Am J Transl Res 2017;9(9):4227-4235
www.ajtr.org /ISSN:1943-8141/AJTR0051890

Original Article

Effects of acrylonitrile-induced oxidative stress on testicular apoptosis through activation of NF-κB signaling pathway in male sprague dawley rats

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Received February 28, 2017; Accepted June 16, 2017; Epub September 15, 2017; Published September 30, 2017

Abstract: Acrylonitrile (ACN) treatment can induce testicular toxicity in Sprague-Dawley (SD) rats, with the toxicity potentially related to apoptosis, mediated by nuclear factor-κB (NF-κB). The present study investigated the potential role of NF-κB in the induction of apoptosis and testicular toxicity in ACN-treated rats. Adult male SD rats were randomly divided into 3 treatment groups: a control group (corn oil), an ACN group (50 mg/kg) in which ACN was administered by gavage, and an ACN and N-acetylcysteine (ACN+NAC) group. The rats were given NAC (300 mg/kg) 30 min prior to the administration of ACN, and ACN was administered by gavage for 90 days. The ACN treatment markedly increased malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activity in the testis. Glutathione (GSH) was significantly depleted in the ACN groups, and the effects of ACN were blocked by the anti-oxidant NAC. The ACN treatment also increased the expression of NF-κB (p65) and phosphorylated-IκB kinase (IKK)-α/β and decreased the expression of an inhibitor of NF-κB (IκB-α). The pretreatment with NAC significantly inhibited the activation of NF-κB. In addition, the expression of Bax increased after the ACN treatment, and the induction of Bax was abolished by NAC. Taken together, the data suggested that ACN-induced oxidative stress activated the NF-κB signaling pathway, which modulated the expression of Bax and contributed to testicular apoptosis.

Keywords: Acrylonitrile (ACN), NF-κB, oxidative stress, testicular apoptosis, antioxidant

Introduction

Acrylonitrile (ACN) is an important intermediary in the synthesis of a variety of organic products, such as artificial fibers, household articles, resins, and plastics [1]. In the workplace, extensive occupational exposure to products containing ACN can lead to neurotoxicity, immunotoxicity, and gastric toxicity [2-4]. Previous research suggested that chronic exposure to ACN was associated with tumor development in various organs [5]. Although there is insufficient evidence to support the carcinogenicity of ACN in humans, ACN is classified as a carcinogenic risk to humans according to the international agency for research on cancer [1].

Although the toxicogenic potential of ACN has been well studied [6], its effects on reproduction need to be further investigated. Epidemiological studies conducted in China reported that there were adverse reproductive effects, such as infertility, testosterone decline, and birth defects, after exposure to ACN [7, 8]. Furthermore, several studies demonstrated that ACN had the potential to induce testicular toxicity [9-12].

A number of studies reported that the level of reactive oxygen species (ROS) was increased after treatment with ACN and that ACN is an oxidative stress inducer [13-15]. In the human body, testicular germ cells are more susceptible to oxidative stress because they are closely related to phagocytic sertoli cells, which generate free radicals [16]. Besides, the germ cell plasma had high levels of polyunsaturated fatty acids, which are sensitive to oxidation by free radicals [17]. Previous studies demonstrated that an elevated level of ROS in the tes-

tis gave rise to marked alterations in tissue physiology or oxidative DNA damage, which can affect reproduction [18, 19].

ROS are generated in many diseases [20] and NF-κB pathway plays an important role in the induction of oxidative stress. A better understanding of the activation of the NF-κB pathway could shed light on the etiology of such diseases and the possibility of using NF-κB as a biomarker of oxidative stress [21]. Other than ROS, many inducers of the NF-κB pathway, such as tumor necrosis factor-α (TNF-α), terephthalic acid (TPA), and UV (Ultraviolet) radiation, can give rise to oxidative stress, suggesting that the generation of ROS is not the only inducer of the NF-κB pathway [22]. Research also showed that the anti-oxidant N-acetylcysteine (NAC) blocked the activation of the NF-κB pathway [23].

There are three main players in the NF-κB pathway: the inhibitor of NF-κB (IκB), the IκB kinase (IKK)-NF-κB essential modulator (NEMO) complex, and NF-κB. The IKK-NEMO complex activates NF-κB via phosphorylation of IκB, eventually leading to its proteasomal degradation [24, 25]. NF-κB is then translocated to the nucleus and activates target genes, such as c-IAP1, c-IAP2, A1 (Bfl1), Bax, Bcl-2, Bcl-XL, Fas/FasL, c-myc, and cyclin D1, which are critical for apoptotic processes [26-28].

The current study evaluated molecular mechanisms of apoptosis in rat testis generated in response to ACN-induced oxidative stress, focusing on IκB phosphorylation, NF-κB activation, and Bax protein regulation.

Materials and methods

Chemicals and reagents

ACN (99% pure) was obtained from Tianjing Sitong Chemical Plant (Tianjin, China). NAC (>99% purity) was purchased from Amresco (Solon, OH, USA). BCA protein assay kits and NF-κB activation nuclear translocation assay kits were purchased from Beyotime Institute of Biotechnology, Inc. (Jiangsu, China). Polyvinylidene difluoride (PVDF) western blotting membrane was purchased from Bio-Rad (CA, USA). Anti-β-actin antibody was purchased from Elabscience Biotechnology Co., Ltd (Wuhan, China). Rabbit anti-rat primary antibodies against NF-κB (p65), IKKα/β, IκBα, and p-IκBα and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody was purchased from Cell Signaling Technology, Inc. (Boston, MA, USA).

Animals

The study consisted of 30 specific pathogen-free (SPF) male two-month-old SD rats, with a weight of 250-300 g. All the animals were obtained from the Laboratory Animal Center of Gansu University of Chinese Medicine (Lanzhou, China). The animals were maintained at 25°C under a photoperiod of 12 h (light/dark cycle) and had free access to water and food. Body weight was measured every four days. All the experiments were approved and conducted according to the guidelines of the ethics committee of Lanzhou University.

Thirty animals were randomly and equally assigned to three group: a control group treated with corn oil; an ACN group treated with ACN (50 mg/kg body weight per day) by gavage solubilized in corn oil [9]; and an ACN+NAC group, which received NAC daily (300 mg/kg body weight per day) 30 min prior to gavage with ACN [29].

Sample collection

After 90 days of gavage, all the rats were anesthetized with diethyl ether before collection of blood from the heart and then were killed by cervical dislocation. The left testis was fixed in 4% paraformaldehyde, processed using an automatic tissue processor, and blocked in paraffin. The 4 mm thick sections were obtained for immunofluorescence analysis. The remaining testicular tissue was frozen in liquid nitrogen and stored at -80°C.

Oxidative stress analysis

For oxidative stress analysis, 1 g of tissue was added to 9 ml of homogenization buffer (0.9% NaCl). The homogenate was then centrifuged at 2500 r/min for 10 min at 4°C and supernatant was collected. The measurement of protein concentrations was performed using BCA protein assay kit (Shanghai, China). Malondialdehyde (MDA) levels were detected by analyzing lipid peroxidation using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activities of glutathione peroxidase (GSH-Px), glutathione/oxidized glu-

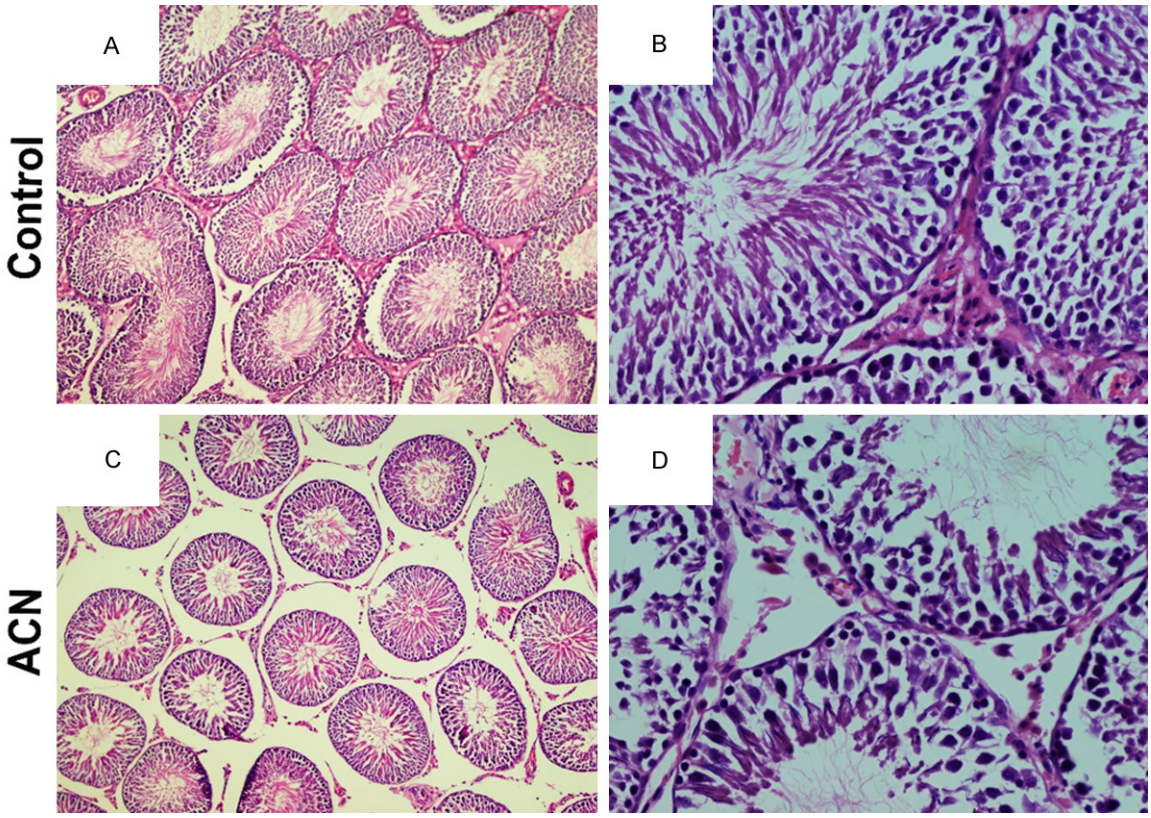

Figure 1. Effect of ACN on the pathology of the testis in the rat detected by H&E staining. ACN was administered at a concentration of 50 mg/kg body weight. A and B: H&E staining of a testis section obtained from the control group. C and D: H&E staining of a testis section obtained from the ACN-treated group.

tathione (GSH/GSSG) and superoxide dismutase (SOD) were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Immunofluorescence

The detection of NF-κB (p65) nuclear translocation was carried out using a specific kit according to the instructions of the manufacturer. Briefly, the testicular tissue paraffin sections were dewaxed and hydrated according to routine methods. The sections were fixed with stationary liquid and blocked with sealing fluid for 1 h at room temperature. The sections were incubated with primary antibody to NF-κB (p65) at 4°C overnight, followed by incubation with Cy3-labeled secondary antibody for 60 min at room temperature. Then cells were stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) solution for 5 min. Every step mentioned was followed by three times wash and 5 min each. Lastly, a fluorescent microscope (Olympus, Japan) was used for the analysis of activation and nuclear translocation of NF-κB (p65) [30].

Western blot

Approximately 100 mg of testicular tissue were homogenized in 1 ml of radio immunoprecipitation assay (RIPA) lysis buffer containing 10 μl of phenylmethanesulfonyl fluoride (PMSF) and centrifuged at 13,000 r/min for 10 min at 4°C [31]. The supernatant was collected, and the protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology, Inc.). Approximately 40 μg of protein were loaded onto SDS-PAGE gels, running at 100 V for 2 h, and then transferred to a PVDF membrane (Bio-Rad). The membranes were then blocked with 5% nonfat milk in Tris-buffered saline (TBS) for 60 min at room temperature and washed three times with TBST (TBS containing 0.1% Tween 20) buffer for 10 min each time. The membranes were incubated with anti-IKK-α/β antibody (1:1,000 dilutions), anti-p-IκB-α antibody (1:1,000 dilutions), anti-IκB-α antibody (1:1,000 dilutions), anti-NF-κB (p65) antibody (1:1,000 dilutions), anti-Bcl-2 antibody (1:1,000 dilutions), and anti-Bax antibody (1:1,000 dilutions) in TBST containing

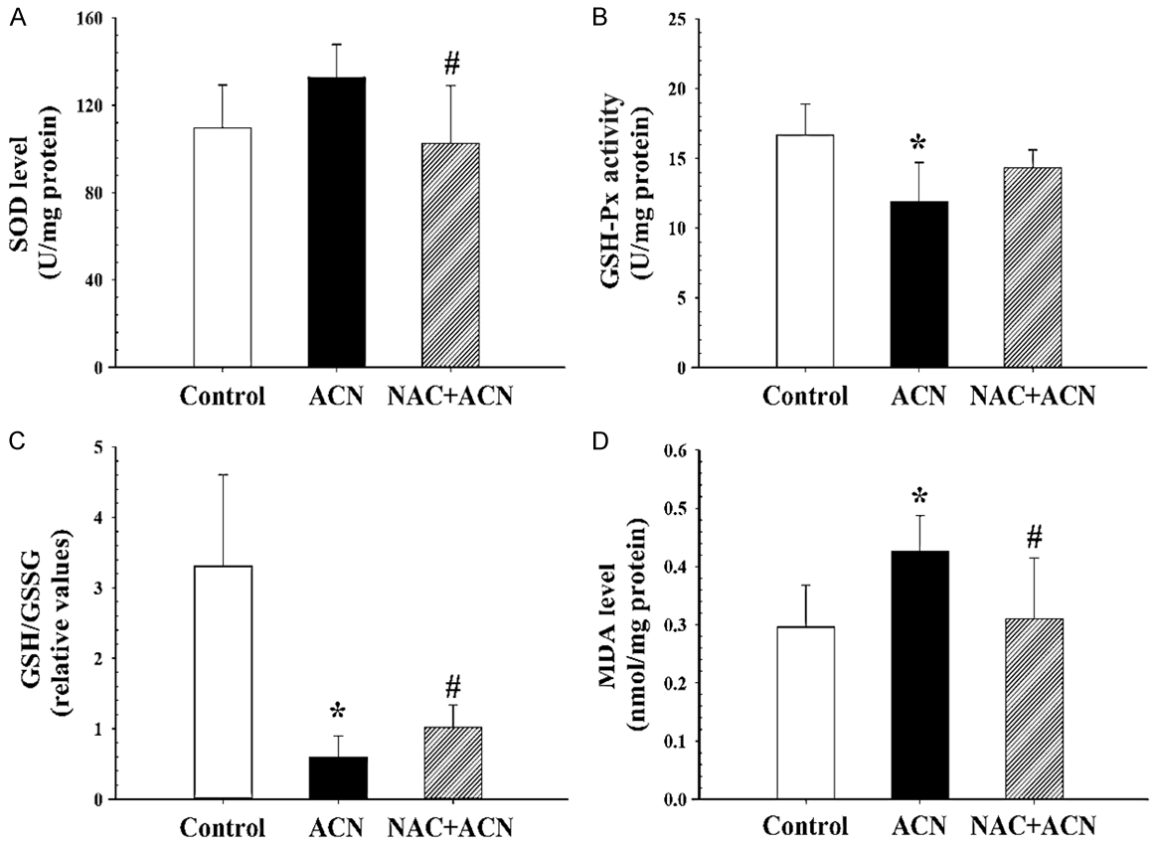

Figure 2. Effect of ACN on SOD level (A), GSH-Px activity (B), GSH/GSSG (C), and MDA level (D) in the testes of the rats. The data obtained are expressed as the means ± SEM of six samples in each group. *P<0.05, comparison with the control (corn oil only) group. #P<0.05, comparison with the ACN group.

5% nonfat milk overnight at 4°C on a three-dimensional rocking table. Anti-β-actin antibody (1:1,000 dilutions) was used as an internal normalizer. The membranes were washed three times with TBST for 10 min each time and then incubated with the HRP-conjugated secondary antibody (1:2,000 dilutions) in antibody dilution buffer for 2 h [32]. Finally, the membranes were washed three times with TBST for 10 min each time and incubated with enhanced chemiluminescence solution (Thermo, MA, U.S.). The membranes were exposed in a Molecular Imager ChemiDoc XRS System (Bio-Rad) to detect the signal of antibodies. Semiquantitative analysis of specific immunolabeled bands was performed using a Fluor S image analyzer (Bio-Rad) and Quantity One 4.6.2 software (Bio-Rad).

Statistical analysis

All the data were analyzed using SPSS vs. 19.0 software (IBM, NY, US) and expressed as means ± SEM of six animals. Statistically significant differences were determined by one-way analysis of variance, and Fisher's Least Significant Difference (LSD) test was used to analyze the differences between groups. A statistically significant difference was considered at a *P* value of P<0.05.

Results

ACN affected testicular pathology

Rat testicular tissue from both the control group and ACN group were isolated for Hematoxylin and eosin (H&E) staining to determine the effect of ACN on the pathology. Regarding seminiferous tubules, the intensity of staining was higher in the control group than in the ACN group. In addition, spermatogenic cells were well ordered and there were many primary spermatocytes in the control group (**Figure 1A**, **1B**). In contrast, in the ACN group, there were sparse seminiferous tubules, with low intensity and a narrow diameter, in addition to a small number of spermatocytes, which

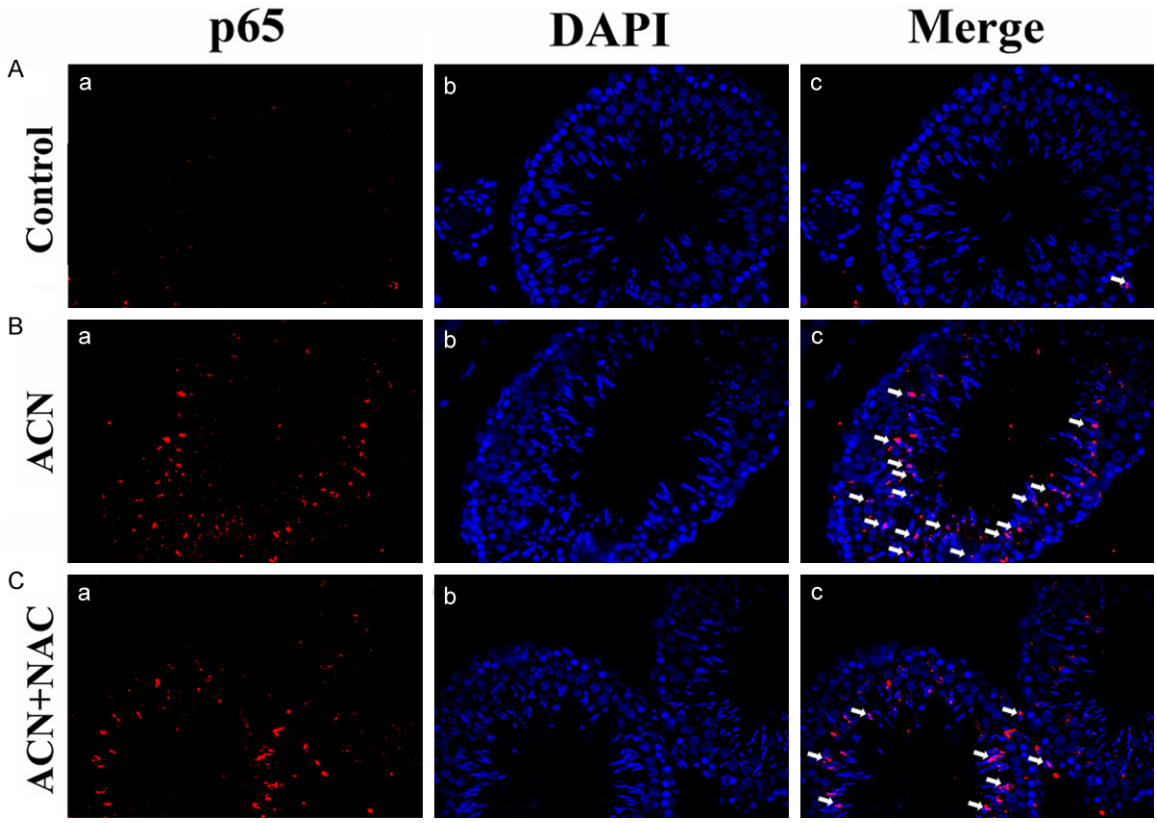

Figure 3. Immunofluorescence microscopic analysis of the localization of the NF-κB (p65) protein in testicular cells. The NF-κB p65 subunit was immunostained with Cy3 (left graph) and nucleus were stained with DAPI (middle graph). The right graph shows the merge of the left and middle graph for each group. The arrows indicated the activation and translocation of the NF-κB p65 subunit. A: The control group; B: The ACN group; and C: The ACN+NAC group. Magnification: 400×.

were disorganized. Furthermore, the testis of the ACN group contained lower numbers of Leydig cells, with unclear outlines (**Figure 1C**, **1D**).

ACN increased oxidative stress in testicular tissue

In order to gain insight into the relation between ACN and oxidative stress, several parameters, including MDA production, SOD and GSH-Px activity, and the GSH/GSSG ratio, were measured. As shown in **Figure 2A**, compared with the control group, the SOD level was increased 1.21 fold in the ACN group, and the SOD level was 30% lower in the ACN+NAC group than in the ACN group. The level of MDA showed a similar pattern (**Figure 2D**). Furthermore, compared with the control group, GSH-Px activity and the GSH/GSSG ratio were significantly decreased in the ACN group. In the ACN+NAC group, the GSH-Px activity and GSH/GSSG ratio were 20% and 71% higher than those in the ACN group (**Figure 2B**, **2C**).

Immunofluorescence analysis of NF-κB p65 activation and localization

The anti-p65 antibody, followed by the Cy3-labeled secondary antibody (red fluorescence), was used to detect cellular subunits of NF-κB-p65. The nucleus was stained with DAPI (blue fluorescence). In the control group, very few testicular cells were labeled with Cy3-labeled red fluorescence (**Figure 3A**). In contrast, in the ACN group, the p65 subunit was significantly activated and was translocated to the nucleus, indicated by the arrows in **Figure 3B**. However, in the ACN+NAC group, fewer cells were stained red, especially in the nucleus (**Figure 3C**), indicating that NAC partially inhibited the nuclear translocation of NF-κB.

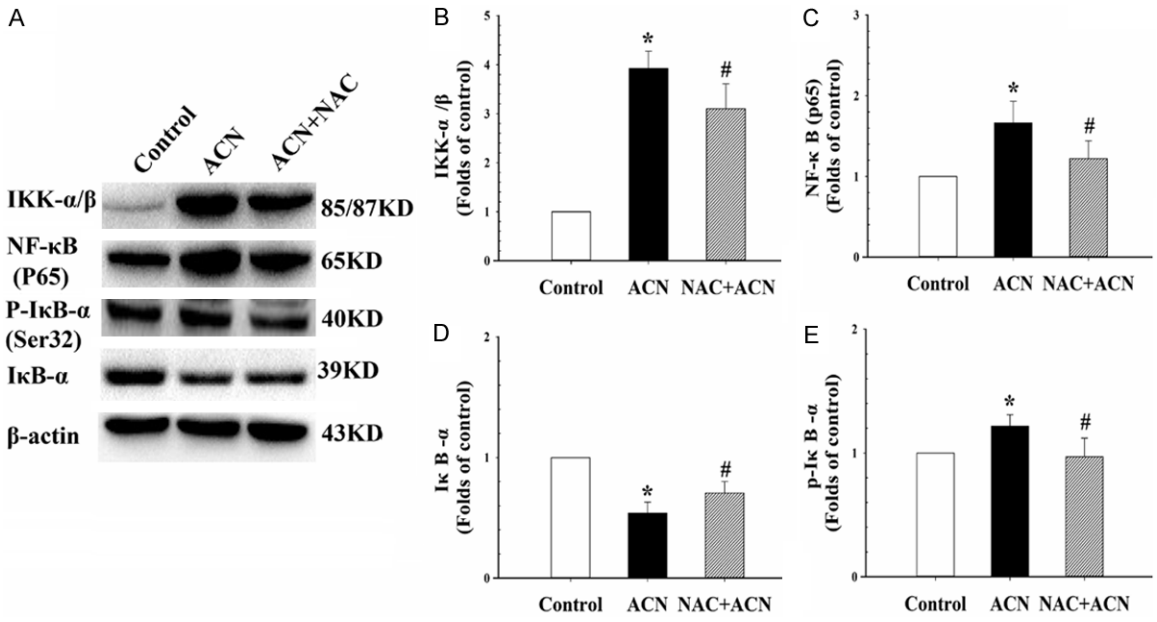

Figure 4. ACN-induced oxidative stress activated the NF-κB signaling pathway. (A) The expression levels of IKK-α/β, NF-κB (p65), IκB-α, and p-IκB-α in the rat testis were detected by western-blot. (B-E) Quantitative analysis of the protein expression levels of IKK-α/β (B), NF-κB (p65) (C), IκB-α (D), and p-IκB-α (E) in different treated groups. The data are expressed as the means ± SEM of three independent experiments. *P<0.05, comparison with the control group. #P<0.05, comparison with the ACN group. (n=6 in each group).

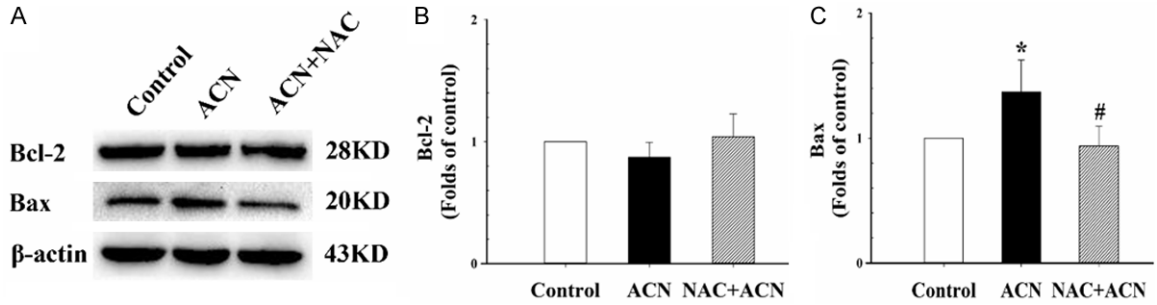

Figure 5. The expression of Bcl-2 family proteins in the ACN group. (A) Expression levels of Bcl-2 and Bax in the rat testis were detected by western blot. (B and C) Quantification of the expression levels of Bcl-2 (B) and Bax (C). The data are expressed as the means ± SEM of three independent experiments. *P<0.05, comparison with the control group. #P<0.05, comparison with the ACN group. (n=6 in each group).

ACN-induced oxidative stress activated the NF-κB signaling pathway

To study the effect of ACN-induced oxidative stress on the NF-κB (p65) signaling pathway, the protein levels of NF-κB (p65), IKK-α/β, IκB-α, and p-IκB-α in the testes were measured. The protein expression of IKK-α/β, NF-κB (p65), and p-IκB-α were increased 3.94-, 1.57-, and 1.22-fold, respectively, in the ACN group compared to the control group but decreased after the pretreatment with NAC (**Figure 4A-C**, **4E**). Compared with the control group, the protein expression of IκB-α was downregulated 0.58-fold in the ACN group, and the co-treatment with NAC reversed this downregulation (**Figure 4A**, **4D**).

NF-κB activation promoted ACN-induced apoptosis

To investigate the potential impact of the activation of the NF-κB signaling pathway on apoptosis-related proteins, the expressions of Bax and Bcl-2, two key proteins activated during apoptosis, were measured. In the ACN group, Bax expression increased 1.37-fold compared with the control group (**Figure 5A**, **5C**). After

the pretreatment with NAC, Bax expression decreased when compared with the ACN group (**Figure 5A**, **5C**). Moreover, the expression of Bcl-2 was not significantly changed in the ACN group compared with the control group (**Figure 5A**, **5B**).

Discussion

Previous studies showed that ACN could induce oxidative stress in neoplastic tissue and cells [33]. Similarly, Syrian hamster embryo (SHE) cell transformation assay demonstrated that oxidative stress increased in response to ACN and the increase could be prevented by co-treatment with antioxidants [34]. In addition, the germ cell plasma membrane of the testes contains a higher level of polyunsaturated fatty acids, which are sensitive to oxidation by free radicals and are more susceptible to oxidative stress [17].

The present study had explored the potential of ACN-induced effects on the testicular apoptosis. Our results demonstrated that ACN induced oxidative stress in testicular germ cells of rats and ultimately resulted in the activation of some transcription factors. Histology analysis showed that the ACN treatment altered the pathology of the testis, suggesting that ACN could damage the reproductive function of the testes. The present study also provided evidence that oxidative stress was indispensable for the activation of the transcriptional factor of NF-κB (p65). In addition, the results demonstrated that NAC affected the ability of oxidative stress to activate NF-κB (p65), indicating that oxidative stress plays a key role in triggering the NF-κB pathway, which translocates the latent transcription factor to the nucleus. Previous studies demonstrated that antioxidants, such as flavonoids, vitamin E, and vitamin C, as well as thiol-containing compounds glutathione, α-lipoic acid, and NAC, inhibited the activation of NF-κB (p65) [35-38].

NF-κB (p65) exists in the cytoplasm as a protein complex with p65-p50-IκB and other proteins [39]. Phosphorylation of IκBα, an inhibitory subunit of NF-κB, is a prerequisite for IκB degradation and the subsequent release of transcriptionally active NF-κB, which is achieved via protein kinases (IKK) [40]. In cells, IKK exists as a complex with catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ [41]. Once the IKK complex is activated, IκB is phosphorylated and thus targeted for poly-ubiquitination and degradation by the 26S proteosome complex [42, 43]. As a result, NF-κB (p65) is liberated from its inhibitor and translocated to the nucleus, where it trans-activates NF-κB (p65)-responsive genes [44, 45]. In the present study, NF-κB (p65) and IKK-α/β were significantly upregulated in the ACN-treated rats, and our data suggested that the phosphorylation of IκBα at Ser32 may be involved in the degradation of IκBα. Furthermore, the NAC pretreatment inhibited the activation of NF-κB (p65). These findings indicated that oxidative stress increased IκBα degradation by upregulation of members of the IKK complex, followed by activation of NF-κB (p65).

The activation of NF-κB (p65) could induce genes that promote apoptosis or prevent apoptosis, depending on the types of the gene [46]. The expression of these genes could be increased through the binding of transcriptional factors to the 5' upstream promoter region [21]. In the present study, Bax was upregulated by ACN. Moreover, Bcl-2 family proteins played a key role in the initiation of apoptosis, implicating Bax in ACN-induced apoptosis.

In summary, the present study demonstrated that oxidative stress induced by ACN triggered the activation of NF-κB (p65), which was translocated to the nucleus of rat testis, resulting in upregulation of the expression of the apoptotic protein Bax. Bax proteins may ultimately contribute to testicular germ cell apoptosis in the rats.

Acknowledgements

We thank Dr. Sun from the School of Public Health of Lanzhou University and Dr. Fu from the School of Medicine of Northwest University for Nationalities for their enthusiastic help in the manuscript review and modification. This work was supported by the Fundamental Research Funds for the Central Universities (lzujbky-2014-221). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure of conflict of interest

None.

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