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N-acetylcysteine alleviates fluoride-induced testicular apoptosis by modulating IRE1α/JNK signaling and nuclear Nrf2 activation

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ABSTRACT

We previously investigated excessive fluoride exposure elicited intracellular endoplasmic reticulum (ER) stress and led to Sertoli cells dysfunction *in vitro*. However, the mechanisms underlying fluoride-mediated male reproductive damage *in vivo* remain largely unknown. Considerable evidence has now revealed ER stress is closely linked with testicular oxidative damage. Hence, we aimed to explore whether ER stress signaling was involved in the testicular protective effects of antioxidant N-acetylcysteine (NAC) against testicular apoptosis induced by fluoride. Male SD rats were oral gavaged with sodium fluoride (NaF) for 7 weeks to induce fluorosis. The animals were pretreatment with or without NAC (150 mg/Bw•d). Our results demonstrated that sub-chronic NaF exposure triggered testicular apoptosis and sex hormonal disturbance in pituitary-testicular (PT) axis, promoted oxidative stress and the expression of ER stress mediators. Antioxidant NAC, however, prevented NaF-induced testicular apoptosis accompanied by activating Nrf2-mediated antioxidant potential. Simultaneously, NAC pretreatment downregulated XBP1 splicing, reduced JNK phosphorylation and further blocked cleavage of caspase-3, all these might contribute to the inhibition of testicular cell apoptosis. Collectively, the present results suggested that prolonged administration of NAC preserved testicular function and normalized sex hormonal disruption induced by NaF *via* the inhibition of Nrf2-associated oxidative damage and Ire1α-JNK-mediated apoptosis in rat testis.

1. Introduction

As a kind of essential trace element, low dose of fluorine is beneficial to body health by maintaining bone homeostasis and preventing dental caries. However, fluoride is also a cumulative toxicant, and long-term exposure can bring on a significant increase in body burden [1]. Globally, the persistence of fluoride in humans and its associated health risk is a matter of serious concerned issue. Although excessive fluoride has been linked etiologically and epidemiologically to the well-known effects of fluorosis on the skeleton and teeth, much interest has been attracted to the cell-type- and tissue-dependent toxicities of fluoride in recent years [2–5], especially the reproductive dysfunction [6,7].

Testis is considered to be more vulnerable to the toxic manifestations of fluoride due to the regulation processes of spermatogenesis. Previous studies revealed that fluoride exposure can lead to marked degenerative changes in the Sertoli cells and spermatogonia [8,9], as well as disturbances of multiple hormone system in human and the rodents [10,11]. Although it is now well accepted that testicular structure loss and seminiferous tubule damage may contribute to the pathogenesis of cell apoptosis in testis [12,13], the underlying cellular and molecular mechanisms regulating fluoride-mediated spermatogenic apoptosis and testicular injury remain largely unknown.

Oxidative stress is considered as the major contribution in mediating the onset of reproductive injury such as spermatogenesis damage caused by excess fluoride. Indeed, our previous study demonstrated that fluoride induced the generation of ROS and oxidative stress in multiple cell types as well as in rat testis [9,14]. As a critical scavenger of hydroxyl radicals, N-acetylcysteine (NAC) modulates intracellular redox

Abbreviations: CHOP, C/EBP homologous protein; eIF2α, eukaryotic initiation factor 2; ER, endoplasmic reticulum; FSH, follicle-stimulating hormone; GRP78/BiP, glucose-regulated protein 78; HO-1, heme oxygenase 1; IHC, immunohistochemistry; IRE1, inositol-requiring enzyme 1; JNK, c-Jun N-terminal kinase; LH, luteinizing hormone; NAC, N-acetylcysteine; NaF, sodium fluoride; NF-κB, nuclear factor-κB; NQO1, NAD (P) H:quinine oxidoreductase 1; Nrf2, NF-E2-related factor 2; PERK, protein kinase-like endoplasmic reticulum kinase; PT axis, pituitary-testicular axis; ROS, reactive oxygen species; UPR, unfolded protein response; XBP1s, spliced X-box-binding protein1; XBP1u, unspliced X-box-binding protein1

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effectors involved in semen quality and testicular morphology. Concurrently, ER stress and oxidative stress could possibly be closely linked events. Although several studies have illustrated the role of NAC in regulating testicular cell apoptosis accompany with the changes of pro-oxidant biomarkers in relation to fluoride exposure [9,15], very limited experiments have shown that NAC treatment could attenuate the negative effect of fluoride via ER stress mediators *in vivo* [4,16].

Endoplasmic reticulum (ER) stress response, which is known to trigger apoptosis, occurs by a variety of physiological states or environmental stimuli including excessive production of reactive oxygen species(ROS), Ca^{2+} balance disorders, hypoxia, toxic chemicals [17–19]. The expression of C/EBP homologous protein (CHOP), a proapoptotic transcription factor induced by ER stress, was correlated with testicular apoptosis [16,20]. Notably, in a relevant *in vitro* model induced by fluoride, we have also shown that NAC may attenuate ER stress and its PERK-eIF2α signaling in the form of increased expression of glucose-regulated protein 78 (GRP78) and CHOP [14]. However, little is known about the role of ER stress and antioxidant signaling in fluoride-mediated testicular damage of rodents.

In present study, we used a rat model to investigate the possible effect of NAC on fluoride-induced testicular injury and focus on the roles of IRE1-mediated ER stress as well as apoptosis. Furthermore, we also specially evaluated whether NAC-mediated NF-E2-related factor 2 (Nrf2) expression contribute to the regulation of antioxidant pathway.

2. Materials and methods

2.1. Experimental animals

A total of 60 male specific pathogen free (SPF) SD rat (125 ± 25 g, aged 4 weeks) were obtained from the Experimental Animal Center of Henan province and group-housed in accordance with the Guide for the Care and Use of Laboratory Animals published by Ministry of Health of People's Republic of China. All experimental procedures and protocols were reviewed and approved by Life Science Ethics Review Committee of Zhengzhou University.

2.2. Establishment of the fluoride-exposed male rat model

After one week of balanced feeding, the experimental mice were randomly divided into four groups of 15 rats each: NAC group, NaF group, NAC + NaF group. For the Control group, rats were fed with a standard diet and given normal saline. The rats of NAC group were oral gavaged with 150 mg/kgBw•d of NAC. For NaF group, the rats were oral gavaged with 25 mg/kgBw•d of NaF. The rats of NAC + NaF group received 150 mg/kgBw•d of NAC 0.5 h prior to oral NaF exposure a single dose of 25 mg/kgBw•d. All solutions were administrated to rats in accordance with the principle of isovolumetric gavage (10 ml per kilogram of body weight) once per day for 7 weeks. Of note, the exposure dose for subchronic toxicity experiments is usually 1/20–1/5 of LD50. In present study, rats in the NaF group were subjected to oral gavage with NaF at a dose of 25 mg/Bw•d for 7 weeks, which was 1/19 of the oral LD_{50} values in rats(471.7 mg/Bw•d NaF) [21]. This NaF dose is equivalent to 11.31 ppm of fluoride over the course of the exposures. The effectiveness of NAC depends on the timing of the application (co- or pre-treatment), fluoride concentration and incubation time. It was convinced that pre-treatment with NAC was more effective in counteracting NaF-induced oxidative damage processes than concurrent treatment with both compounds by Pawłowska-Góral et al [22]. An *in-vitro* study also confirmed the way of NAC pretreatment was feasible [23]. Although some scholars may prefer the simultaneous addition of NAC and exposure factor, the NAC pretreatment was introduced in present and other *in-vivo* studies in order to light its possible preventive role, especially focus on substantial reproductive protection with pronounced efficacy against environmental insult [24,25]. The NAC dose was selected from studies that have been published previously as the level that in adult male rats exerts potent antioxidant effects [26–28]. The rats' body weights were recorded on the day of sacrifice. Blood samples were obtained from abdominal aorta after being anesthetized by chloral hydrate. Testis and epididymis were immediately removed and organ weights were measured by electronic balance. The testis was bifurcated: one part was kept at −80 °C for RT-qPCR and Western blotting, another part was immersed in formaldehyde liquid for 24 h for testicular histology and apoptosis analysis.

Table 1
List of quantitative PCR primers.

Gene	GenBank No.	Sequence
GAPDH	NM_001289746.1	Forward: gtcggtgtgaacggattt Reverse: actccacgacgtactcagc
IRE1	NM_001433.3	Forward: tggacggacagaatacacca Reverse: tggacacaaagtgggacatc
XBP1	NM_005080.3	Forward: tttgggcattctgaacaagt Reverse: aagggaggctggtaaggaac
JNK	NM_053829.1	Forward: agtgtagagtggatgcatga Reverse: atgtgcttcctgtggtttac
Nrf2	NM_031789.2	Forward: ccttcctctgctgccattagtc Reverse: gaactccaccgtgccttcag
NQO1	NM_017000.3	Forward: tgtctgggaggacttcacca Reverse: ttgtcggctggaatggac
HO-1	NM_012580.2	Forward: cattgagctgtttgaggagctg Reverse: gcggtgtctgggatgaacta

Table 2
Body weight, organ coefficient and fluorine levels of testis.

Groups	Body weight (g)	Testicular organ coefficient (%)	Testicular fluorine (μg/g)
Control	475.15 ± 8.98	0.36 ± 0.01	5.69 ± 0.11
NAC	472.23 ± 5.50	0.36 ± 0.01	5.87 ± 0.05
NaF	442.18 ± 8.02*	0.36 ± 0.01	10.95 ± 0.24*
NAC + NaF	459.54 ± 8.91	0.36 ± 0.01	10.72 ± 0.09*

Values are expressed as means ± SEM, n = 10. *$P < 0.05$ compare to Control group.

2.3. Assessment of testicular organ coefficient

At the end of 7-week exposure, the body weights of rats from each group were measured by electronic balance. The testis was removed immediately and measured when the rat was sacrificed by cervical dislocation. The organ coefficient of testis was calculated according to following formula: organ coefficient = wet weight of organ (g)/body weight (g) × 100%.

2.4. Testicular fluorine content analysis

The testis was taken out from the freezer (−80 °C) and thawed. Preheat oven to 60 °C and bake testis for 24 h until it was dried to constant weight, then measure the weight. The testicular tissue was homogenized in hydrochloric acid using grinders and centrifugation to obtain supernatant. Then the fluoride elemental content was analyzed by ion selection electrode (Shanghai Exactitude Instrument Company, China) method.

2.5. Determination of sex hormone levels

The levels of sex hormones concerned to androgen synthesis in serum and testis of rat were quantified using available commercial ELISA kits specific for rats namely testosterone((Hengyuan Biotech Co., China), FSH and LH (Hangzhao Eastbiopharm Co., China)) as per the manufacturer's instruction (Hengyuan Biotech Co., Ltd, Shanghai, China). The intra-assay coefficients of variation were less than 5% for

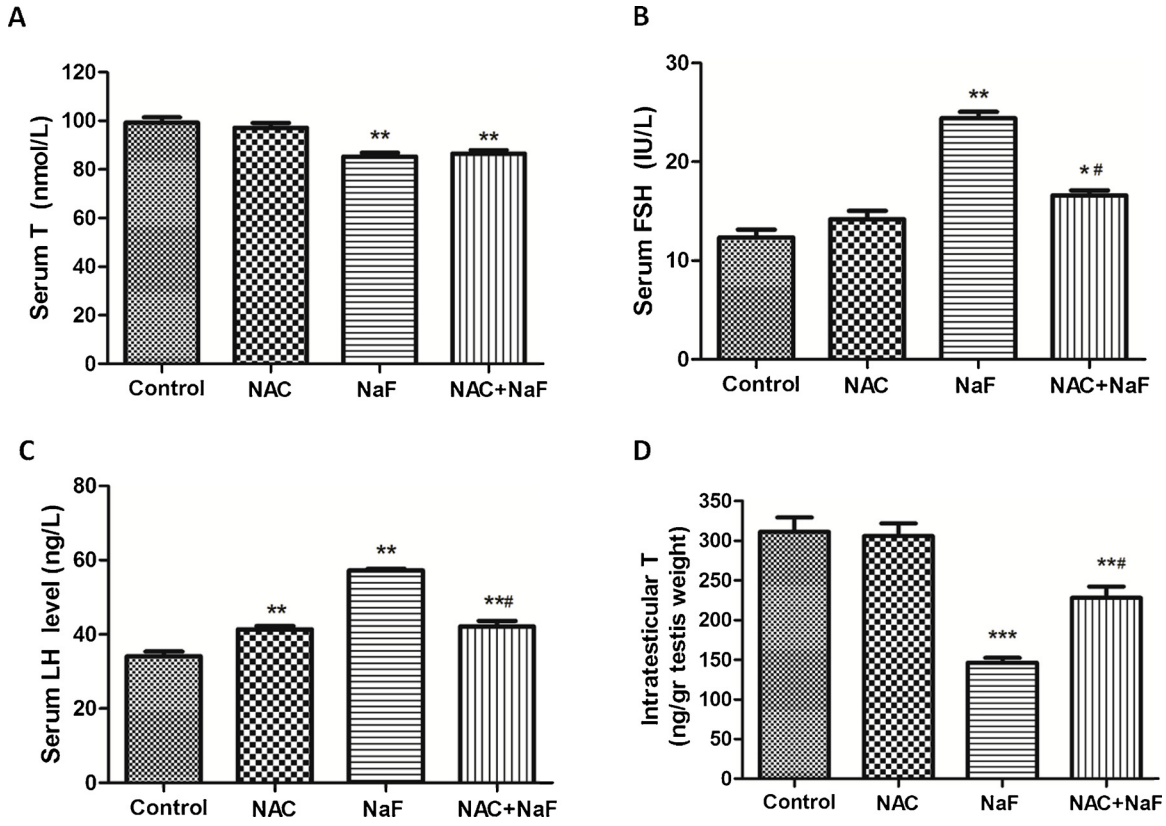

Fig. 1. Effects of NaF and/or NAC on sex hormone levels in serum and testis of rat. The levels of **(A)** testosterone, T, **(B)** FSH, and **(C)** LH level in serum and **(D)** T content in testis was determined in **rat** of each group by ELISA. Values are expressed as mean ± SEM (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *compared to Control group;* $^{\#}P < 0.05$ *compared to NaF group.*

these assays. Inter-assay variation was avoided by assaying all the samples on the same day.

2.6. TUNEL analysis

The sections from each rat testis were embedded in paraffin, and the paraffin- embedded sections were used to identify apoptotic cells by TUNEL assay using an in situ cell death detection kit (Nanjing Jiancheng Bioengineering Inst., China). Apoptotic cells were identified either as cells with brown-stained nuclei using a light microscope. The number of TUNEL-staining cells was enumerated for five random fields per tissue section at 400× or 200× magnification or by three reviewers who were blinded to the sample origin. Values for all three sections from each block of tissue were averaged and expressed as the apoptotic rates in per tubule or percentage of seminiferous tubule containing TUNEL positive cells for each rat.

2.7. Quantitative real-time PCR

The total cellular RNA from testicular tissue was extracted with TRIzol reagent (Invitrogen, USA). First-strand cDNA was synthesized using HiScript®II1st strand cDNA Synthesis Kit (Vazyme Biotech., Nanjing, China) and stored at −80 °C for further use. The mRNA expression of IRE1, JNK, XBP1 Nrf2, and NQO1 and HO-1 was inspected using cDNA as a template for amplification utilizing the corresponding primers (Supplementary Table 1). The expression levels of each sample were normalized against GADPH and calculated using the comparative CT method ($2^{-\Delta\Delta CT}$).

2.8. Protein extraction and Western blot analysis

The cytoplasmic and nuclear protein fractions were extracted from testicular tissue using NE-PER™ extraction reagents (Thermo Scientific, New York, USA) according to the manufacturer's instructions. In brief, 10 μL of protease inhibitor cocktail was added in a pre-chilled tube with 990 μL of Cytoplasmic Extraction Reagent I (CER I). Then 100 mg of testicular tissue was homogenized in the above mixture using a tissue grinder on ice. The tube with tissue homogenate was put under vortex movement for 15 s and placed on ice for 10 min. Next, ice-cold CER II was also added into the tube and mix thoroughly. After centrifuge at 16,000 *g* for 5 min, the supernatant in the tube was collected and labeled as cytoplasmic extract. Finally, the insoluble fraction in the tube was suspended with ice-cold Nuclear Extraction Reagent (NER) and incubation on ice for 40 min. The supernatant fraction which contained nuclei was extracted and collected as nuclear extract. Immunochemical analysis of protein level and phosphorylation status of the testicular tissue was performed by Western blotting in standard conditions. Briefly, equal amounts of samples were subjected to 8%–12% SDS-PAGE gels and transferred onto the PVDF membrane. Next, the membranes were blocked with 5% non-fat milk and then incubating the membranes with the GRP78 (Cell Signaling Technology, MA, USA), IRE1 (Abcam, Cambridge, UK), p-IREI and XBP1 (Santa Cruz Biotech., CA, USA), JNK and p-JNK(CST, MA, USA), Nrf2 (Proteintech, USA), NQO1 (Bioss, Beijing, China), HO-1 and caspase-3 (Abcam, Cambridge, UK) primary and secondary antibodies (Beyotime, Shanghai, China). GADPH and Lamin B were used as loading control for total/cytosolic and nuclear proteins respectively. For densitometric analysis, the blots were scanned with Amersham Imager 600 and the pixel intensities of each band of interest were quantified using Image Quant TL (7.0 version, GE Healthcare, USA).

2.9. IHC staining for GRP78 expression

Immunohistochemistry (IHC) staining with GRP78 (Cell Signaling

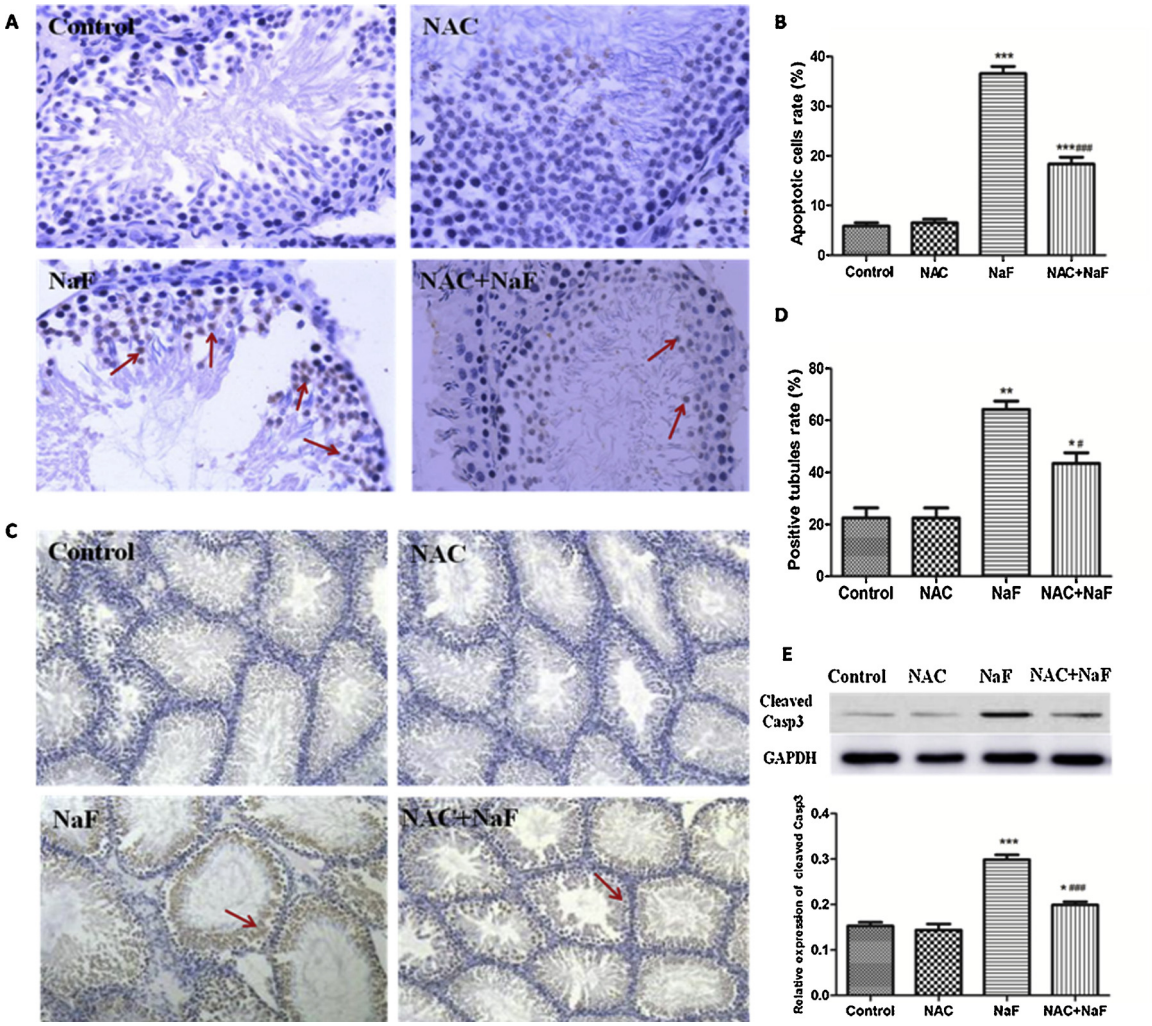

Fig. 2. NAC prevented apoptosis and caspase-3 activation in testis of NaF-exposed rat. Apoptotic morphological changes were assessed by TUNEL staining. Series of representative morphological image are present at different magnifications(**A**, 400× ; **C**, 200×), and the apoptotic rates in per tubule (**B**) as well as percentage of seminiferous tubule containing TUNEL positive cells (**D**) are expressed as mean ± SEM respectively. **(E)** Representative Western blot and quantitative results for cleaved caspase-3 level in the testes. The densities are normalized to GADPH. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *compared to Control group;* $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *compared to NaF group.*

Technology, MA, USA) was used to evaluate the phenotypic expression of testicular GRP78. Deparaffinized testis sections were hydrated by serial set of ethanol and washed in distilled water, and then sections were kept in pre-heated Tris-EDTA buffer (pH 9.0) for reduction of endogenous peroxidases. Subsequently, the sections were blocked in 10% BSA for 1 h and were stained according to the standard protocol and visualized GRP78-positived cells with diaminobezidin (DAB) kit. Three sections per testis were used for analyzing the mean optical density (OD) of GRP78-labeled cells by IPP 6.0 software from five views under the light microscope (Nikon, 50i, Japan).

2.10. Statistical analysis

All statistical analyses were performed using the *SPSS* software (version 17.0) and GraphPad Prism 5 for Windows was used for graph fitting. One-way *ANOVA* and *Tukey's post hoc* tests were performed for parametric data. All data in the text and figures were expressed as mean ± standard error of the mean (SEM), with n representing the number of animals used in each experiment. Statistical differences were defined significant at the 95% confidence level ($P < 0.05$).

3. Results

3.1. Body weight, organ coefficient and fluorine levels of testis

As shown in Table 1, rats exposed to fluoride showed lower body weight compared with those of the control group or NAC + NaF group ($P < 0.05$). Following the exposure period, testicular fluorine content in the NaF treated group and NAC+NaF group was significantly increased compared with the control group ($P < 0.05$ respectively). Rats treated with NaF for 7 weeks demonstrated a reduction in body weight, and it was improved following pretreatment with NAC. However, the rats did not show any significant change in testicular organ coefficient after either fluoride exposure or NAC pretreatment (Table 2).

3.2. Effect of NaF exposure on sex hormones levels

ELISA was performed to evaluate sex hormone levels in serum and testis of rat related to testicular function following exposure to NaF with or without NAC gavage and the results were summarized in Fig. 1. The results showed that there was decreased circulatory testosterone production and increased levels of LH and FSH in the NaF group ($P < 0.01$ respectively, Fig. 1**A-C**). Administration of NAC distinctly partly restored the expression of both LH and FSH whereas failed to alter the

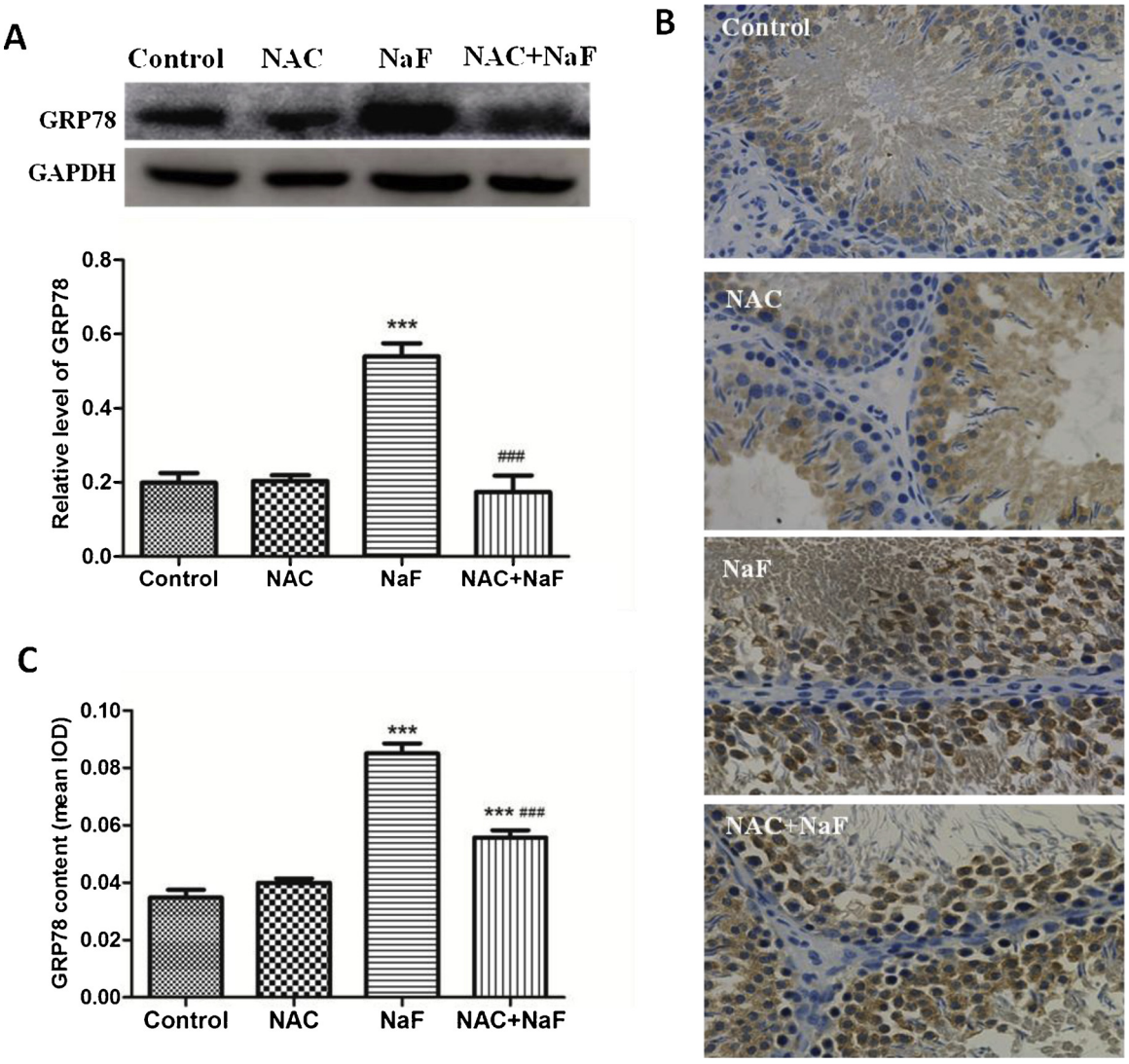

Fig. 3. NAC decreased NaF-mediated accumulation of GRP78 in the testis. **(A)** Representative Western blot and quantitative results for testicular GRP78 protein in **rats** of each group (n = 6). The densities are normalized to GADPH. **(B)** Representative photomicrographs of GRP78 positive staining were observed by IHC method (400×). **(C)** Quantitative results of GRP78 content in the testicular tissue of each group (n = 3). $^{***}P < 0.001$ *compared to Control group;* $^{\#\#\#}P < 0.001$ *compared to NaF group.*

level of serum testosterone in response to NaF. Moreover, there was also increased serum LH levels upon NAC treatment alone ($P < 0.01$, Fig. 1C). By contrast, NaF exposure led to reduction of testosterone in testis of rat ($P < 0.001$, Fig. 1D), which was significantly reversed by the pretreatment with NAC ($P < 0.05$).

3.3. NAC alleviated NaF-induced testicular cell apoptosis and caspase-3 activation

The effect of fluoride exposure on testicular injury was analyzed by TUNEL assay (Fig. 2A, C). Observed at low magnification, prolonged exposure of NaF increased the percentage of seminiferous tubules with TUNEL positive cells in the testis ($P < 0.01$, Fig. 2D). At high magnification, only a small amount of intrinsic apoptosis was detected in testis of the control group or NAC group, there were a large number of tan and brown-like apoptotic cells in the testicular tissue sections treated with NaF. Further analysis showed that the number of apoptotic cells in per tubule was also significantly increased in the testis ($P < 0.001$, Fig. 2B). In addition, pre-treatment with NAC markedly decreased both the number of TUNEL positive cells per tubule ($P < 0.001$, Fig. 2B) and the percentage of tubules with TUNEL positive cells ($P < 0.05$, Fig. 2D), although which has not yet reached the level of the control group.

Active caspase-3 directly triggers apoptosis of Sertoli cells as well as Leydig cells following testicular injury and indicates the levels of apoptosis. Hence, we further detected the cleaved caspase-3 level of each group. As shown in Fig. 2E, exposure to NaF caused a robust induction in caspase-3 cleavage ($P < 0.001$). In contrast, similar to percent of TUNEL positive cells, caspase-3 activation was almost the same between the control group and NAC group. NAC administration showed a reversal of high level of cleaved caspase-3 ($P < 0.001$).

3.4. NAC decreased NaF-mediated accumulation of GRP78 in testis of rat

To explore whether NaF induce ER stress in testicular tissue, we analyzed the expression of GRP78, which was a central regulator of ER stress and has been used as a marker of ER stress/ unfolded protein response (UPR). As shown in Fig. 3A, the level of GRP78 protein was found to be upregulated in NaF group compared to control or NaF + NAC group ($P < 0.001$ respectively, Fig. 3A). Additionally, we analyzed the effects of NaF and NAC on the phenotypic expression of testicular GRP78 using IHC staining (Fig. 3B and C). As expected,

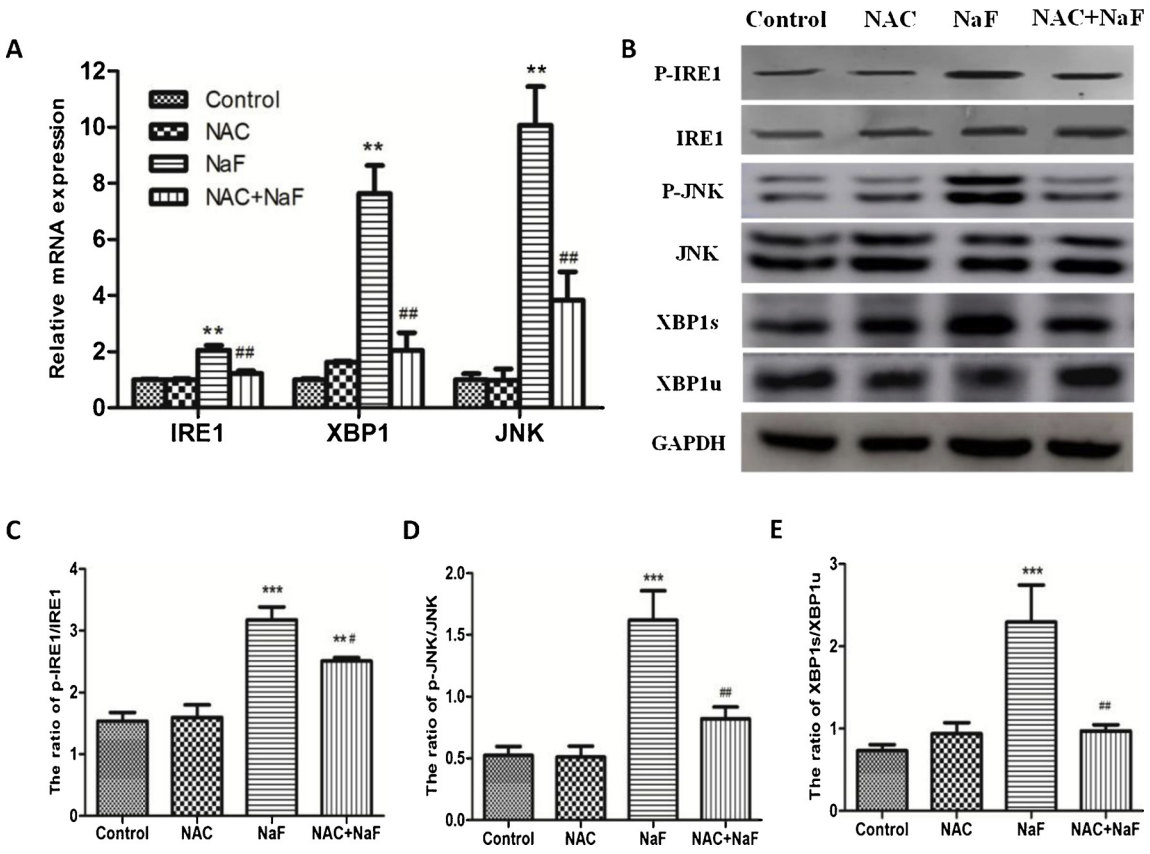

Fig. 4. NAC attenuated NaF-induced testicular ER stress in the form of decreased expression of ER stress mediators. **(A)** The endogenous IRE1, XBP1 and JNK transcript levels were determined by RT-qPCR from rat testicular homogenates of each group. Quantitative results for each sample normalized by GADPH. **(B–E)** Representative Western blot and quantitative results for phosphorylated IRE1 and its downstream markers: p-JNK/JNK and XBP1s / XBP1u ratio. The densities are normalized to GADPH (n = 8). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to NaF group.

increased GRP78 expression was found in NaF group rather than control group and the presence of NAC partly eliminated the induction of GRP78 mediated by NaF in rat testis.

3.5. NAC attenuated NaF-induced ER stress activation via inhibiting IRE1-JNK signaling pathway

The IRE1-JNK pathway, downstream of GRP78, is the main apoptotic pathway of ER stress. These ER stress molecules including IRE1, XBP-1 and JNK are expressed at low levels under physiological conditions, but are induced strongly during prolonged severe ER stress. As shown in Fig. 4**A**, both IRE1 and JNK gene expressions were significantly elevated in the testis during NaF exposure. The activation of these two genes was also determined by Western blotting (Fig. 4B-D). Exposure to NaF resulted in significant increase in the ratio of pIRE1/ IRE1 or pJNK/ JNK when compared to control rat ($P < 0.001$ respectively). In contrast, NAC prevented the NaF-induced phosphorylation of IRE1or JNK in the testis ($P < 0.05$ or $P < 0.01$).

In addition, activated IRE1α leads to a translational frame shift, resulting in the production of the spliced/activated form of XBP1 protein. XBP1 splicing was detected in rat experiencing NaF exposure. As shown in Fig. 4B and 4E, the XBP1s / XBP1u ratio of rat from NaF group was significantly higher than that of control group ($P < 0.001$). In contrast, NAC could suppress XBP1 activation when compared to the NaF group ($P < 0.01$).

3.6. Effect of NAC on NaF-induced stress sensitive redox molecules in testis of rat

Given that NAC was reported to be a potential activator of Nrf2 and could protect Sertoli cell against NaF-induced oxidative stress, we detected the involvement of Nrf2 in mounting the protective effect of NAC using Western blotting and RT-qPCR. As shown in Fig. 5A, either NAC or NaF alone was able to potentiate testicular Nrf2 in nuclei ($P < 0.001$ or $P < 0.01$). Meanwhile, NAC pretreatment further promoted its nuclear expression in NaF-intoxicated rat ($P < 0.001$). In comparison, both Nrf2 mRNA expression (Fig. 5B) and cytoplasmic Nrf2 were depleted by NaF exposure. NAC administration partly restored the reduction of cytoplasmic Nrf2 ($P < 0.05$) along with promotion of its nuclear form.

The mRNA level of Nrf2 target genes, NQO1 and HO-1, was increased in rat from NaF group (Fig. 5B). The rat also displayed an increase in protein expression of these two genes in response to NaF (Fig. 5C). Moreover, NAC pretreatment further promoted the NaF-induced protein level of NQO1 ($P < 0.01$), but not the HO-1, in the testes.

4. Discussion

We previously showed that rodents with long-term fluoride exposure were at increased risk for testicular abnormalities, mainly manifesting as testicular structure loss, impaired spermatogenesis and reduced sperm motility [15]. Alternatively, apoptosis can also contribute to defective spermatogenesis, resulting in diminished sperm output. Similarly, the current data demonstrated that testicular cell apoptosis was induced in NaF-intoxicated rat which displayed increased TUNEL positive cells in seminiferous tubule. Moreover, oxidative stress is also strongly associated with testicular cell apoptotic cascades companying sex hormonal disruption [11,13]. As its prominent antioxidant, NAC is critical in reducing or preventing testicular oxidation induced by

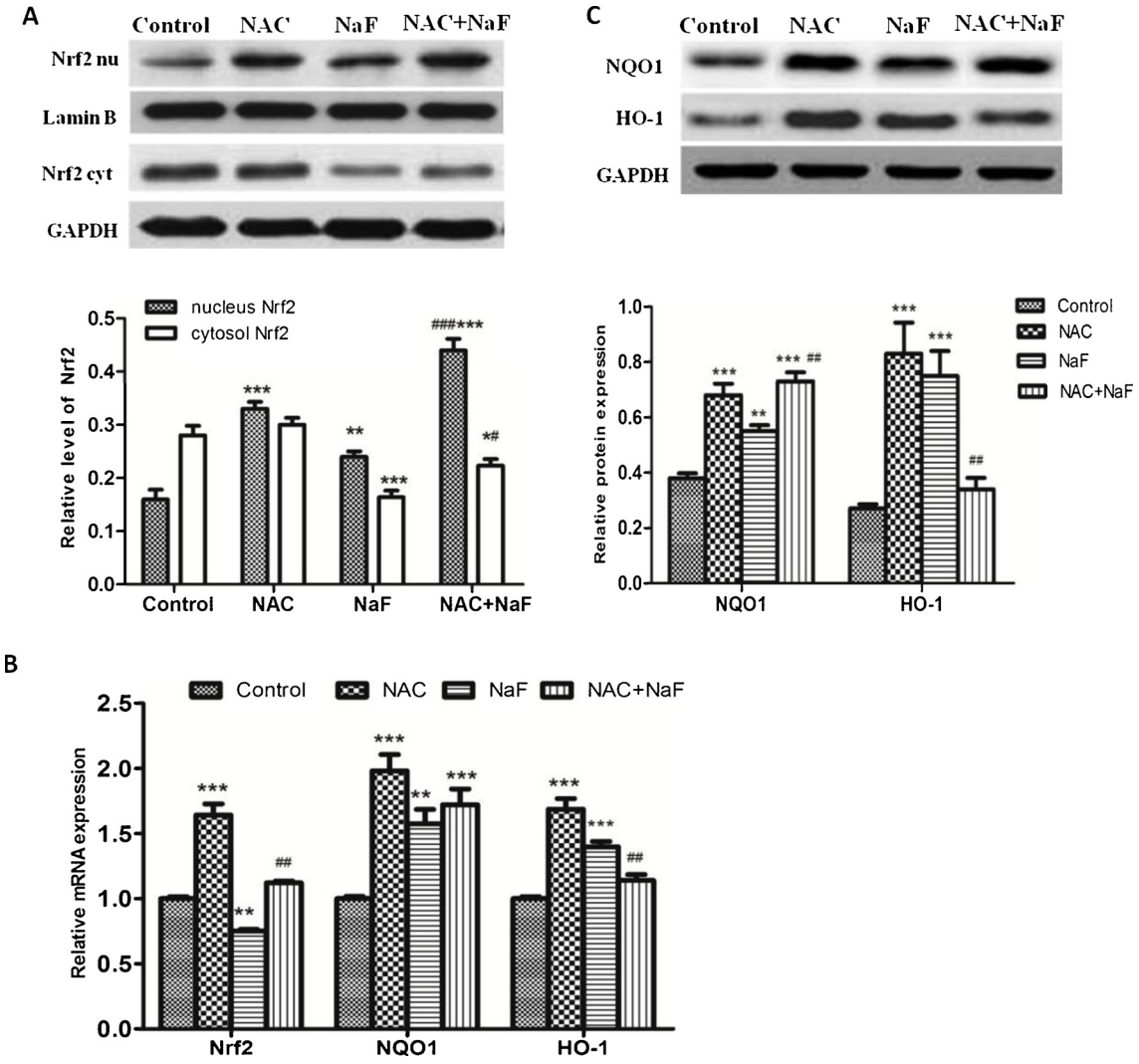

Fig. 5. Effect of NAC on NaF-induced stress sensitive redox molecules in testes. **(A)** Representative Western blot and quantitative results for Nrf2 level in nucleus and cytosol of testis. The densities are normalized to Lamin B or GADPH respectively. **(B)** The endogenous Nrf2, NQO1 and HO-1 transcript levels were determined by RT-qPCR from rat testicular homogenates of each group. **(C)** Representative Western blot and quantitative results for the levels of Nrf2 target genes, NQO1 and HO-1. Values are expressed as mean ± SEM (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *compared to Control group;* $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *compared to NaF group.*

environmental insults [29,30]. Here we found that NAC treatment was able to alleviate testicular cleaved caspase-3 level, which was in line with the TUNEL results.

Apart from apoptosis, there is of great interest in NaF-mediated hormones profiles concerned to androgen synthesis. Although it is generally accepted that the physiological role of GnRH is to trigger the activity of reproductive hormones regulation, its expression in serum was fail to determine due to its pulsatile secretion and short half-life [31]. We therefore mainly observed the level of gonadotropins (i.e. FSH and LH) as well as and testosterone secretion in present study. The testicular dysfunction was clinically caused by the improper FSH levels, then led to hypersecretion of LH levels [32], with marked degenerative changes in the Sertoli cells and spermatogonia. Elevated FSH level in serum reportedly up-regulates inducible testicular spermatogenic dysfunction by fluoride. As expected, the pituitary secretion of FSH and LH were significantly enhanced after fluoride exposure in present study. Accordingly, an inverse relationship between circulating T and FSH as well as LH was clearly observed, which indicated a clear disruption of the feedback effect between gonadotropins and testosterone in response to fluoride. As a recognized antioxidant, NAC is tried mostly in animal studies to protect the reproductive function against chemicals with variable efficacies [24,29]. The inhibitory effect of NAC on serum FSH and LH was evident in the backdrop of significant reduction of both hormones in NaF-exposed rat, as presently observed. It is tempting to appear that the disorder of FSH and LH, which acted as a negative feedback signal to testosterone at the pituitary level, were partly rescued by NAC. However, the rats following NAC pretreatment showed reduced circulating levels of testosterone despite increased testicular secretion of testosterone. The failure in reversal of decreased serum testosterone might partly attribute to decreased blood flow induced by fluoride, and finally normal testosterone to release blood vessels was disrupted. As a whole, these findings revealed that the restoration of sex hormonal disruption in pituitary-testicular axis and inhibition of testicular caspase-3 cleavage were important for the action of NAC on maintaining testicular functions in cases of fluoride exposure.

In addition to oxidative stress, some studies demonstrated the induction of ER stress in the onset of testicular injury [33]. ER stress is also regarded as an adaptive response to fluoride-induced cell apoptosis [16,33,34]. Recently indeed, the study from our lab has been shown that NAC could effectively block or attenuate Sertoli cells apoptosis due to its ability to remove molecular chaperone GRP78 and to initiate ROS-mediated ER stress pathway *in vitro* [14]. Disruption of ER homeostasis

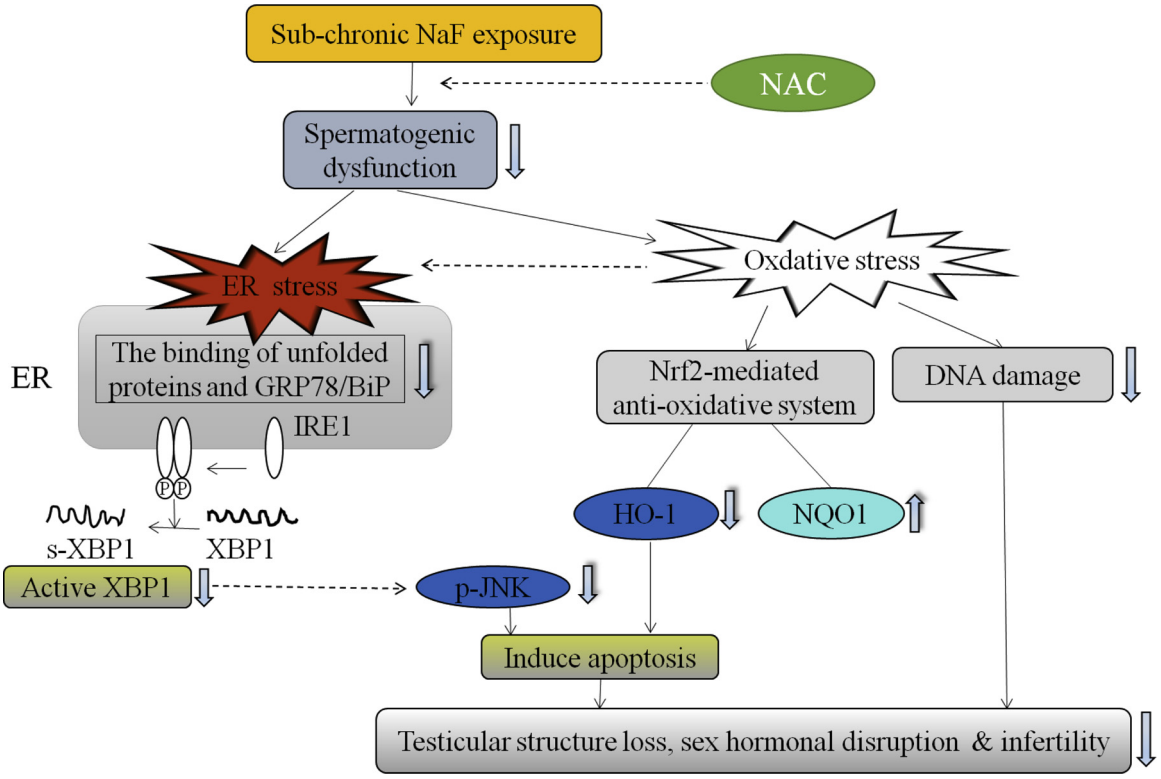

Fig. 6. Model for the mechanism by which NAC attenuate testicular lesion in NaF-intoxicated rat. Sub-chronic NaF exposure causes spermatogenic dysfunction. Both ER stress and oxidative stress mediate the disorder of spermatogenesis. The elevated IRE1α phosphorylation as well as XBP1 splicing caused by ER stress may induce JNK-mediated apoptosis. Simultaneously, the alteration of the Nrf2 antioxidant pathway along with enhanced DNA damage during testicular oxidation also contributes to apoptosis. These sequential responses may elicit testicular structure loss as well as sex hormonal disruption, and subsequent infertility. NAC pretreatment may normalize the processes of spermatogenesis by attenuating cellular endoplasmic reticulum and oxidative stress-mediated apoptotic death, resulting in the inhibition of reproductive abnormalities.

triggers the UPR signaling, an ER-specific stress response. Of note, the inositol-requiring enzyme 1 (IRE1) pathway, the most evolutionarily conserved pathway of the UPR, has a pro-apoptotic function that contribute to the activation of binding protein (BiP/GRP78), spliced X-box-binding protein 1 (sXBP-1), and apoptotic mediator JNK [35,36].In the present study, treatment with NAC effectively reversed testicular GRP78 level as well as phenotypic expression thus was associated with UPR activation. We further found the increase of testicular XBP1 and IRE1α in transcript and protein levels induced by fluoride. In comparison, both elevated XBP1 splicing and IRE1α phosphorylation were rescued by NAC in the testes, thus confirming IRE1α-mediated modulation of ER stress. Indeed, the IRE1/XBP1 pathway influences both the UPR-mediated survival response and the apoptotic cell death response to ER stress [37]. Several studies have demonstrated that JNK-signaling is an important outcome of IRE1 activation in ER stress-induced apoptosis [38–40] Our data showed that the level of phosphorylated JNK was also markedly increased with subsequent caspase-3 activation during NaF exposure, although did not confirming whether JNK act downstream of IRE1a. Importantly, NAC almost completely reversed NaF-induced JNK phosphorylation in the testis. Overall, it is reasonable to assume that NAC ameliorates apoptotic effect of fluoride on rats' testes, partly through inhibition of the ER stress response via repressing IRE1/JNK pathway activation.

The connection between fluoride-induced apoptosis and testicular oxidation is also complex. It has been reported that oxidative stress-induced steroid cells apoptosis plays a critical role in the pathogenesis of testicular lesion. In previous studies, excessive fluoride induced oxidative stress and characterized by the elevated generation of MDA and increased expression of 8-OHdG [14]. The expression of anti-oxidative enzymes and detoxifying enzymes is regulated by Nrf2, a prime molecular target against chemical/oxidative stress [41–43]. Of note, the primary control of Nrf2 transcriptional activation of target gene induction relies on subcellular distribution in response to oxidative stress. It is also reported that NAC can promote nuclear translocation of Nrf2 and ultimately lead to activation of Nrf2/ARE mediated anti-oxidant response [44,45]. We consequently assessed the expression of Nrf2 protein in both cytoplasmic and nuclear forms. Here, our results showed NAC treatment alone increased the level of nuclear Nrf2 without affecting its expression in cytosol. Interestingly, a single dose of NaF exposure also promoted the induction of nuclear Nrf2, consisted with our earlier findings *in vitro* that reported nuclear translocation of Nrf2 to be activated during fluoride exposure [14]. However, the decreased Nrf2 mRNA expression as well as depletion of cytoplasmic Nrf2 suggested that Nrf2 synthesis was inhibited during NaF exposure. This was likely to because the extended stress induced by NaF promoted cytoplasmic activation and then increased nuclear accumulation of Nrf2. Moreover, there were many of transcription factors antagonizing Nrf2 activation, such as nuclear factor-κB (NF-κB) and estrogen receptor α [46,47]. Therefore, the decreased Nrf2 expression in transcription level caused by fluoride might be attributed to other associated pathways and impaired nuclear translocation.

Further, we also attempted to determine the possibility that NaF-induced nuclear Nrf2 accumulation contributed to abnormality of redox response. Here, mRNA expression of Nrf2 target genes, NQO1 and HO-1 showed a slight upregulation after NaF exposure. Indeed, the protein expression of these two genes manifested the same variation tendency with nuclear Nrf2 protein, which confirmed the activation of the Nrf2 pathway and its role in protection against NaF-induced testicular oxidation. In comparison, NAC administration led to promotion of Nrf2 protein in its nuclear form along with a partly reversal of low level in cytosol induced by NaF, indicating that NAC treatment was effective to sequester allocate testicular Nrf2 into the nucleus in response to NaF

and resulted in reinforcing nuclear transport. Although cytosolic protein expression of Nrf2 was not fully restored along with dramatic induction in nuclei, some pro-oxidant and anti-oxidant biomarker, including MDA and CAT, showed trends toward partial reduction or elevation [14]. Additionally, NAC also promoted the content of NQO1, but not HO-1 in testicular tissue of NaF-exposed rat. In addition to antioxidant potential, an increased expression of HO-1 has been complicated in testicular apoptosis initiated by environmental insult such as stress and Cd [20]. Herein, NAC inhibited the NaF-induced upregulation of testicular HO-1, which paralleled with the decline in cells apoptotic changes. Thus, our current study does not exclude the role of HO-1 in NaF-mediated testicular apoptosis. Collectively, the present results indicated that systemic administration of NAC was able to protect and even rescue NaF-induced apoptosis, an effect that could be explained, at least in part, by Nrf2-mediated antioxidant protection.

5. Conclusions

Based on the present and recent results, we propose a modulatory mechanism by which NAC attenuate testicular lesion in our NaF-intoxicated rat model (Fig. 6). Sub-chronic NaF exposure causes spermatogenic dysfunction. Both ER stress and oxidative stress mediate the disorder of spermatogenesis. The elevated IRE1α phosphorylation as well as XBP1 splicing caused by ER stress may induce JNK-mediated apoptosis. Simultaneously, the alteration of the Nrf2 antioxidant pathway along with enhanced DNA damage during testicular oxidation also contributes to apoptosis. These sequential responses may elicit testicular structure loss as well as sex hormonal disruption, and subsequent infertility. NAC pretreatment may normalize the processes of spermatogenesis by attenuating cellular endoplasmic reticulum and oxidative stress-mediated apoptotic death, resulting in the inhibition of reproductive abnormalities. Thus, the protective effects of NAC not only reflect its confirmed antioxidant effects, but also are caused by regulation of ER stress molecules and prevention of related apoptosis development in cases of fluoride exposure.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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