

ORIGINAL ARTICLE

Protective Effect of Apigenin on Acrylonitrile-Induced Inflammation and Apoptosis in Testicular Cells *via* the NF- κ B Pathway in Rats

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Abstract— Apigenin (AP) as a plant flavonoid is found to attenuate acrylonitrile (ACN) toxicity by reducing ROS production and inhibiting apoptosis. Therefore, the present study aimed to evaluate the role of AP on ACN-induced inflammation and apoptosis in germ cells and whether it is through the NF- κ B signaling pathway. AP increased the concentrations of lactate dehydrogenase isozyme (LDH) and sorbitol dehydrogenase (SDH), while the concentrations of interleukin β (IL-1 β), tumor-necrosis factor- α (TNF- α), and interleukin-6 (IL-6) were significantly reduced. AP could downregulate the expression of the nuclear factor of kappa B (NF- κ B) and inhibit phosphorylation of the inhibitory κ B α (I κ B α). Cleaved caspase-3 was also upregulated by AP, and the apoptotic were less than those in the ACN group. These results suggest that AP might improve maturation and energy metabolism of testes, inhibit NF- κ B activation. Then AP could further downregulate NF- κ B signal and inhibit the germ cell apoptosis and reduce inflammatory caused by ACN.

KEY WORDS: apigenin; acrylonitrile; inflammation; apoptosis; NF- κ B pathway; testicular cells in rat.

INTRODUCTION

Acrylonitrile (ACN, CH₂=CH-CN) is widely used in commercial manufacturing. Exposure to acrylonitrile is primarily through occupational exposure. In life, acrylonitrile can be found in secondhand smoke [1], kitchen supplies [2], and even children's toys [3]. Studies have

shown that occupational exposure to ACN can lead to neurotoxicity [4], immunotoxicity [5], and gastric toxicity [6]. Epidemiological studies reported that there are adverse reproductive effects, such as infertility, sex hormone decline and birth defects, after exposure to ACN [7, 8]. Furthermore, previous studies demonstrated that ACN has the potential to induce testicular toxicity [9, 10].

Apigenin (AP, 4', 5, 7-trihydroxy flavonoid) is an abundant dietary flavonoid and is widely distributed in fruits and vegetables [11]. AP has been shown to possess many pharmacological effects, such as anti-inflammatory, antioxidant, and anticancer benefits [12]. Furthermore, studies have shown that the toxicity of AP is very minor, and the No Observed Adverse Effect Level (NOAEL) of AP is 8000 mg kg⁻¹ for rats [13]. The results showed that the median lethal dose (LD50) of the methanol extract of *Adonis coerulea* (main ingredients: AP and luteolin) was estimated to be more than 5000 mg kg⁻¹ in mice [14]. In our earlier preliminary studies, AP showed antioxidant

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activity at 234 and 468 mg/kg; the main manifestations were total antioxidant capacity of testes increased and free radicals or reactive oxygen species (ROS) decreased, but AP showed pro-oxidant activity in rat testes at 936 mg/kg [15]. AP had a protective effect on ACN-induced lipid peroxidation, by mainly increasing the ratio of reduced glutathione and oxidized glutathione (GSH/GSSG) and glutathione (GSH) and reducing the elevation of malondialdehyde (MDA) in rat testes [9]. In the rotenone-induced rat model of Parkinson's disease, AP significantly attenuated the upregulation of the nuclear factor of kappa B (NF- κ B) gene expression, prevented neuroinflammation, and inhibited the release of proinflammatory cytokines, tumor-necrosis factor- α (TNF- α), interleukin-6 (IL-6), and the proinflammatory enzyme inducible nitric oxide synthase (iNOS-1) [16]. In some cancer cells, AP decreased the phosphorylation levels of the inhibitory κ B α (I κ B α) in the cytosol and abrogated the nuclear translocation of p65 within the nucleus, suggesting that it blocks the activation of the NF- κ B signaling pathway [17, 18]. Previous studies have shown that ACN-induced oxidative stress activated the NF- κ B signaling pathway, which modulated the expression of Bax and contributed to testicular apoptosis [19]. We also found that, at a certain concentration, AP reduces ACN-induced decrease of sperm density through the reduction of apoptosis of testicular germ cells [20]. Therefore, our present study aimed to evaluate the role of AP on ACN-induced germ cell apoptosis and whether it is through the NF- κ B signaling pathway.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats aged 50 to 56 days and weighing 250–300 g were obtained from the Animal Care Center, Gansu University of Traditional Chinese Medicine, China. The rats were randomly divided into four groups: control group with corn oil, ACN group with 50 mg kg⁻¹ ACN [21], ACN + AP1 group with 50 mg kg⁻¹ ACN + 234 mg kg⁻¹ AP, and ACN + AP2 group with 50 mg kg⁻¹ ACN + 468 mg kg⁻¹ AP [22]. All the rats were gavaged 6 days per week for 12 weeks of treatment. AP1 represents 234 mg/kg of AP, and AP2 represents 468 mg/kg. AP and ACN were dissolved in corn oil [23]. The study was approved by the Animal Ethics Committee of the Experimental Animal Care Center, and the rats were housed with a 12-h light/dark cycle and had free access to water and a normal chow diet. The rats were euthanized on

the day following the last administration. All the testes were immediately frozen in liquid nitrogen and stored at -80 °C.

Wet Weight and Organ Coefficients

We weighed the testes, epididymis, prostates, and seminal vesicles using a filter paper. Organ coefficient (%) = weight of organs (g)/weight of rat (g) \times 100%.

Enzymes Involved with Testicular Metabolism

The testes were homogenized in 9 ml of homogenization buffer (0.9% NaCl) and centrifuged at 2500 r/min for 10 min at 4 °C. The concentrations of acid phosphatase (ACP), alkaline phosphatase (AKP), lactate dehydrogenase isozyme (LDH), sorbitol dehydrogenase (SDH) and glucose-6-phosphate dehydrogenase (G-6-PD) were determined using commercially available kits, according to the instructions by the manufacturer (Nanjing Jian Cheng Bioengineering Institute, China). The results of the assays were normalized to the total amount of protein, as measured by the bicinchoninic acid (BCA) method.

Inflammatory Cellular Factors and 8-Hydroxy-2-Deoxyguanosine

The inflammatory cellular factors, such as interleukin β (IL-1 β), tumor-necrosis factor- α (TNF- α) and interleukin-6 (IL-6), in rat testes were determined using commercially available kits, according to the instructions by the manufacturer (Elabscience Biotechnology Co., Ltd); 8-hydroxy-2-deoxyguanosine (8-OHDG) in rat testes was determined (Nanjing Jian Cheng Bioengineering Institute, China). All detection methods were by ELISA.

Immunofluorescence Analysis of NF- κ B (p65) Activation and Localization

Briefly, the testicular sections were fixed with stationary liquid and blocked with sealing fluid for 1 h. They were incubated with primary antibody of NF- κ B (p65) at 4 °C overnight, followed by incubation with Cy3-labeled secondary antibody for 60 min. Then, they were stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) solution for 5 min. The analysis of activation and nuclear translocation of NF- κ B (p65) was examined by fluorescence microscope (Olympus, Japan). The detection was carried out according to instructions of the manufacturer (Beyotime Institute of Biotechnology, Inc. Jiangsu, China).

Table 1. Primer Sequences and Amplification Lengths of Destination Fragments for Real-time PCR Analyses

Gene	Primer sequences(5' → 3')		Product size/bp
<i>β-actin</i>	F	GGAGATTACTGCCCTGGCTCCTA	150
	R	GACTCATCGTACTCCTGCTTGCTG	
<i>Ikk</i>	F	GGTCATCTAATGTCCCAGCCTTC	183
	R	CTCCATCTGTAAACCAGCTCCAGTC	
<i>Nf-κb</i>	F	CGACGTATTGCTGTGCCTTC	139
	R	TTGAGATCTGCCCAGGTGGTA	
<i>Ikb-α</i>	F	TGACCCATGGAAGTGATTGGTCAG	95
	R	GATCACAGCCAAGTGGAGTGGGA	

Western Blot Analysis

Protein levels of inhibitory protein of NF-κB (IKK), NF-κB (p65), the phosphorylation inhibitory κBα (P-IκBα), IκBα and caspase-3 in rat testicles were analyzed by Western blot. The proteins of rat testicles (three per group) were determined using BCA Protein Assay Kit (Thermo Scientific, USA). 60 μg proteins and 2.5 μl rainbow prestained protein marker were separated by 10–15% sodium dodecyl sulfate polyacrylamide gels, followed by electrophoresis. Separated target proteins were transferred onto the polyvinylidene difluoride membrane (constant current of 200 mA for 75 min and 220 mA for 75 min). The membranes were incubated in 5% non-fat milk (Tris-buffered saline with tween-20) for 2 h, then incubated with primary antibodies against β-actin (1:1000, Elabscience), IKK, NF-κB (p65), P-IκBα, IκBα, and caspase-3 (1:1000, CST) in TBST overnight at 4 °C and then washed five times with Tris-buffered saline and tween-20 for 25 min. Subsequently, using horseradish peroxidase-conjugated secondary antibody, the blots were incubated for 2 h, and then the membranes were washed and detected using enhanced chemical

luminescence detection solution. The electrophoresis system and gel imaging exposure meter were from Bio-Rad, USA. The relative density of the blots was analyzed by Quantity One 4.6.2 software.

RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA of the testes tissues was extracted using the TRIzol reagent. The total RNA purity was assessed by the quotient of optical density at 260/280 nm (1.8–2.2). For the internal standard control, the expression level of β-actin was simultaneously quantified. Real-time PCR was performed using the iQ5 multicolor real-time PCR detection system (Bio-Rad, USA). Reverse transcription was performed using the Takara Prime Script RT reagent kit. The primers used in this study are listed in Table 1. The specific operating conditions were as follows: 95 °C for 2 min, followed by 50 cycles of 95 °C for 10 s, 55–64 °C for 30 s, and 72 °C for 30 s; then, the temperature was increased to 60 °C for 15 s for 71 cycles for the melting curve formation. The mRNA expression levels were calculated with the Pfaffl method.

Table 2. The Wet Weights and Organ Coefficients of the Genital Organs

Testes		Epididymis		Prostate glands		Seminal vesicles	
Weights (g)	Coefficients (g kg ⁻¹)	Weights (g)	Coefficients (g kg ⁻¹)	Weights (g)	Coefficients (g kg ⁻¹)	Weights (g)	Coefficients (g kg ⁻¹)
3.38 ± 0.12	0.79 ± 0.10	1.42 ± 0.17	0.33 ± 0.03	0.79 ± 0.13	0.19 ± 0.03	2.3 ± 0.26	0.54 ± 0.07
3.23 ± 0.13	0.79 ± 0.11	1.39 ± 0.22	0.45 ± 0.03	0.73 ± 0.09	0.18 ± 0.03	2.08 ± 0.22	0.51 ± 0.08
3.45 ± 0.11	0.82 ± 0.08	1.37 ± 0.2	0.26 ± 0.05	0.88 ± 0.22	0.21 ± 0.05	2.26 ± 0.63	0.53 ± 0.11
3.37 ± 0.12	0.84 ± 0.06	1.27 ± 0.21	0.34 ± 0.06	0.77 ± 0.20	0.19 ± 0.05	2.00 ± 0.58	0.49 ± 0.13

Note: (n = 6)

Table 3. Effect of ACN and ACN + AP on the Levels of ACP, AKP, LDH, SDH, and G-6-PD in Rat Testes

	ACP (U/gprot)	AKP (U/gprot)	LDH (U/gprot)	SDH (ng/ml)	G-6-PD (ng/ml)
Control	59.09 ± 13.99	89.23 ± 8.56	402.3 ± 71.25	4.61 ± 0.78	7.79 ± 2.83
ACN	51.71 ± 9.84	68.61 ± 16.47	288.4 ± 54.02 ^a	3.51 ± 0.63 ^a	7.23 ± 2.03
ACN + AP1	61.03 ± 12.69	70.96 ± 21.94	364.7 ± 44.45	4.63 ± 0.62 ^b	7.55 ± 2.37
ACN + AP2	63.9 ± 9.18	75.65 ± 30.57	450.97 ± 76.99 ^b	3.80 ± 0.36	8.34 ± 1.59

Note: (n = 6)

^aP < 0.05 vs. the control group

^bP < 0.05 vs. the ACN group

The Apoptotic Signal of Testicular Cells Was Detected by TUNEL

Rat testes paraffin slices were prepared for terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) assay, which was performed with a commercial kit in accordance with the instructions by the manufacturer. Briefly, the wax was removed from the slices, put in 3% freshly prepared

hydrogen peroxide and treated at room temperature for 10 min, then washed. The fixed sections were incubated with 0.01 M TBS, 1:200 proteinase K solution for 12 min. 1 μl of TdT and DIG-d-UTP was mixed with 18 μl of the labeling buffer, and added to each slice. The samples were placed in a wet box and incubated at 37 °C for 2.5 h., then washed with 0.01 M TBS for 5 min, three times. Closed solution was added

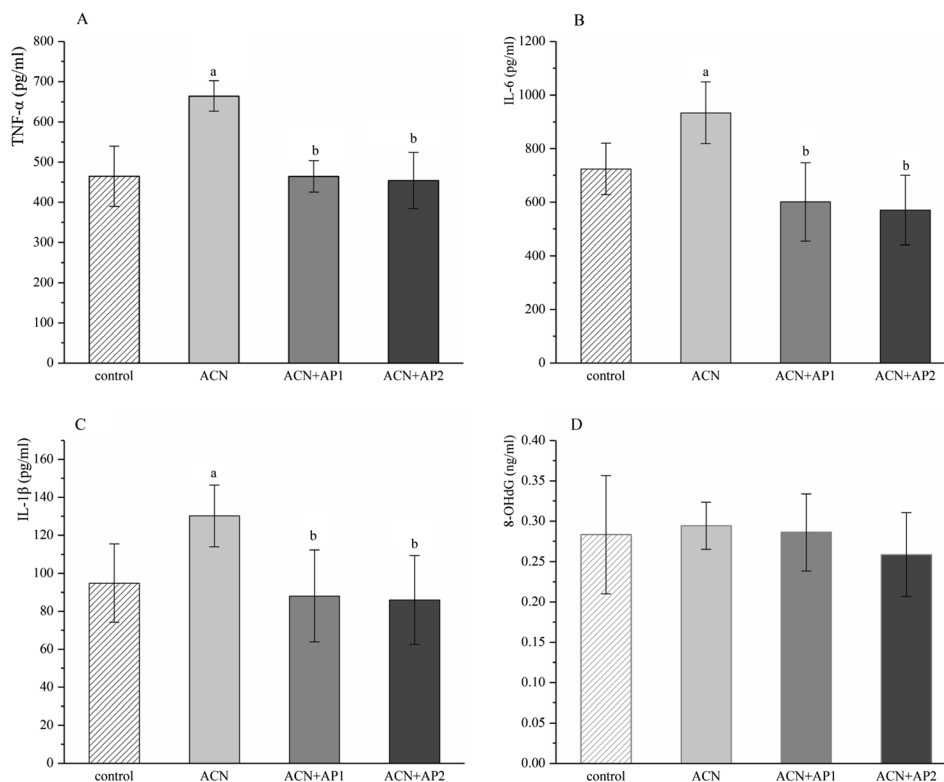


Fig. 1. Effect of ACN and ACN + AP on the levels of TNF-α (a), IL-6 (b), IL-1β (c), and 8-OHdG (d) in rat testes (n = 6). Note: Data are means ± SD. ^aP < 0.05 vs. the control group. ^bP < 0.05 vs. the ACN group.

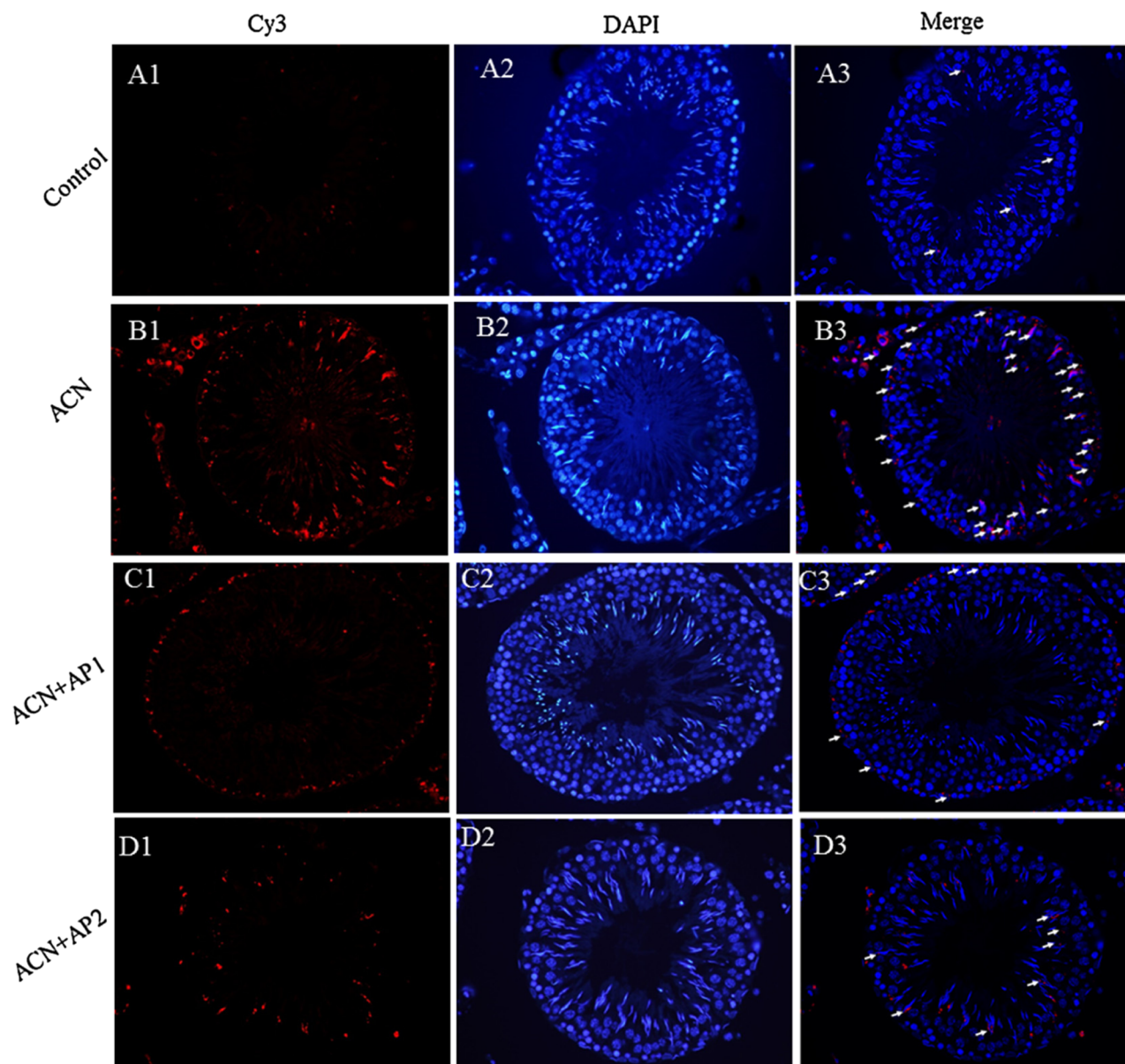


Fig. 2. Immunofluorescence microscopic analysis of the localization of p65 protein in testicular cells. The immunofluorescence staining of sections showed that the p65 subunit was labeled by Cy3 (red, left graphs) and the nuclei were dyed by DAPI (blue, middle graphs). The merged are the right graphs. The arrows indicate the activation and translocation of the p65 subunit. Magnification, $\times 400$.

(50 $\mu\text{l/slice}$) and incubated in the 37 $^{\circ}\text{C}$ incubator for 30 min; and then the liquid was removed, and the slices were not washed. The slices were then labeled with biotin anti-Digoxin antibody, DAB dyeing, and hematoxylin counterstaining. The labeled slices were washed and then imaged under the microscope (Olympus, Japan). All the reagents were provided by Boster Biological Technology Co., Ltd., China.

Statistical Analyses

Results are expressed as the mean values \pm standard deviation (SD) and were analyzed using the statistical software, SPSS v. 22.0. The data were calculated by one-way analysis of variance (ANOVA). If the variances were equal, least significant difference (LSD) would be used; if not, Dunnett's T3 would be used. P values < 0.05 were considered significant.

Table 4. Effect of ACN and ACN + AP on mRNA Expression of the Genes Related to the NF- κ B (p65) Signaling Pathway

	<i>Ikk</i>	<i>Nf-κb</i> (p65)	<i>Ikb</i>
control	0.58 \pm 0.09	0.71 \pm 0.09	1.02 \pm 0.24
ACN	0.85 \pm 0.04 ^a	0.83 \pm 0.06 ^a	0.70 \pm 0.1 ^a
ACN + AP1	0.48 \pm 0.05 ^b	0.53 \pm 0.04 ^b	0.99 \pm 0.28 ^b
ACN + AP2	0.65 \pm 0.06 ^b	1.30 \pm 0.12 ^{ab}	1.15 \pm 0.23 ^b

Note: (n = 6)

^a P < 0.05 vs. the control group

^b P < 0.05 vs. the ACN group

RESULTS

The Growth of Rats, Wet Weights and Organ Coefficients of the Genital Organs

With the increase of exposure time, the weight of rats increased, and there were no significant differences of the body weight in each group. The wet weight and organ coefficients of testes, epididymis, prostate glands and seminal vesicles were not significantly different in each group (Table 2).

Testes Biochemical Indicator Analysis

The concentrations of ACP, AKP and G-6-PD in the testes were almost unaffected by both ACN and AP (Table 3). The ACN group showed a decrease in the concentration of LDH, compared with the control group (P < 0.05), whereas AP2 significantly increased the concentration of LDH (P < 0.05) (Table 3). The concentration of SDH was significantly decreased in the ACN group, compared with that of the control group (P < 0.05), whereas AP1 reversed the decreased concentration of SDH (Table 3).

Inflammatory Cellular Factors and 8-Hydroxy-2-Deoxyguanosine in Rat Testes

In this study, we tested the concentrations of the inflammatory factors, TNF- α , IL-6, and IL-1 β , which were proved to be associated with the NF- κ B signaling pathway. Compared with the control group, ACN significantly increased TNF- α and IL-6 levels in the testes (P < 0.05), while AP1 and AP2 obviously decreased the increase of TNF- α and IL-6 (Fig. 1a, b). ACN could increase IL-1 β levels in the testes, but both AP1 and AP2 obviously decreased the concentrations of IL-1 β , compared with the control group (Fig. 1d); 8-OHdG is a biomarker for oxidative damage of DNA caused by endogenous and exogenous factors. Testing 8-OHdG can assess the degree of oxidative damage and repair and the

relationship between oxidative stress and DNA damage. Both ACN and ACN + AP did not have an effect on 8-OHdG (Fig. 1d).

Immunofluorescence Analysis of NF- κ B (p65) Activation and Localization

The anti-p65 antibody was red fluorescently labeled by the Cy3-labeled secondary antibody, which was used to detect cellular subunits of NF- κ B (p65). The nuclei were stained with blue fluorescence (stained by DAPI) (Fig. 2). In the control group, very few testicular cells were labeled with Cy3, but there were many in the ACN group, and the p65 subunit was significantly activated and translocated to the nucleus, indicated by the arrows in the merged figure (Fig. 2, right). However, in the ACN + AP1 and ACN + AP2 group, the cells stained red were decreased, especially in the nuclei, indicating that AP might partially inhibit the nuclear translocation of NF- κ B.

mRNA Expression and Protein Levels of the NF- κ B Signaling Pathway

We found that ACN significantly increased the mRNA expression of *Ikk* and *Nf- κ b* but decreased that of *Ikb* (Table 4). However, AP1 could possibly reverse this change. AP2 reversed the change of *Ikk* and *Ikb*, but it significantly increased *Nf- κ b*, compared with the control group and the ACN group (Table 4).

Compared with the control group, the protein levels of IKK were increased, but they were reduced in the ACN + AP1 and ACN + AP2 groups, compared with the ACN group (Fig. 3a, b). Compared with the control group, the protein levels of NF- κ B and P-I κ B α were increased in the ACN group. However, in the ACN + AP1 and ACN + AP2 groups, they were significantly decreased, not only compared with the ACN group but also compared with the control group (Fig. 3a, c, d). Compared with the control group, the protein levels of I κ B α were reduced in the ACN group, but in the ACN + AP1 and ACN + AP2 groups, they were significantly increased (Fig. 3a, e). The rates of P-I κ B/I κ B were significantly decreased in ACN + AP1 and ACN + AP2 groups, but in the ACN group, P-I κ B/I κ B was increased compared to the control group.

Protein Levels of Caspase-3 in Rat Testes

Compared with the control group, the protein level of uncleaved caspase-3 in the ACN group was significantly

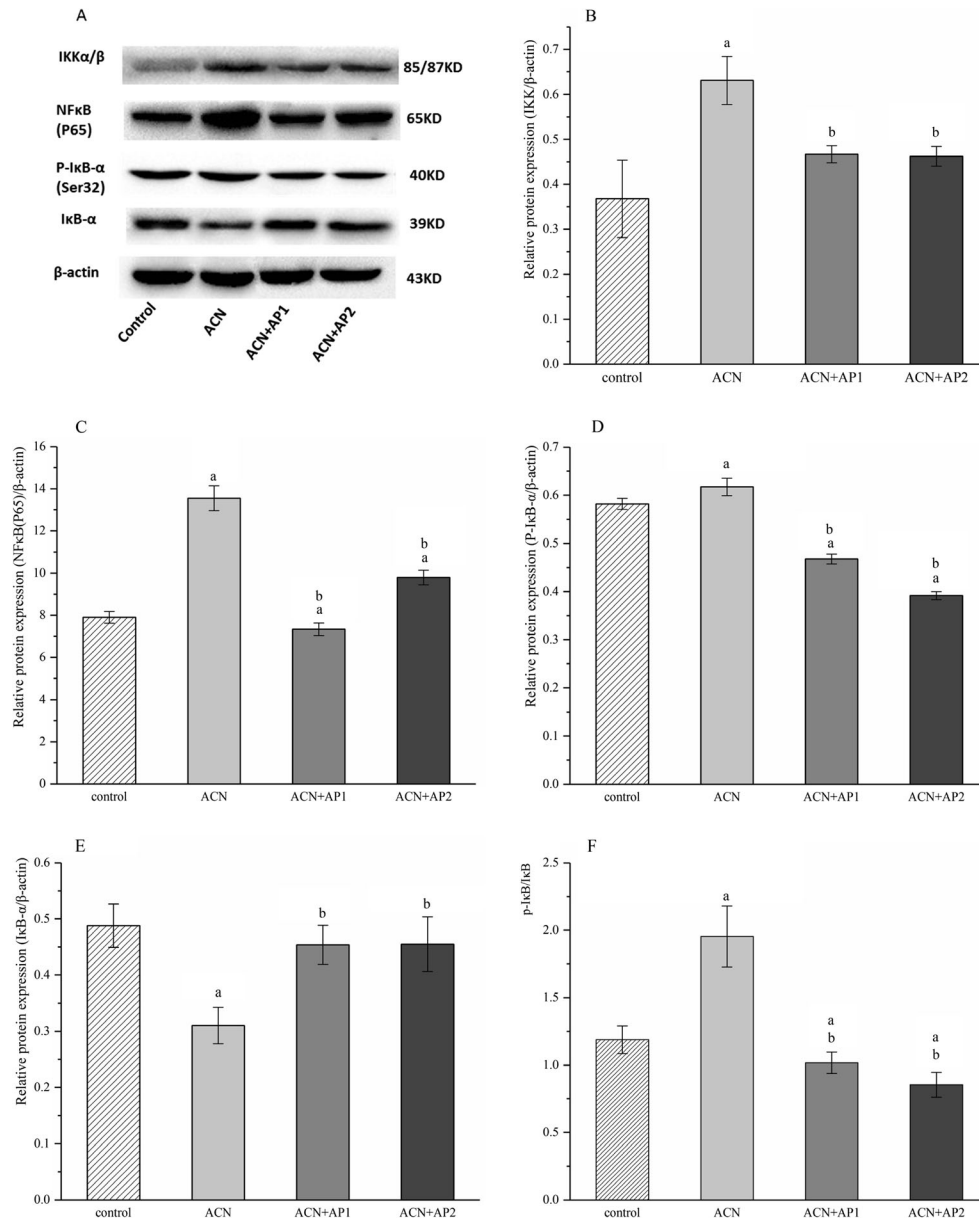


Fig. 3. Levels of IKK- α/β , NF- κ B (p65), p-I κ B α , and I κ B α in testicular tissue in rats. The expression levels in the rat testes were detected by Western blot (a). Quantitative analysis of the protein expression levels of IKK- α/β (b), NF- κ B (p65) (c), p-I κ B α (d), I κ B α (e), p-I κ B/I κ B (f). One image of three independent experiments is shown. ^a $P < 0.05$ vs. the control group, ^b $P < 0.05$ vs. the ACN group.

reduced, but in the AP + ACN groups was unchanged. Cleaved caspase-3 was significantly upregulated in every group, but compared with the ACN group, cleaved caspase-3 was significantly downregulated in each AP + ACN group (Fig. 4).

The Apoptotic Signal of Testicular Cells Was Detected by TUNEL

From Fig. 5 we found that the TUNEL positive cells in the ACN group and ACN + AP group were increased, compared to the control group. However, the positive cells

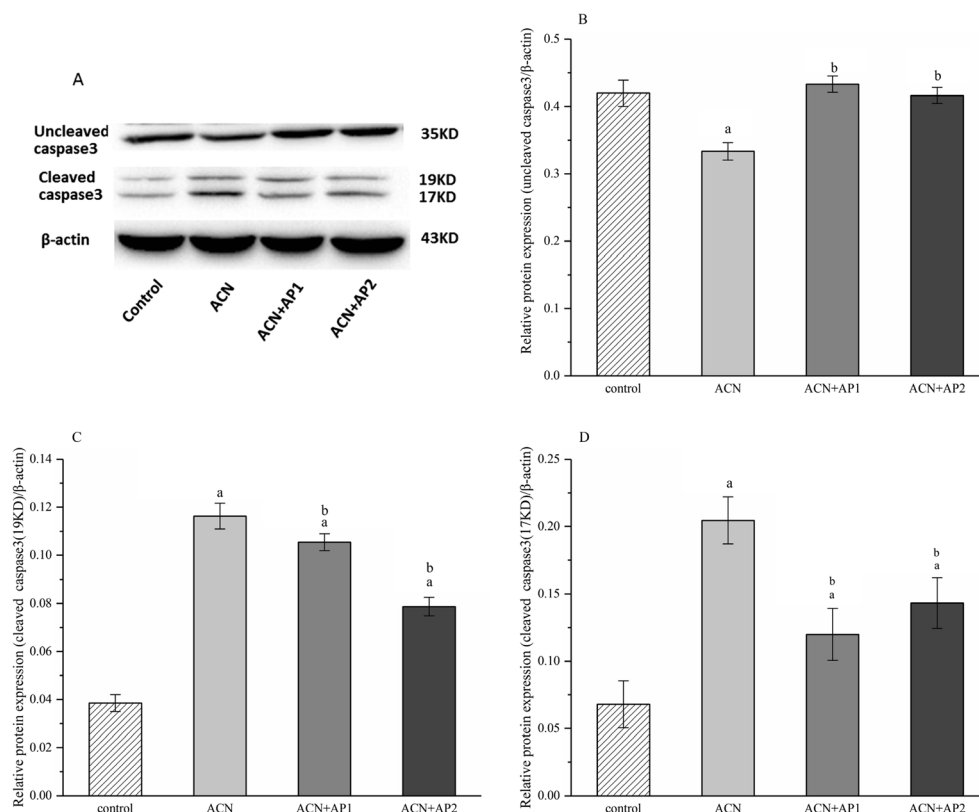


Fig. 4. Levels of caspase-3 in testicular tissue in rats. Uncleaved caspase-3 (35 kDa) and cleaved caspase-3 (19 kDa and 17 kDa) were detected by Western blot (a). Quantitative analysis of the protein expression levels of uncleaved caspase-3 (b), cleaved caspase-3 19 kDa (c) and 17 kDa (d). One image of three independent experiments is shown. ^a*P* < 0.05 vs. the control group, ^b*P* < 0.05 vs. the ACN group.

in the ACN + AP1 and ACN + AP2 groups were less than the ACN group. The quantitative analysis also confirmed the results (Fig. 5e).

DISCUSSION

In the testes, AKP is associated with the division of spermatogenic cells and the transportation of glucose to spermatogenic cells. ACP is one of the markers of dyszoospermia that is associated with the denaturation of seminiferous epithelium and phagocytosis of Sertoli cells. In the present study, the activity of ACP and AKP was not changed in all of groups. The results indicated that ACN and AP would probably do little to impact the lipid and energy metabolism in the testes. LDH and SDH are widely distributed and located in the seminiferous tubules and germ cells, which are associated with the maturation of

spermatogenic cells and testes and the energy metabolism of spermatozoa [24, 25]. Our study showed that the activity of LDH and SDH was significantly decreased in the ACN group. However, the activities of SDH and LDH were increased in the AP + ACN group. The results suggested that AP may improve the maturation and energy metabolism of spermatogenic cells and spermatozoa, which were previously damaged by ACN. G-6-PD is mainly located in the endoplasmic reticulum, which is the enzyme marker of the endoplasmic reticulum.

TNF- α is a multifunctional cytokine with effects not only in immunoregulatory responses but also in the proinflammatory response and apoptosis [26]. TNF- α in the testes is produced by round spermatids, pachytene spermatocytes, and testicular macrophages. Numerous studies suggest a paracrine mode of action for TNF- α in the normal testes. IL-1 α and IL-1 β are secreted by macrophages. IL-1 is known to cause inflammation and induce

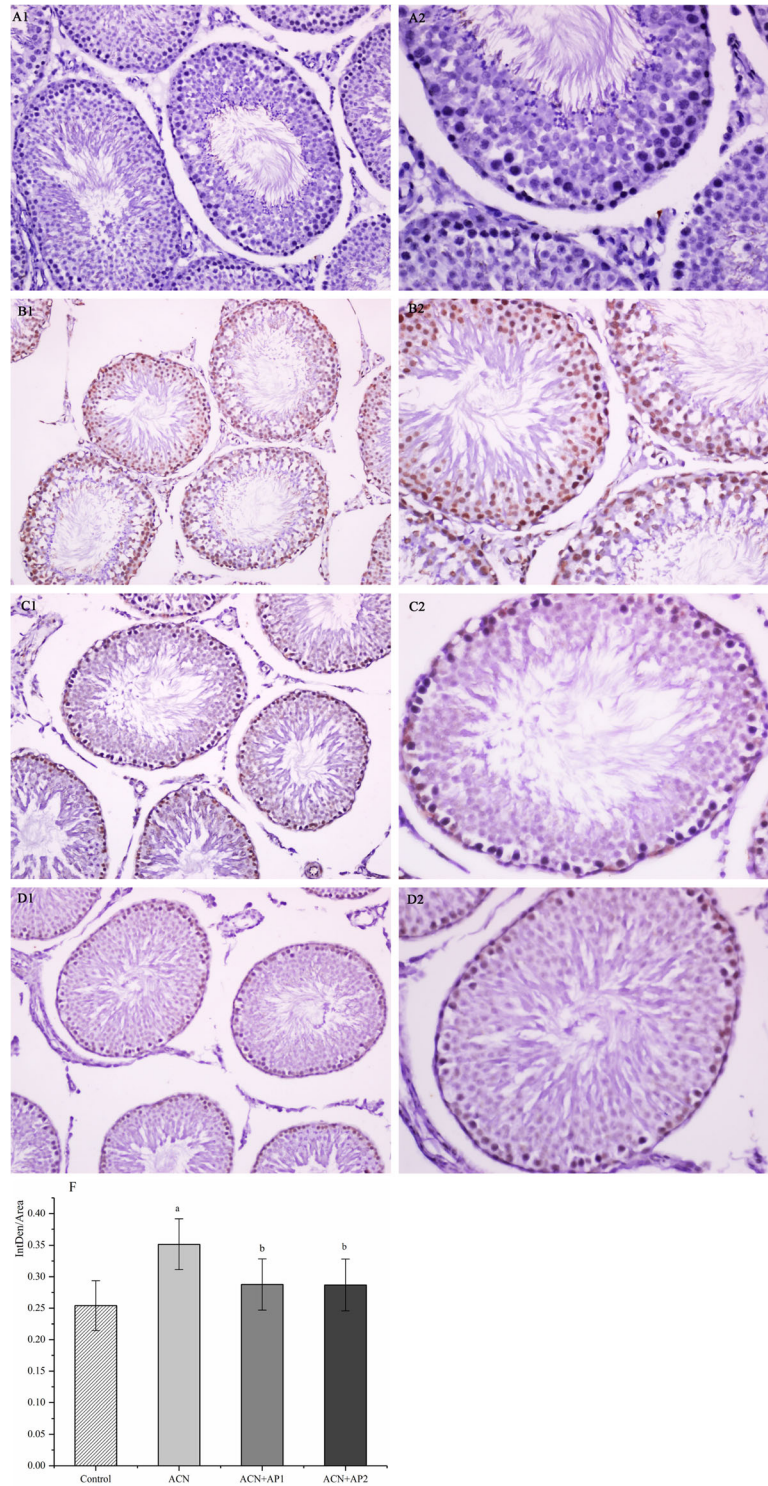


Fig. 5. Effects of AP on ACN-induced apoptosis of testicular cells. TUNEL stained testicular sections and brown granules indicate positive cells. The control group (a), the ACN group (b), the ACN + AP1 group (c), and the ACN + AP2 group (d). The original magnification (A1, B1, C1, D1), $\times 200$, (A2, B2, C2, D2), $\times 400$. Quantitative analysis was performed using NIH ImageJ software (e). ^a $P < 0.05$ vs. the control group. ^b $P < 0.05$ vs. the ACN group.

the expression of proinflammatory peptides, such as inducible nitric oxide synthase, IL-6, and TNF- α [27]. TNF- α was able to increase IL-6 production from cultures of rat Sertoli cells [28]. In the present study, ACN significantly increased IL-1 β , TNF- α and IL-6 in the testes, while in the ACN + AP1 and ACN + AP2 group, they were decreased. This result is consistent with the study of the anti-inflammatory effects of AP. While these proinflammatory cytokines have important roles in normal testicular homeostasis, an elevation of their expression can lead to testicular dysfunctions. Under physiologically low concentrations of TNF- α produced by Sertoli cells, this acts as a survival factor for germ cells, whereas at high concentrations of TNF- α , such as during inflammation, TNF- α induces membrane bound Fas, which primes the Sertoli cells for FasL-induced cell death. TNF- α can stimulate activation of the transcription factor, NF- κ B [29]. Recent data have demonstrated an increase in TNF- α and IL-1 β expression after reperfusion of the testes and that they may be responsible for stimulating a stress-related kinase signaling pathway. AP significantly inhibited TNF- α and IL-1 β -induced activation of NF- κ B in macrophages [30]. The activation of NF- κ B, a marker of inflammation, occurs due to the activation of oncogenes and inflammatory stimuli.

The most abundant form of NF- κ B consists of a heterodimer of RelA (p65) and p50 subunits [31]. NF- κ B is located in the cytoplasm in an inactive form, in which the heterodimer is bound to the I κ B α protein. I κ B α has been shown to mask the nuclear localization sequence, thereby preventing nuclear entry of both DNA-binding subunits [32]. Stimulation with IL-1 β and TNF- α leads to phosphorylation and subsequent degradation of I κ B α by I κ B kinases (IKK α and IKK β). There, released NF- κ B is then translocated into the nucleus where it binds to specific consensus DNA sequences of target genes and initiates transcription. However, NF- κ B activity is regulated by I κ B α [33]. AP regulates inflammatory mediators, including IL-1 β and TNF- α , in both human and mouse cell lines [34, 35]. AP reduces proinflammatory cytokine production by inhibiting NF- κ B phosphorylation in macrophages and reducing neutrophil chemotaxis *in vitro* [36, 37]. In the present study, ACN significantly increased the mRNA expression of *Ikk* and *Nf- κ b*, but decreased that of *Ikb*, and the protein levels of NF- κ B (p65) and IKK- α / β were significantly upregulated in the ACN-treated rats. Furthermore, AP1 pretreatment inhibited the activation of NF- κ B (p65), and IKK- α / β and p-IKB- α were downregulated. However, the mRNA expression of NF- κ B (p65) was not decreased, while the protein level of NF- κ B (p65) was downregulated, which might be when the concentration

of AP was increased. These results were also shown in the immunofluorescence analysis of NF- κ B (p65) activation and localization. Therefore, we thought that AP could regulate NF- κ B activated by ACN through changing TNF- α and IL-1 β . When NF- κ B is activated, it translocates to the nucleus and further stimulates gene transcription of several proinflammatory factors, including inducible nitric oxide synthase, IL-1 β , IL-6, cyclooxygenase-2, and TNF- α [38].

NF- κ B activation regulates or controls the expression of genes that mediate cell transformation, proliferation, invasion, angiogenesis, apoptosis and metastasis. Caspase-3 plays a crucial role in the apoptotic chromatin condensation and DNA fragmentation in all cell types examined. In our previous study [10], we reported that ACN significantly increased the mRNA expression of Bax and caspase-3 but decreased that of Bcl-2. AP could possibly reverse this change; the protein levels of Bcl-2 and Bax were also regulated. In the present study, cleaved caspase-3 was upregulated in the ACN group, while it was also upregulated in the AP + ACN groups but was reduced when compared to the ACN group. In addition, the results showed that AP might reduce acrylonitrile-induced apoptosis of testicular cells. The results of the TUNEL analysis further evidenced the role of AP in the apoptosis of testicular cells (Fig. 5).

CONCLUSION

AP might improve maturation and energy metabolism of testes, inhibit NF- κ B activation. Then AP could further downregulate NF- κ B signal and inhibit the germ cell apoptosis and reduce inflammatory caused by ACN. Therefore, AP has a protective effect on ACN-induced rat reproductive damage, although AP1 and AP2 do not always get the exact same results on all of the parameters and the most effective dose of AP should be detected in the future studies, we can still propose an appropriate dietary AP to prevent male reproductive damage.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of Interest. The authors declare no conflict of interest.

Ethics Statement. All animal experiments were performed in accordance with the Institutional Animal Care Committee of Gansu University of TCM.

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