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Procyanidin A_1 and its digestive products inhibit acrylamide-induced IPEC-J2 cell damage: apoptosis and cell cycle arrest

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ABSTRACT

Our previous study has demonstrated that procyanidin A_1 (A_1) and its simulated digestive product (D-A_1) can prevent acrylamide (ACR)-induced cytotoxicity in small intestine cells. However, the potential mechanism remains poorly understood. In this study, ACR treatment was found to increase the levels of 8-hydroxy-deoxyguanine (8-OHdG) and phosphorated histone H_2AX (γH_2AX), two DNA damage markers, thereby resulting in cell cycle arrest in the G2/M phase; whereas both A_1 and D-A_1 could prevent the phosphorylation of ataxia telangiectasia mutated (ATM) and checkpoint kinase 2 (Chk2), and then regulate the expression of G2/M phase-related proteins, finally maintaining normal cell cycle progression. Moreover, A_1 and D-A_1 could increase the B cell lymphoma 2 (Bcl-2)/Bcl2-associated X (Bax) ratio and decrease the expression of cleaved caspase-3 and cleaved caspase-9 proteins to alleviate ACR-induced cell apoptosis, which might be related to the inhibition of the mitogen-activated protein kinase (MAPK) pathway. More importantly, A_1 showed no remarkable variation in inhibitory effect before and after digestion, indicating that it can endure gastrointestinal digestion and may be a promising phytochemical to alleviate ACR-induced intestinal cell damage.

1. Introduction

Acrylamide (ACR) is a potential carcinogen formed during food thermal processing, and is extensively detected in different foods such as chips, coffee, breads and cakes[1]. It has been evaluated that the average human intake of ACR varies from 0.2 μg/(kg·day) to 1.0 μg/(kg·day), and for some high-level consumers, it can even reach up to 4 μg/(kg·day), which far exceeds the acceptable daily intake of ACR (1 μg/day)[2]. Recent *in vivo* data have demonstrated that dietary ACR is easily absorbed[2], and then induces small intestinal damage with disorderly and sparsely arranged epithelial cells, lower villus height/crypt depth ratio, and higher intestinal permeability[3].

In human body, ACR is preliminarily metabolized to glycidamide, and then ACR or glycidamide can form adducts with glutathione (GSH), resulting in the accumulation of reactive oxygen species (ROS) and further DNA oxidation[4-5]. Besides, both ACR and glycidamide can bind to DNA forming adducts to induce DNA damage[5-6]. To repair the damaged DNA, the DNA damage response system will be activated, which further leads to cell cycle arrest by mediating cell cycle-related regulatory kinases[7]. However, when the DNA damage is too severe to be repaired, this system will activate cell apoptosis to remove the damaged cells. Besides, ACR-induced excessive ROS production can cause cell apoptosis by promoting cell

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signaling transduction, such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase, nuclear factor erythroid-2-related factor 2 (Nrf2), and nuclear factor-kappa B[8-11]. Previous studies have reported that ACR exposure can activate MAPK signaling, which plays important roles in various cellular processes including proliferation, differentiation, and apoptosis[12-13]. In the intestinal tissues or cells, ACR treatment may boost ROS accumulation and subsequently regulate the phosphorylation of MAPK, thereby causing mitochondrial dysfunction and activating caspase-3 dependent cell apoptosis[14-15]. Therefore, the MAPK signaling pathway may be a potential target for effective alleviation of ACR-induced cell damage, and it is important to develop some effective strategies to inhibit ACR-induced small intestinal damage.

Some studies have demonstrated that allicin, *N*-acetylcysteine, *Ganoderma atrum* polysaccharide, and crocin can suppress ACR-induced cell apoptosis, oxidative stress, and inflammation in intestinal epithelial cells[16-18]. However, considering their poor dietary sources, these compounds may not be the most appropriate antidotes against ACR-induced intestinal cell damage. Procyanidins (PCs) are widely present in human daily diets such as cereals, nuts, and fruits, whose average intake is 57.5–95.0 mg/day in the USA, and is as high as 450 mg/day in Spain[19]. PCs possess valuable biological activities, and may be a class of excellent dietary antidotes for mitigating ACR-induced intestinal cell damage[20]. Rodriguez-Ramiro et al. [14] have reported that PCs can protect the small intestinal epithelial cells from ACR-induced toxicity, but they ignored that the inhibitory effect of PCs may be altered or even decreased after being absorbed in the gastrointestinal system. Our previous study has preliminarily evaluated the inhibitory effect of PCs on ACR-induced intestinal cytotoxicity, and found that procyanidin A_1 (A_1) has a superior effect compared with catechin, epicatechin, procyanidin B_3 and a procyanidin A-type trimer, and the effect is almost equal before and after digestion[21]. These findings indicate that A_1 may be an excellent phytochemical that can endure gastrointestinal digestion to prevent ACR-induced small intestinal cell damage.

In this study, we evaluated the inhibitory effect of A_1 before and after digestion on ACR-induced small intestinal cell damage, and revealed the related molecular mechanism. Our findings provide some important implications for revealing the effect of gastrointestinal digestion on PCs in alleviating ACR-induced small intestinal cell damage.

2. Materials and methods

2.1 Materials and reagents

Dulbecco's modified Eagle medium (DMEM)/F12 was provided by HyClone (Logan, UT). Fetal bovine serum (FBS) was obtained from Gibco (Grand Island, USA). Penicillin/streptomycin, 0.25% trypsin, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sinopharm (Wuhan, China). ACR was obtained from Aladdin (Shanghai, China). Cell cycle detection, Hoechst 33342, and bicinchoninic acid (BCA) protein assay kits were provided by Nanjing Jiancheng Biology Engineering (Nanjing, China). 8-Hydroxy-deoxyguanine (8-OHdG) enzyme linked immunosorbent assay (ELISA) kit was from Shanghai Hengyuan Biotechnology Co., Ltd. (Shanghai, China). The primary antibodies against ataxia telangiectasia mutated (ATM) (No. 382214), extracellular regulated protein kinase (ERK) 1/2 (No. R66285), p-ERK1/2 (No. 301245), c-Jun N-terminal kinase (JNK) 1/2/3 (No. R24780), p-JNK1/2/3 (No. 340778), p38 (No. R25239), and p-p38 (No. 310069) were provided by Zhengneng Biological (Chengdu, China). The primary antibodies against phosphorated histone H_2AX (γH_2AX) (No. AF5836), cell division cycle 25 homolog C (CDC25C) (No. AF6462), cyclin dependent kinase 1 (CDK1) (No. AF0111), cyclin B1 (No. AF6627), growth arrest and DNA damage 45A (GADD45A) (No. AF6954), p-ATM (No. AF5743), checkpoint kinase 2 (Chk2) (No. AF6501), p-Chk2 (No. AF5776), and tumor protein 53 (p53) (No. AF7671) were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). The primary antibodies against *β*-actin (No. YM3028), cleaved caspase-9 (No. RLT0662), cleaved caspase-3 (No. RLT0653), and B cell lymphoma 2 (Bcl-2) (No. RML3041) were from Ruiying Biological (Suzhou, China). The primary antibody against Bcl2-associated X (Bax) (No. A0207) was obtained from ABclonal Technology (Wuhan, China). RIPA lysis buffer, enhanced electrochemiluminescence (ECL) assay kit, and horseradish peroxidase (HRP)-conjugated secondary antibodies were provided by Sinopharm (Wuhan, China).

2.2 Preparation of A_1 and its simulated digestive product (D-A_1)

A_1 was isolated from peanut skin in our laboratory, whose purity was higher than 98% as analyzed by high performance liquid chromatography (HPLC). D-A_1 was prepared with the method reported in our previous study[21]. Briefly, A_1 was subjected to simulated salivary, gastric and intestinal digestion. Then, the samples were centrifuged to obtain the supernatant, which was finally freeze-dried to obtain D-A_1. D-A_1 was mainly composed of three different procyanidin A-type dimers as indicated by high performance liquid chromatography-quadrupole-time of flight-mass spectrometry (HPLC-Q-TOF-MS^2) analysis.

2.3 Cell culture

Porcine epithelial cell line IPEC-J2 cells were provided by the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. According to our previous results[21], cells were seeded and pretreated with A_1 or D-A_1 (0.2, 1, or 2 μmol/L) for 4 h, followed by treatment with ACR (7.5 mmol/L) for 4–20 h in this study.

2.4 MTT analysis

IPEC-J2 cells were seeded in 96-well plates at the density of 1×10^4 cells/well for 24 h. Cells were preincubated with A_1 or D-A_1 for 4 h before exposure to ACR for 20 h. After treatment, the cells were cultured with 0.5 mg/mL MTT solution for 4 h. Then, the medium was removed, and 150 μL dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Finally, the absorbance was measured at 490 nm by a microplate reader (Thermo Fisher Scientific, USA).

2.5 Cell morphology observation

IPEC-J2 cells were grown in 6-well plates (3×10^5 cells/well) for 24 h. Cells were pretreated with A_1 or D-A_1 for 4 h, and then exposed to ACR for 20 h. Afterwards, the cell morphology was observed and photographed by an optical inverted microscopy (Nikon, Japan).

2.6 Hoechst 33342 staining assay

IPEC-J2 cells were seeded in 6-well plates (3×10^5 cells/well). Cells were pretreated with A_1 or D-A_1 for 4 h before exposure to ACR for 20 h. After treatment, cells were washed with phosphate buffered saline (PBS) twice and stained with Hoechst 33342 at room temperature for 5 min in the dark. Then, cells were washed twice and photographed using a fluorescence microscope (Nikon, Japan).

2.7 Cell cycle analysis

IPEC-J2 cells were grown in 6-well plates at a density of 3×10^5 cells/well for 24 h. Cells were incubated with A_1 or D-A_1 for 4 h, and then treated with ACR for 20 h. After treatment, the cells were collected and fixed in 70% ethanol solution at −20 °C overnight. Then, the cells were washed twice with PBS and stained with propidium iodide (PI)/RNase A solution at room temperature for 1 h in the dark. Ultimately, the cell cycle analysis was conducted with flow cytometry (Backman Counter, USA), and the distribution of cell cycle was analyzed using FlowJo software (Ashland, USA).

2.8 Detection of 8-OHdG

IPEC-J2 cells were seeded in 6-well plates at a density of 1×10^5 cells/well for 24 h. After pretreatment with A_1 or D-A_1 for 4 h and exposure to ACR for another 20 h, the medium was collected. The level of 8-OHdG in the medium was measured with ELISA kit according to the manufacturer's instructions.

2.9 Western blot analysis

After the indicated treatments, cells were harvested with RIPA buffer, and the protein content was quantified with BCA protein assay kit. Proteins were mixed with loading buffer and denatured by being boiled at 100 °C for 10 min. Equal content of proteins (20 μg) was separated by 8%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto 0.22 μm polyvinylidene fluoride (PVDF) membranes. Afterwards, the membrane was blocked with 5% BSA solution at room temperature for 2 h, and then incubated with primary antibodies (diluted ratio: γH_2AX, CDC25C, CDK1, cyclin B1, GADD45A, p-ATM, p-Chk2, Chk2, p53, Bcl-2, cleaved caspase-3, and cleaved caspase-9, 1:500; ATM, Bax, p-p38, p38, p-ERK1/2, ERK1/2, p-JNK1/2/3, and JNK1/2/3, 1:1 000; *β*-actin, 1:3 000) overnight at 4 °C. Then, the membranes were incubated with secondary antibodies at room temperature for 2 h. After enhancement with a chemiluminescence ECL assay kit, the immunoreactive bands were visualized using the Odyssey system (LI-COR Biosciences, USA), whose densities were quantified by ImageJ software.

2.10 Statistical analysis

Statistical analysis was performed using the SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Duncan's test were performed to compare the differences among multiple groups, and the results were shown as mean ± standard error of the mean (SEM). $P < 0.05$ was considered as statistically significant.

3. Results

3.1 A_1 and D-A_1 inhibit ACR-induced IPEC-J2 cell damage

To investigate the inhibitory effect of A_1 and D-A_1 on ACR-induced small intestinal cell damage, IPEC-J2 cells were pretreated with different concentrations of A_1 or D-A_1 (0.2, 1 or 2 μmol/L) for 4 h before exposure to ACR for 20 h. The cell viability was evaluated by MTT assay. As shown in Fig. 1A, ACR significantly decreased the cell viability ($P < 0.000\ 1$), whereas both A_1 and D-A_1 could alleviate the decrease in a concentration-dependent manner ($P < 0.05$). In addition, cell morphology examination also showed that ACR-treated cells had the characteristics of shrinkage, deformation, blebbing, and detachment from the surface of plates, while pre-treatment with A_1 or D-A_1 could obviously maintain the normal cell morphology (upper part in Fig. 1C). Furthermore, a Hoechst 33342 staining assay was performed to confirm the prevention effect of A_1 and D-A_1 on ACR-induced cell damage. As expected, ACR treatment caused nuclear condensation with bright blue fluorescence intensity in IPEC-J2 cells, indicating the occurrence of DNA damage and cell apoptosis, while this phenomenon could be inhibited by pretreatment with A_1 or D-A_1 (Fig. 1B and lower part in Fig. 1C).

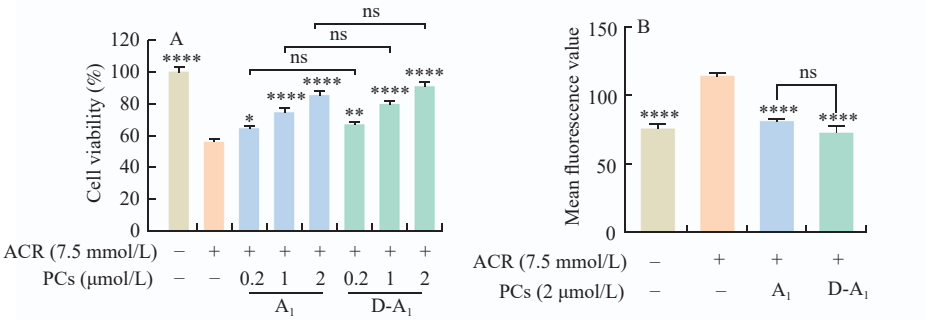

Fig. 1 A_1 and D-A_1 inhibit ACR-induced IPEC-J2 cell damage. IPEC-J2 cells were incubated with A_1 or D-A_1 (0.2, 1 or 2 μmol/L) for 4 h before treatment with ACR (7.5 mmol/L) for additional 20 h. (A) The cell viability was determined by MTT assay. (B) The mean fluorescence value of cells examined by Hoechst 33342 staining. (C) The morphology of IPEC-J2 cells was observed by an optical inverted microscopy (upper). Cell apoptosis was examined by Hoechst 33342 staining (lower). Scale bar: 50 μm. Data are represented as mean ± SEM, $n = 4–6$. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.000\ 1$ *vs*. ACR-treated alone group; not significant (ns) $P > 0.05$ in comparison with the indicated group; same as below.

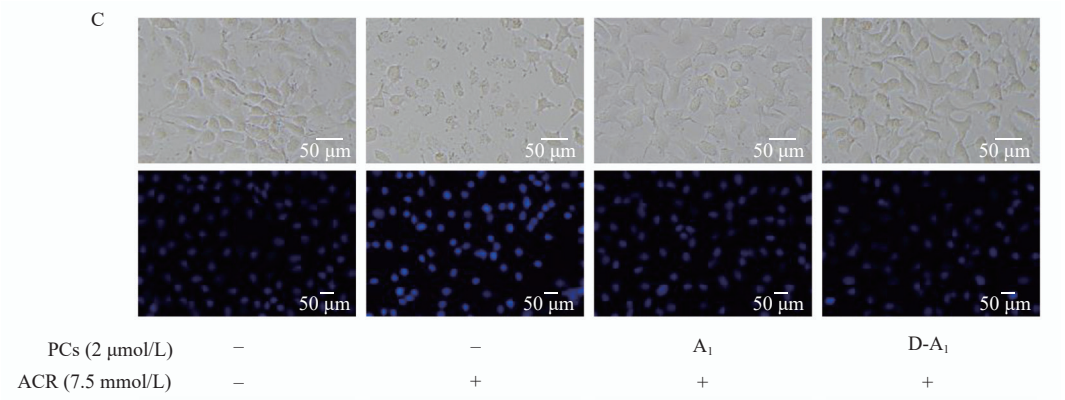

Fig. 1 (*Continued*)

3.2 *A_1 and D-A_1 alleviate ACR-induced DNA damage*

To explore the inhibitory effect of A_1 and D-A_1 on ACR-stimulated DNA damage, we determined the level of 8-OHdG, a DNA damage marker[22]. As shown in Fig. 2A, treatment with ACR alone remarkably boosted the level of 8-OHdG compared with the blank treatment ($P < 0.000\ 1$), whereas A_1 or D-A_1 pre-treatment could inhibit the increase ($P < 0.001$), with D-A_1 exhibiting a stronger effect than A_1 ($P < 0.001$). The expression of the γH_2AX protein, an indicator for the degree of DNA damage[23], was detected to confirm the inhibitory effect of A_1 and D-A_1 on ACR-induced DNA damage. The results indicated that A_1 and D-A_1 pretreatment significantly decreased the expression of γH_2AX relative to treatment with ACR alone ($P < 0.000\ 1$) (Fig. 2B). Similarly, D-A_1 exhibited a superior inhibitory effect relative to A_1 ($P < 0.01$). Taken together, it could be concluded that A_1 and D-A_1 can suppress ACR-stimulated DNA damage, and A_1 possesses an excellent inhibitory effect after undergoing gastrointestinal digestion.

3.3 *A_1 and D-A_1 prevent ACR-induced cell cycle arrest*

When DNA damage occurs, cell cycle arrest may be triggered to repair the damaged DNA[23]. Thus, the distribution of cell cycle was examined by a flow cytometry analysis. As demonstrated in Fig. 3, compared with the control treatment, ACR treatment significantly reduced the percentage of cells in the G0/G1 phase ($P < 0.000\ 1$), and led to no variation of that in the S phase, but remarkably increased the percentage in the G2/M phase ($P < 0.000\ 1$), indicating the occurrence of cell cycle arrest in the G2/M phase. Pretreatment with A_1 or D-A_1 (2 μmol/L) on IPEC-J2 cells significantly increased ($P < 0.000\ 1$) the percentage of cells in the G0/G1 phase, and at the same time significantly decreased ($P < 0.001$) that in the G2/M phase compared with the treatment with ACR alone. More importantly, there was no significant difference between A_1 and D-A_1 pretreatment ($P > 0.05$).

3.4 *A_1 and D-A_1 regulate the expression of cell cyclin-related proteins in ACR-induced cells*

Cell cycle progress is modulated by cyclins and their kinase CDKs[24]. To explore the molecular mechanisms for A_1 and D-A_1 to regulate cell cycle distribution, the levels of G2/M phase-associated proteins (such as CDC25C, GADD45A, CDK1, and cyclin B1) were examined (Figs. 4A-E). The results demonstrated that ACR

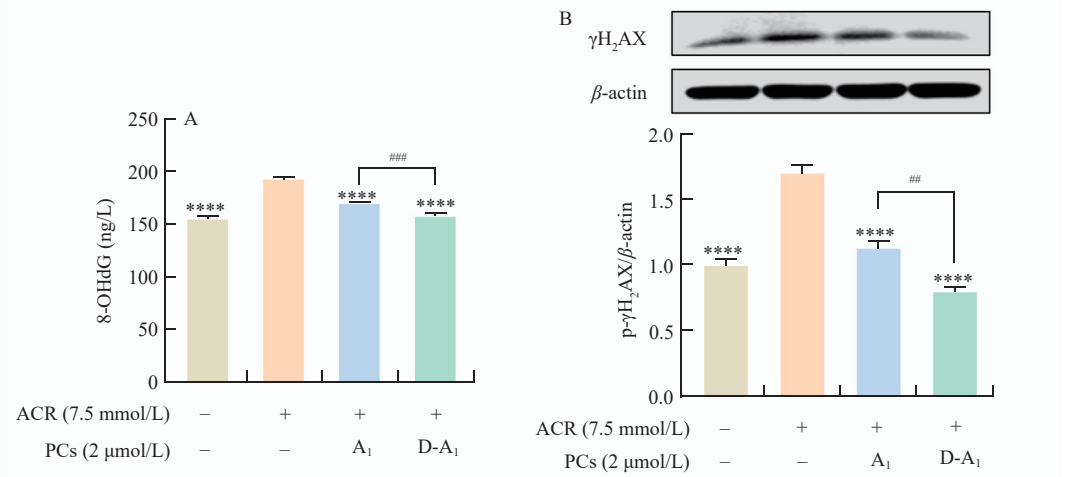

Fig. 2 A_1 and D-A_1 prevent ACR-induced IPEC-J2 cell DNA damage. IPEC-J2 cells were pretreated with A_1 or D-A_1 (2 μmol/L) for 4 h before exposure to ACR (7.5 mmol/L) for 20 h. (A) Level of 8-OHdG in the medium. (B) Western blotting was performed to measure the expression of γH_2AX protein. β-Actin was used as a loading control. Data are represented as mean ± SEM, $n = 3$. ****$P < 0.000\ 1$ *vs*. ACR-treated alone group; $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ in comparison with the indicated group.

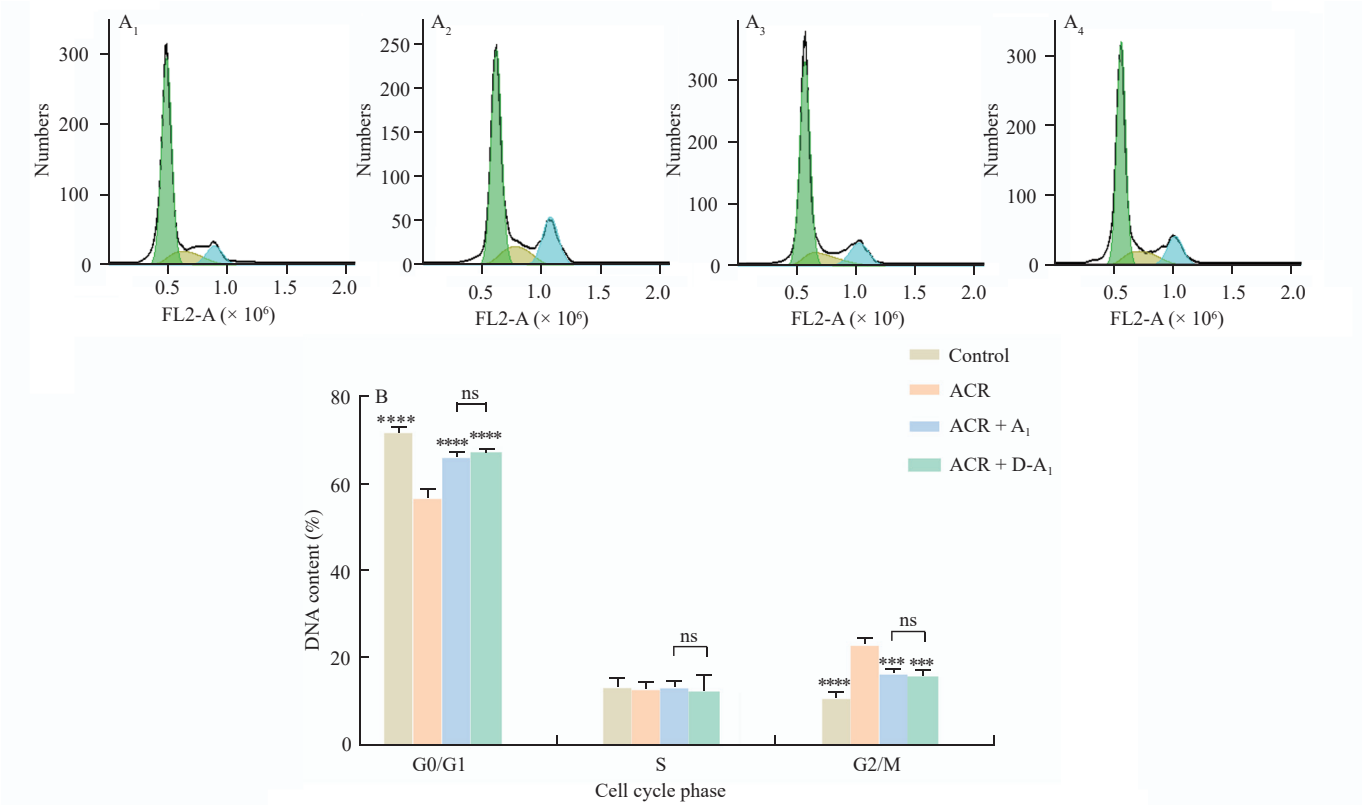

Fig. 3 A_1 and D-A_1 modulate ACR-induced IPEC-J2 cell cycle distribution. Cells were pre-incubated with A_1 or D-A_1 (2 μmol/L) for 4 h, and then cultured with ACR (7.5 mmol/L) for 20 h. (A) Flow cytometry analysis of cell cycle of IPEC-J2 cells, the subscripts 1 to 4 are control, ACR, ACR + A_1, ACR + D-A_1, respectively. (B) Histogram of cell cycle phase distribution of IPEC-J2 cells. Data are represented as mean ± SEM, $n = 3$. ***$P < 0.001$, and ****$P < 0.000\ 1$ *vs*. ACR-treated alone group; not significant (ns) $P > 0.05$ in comparison with the indicated group; same as below.

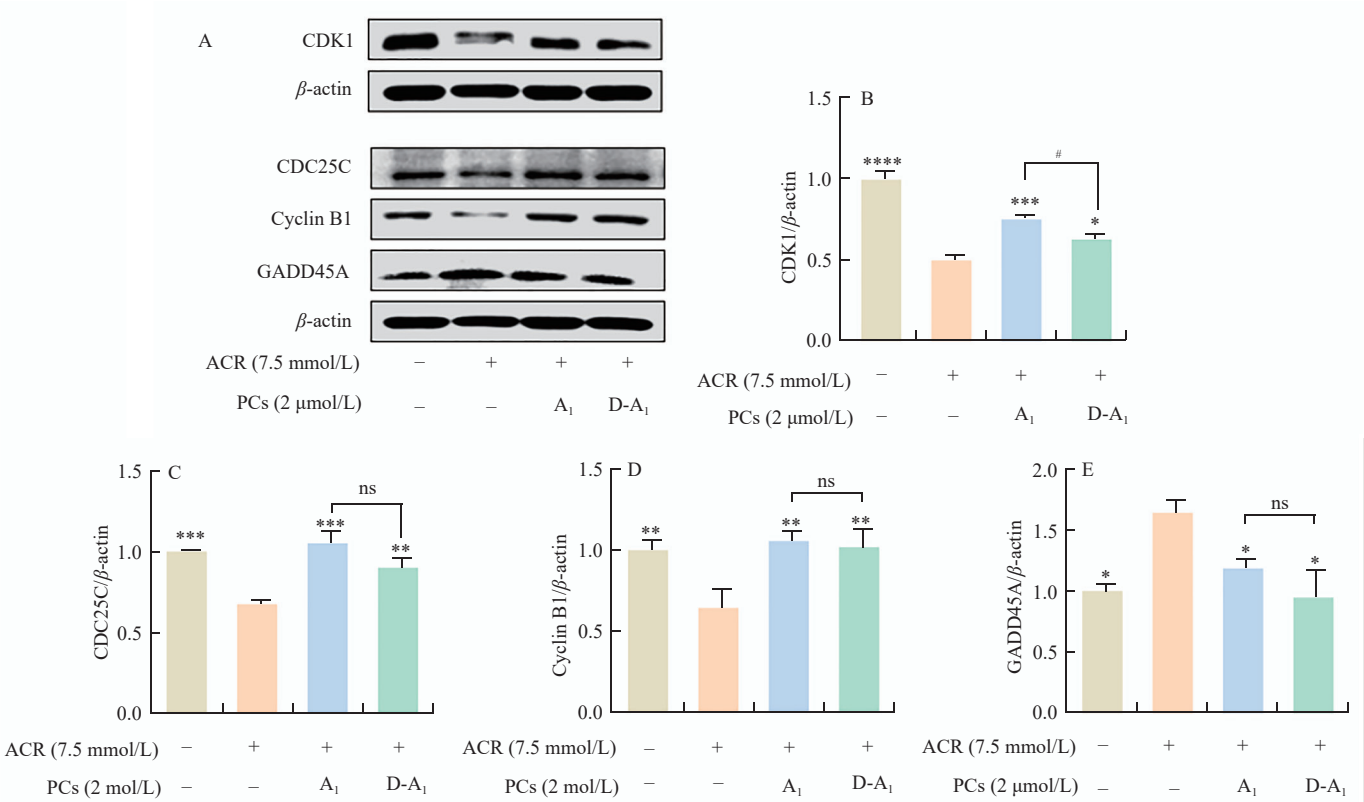

Fig. 4 Effects of A_1 and D-A_1 on cell cycle-related proteins. Cells pretreated with A_1 or D-A_1 (2 μmol/L) for 4 h and exposed to ACR (7.5 mmol/L) for 20 h, and the protein expression was analyzed by Western blot. β-Actin was used to normalize the expression of these proteins. (A) Images of Western blot bands. Expression of (B) CDK1, (C) CDC25C, (D) cyclin B1 and (E) GADD45A proteins.

treatment alone significantly reduced the expression of CDK1, cyclin B1, and CDC25C proteins ($P < 0.000\ 1$, $P < 0.01$, $P < 0.001$), as well as substantially promoted the expression of GADD45A protein ($P < 0.05$). However, A_1 or D-A_1 pre-treatment could reverse these changes ($P < 0.05$), and D-A_1 showed a stronger promoting effect on the expression of CDK1 protein than A_1 ($P < 0.05$). These results indicated that A_1 and D-A_1 could reverse ACR-induced cell cycle arrest by regulating the expression of G2/M phase-associated proteins.

3.5 *A_1 and D-A_1 suppress ACR-induced activation of ATM/Chk2*

It has been documented that the ATM/Chk2 pathway is involved in regulating the expression of cell cycle-associated proteins[25]. Therefore, we monitored the phosphorylation status of ATM and Chk2 (Figs. 5A and B). The phosphorylation level of ATM was obviously promoted after ACR treatment for 8 h ($P < 0.01$), accompanied by an increase in the phosphorylation of Chk2 ($P < 0.001$), a main target of ATM. However, cells pretreated with A_1 or D-A_1 showed inhibited phosphorylation reaction ($P < 0.001$), and D-A_1 showed a stronger inhibitory effect on the phosphorylation of Chk2 than A_1 ($P < 0.05$). In addition, ACR exposure resulted in significant upregulation of p53 protein ($P < 0.01$), which could be inhibited by A_1 or D-A_1 pre-treatment ($P < 0.01$, $P < 0.001$) (Fig. 5C).

3.6 *A_1 and D-A_1 regulate the level of cell apoptosis-associated proteins in ACR-induced cells*

When the damaged DNA cannot be repaired, the DNA damage response system will activate cell apoptosis to remove the damaged cells[7]. Fig. 1 clearly shows that A_1 and D-A_1 can attenuate ACR-induced cell apoptosis in IPEC-J2 cells. To reveal the molecular mechanism for the inhibitory effect of A_1 and D-A_1 on ACR-induced cell apoptosis, the expression of cell apoptosis-related proteins was measured (Figs. 6A-E). The results indicated that ACR treatment alone could promote the protein expression of Bax, cleaved caspase-9, and cleaved caspase-3, but decrease that of Bcl-2 ($P < 0.01$), resulting in a decrease in the Bcl-2/Bax ratio ($P < 0.000\ 1$). Importantly, compared with the treatment with ACR alone, pretreatment with both A_1 and D-A_1 could significantly reverse the changes

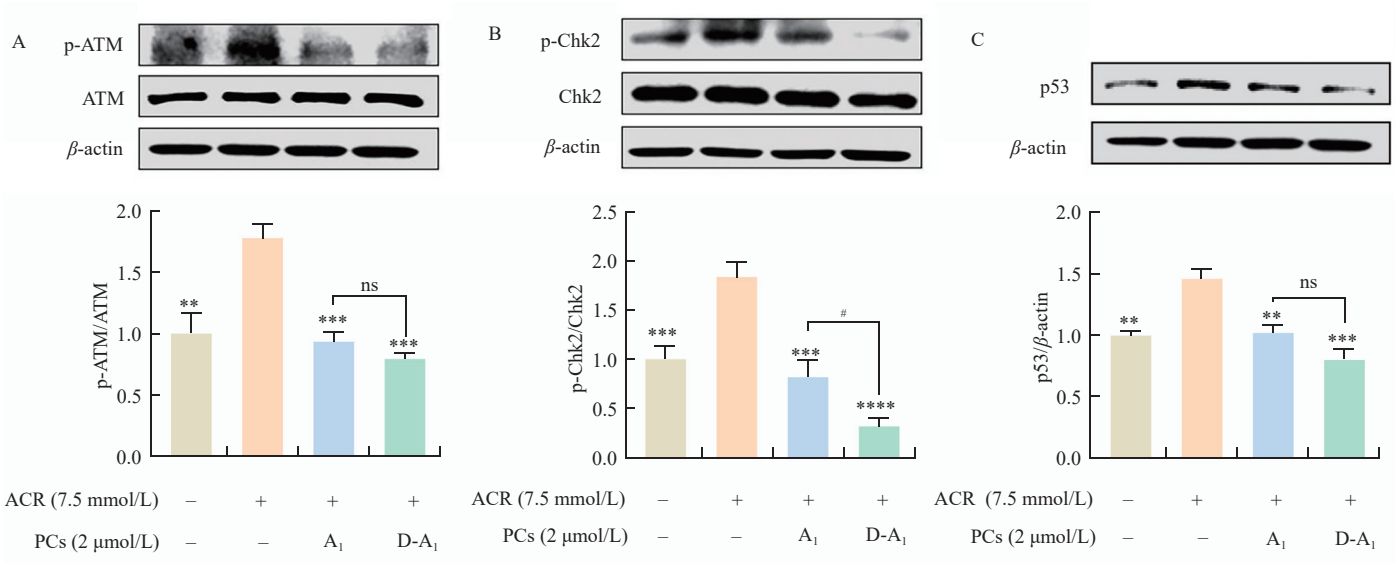

Fig. 5 Effects of A_1 and D-A_1 on ATM pathway in IPEC-J2 cells. IPEC-J2 cells pretreated with A_1 or D-A_1 (2 μmol/L) for 4 h and then exposed to ACR (7.5 mmol/L) for 8 h. The protein expression was analyzed by Western blot. β-Actin was used to normalize the expression of these proteins. The ratios of (A) p-ATM/ATM, (B) p-Chk2/Chk2 and (C) p53/β-actin.

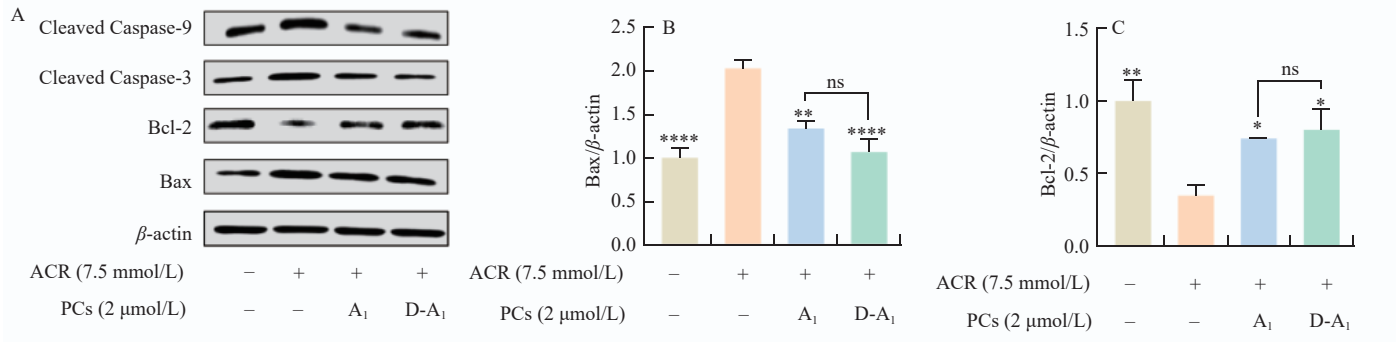

Fig. 6 Effects of A_1 and D-A_1 on cell apoptosis-related proteins. Cells pretreated with A_1 or D-A_1 (2 μmol/L) for 4 h and exposed to ACR (7.5 mmol/L) for 20 h, and the protein expression was analyzed by Western blot. β-Actin was used to normalize the expression of these proteins. (A) Images of Western blot bands. Expression of (B) Bax, (C) Bcl-2, (D) cleaved caspase-3 and (E) cleaved caspase-9 proteins. (F) Bcl-2/Bax ratio.

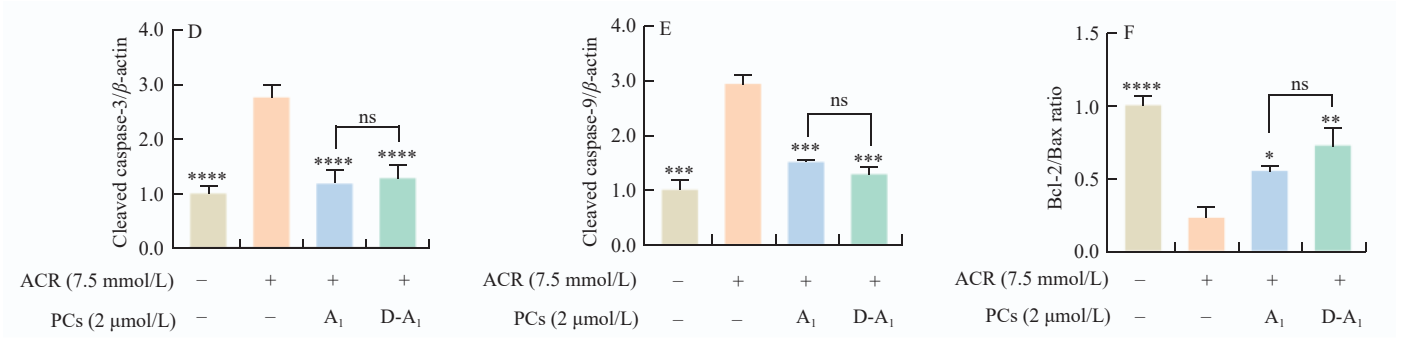

Fig. 6 (*Continued*)

in the expression of these proteins ($P < 0.05$, $P < 0.01$), and there was no significance between A_1 and D-A_1 treatments ($P > 0.05$). These results demonstrated that A_1 and D-A_1 can attenuate ACR-induced IPEC-J2 cell apoptosis by regulating apoptosis-associated proteins.

3.7 A_1 and D-A_1 prevent the ACR-induced activation of MAPK

Since ACR-induced ROS accumulation can mediate the MAPK pathway and subsequently cause cell apoptosis[10], the expression of MAPK pathway-related proteins was measured by Western blot to investigate the role of the MAPK pathway in the inhibitory effect of A_1 and D-A_1. As shown in Figs. 7A-D, treatment with ACR alone resulted in significant phosphorylation of p38, ERK1/2 and JNK1/2/3 in IPEC-J2 cells ($P < 0.01$), which could be equally abolished by A_1 or D-A_1 pretreatment ($P < 0.05$, 0.01).

4. Discussion

ACR is a typical processing-induced pollutant extensively existing in thermally processed foods, such as roasted coffee beans, breakfast cereals, and chips[26]. Thus, life-long exposure to ACR through dietary intake seems to be inevitable. Many studies have demonstrated that ACR can induce small intestinal damage with wider villus spacing, shorter villus length and necrosis of epithelial cells in rats[3,27]. Our previous study has demonstrated that ACR can cause damage to small intestinal epithelial IPEC-J2 cells, which could be alleviated by PCs and their digestive products, particularly A_1 and D-A_1. However, the underlying mechanism remains poorly understood[21]. Here, we explored the molecular mechanisms for A_1 and D-A_1 to inhibit ACR-induced damage to small intestinal epithelial IPEC-J2 cells.

In the body, ACR is easily coupled with GSH, resulting in the consumption of GSH and subsequent accumulation of ROS, which is an initial signal for various ACR-induced toxicities, such as

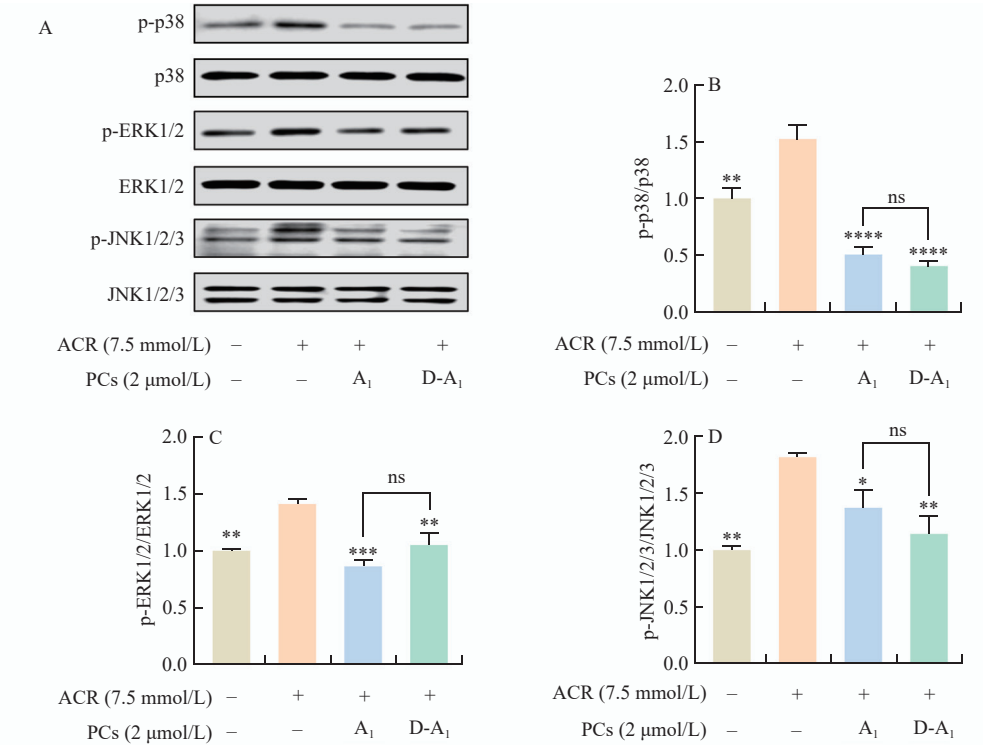

Fig. 7 Effects of A_1 and D-A_1 on the MAPK pathway in IPEC-J2 cells. IPEC-J2 cells treated with A_1 or D-A_1 for 4 h and then exposed to ACR (7.5 mmol/L) for additional 4 h. The protein expression was analyzed by Western blot. *β*-Actin was used to normalize the expression of these proteins. (A) Images of Western blot bands of p-p38, p38, p-ERK1/2, ERK1/2, p-JNK1/2/3, and JNK1/2/3 proteins. The ratios of p-p38/p38 (B), p-ERK1/2/ERK1/2 (C) and p-JNK1/2/3/JNK1/2/3 (D).

oxidative stress damage, apoptosis, inflammation, and autophagy[28-31]. In our previous studies, we have demonstrated that ACR can lead to decrease in GSH and accumulate ROS in IPEC-J2 cells[32]. However, excessive ACR-induced ROS can directly attack the guanine residues on DNA to produce 8-OHdG or other DNA adducts, resulting in DNA damage[33-34]. Besides, both ACR and its primary metabolite (glycidamide) can bind to DNA and eventually cause DNA damage[5-6]. In this study, ACR treatment could induce chromatin condensation and increased the content of 8-OHdG in the medium, indicating that ACR can cause DNA damage to IPEC-J2 cells. The damaged DNA bases are either repaired or removed by the base excision repair system, resulting in DNA strand breaks and finally promoting the phosphorylation of H_2AX at Ser139[23]. In this study, ACR treatment alone boosted the level of γH_2AX protein in IPEC-J2 cells, which could be effectively prevented by A_1 or D-A_1, particularly by D-A_1.

The ATM/Chk2 signaling pathway is a vital initiator of DNA damage response, and is activated during DNA strand breaks, which then repairs the damaged DNA by regulating the downstream proteins related to cell cycle[35]. Upon the occurrence of DNA damage, the ATM protein is phosphorylated at Ser1981, which subsequently leads to phosphorylation of the Chk2 protein at Thr68 or activation of p53, resulting in decreases in the level of CDC25C protein and downstream CDKs/cyclins complexes and eventually cell cycle arrest[23-24]. Cell cycle progression is regulated by the interaction between CDKs and corresponding cyclins[36]. In this process, the CDK1/cyclin B1 complex plays a crucial role in the G2/M phase checkpoint. The level of this complex will increase upon the transition of cells from the G2 phase to the M phase, while the loss or deactivation of CDK1 or cyclin B1 can cause cell cycle arrest in the G2/M phase[36]. Besides, GADD45A can be activated by p53 and then bind to CDK1, and is therefore considered as an inhibitor of CDK1/cyclin B1 complex[37]. Our results suggested that ACR could activate the ATM/Chk2 pathway by promoting the phosphorylation of ATM and Chk2 as well as increasing the level of p53 protein, and then boost the expression of GADD45A protein and inhibit that of CDC25C, CDK1 and cyclin B1 proteins, finally resulting in cell cycle arrest in the G2/M phase in IPEC-J2 cells. This phenomenon was also observed in HepG2 cells[5]. Inconsistently, ACR can induce cell cycle arrest in different phases of various cells by regulating different targeted proteins. Yang et al.[38] have reported that ACR increases the expression of forkhead box O3 protein and decreases that of its downstream cyclin D1, which is a regulator of the cell cycle transition from the G1 phase to the S phase, eventually leading to cell cycle arrest in G1/S phase BRL-3A cells. Similarly, Chen et al.[39] also revealed that ACR can induce G0/G1 phase arrest of human astrocytoma cells, which is associated with the reduction in CDK4 and cyclin D1 proteins. Further exploration is needed to illustrate the mechanism for ACR-induced cell cycle arrest in different cells. Importantly, in the present study, we observed that A_1 and D-A_1 pre-treatment could inhibit the phosphorylation of ATM and Chk2, and then increase the expression of CDK1 and cyclin B1 proteins, which may contribute to the alleviation effect of A_1 and D-A_1 on ACR-induced cell cycle arrest in the G2/M phase. Once the damaged DNA is repaired, the DNA damage response system will be closed, resulting in recovery to normal cell cycle progression[7].

In this study, ACR-induced cell apoptosis was observed through Hoechst 33342 staining assay. Some studies have verified that cell cycle arrest in the G2/M phase indicates that the DNA damage may be too severe to be repaired, which will activate the intrinsic apoptosis pathway[40]. Additionally, our previous study has reported that ACR-induced ROS accumulation could cause mitochondrial dysfunction and subsequently cell apoptosis[32]. Therefore, it could be inferred that ACR may induce cell apoptosis through the intrinsic pathway, resulting in the release of pro-apoptotic mediators from mitochondria and activation of caspase family proteins[41]. Our results indicated that ACR treatment could decrease the Bcl-2/Bax ratio, and then cleaved caspase-9 is activated as an initiator caspase to further activate cleaved caspase-3 as an effector caspase, which is a typical symbol of cell apoptosis. Fortunately, A_1 and D-A_1 could reverse the ACR-induced imbalance of pro-apoptotic and anti-apoptotic proteins, and subsequently inhibit cell apoptosis. Moreover, excessive ROS can also promote cell apoptosis by altering various signaling pathways[42]. The MAPK family members, particularly JNK, ERK, and p38, have certain regulatory effects on mitochondrion-mediated cell apoptosis, possibly through the caspase-3 dependent pathway[43]. Some studies have indicated that ACR can induce cell apoptosis through activation of the MAPK pathway[8]. Pan et al.[43] reported that ACR treatment could quickly activate MAPK signaling within 0.5 h, and the effect is sustainable. In this study, ACR rapidly boosted the phosphorylation of JNK1/2/3, ERK1/2 and p38 and then activated the MAPK signaling pathway in IPEC-J2 cells, which can be inhibited by A_1 and D-A_1.

PCs are a class of polyphenols condensed by the monomeric flavan-3-ol units, and can ameliorate inflammation, oxidative stress damage, and apoptosis by directly scavenging ROS or regulating various signaling pathways[44]. Due to their unique structure with multiple hydroxyl groups in the B ring, PCs can directly scavenge ROS, and mediate the activation of the Nrf2 pathway to upregulate endogenous antioxidant enzymes. Therefore, they have much stronger antioxidant activity and inhibitory effect on tissue oxidation and DNA damage than vitamin C and vitamin E[45]. Besides, previous studies have demonstrated that procyanidin A-type dimers can prevent cell apoptosis, which may contribute to its inhibitory effect on the MAPK pathway[14,46-47]. Rodriguez-Ramiro et al.[14] have demonstrated that PCs can inhibit ACR-induced toxicity in intestine cells by inhibiting the JNK pathway implicated in apoptosis and cell survival, and procyanidin B_2 has a better antiapoptotic effect than catechin and epicatechin. These findings imply that PCs may regulate the MAPK pathway for eliminating ACR-induced cell apoptosis. In the present study, A_1 could suppress the activation of the MAPK pathway, and then prevent ACR-triggered cell apoptosis. Interestingly, A_1 exhibited a similar inhibitory capability before and after simulated gastrointestinal digestion.

Although PCs have been demonstrated to possess valuable biological properties, their activities after absorption and digestion remain poorly understood. It is difficult to quantify and detect most metabolites of PCs, making it a great challenge to explain the potential mechanism for the activity of PCs. In particular, some studies have reported that gastrointestinal digestion may have some adverse effects on the biological activities of PCs[48-49]. Our previous study has indicated that simulated gastrointestinal digestion could greatly alter the inhibitory effects of catechin and epicatechin on ACR-induced IPEC-J2 cell damage[21]. In this study, D-A_1 showed slightly stronger capability than A_1 in inhibiting ACR-induced DNA damage, and no significant difference was observed in the inhibitory effect of A_1 and D-A_1 on ACR-induced cell apoptosis and cell cycle

arrest in IPEC-J2 cells, which is consistent with our previous finding of their protective effect on ACR-induced Caco-2 cell intestinal barrier damage[47]. This is because A_1 will undergo isomerization reaction to produce two new procyanidin A-type dimers after simulated gastrointestinal digestion, and there is no obvious variation in their active functional groups with a catechol structure in the B ring. Therefore, it can be concluded that A_1 is an excellent detoxicant against ACR-induced cytotoxicity in IPEC-J2 cells, as it can resist the negative effect of gastrointestinal digestion. Due to the possibly different absorption and metabolism of PCs in *in vivo* and *in vitro* experiments, it is essential to explore the inhibitory effect and mechanism of A_1 on ACR-induced small intestinal damage in animal models.

5. Conclusion

Our results revealed that A_1 and D-A_1 can inhibit ACR-induced cell cycle arrest and cell apoptosis in IPEC-J2 cells, probably by suppressing the activation of the ATM/Chk2 and MAPK pathways and then regulating the levels of G2/M phase- and apoptosis-related proteins. More importantly, A_1 exhibited an excellent detoxification effect against ACR after gastrointestinal digestion. Therefore, A_1 may be a valuable dietary supplement for eliminating ACR-induced small intestinal damage.

Conflict of interest

Si Qin is an editorial board member for *Food Science and Human Wellness* and was not involved in the editorial review on the decision to publish this article. 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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