Available online at www.sciencedirect.com
ScienceDirect
*Chinese Journal of Natural Medicines* 2020, **18**(12): 934-940
doi: 10.1016/S1875-5364(20)60037-1
Chinese Journal of Natural Medicines

•Research article•

# Ganoderic acid A protects lens epithelial cells from UVB irradiation and delays lens opacity

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Available online 20 Dec., 2020

**[ABSTRACT]** A contributory role of oxidative stress and protection by antioxidant nutrients have been suspected in cataract formation. Ganoderic acid A (GAA), an effective lanostane triterpene, is widely reported as an antioxidant. The aim of this study is to investigate the potential effects of GAA on cataract formation. After lens epithelial cells (LECs) were exposed to UVB radiation for different periods, cell viability, apoptosis-related protein levels, malondialdehyde (MDA) and superoxide dismutase (SOD) activities were monitored. We found that cell viability, the Bcl-2/Bax ratio and SOD activity were increased, while Cleaved caspase-3 levels and MDA activity were decreased compared with those in UVB-impaired LECs after GAA treated. Furthermore, GAA activated PI3K/AKT in UVB-impaired LECs and effectively delayed the occurrence of lens opacity *in vitro*. In conclusion, these findings demonstrated that GAA exhibited protective functions in SRA01/04 cells and rat lenses against UVB-evoked impairment through elevating cell viability and antioxidant activity, inhibiting cell apoptosis, activating the PI3K/AKT pathway and delaying lens opacity.

**[KEY WORDS]** Ganoderic acid A; Cataract; Lens epithelial cells; Cell vitality; Apoptosis
**[CLC Number]** R965 **[Document code]** A **[Article ID]** 2095-6975(2020)12-0934-07

## Introduction

Cataract is the most common blinding ocular disease in China and around the world. As the population ages, cataract poses a serious economic burden to families and society [1]. Although the etiology and pathogenesis of cataract remain unclear, oxidative damage to lens epithelial cells (LECs) is the most important factor [2]. LECs play a vital role in maintaining the transparency of the entire lens and the stability of the internal environment [3]. Epidemiological data suggest that ultraviolet (UV) radiation is an important risk factor in the etiology of cataract [4]. However, there are no effective methods to prevent cataractous lenses. Nevertheless, advances in the surgical removal of cataracts, including small-incision surgery, use of viscoelastics, and the development of intraocular lenses, have made treatment very effective and visual recovery rapid in most cases. Despite these advances, cataract continues to be a leading public health issue that will grow in importance as the population increases and life expectancy is extended worldwide. Hence, more researchers are focusing on natural effective agents with minimal adverse effects for the prevention and treatment of cataract.

Ganoderic acid A (GAA), isolated from *Ganoderma lucidum*, is the main effective component of lanostanoid triterpenoid extracts and exhibits various protective effects [5, 6]. For example, GAA showed a protective effect in hypoxia-induced H9c2 rat cardiomyocyte cells, as cell viability and proliferation were enhanced, whereas apoptosis was repressed after GAA treatment [7]. GAA was found to potentiate an antioxidant effect, protect mitochondrial membranes, improve superoxide dismutase (SOD) activity and reduce apoptosis in primary hippocampal neurons [8]. GAA might be a potential therapeutic agent in the treatment of renal fibrosis, as treatment with GAA significantly decreased epithelial-mesenchymal transition in human proximal tubular epithelial cells [9]. However, the antioxidant effects of GAA on other epithelial cells, including LECs after UVB irradiation, and the relevant signaling pathways remain unknown.

PI3Ks and Akt constitute the classic phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which plays an essential role in numerous cellular processes, such as cellular survival, proliferation and apoptosis [10]. Previous experiments showed enhanced PI3K/Akt signaling pathway-mediated cell survival and cell antioxidant reactions following UVB irradiation [11]. GAA encouraged cell growth and inhibited invasion/migration and apoptosis in U251 glioma cells *via* activation of the

**[Received on]** 24-Jun.-2020
**[Research funding]** The study was supported by the National Natural Science Foundation of China (Nos. 81770906 and 81974129) , the Science and Technology Project of Nantong Municipality (Nos. JCZ19088 and JCZ19077), and the Project of Nantong Municipal Health Commission (No. QA2019061).
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These authors have no conflict of interest to declare.

PI3K/AKT pathway [12]. However, the detailed mechanism by which GAA protects against UVB radiation-induced LEC oxidative damage and apoptosis requires clarification.

Therefore, the aim of the present study was to determine the effect of GAA on human LECs cell survival, apoptosis, and oxidation and to elucidate the mechanisms underlying its effects on UVB-mediated damage. Determining these details will aid in gaining a better understanding of the effects of GAA so that GAA might be used as a natural, preventive anticataract reagent in the future.

## Materials and Methods

### *Reagents*

A human lens epithelial cell line (SRA01/04 cells) was purchased from RIKEN (Japan) and authenticated by short tandem repeat (STR) loci plus sex-determining locus analysis (GTB Technical Support, Suzhou, China). GAA (HPLC-grade, ≥ 98% pure) was purchased from Merck Biotechnology Co., Ltd. (Germany). Cell Counting Kit-8 (CCK-8) detection solution was obtained from Dojindo (Kyushu Island, Japan). Malondialdehyde (MDA, A003-4-1) levels and SOD activity (A001-1) were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Antibodies against cleaved caspase-3 (ab2302), B cell lymphoma-2 (Bcl-2, ab196495), Bcl-2-associated X protein (Bax, ab53154), PI3K (ab133595), phospho-PI3K (p-PI3K, ab182651), and *β*-actin (ab8229) and peroxidase-conjugated secondary antibodies were obtained from Abcam (Cambridge, UK). Antibodies against AKT (9272), phospho-AKT (p-AKT, 9271), p70S6K (9202), and phospho-p70S6K (p-p70S6K, 9205) were purchased from Cell Signaling Technology (Beverly, MA, USA).

### *Cell culture and treatment*

SRA01/04 cells, a line of LECs of human origin, were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen-Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 1% penicillin, and 1% streptomycin in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 ℃ following the protocol in our previous report [13]. Once they had grown to 70%−80% confluence, the cells were either irradiated with UVB for different times (0−120 min) according to our previous study [14] or incubated with GAA at different concentrations (0−100 μmol·L$^{-1}$) for 24 h, followed by UVB irradiation for 15 min. The UVB narrowband lamp had a wavelength spectral distribution of 275−400 nm with a peak wavelength at 310 nm. The irradiance of the UVB light was 0.09 mW·cm$^{-2}$, After removing the PBS and adding culture medium, the culture dish containing SRA01/04 cells were incubated at 37 ℃ in 5% $CO_2$ for 24 h. Un-irradiated PBS-treated SRA01/04 cells were used as a control.

### *CCK-8 assay*

Cell viability was evaluated by CCK-8 assay according to our previous study [14]. CCK-8 detection solution (10 μL) was added to cells in each well of a cell culture plate and then incubated for 1 h. The absorbance at 450 nm (OD450) of each well was measured. Each experiment was repeated in triplicate.

### *Western blot analysis*

Proteins were extracted from cell pellets in lysis buffer, separated on 12% and 6% SDS-polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk, and reacted with anti-Cleaved caspase-3 (1 : 2000), anti-Bcl-2 (1 : 2000), anti-Bax (1 : 2000), anti-PI3K (1 : 2000), anti-p-PI3K (1 : 2000), anti-AKT (1 : 2000), anti-p-AKT (1 : 2000) and anti-*β*-actin (1 : 2000) antibodies at 4 ℃ overnight. After washing, the membranes were incubated with horseradish peroxidase (HRP)-coupled goat anti-rabbit or goat anti-mouse IgG (1 : 2000) at 25 ℃ for 2 h, and the blots were visualized by enhanced chemiluminescence (Immobilon Western HRP, Millipore Biotechnology, Billerica, MA, USA) according to our previous study [15].

### *Measurement of the MDA content*

To test the protective effect of GAA on SRA01/04 cell viability, lipid peroxidation (*via* MDA, an end product of lipid peroxidation) was detected. The MDA level was evaluated with an MDA ELISA Kit according to the manufacturer's instructions. Briefly, SRA01/04 cells were seeded on plates and cultured in cell medium until they reached 70%−80% confluency. After treatment with UVB or GAA, the assay mixture (containing the cell lysis supernatant) was heated in boiling water for 40 min. After cooling to room temperature, the mixture was centrifuged at 4000 g for 10 min. The absorbance of the resulting product was read at 532 nm. The concentration of MDA was determined using a standard curve, and MDA levels are expressed as μmol· mg$^{-1}$ protein.

### *SOD assay*

SOD activity in SRA01/04 cells was measured with the xanthine oxidase method following the manufacturer's instructions. The assay mixture (containing cell supernatant) was incubated at 37 ℃ for 40 min and room temperature for 10 min, after which the absorbance at 550 nm was then analyzed on a BioTek microplate reader. The results are expressed as U·mg$^{-1}$ protein.

### *Animals and surgery*

Adult male Sprague-Dawley (SD) rats weighing from 180 to 200 g were obtained from the Center of Laboratory Animals at Nantong University. All rats were deeply anesthetized, and their eyeballs were carefully and quickly removed. All surgical procedures were performed in accordance with the Jiangsu Branch of the Chinese National Committee to the Use of Experimental Animals for medical purposes.

### *Rat lens culture*

Lenses extracted from the eyeballs were cultured in M199 medium (Invitrogen-Gibco, Carlsbad, CA, USA) supplemented with 1% penicillin/streptomycin (Invitrogen-Gibco) at 37℃ in a humidified incubator with 5% $CO_2$ for 1 day in a 24-well plate. Transparent lenses without surgical

damage were transferred to a 24-well plate and maintained in M199 medium under various incubation conditions; the medium was replaced with fresh medium every 12 h. GAA was dissolved in M199 medium with the assistance of DMSO (the final proportion of DMSO in the medium was lower than 0.1%). The lenses were incubated under four conditions and set as the Control, GAA, UVB and UVB + GAA groups. The first group was cultured in only M199 daily ($n$ = 6), and the second group was incubated with 100 μmol·L$^{-1}$ GAA for 24 h ($n$ = 6), the third group was irradiated with UVB for 20 min every day ($n$ = 6), the forth group was incubated with 100 μmol·L$^{-1}$ GAA and irradiated with UVB for 20 min every day ($n$ = 6). The medium was replaced daily. The method of UVB exposure was based on our previous paper, which also contains detailed information on the UVB lamp used [14].

*Visual assessment*

Images of all lenses were taken with a Nikon D90 camera at the largest magnification under the same lighting environment. The transparency of the total lens area was observed; black areas on an image represented areas of transparent lens, and gray areas on the image represented areas of opaque lens. The specific methods and imaging steps were in accordance with our previous research [16].

*Statistical analyses*

Statistical analyses were performed with Stata 11.0 (Stata Corp., College Station, TX, USA). The results of CCK-8 assays, Western blotting, and MDA and SOD assays are expressed as the mean ± SD. All experiments were performed at least three times. Statistical calculations between groups were undertaken using one-way ANOVA. Significance levels were defined as $^{*}P < 0.05$, $^{\#}P < 0.05$, $^{**}P < 0.01$.

## Results

*The effects of GAA on the viability of SRA01/04 cells*

To investigate whether GAA possesses any potential toxic side effects, we examined its effect on the proliferation of SRA01/04 cells. Treatment with GAA at different concentrations (0, 10, 20, 50, 100 μmol·L$^{-1}$) alone did not significantly affect the proliferation of SRA01/04 cells (Fig. 1B). To further investigate whether GAA alters the effects of UVB irradiation of SRA01/04 cells, a CCK-8 assay was performed to examine the effects of GAA on the viability of SRA01/04 irradiated with UVB. The results revealed a reduction in SRA01/04 viability as the UVB exposure time increased, and the differences in viability between the control group and each experimental group were significant ($P < 0.05$), except for the groups treated with UVB for 5 and 10 min (Fig. 2A). This effect was reversed by GAA at different concentrations, and the differences in cell viability between the control group and each experimental group were significant ($P < 0.05$), except for the groups treated with 50 μmol·L$^{-1}$ and 100 μmol·L$^{-1}$GAA(Fig.2B).BasedontheCCK-8assayresults,wechose 15 min of UVB exposure and 100 μmol·L$^{-1}$ GAA as the best effective treatment conditions for further experiments.

*GAA inhibited UVB-triggered caspase-3 activation, Bax upregulation and Bcl-2 downregulation*

To further confirm the inhibitory effects of GAA on UVB-induced apoptosis, we analyzed the protein levels of Cleaved caspase-3, a crucial mediator of apoptosis through its protease activity, Bax, a proapoptotic protein, and Bcl-2, an antiapoptotic protein, by Western blot analysis. As shown in Fig. 3, UVB treatment led to caspase-3 activation, as indicated by an increase in Cleaved caspase-3 fragments compared with those in the control cells ($P < 0.01$), and a significant increase in Bax protein and reduction in Bcl-2 protein, resulting in a decrease in the Bcl-2/Bax ratio ($P < 0.01$). Compared with the UVB-treated cells, cells pretreated with GAA showed a pronounced reversal of UVB-induced caspase-3 activation ($P < 0.05$; Fig. 3A), Bax upregulation, and Bcl-2 downregulation, resulting in an increase in the ratio of Bcl-2/Bax, which reached the level in the control group ($P < 0.05$; Fig. 3B).

*GAA reduced UVB-induced lipid peroxidation*

The levels of MDA, a lipid peroxidation product, can reflect the extent of lipid peroxidation induced by oxidative stress. As shown in Fig. 4A, compared with the control treatment, UVB treatment caused a significant increase in the MDA level ($P < 0.05$). Pretreatment with GAA significantly reduced the level of MDA induced by UVB treatment ($P < 0.05$).

*GAA prevented UVB-induced decreases in antioxidative enzymatic activity*

To study the effects of GAA on antioxidant activity, the

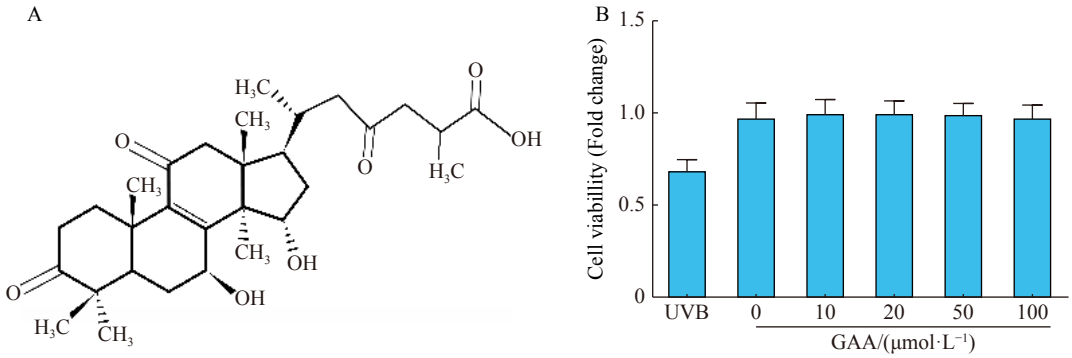


**Fig. 1 The effect of GAA on the viability of SRA01/04 cells was measured by CCK-8 assay. (A) Structure of GAA. (B) SRA01/04 cells on a 96-well plate were stimulated with GAA at different concentrations (0−100 μmol·L$^{-1}$) for 24 h. UVB treatment as a positive control for cell viability. The values shown are the means ± SDs ($n$ = 3)**

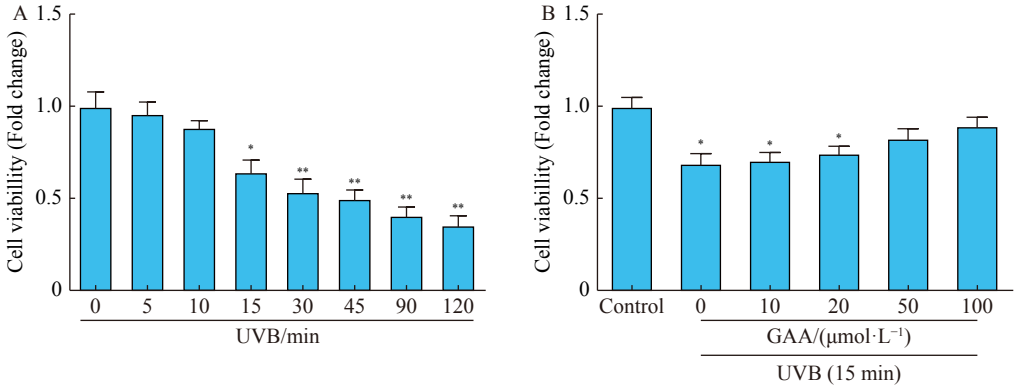


**Fig. 2 The viability of SRA01/04 cells after UVB irradiation and GAA treatment was measured by CCK-8 assay. (A) The effect of UVB irradiation for different periods (0−120 min) on SRA01/04 cells was assessed. (B) Cells were pretreated with GAA at different concentrations (0−100 μmol·L$^{-1}$) for 24 h and then irradiated with UVB for 15 min. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* the non-UVB-treated group or control group ($n = 3$)**

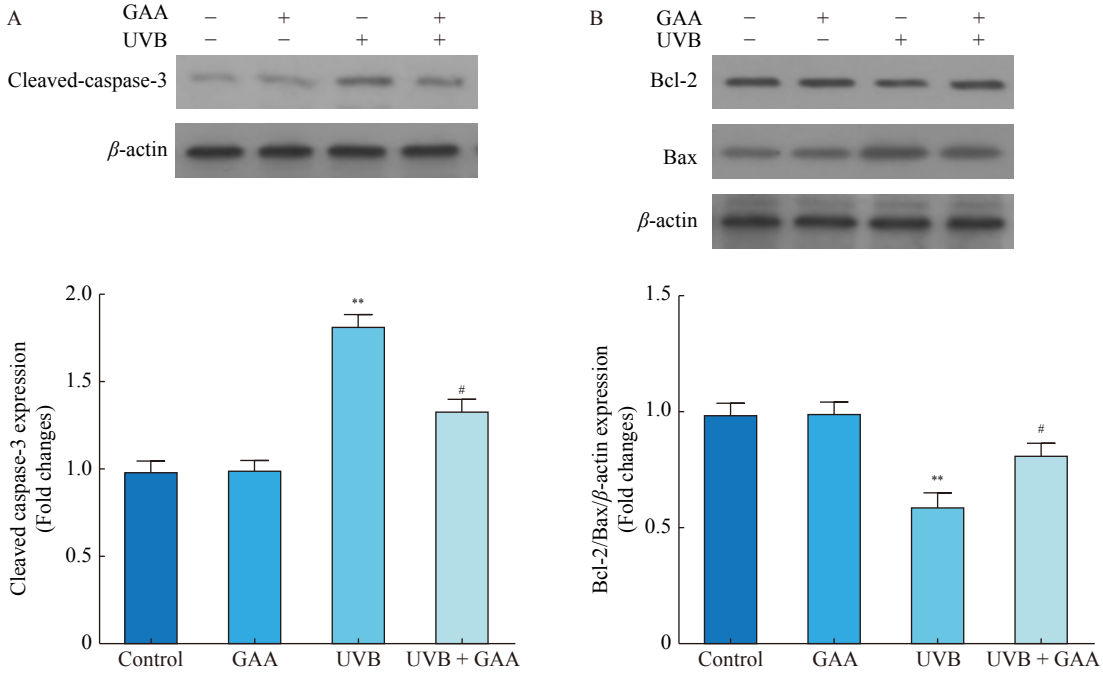


**Fig. 3 The effects of 100 μmol·L$^{-1}$ GAA on the levels of Cleaved caspase-3 (A), Bcl-2 (B), and Bax (B) in SRA01/04 cells treated with 15 min UVB were determined. A representative immunoblot showing Cleaved caspase-3, Bcl-2, Bax and *β*-actin were detected by Western blot, and protein quantities expressed as the ratio (percentage) compared to *β*-actin expression were shown. $^{**}P < 0.01$ *vs* the Control group; $^{\#}P < 0.05$ *vs* the UVB treated group ($n = 3$)**

activity of SOD was measured. As shown in Fig. 4B, UVB treatment led to a significant decrease in SOD activity ($P < 0.01$) compared with that of control cells. Pretreatment with GAA significantly protected against the UVB-induced decrease in SOD enzymatic activity ($P < 0.05$).

*GAA protected SRA01/04 cells from UVB-induced cell injury through activating the PI3K/AKT pathway*

To reveal the underlying mechanisms of GAA, the phosphorylation levels of key kinases involved in the PI3K/AKT pathway were estimated. As shown in Fig. 5A, the phosphorylation levels of both PI3K and AKT were decreased by UVB irradiation. Meanwhile, the phosphorylation level of PI3K and AKT was clearly increased by 100 μmol·L$^{-1}$ GAA compared with that in the UVB-treated group. Moreover, the levels of both p-PI3K and p-AKT in SRA01/04 cells treated with UVB and 100 μmol·L$^{-1}$ GAA were downregulated by the addition of LY294002 (a PI3K inhibitor) (Fig. 5B). Immunoblotting results illustrated that the PI3K/AKT pathway in UVB-induced SRA01/04 cells was activated by GAA.

*Rat lenses exhibited delayed opacification after pretreatment with GAA*

To determine whether GAA regulates cataract pathogenesis and progression, we extracted lenses from rats and cultured them in M199 medium under four lens incubation con-

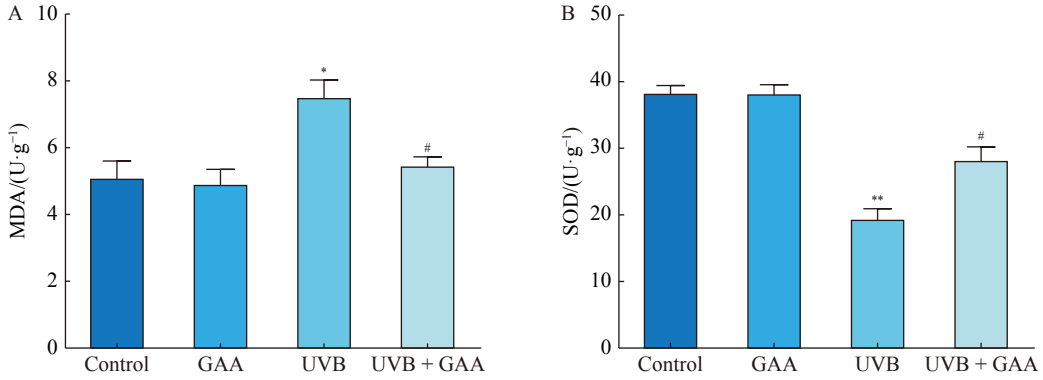


**Fig. 4 Effects of GAA on MDA (A) and SOD (B) activity in SRA01/04 cells exposed to UVB for 15 min. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* the control group; $^{\#}P < 0.05$ *vs* the UVB treated group ($n = 3$)**

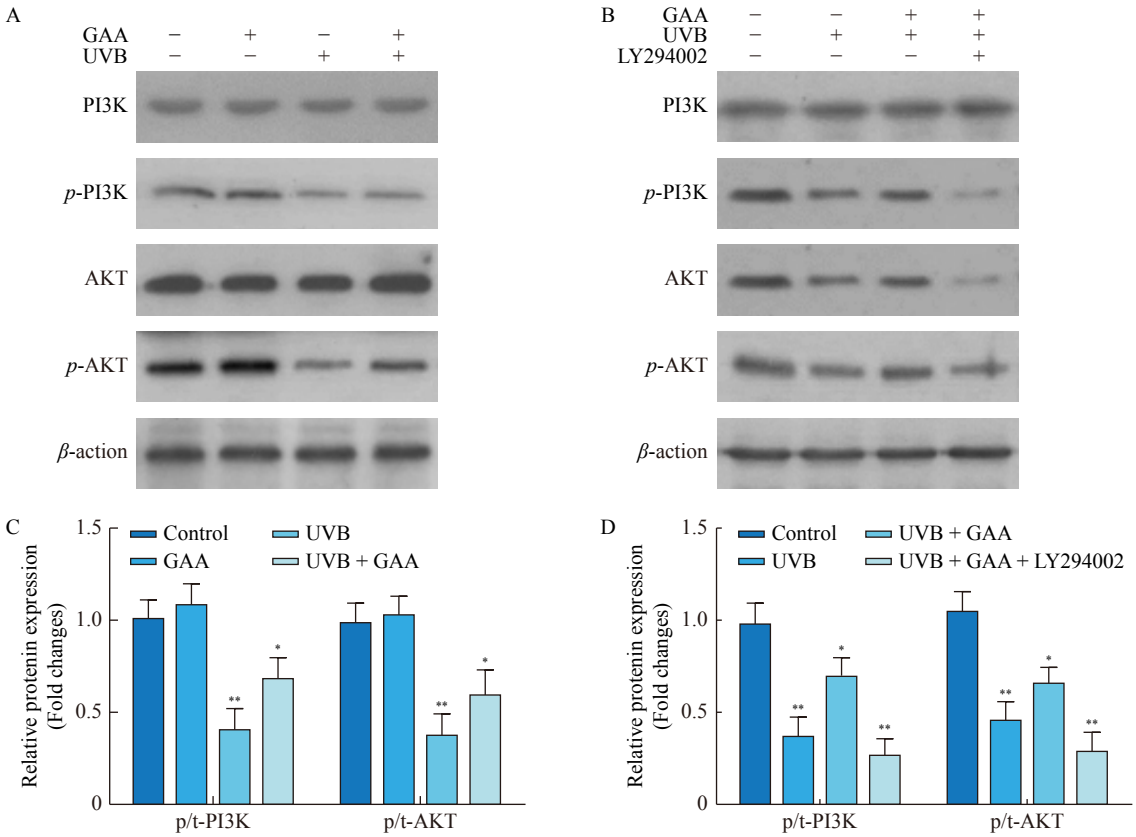


**Fig. 5 GAA activated the PI3K/AKT signaling pathway in SRA01/04 cells. (A) Cells were pretreated with 100 μmol·L$^{-1}$ GAA and then irradiated with UVB for 15 min. (B) Cells were incubated with 100 μmol·L$^{-1}$ GAA with or without a PI3K inhibitor (LY294002). The expression of key proteins in the PI3K/AKT signaling pathway was assessed by Western blot analysis. A−B, representative protein expression is shown. C−D, relative fold changes in expression are shown. $^{*}P < 0.05$ *vs* the control group, $^{**}P < 0.01$ *vs* the control group ($n = 3$)**

ditions. After 1 day of culture, all lenses in the four groups (Control, GAA, UVB, UVB + GAA) were transparent. After 3 days of culture, all the lenses in the UVB group developed moderate opacity, which was consistent with our previous research [14]. However, all the lenses in the UVB + GAA group developed only slight opacity, which was less than that observed in the lenses treated with only UVB. Pretreatment with GAA effectively delayed the occurrence of lens opacity, although it failed to prevent opacity (Fig. 6).

## Discussion

Although the antioxidant activity of *G. lucidum* extract has been reported [17-19], the effects of GAA, an important active component of *G. lucidum*, on UVB radiation-induced oxidative damage have not yet been well studied. Herein, we constructed an *in vitro* cell model subjected to UVB irradiation using human LECs (SRA01/04) and used rat lenses to simulate cataract. Interesting, we found that GAA could ef-

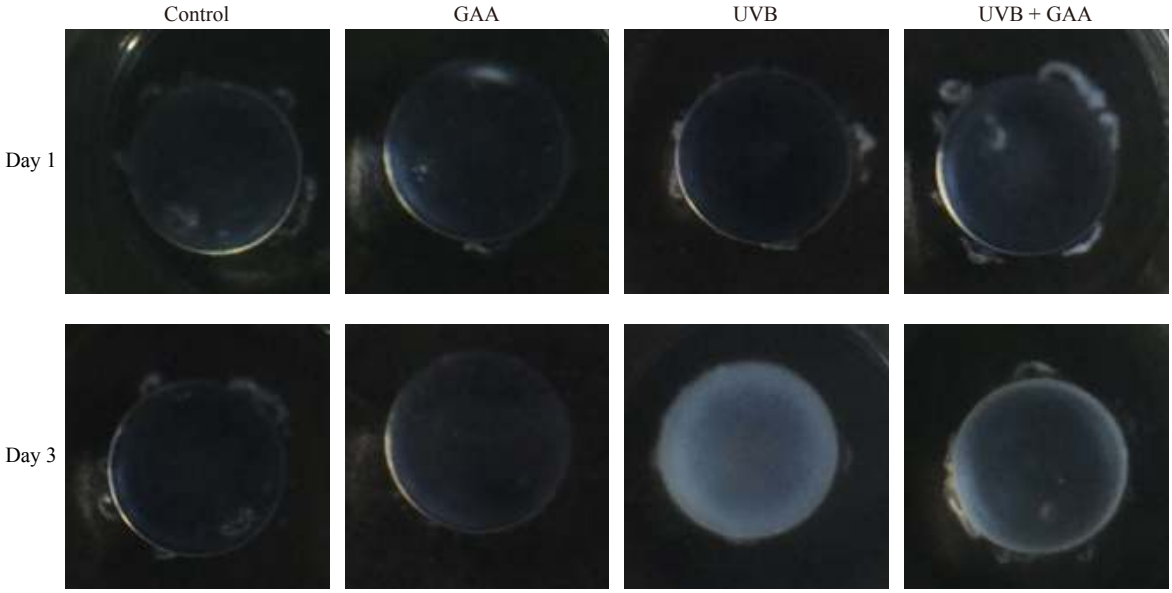


**Fig. 6 Changes in lens turbidity after treatment with GAA. Compared with the control lenses, lenses exposed to UVB became more opaque at 3 days. However, lenses pretreated with GAA before exposure to UVB showed an effective delay in the occurrence of lens opacity, and lens treated with only GAA did not become opaque. Bar = 2 mm**

fectively attenuate UVB-induced injury, as it promoted cell viability and antioxidant activity and had inhibitory effects against apoptosis in UVB-irradiated SRA01/04 cells and rat lenses. Moreover, GAA exerted prosurvival and antioxidant activities by activating the PI3K/AKT pathway in UVB-irradiated SRA01/04 cells and rat lenses.

UVB contributes to injuries of the human eye, resulting in LEC apoptosis and thereby the development of cataract [20-22]. Because LECs are the major cell type in the lens that can maintain the transparency of the entire lens and the stability of the internal environment, we explored a potential preventive compound for cataract in UVB-treated LECs.

Previous studies have proposed that Cleaved caspase-3, Bcl-2 and Bax are related to LEC apoptosis [23]. Cleaved caspase-3 participates in apoptosis as the main executor in the caspase cascade, and the ratio of Bax to Bcl-2 increases during LEC apoptosis [24]. GAA alleviated hypoxia-induced Cleaved caspase-3 expression and cell apoptosis in neural stem cells [25]. GAA also blocked cyclophosphamide-induced Cleaved caspase-3 and Bax expression and increased the expression of Bcl-2 [26]. In this study, we found that UVB significantly increased the expression of Cleaved caspase-3 and Bax and reduced the expression of Bcl-2. Interestingly, GAA intervention partially restored UVB-induced changes to apoptosis, suggesting that GAA blocks UVB-induced LECs apoptosis.

To explore whether GAA could regulate UVB radiation-induced oxidative damage, we observed alterations in MDA and SOD activity. MDA, the end product of lipid oxidation and a reliable indicator of oxidative damage to cell membranes, reflects the extent of lipid peroxidation in LECs [27]. SOD, an antioxidant metal-containing enzyme, plays an important role in protecting the lens from oxidative stress-induced cataract [28]. GAA was suggested to be related to the downregulation of MDA activity and upregulation of SOD activity in mouse serum [29]. Our results showed that UVB could significantly increase MDA activity and reduce SOD activity. However, GAA treatment restored UVB-induced oxidative damage, suggesting that GAA can reduce lipid oxidation and shows antioxidant activity.

Previous studies showed that the PI3K/AKT signaling pathway enhanced cell survival and antioxidant response after UVB irradiation [11]. The PI3K/AKT signaling pathway was found to be involved in the proliferation and apoptosis of human LECs and cataract tissue [30]. And GAA could activate the PI3K/AKT signaling pathway through mediating miR-153 expression in hypoxia-impaired PC12 cells [31]. Based on our findings that GAA could inhibit the UVB-induced apoptosis of LECs, we investigated whether GAA exerted its functions *via* PI3K/AKT signaling pathway. Herein, we discovered that GAA obviously activated the PI3K/AKT signaling pathway in UVB-exposed LECs. However, the effects of GAA on the PI3K/AKT signaling pathway were reversed when PI3K was inhibited by LY294002. These findings provide evidence that the PI3K/AKT pathway is involved in the protective functions of GAA in UVB-impaired LECs.

Finally, we investigated whether GAA played an important role in cataract formation. By performing an established UVB-induced cataract model [14], we found that GAA delayed the occurrence of lens opacity and might play a preventive role in cataract formation.

To summarize, we report that GAA could protect LECs and rat lenses against UVB irradiation. These results indicate that GAA might elevate cell viability and repress apoptosis and oxidative stress through activating the PI3K/AKT pathway. These data demonstrate that GAA, a natural compound from *G. lucidum,* has the capability to protect the lens against UVB-induced cataract. Chemical prevention is a promising

strategy for the management of cataract. Natural products provide an ideal source and should be pursued [32]. The future application of 100 μmol·L$^{-1}$ GAA as a preventive compound should be discussed through *in vivo* experiments and human studies. This might be meaningful for the clinical pharmacotherapy of cataract in the future.

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**Cite this article as:** KANG Li-Hua, ZHANG Guo-Wei, ZHANG Jun-Fang, QIN Bai, GUAN Huai-Jin. Ganoderic acid A protects lens epithelial cells from UVB irradiation and delays lens opacity [J]. *Chin J Nat Med*, 2020, **18**(12): 934-940.