induced cytoprotection through inhibiting the JNK and p38 pathways and activating the ERK1/2 pathway.

From our results, AA exposure down-regulated Phospholipase A2 expression and up-regulated KGF, Gadd45a, c-Fos and Dusp5 expression. Interestingly, pretreatment with allicin reversed the effect of AA on the expression of these proteins in BRL-3A cells. KGF is a protective drug to protect cells from various toxic injuries. It can regulate inflammation and promote the repair of injured liver cells and liver tissue [44]. Gadd45a exhibits protective effects against oxidative stress and plays a crucial role in healing hepatocyte injury [45]. Stress-inducible GADD45-like proteins activates the MAPK signaling pathway and

regulates cellular stress and inflammatory cytokines in the body [46]. Accumulating evidence indicates that *c-fos* is involved in hepatotoxicity, which is mediated by the MAPK pathway [47]. Moreover, up-regulation of Dusp5 (a tumor suppressor) is able to negatively regulate the MAPK signaling pathway to promote the development of liver tumors [48]. Phospholipase A2, linked with MAPKs in different cell lines, is involved in oxidative and inflammatory signaling pathways [49]. Our results demonstrate that allicin supplementation plays an ameliorative role in hepatocyte injury via mediating the MAPK pathway.

5. Conclusions

We have pioneeringly revealed that allicin can alleviate oxidative stress induced by AA in BRL-3A cells. Such protective effects are accompanied by increased antioxidant enzyme activities, which consequently modulate intracellular redox balance through the MAPK signaling pathway. Thus, the results provide a cellular and molecular basis for ameliorative mechanism of allicin against AA-induced oxidative stress. Further investigation is needed to elucidate specific mechanisms of allicin on AA-induced injury.

Declaration of Competing Interest

The authors declare no competing financial interest.

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