Mutation Research 792 (2015) 35–45

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Ameliorative effects of *N*-acetylcysteine on fluoride-induced oxidative stress and DNA damage in male rats' testis

Demin Feng[a], Hui Huang[a], Yang Yang[a], Ting Yan[a], Yuefei Jin[b], Xuemin Cheng[a], Liuxin Cui[a,*]

[a] Department of Environmental Health, School of Public Health, Zhengzhou University, Zhengzhou, Henan, People's Republic of China
[b] Department of Occupational Health, School of Public Health, Zhengzhou University, Zhengzhou, Henan, People's Republic of China

ARTICLE INFO

Article history:
Received 13 May 2015
Received in revised form
11 September 2015
Accepted 15 September 2015
Available online 25 September 2015

Keywords:
Sodium fluoride
Oxidative stress
DNA damage
N-acetylcysteine
Spermatogenesis

ABSTRACT

This study was to elucidate DNA damage in rats treated with sodium fluoride (NaF) by performing 8-Hydroxy-2-deoxyguanosine (8-OHdG) immunohistochemical staining assays on seminiferous tubules of rats' testis, and also to evaluate the protective effects of *N*-acetylcysteine (NAC) on spermatogenesis. Male Sprague Dawley (SD) rats were exposed to a single dose of NaF (25 mg/kg/day) with or without NAC (150 mg/kg/day) for 7 weeks (7W) by gastric gavage. Testicular fluorine content was detected by fluorine ion selective electrode method. Oxidative damage to DNA was evaluated by measuring the increase in 8-OHdG formation in testicular tissue through immunohistochemical staining assays and also the effects of NAC pretreatment. The biochemical indicators about oxidative stress were detected by colorimetric assays, sperm parameters and the morphological changes of testis were studied. NaF significantly increased serum levels of oxidative stress, markedly elevated testicular fluorine and 8-OHdG expression levels as well as the rate of sperm aberration compared to saline group. Testosterone in serum, sperm counts and the mobility of sperm were lower than those of the rats in control group. The pathological morphological changes in testicular seminiferous tubule were also obvious in the rats with NaF treatment. Pretreatment with NAC did not reduce the contents of fluoride content in testis, but significantly reduced 8-OHdG formation and lipid peroxidation. This study suggests that NAC may have certain antagonism on the reproductive damage induced by NaF.

1. Introduction

As we all know, excessive fluorine could induce skeletal toxicity, such as dental fluorosis and skeletal fluorosis [1]. Besides, excessive fluoride consumption could cause damage to non-skeletal tissue, such as brain, kidney, liver and testis, especially to male reproductive system. Animal experimental studies reported fluoride exerted an adverse effect on sperm quantity and quality, sperm chemotaxis [2], serum testosterone, follicle stimulating hormone and luteinizing hormone levels, spermatogenesis and steroidogenesis [3,4], testicular cell cycle [5]. Moreover, high doses of fluoride could result in apoptosis of Leydig cells [6], and vacuolar dystrophy in seminal cells and necrosis in mice [7]. Besides, excessive fluoride could induce disruption of reproductive hormones and low birth rates in humans [8,9]. Due to exposure to environmental pollutants and physical stress, the rate of male infertility has been increasing, which has become a very serious social problem [10,11]. Therefore, it is vitally significant to further investigate the molecular mechanism of reproductive toxicity induced by fluorosis and explore the proper antidote.

Currently, oxidative stress induced by NaF is still recognized to be associated with fluorosis formation. Researches suggested that fluorosis may cause excessive oxidative stress which impaired the balance of antioxidant system, hence induced oxidative damage to rat testes [12,13]. Recent study [14] showed that reactive oxygen species (ROS) caused the oxidation of DNA, proteins, and lipids, resulting in the formation of 8-OHdG and MDA. 8-OHdG is a product of oxidative damage following specific enzymatic cleavage after 8-hydroxylation of the guanine base, which has a biological role, in that it is capable of inducing G:C to T:A conversion during DNA replication. 8-OHdG could be used as a typical biomarker of oxidative DNA damage produced by ROS [15]. Some researches discovered that 8-OHdG was a biomarker of oxidative damage in benzopyrene carcinogenesis [16], diabetes mellitus [17], ageing [18]. Besides recent studies showed that 8-OHdG was a novel biomarker of inflammatory activity in patients with car-

* Corresponding author.
E-mail address: m13703980133@163.com (L. Cui).

http://dx.doi.org/10.1016/j.mrgentox.2015.09.004
1383-5718/

Table 1
Body weight and major organs coefficient after 7 weeks exposure ($n = 15$; mean ± SD).

Groups	Body weight (g)	Testis weight (g)	Epididymis weight (g)	Organs coefficient	
				Testis/body weight (%)	Epididymis/body weight (%)
Control	475.15 ± 34.77	1.71 ± 0.17	0.63 ± 0.05	0.36 ± 0.04	0.13 ± 0.01
NAC	472.23 ± 21.32	1.72 ± 0.16	0.61 ± 0.02	0.36 ± 0.03	0.13 ± 0.01
NaF	442.18 ± 31.06*	1.56 ± 0.12*	0.53 ± 0.07*	0.36 ± 0.03	0.13 ± 0.02
NaF+ NAC	459.54 ± 34.51	1.69 ± 0.18	0.59 ± 0.06	0.36 ± 0.05	0.13 ± 0.02

* $P < 0.05$, compared with the control group.

diac sarcoidosis [19]. Animal and human studies revealed that the prevalence of 8-OHdG-immunostained germ cells was positively correlated with clinical grades of varicocele [20,21]. Oxidative damage in testicular DNA is associated with poor semen quality, reduced fertility and increased risk of stillbirths and birth defects. Therefore, the study was conducted to evaluate the genetic toxic effect of NaF on male reproductive system by immunohistochemistry for 8-OHdG expression in testicular tissue, and speculate whether NaF could cause DNA damage.

N-Acetylcysteine (NAC) is widely used as a mucolytic agent and an antidote for acetaminophen overdose in the clinic. Besides, NAC could be involved in the regulation of oxidative stress related gene expression, hence it had an antagonistic effect on oxidative damage [22]. It was reported that NAC may prevent the exogenous toxicant-induced male reproductive injury through antagonizing oxidative damage [23,24]. However, the related domestic and foreign literatures about the regulating role of NAC in the NaF-induced male reproductive toxicity were few, and the specific mechanism of action was not clear yet.

The purpose of this study was to explore whether NaF induce oxidative stress and DNA damage in rats' testis, and examine the protective effects of antioxidant on the development of testicular dysfunction under such conditions. Through establishing animal model of sub-chronic fluorosis and using NAC as intervention agent, this research provided theoretical basis for revealing the pathogenetic mechanism of male reproductive toxicity induced by NaF and exploring the possible antidotes relieving fluorosis.

2. Materials and methods

2.1. Materials and chemicals

NaF and NAC were purchased from Sigma–Aldrich (St. Louis, MO, USA). Oxidative stress related biochemical indicator detection kits for SOD, CAT and MDA were provided by the Nanjing Jianchen Institute of Biotechnology (Nanjing, China). Serum testosterone ELISA test kit was purchased from ShangHai HengYuan Biological Technology Co., Ltd. (Shanghai, China). Rabbit anti-8-OHdG antibody was purchased from Bioss (Beijing, China). Computer-aided Semen Analysis System (CASA) was purchased from Hamilton Thorne Biosciences (Cummings Center, USA). PF—type 1 fluorine ion selective electrode was purchased from Shanghai Reunion Scientific Instrument Co., LTD (Shanghai, China). ELx808 Absorbance Reader was obtained from BioTek Instruments, Inc. (USA).

2.2. Animals and treatment

Sixty SD male specific pathogen free (SPF) rats that were 28 days old, weighing 100–150 g, were obtained from the Experimental Animal Center of Henan province (Zhengzhou, China) [License No. SCXK (Yu) 20100002]. Animals were housed in sterilized plastic cages with corncob bedding in a controlled-environment animal room (temperature, 23 ± 1 °C; relative humidity, 50 ± 10%; photoperiod, 12 h light/dark cycle). Free access to distilled water and sterilized food was allowed at all the time. Animals were allowed to acclimatize for the inspection and quarantine for 7 days prior to treatment. All animal procedures were performed in compliance with the regulations and guidelines of the international ethics committee on animal welfare.

In this experiment, 60 rats were randomly divided into four different treatment groups (15 rats per group). 0.09% physiological saline was used as solvent to prepare 2.5 g/L NaF solution and 15 g/L NAC solution, respectively. 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 7W. Animals in group 1(saline, saline control group) were administrated orally with saline(10 ml/kg) to determine the basal values for biochemical comparisons; the group 2 (NaF treated group) in which rats received a single dose of 2.5 g/L NaF, corresponding to 25 mg/kg/d, in order to induce sub-chronic fluorosis features; A single dose of 15 g/L NAC solution, corresponding to 150 mg/kg/d, was given to the third group (NAC treated group), in order to act as biochemical comparisons with the fourth group; the fourth group (the protective group, NAC + NaF) which received 150 mg/kg/day of NAC 0.5 h prior to oral NaF exposure (25 mg/kg/d). Since one spermatogenic cycle in the rat is 50 ± 2 days [25], in this way, we ensured that the fluoride-exposure duration contained at least a complete period of spermatogenesis in the rat. The symptom and mortality were observed and recorded carefully throughout the experimental schedule. Final body weights of the animals were recorded on the day of sacrifice after being anesthetized by chloral hydrate. Blood samples were obtained from abdominal aorta using vacuum tube after light anesthesia. Serum samples were separated by centrifugation and stored at −20 °C until determination of plasma testosterone. Testes and epididymides were dissected out and organ weights were measured by electronic balance. Sperms were quickly flushed out from the caudal part of both epididymides of each rat with 10 ml suspension medium and the resulting sperm suspension was used for determination of the quantity, the activity and malformation rate of sperm. Testes were frozen at −80°C.

2.3. Body weight, organ coefficient of testis and epididymis analysis

At the end of 7-week exposure, the body weights of all the rats were measured, the weights of testis and epididymis were measured when the rats were sacrificed and the organ coefficients of testis and epididymis were calculated according to the equation: organ coefficient = wet weight of organ (g)/body weight (g) × 100%.

2.4. Sperm quality assessment

Sperm from the cauda epididymis and vas deferens were allowed to disperse into HTF medium at 37 °C. Sperm were diluted to 5×10^6/ml in phosphate-buffered saline (PBS) and incubated at 37 °C, 5% CO_2 for further experiments. Direct smear of sperm suspension was conducted with computer-aided semen analysis system (CASA) to detect sperm count, sperm motility ratio. For sperm morphology, a drop of sperm suspension in medium was smeared on a slide, air dried, and fixed with methanol. After fix-

Table 2
Sperm quality after 7 weeks exposure ($n = 15$, mean ± SD).

Groups	Sperm count (10^6)/ml	Sperm motility (%)	Sperm malformation ratio (%)
Control	10.69 ± 2.40	66.67 ± 4.07	2.14 ± 0.65
NAC	10.71 ± 2.58	70.45 ± 3.50	2.32 ± 1.24
NaF	5.71 ± 1.47**	33.33 ± 3.42**	6.68 ± 1.27**
NaF+ NAC	8.42 ± 2.13*	50.62 ± 3.14*	5.37 ± 1.35*

* $P < 0.05$, compared with the control group.
** $P < 0.01$, compared with the control group.

ation, the sample was stained with 1% Eosin-Y solution for 1 h. The morphological change of sperm was observed with a microscope. Sperms differing from normal structure can be considered as abnormal sperms. Two hundred sperms from each sample were examined to assess the percentage of morphologically abnormal sperms and the percent of abnormality was calculated according to the equation: sperm abnormality rate = abnormal sperm count/total sperm count × 100%.

2.5. Testis fluorine content analysis

The testes were removed from the freezer (−80 °C) and thawed. Preheat oven to 60 °C and bake testes for 24 h in the oven until testes were dried to constant weight, then measure the weight. The testicular tissue was cut up with ophthalmic scissors and homogenized with grinders. Then it was digested with 1 mol/l HCL solution and analyzed for the fluoride elemental content by fluoride selection electrode.

2.6. Determination of testosterone levels

The serum levels of testosterone were assayed by the method of double- antibody sandwich ELISA using rat-specific ELISA Kits (HengYuan Biotech Co., Ltd, ShangHai, China). All the operations followed the protocols provided in the kits. The sensitivity of the assays for testosterone was 0.5 nmol/L and the detectable range was 5 nmol/L–160 nmol/L. All samples were measured in duplicate and run in a single assay, which had an inter-assay coefficient of variation (CV) of 9.0% and an intra-assay CV of 11.0%.

2.7. Biochemical analysis of serum

The CAT activity determination method was based on the determination of the rate constant of the H_2O_2 decomposition rate at 405 nm. The principle of the SOD activity determination method is based on the inhibition of nitroblue tetrazolium reduction by the xanthine–xanthine oxidase system as a superoxide radical generator. One unit of SOD was defined as the enzyme activity causing 50% inhibition of the nitroblue tetrazolium reduction rate. The results of CAT and SOD activity are expressed as units per milliliter. The MDA levels in serum were analyzed by a method based on the reaction with thiobarbituric acid at 90–100 °C. In the thiobarbituric acid test reaction, MDA or MDA-like substances and thiobarbituric acid react together to produce a pink pigment with an absorption maximum of 532 nm. Serum MDA level was expressed as nanomole per milliliter.

2.8. Observation of testicular morphological structure

First, those chosen testicular tissue were routinely fixed by 4% paraformaldehyde and then embedded in paraffin. The paraffin-embedded samples were sectioned at a thickness of 4 μm. Some sections were stained with hematoxylin and eosin for observing testicular morphological structure, and the other sections were used for immunostaining of 8-OHdG. Those sections which were stained with hematoxylin and eosin were used to observe the condition of the testicle histomorphology under 200 and 400 magnified visual fields with ordinary light microscope.

2.9. Determination of 8-OHdG content in rat testis by immunohistochemistry

Some sections were used for immunostaining of 8-OHdG. The expression degree of 8-OHdG and its localization in the testes were detected by immunohistochemistry using anti-8-OHdG antibody (rabbit polyclonal IgG, affinity purified by protein A; reacts with: 8-OHdG; immunogen: KLH conjugated to 8-OHdG; predicted mocular weight: 0.283 kDa). After antigen repairing, preventing endogenous peroxidase and blocking with normal goat serum, the tissue sections were incubated with anti-8-OHdG antibody (dilution, 1:100) overnight at 4 °C, Following incubation in the secondary antibody at a concentration of 1:100 at 37 °C for 30 min. The reacting results were colored with diaminobenzidine (DAB) as chromogenic reagent and then counterstained the cells by hematoxylin. Image-manipulation system IPP6.0 was used to analyze the mean optic density of 8-OHdG positive cells with strong or moderate brown staining through selecting the mesurements (Area, Average Optical Density, Integrated Optical Density) and setting corresponding parameters (H: 0–30; S: 0–255; I: 0–230) in the window of selecting colors, in this way could the brown–yellow staining solely be analyzed by blocking out other colors during the quantification. In regard to the immunohistochemical control, we could appeal to the document [14]. Two sections were chosen randomly from each rat, 5 randomly selected high power fields in each slice was observed in light microscope, which was to investigate the distribution and expression of immunoreactive 8-OHdG in the rat testis.

2.10. Statistical analysis

All parameters were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA). Differences between groups were evaluated by one-way ANOVA followed by Students–Newman–Keuls (SNK)-q test using SPSS 21.0 software (USA). The correlation between the two variables was analyzed by line correlation analysis. A two-tailed P value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of NAC on body weight, major organ coefficient of NaF-treated rats

After 7 weeks exposure, NaF exposure induced a significant decrease in body weight, testis and epididymis weight compared with the control group ($P < 0.05$) (Table 1). Through NAC treatment, the body weight, testis and epididymis weight increased but not with significant difference compared with the NaF treated group (Table 1). Furthermore, no significant difference in organ coefficient of testis and epididymis was observed between four groups ($P > 0.05$).

3.2. Effects of NAC on sperm quality of NaF-treated rats

Effects of NAC on sperm count, sperm motility and sperm malformation ratio in experimental NaF-treated rats was shown in Table 2. In the sperm quality examination, compared with the control group, a significant decrease in sperm count and sperm motility

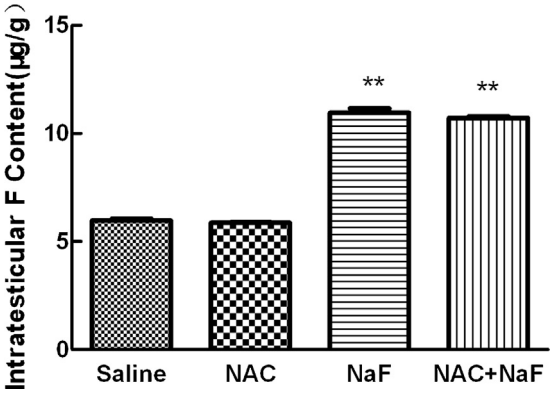

Fig. 1. Levels of fluoride in the rat testis from each group. Notes: F, fluorine element. Levels of fluoride were expressed as μg of fluorine per gram of wet tissue. Values represented mean ± SD. The symbol (*) upon the bars indicated that the mean fluorine level in fluoride exposure group was significantly greater than control group. Significant level was at 0.05. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group. Number of rats in each group was 15.

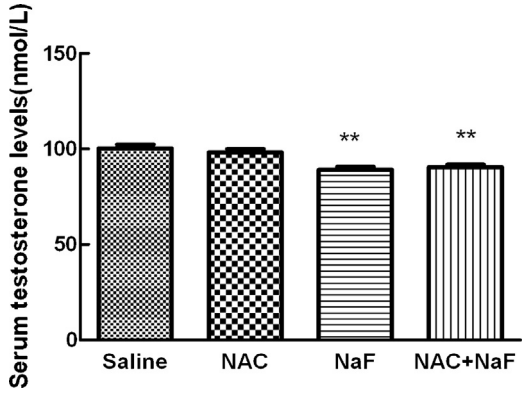

Fig. 2. Effects of NAC on serum testosterone level of NaF-treated rats. The unit of serum testosterone level was expressed by nmol/L. Serum testosterone level in NAC treated group was comparable to that of the control group. NaF significantly lowered serum testosterone levels compared with the control group. Serum testosterone level in NaF+ NAC group was similar to NaF group ($P > 0.55$), but lower than the control group significantly. $^{*}P < 0.05$, $^{**}P < 0.01$, comparing to control. Data were presented as mean ± SD.

from NaF and NaF+ NAC group was observed, the sperm malformation ratio showed significant increase; No statistical difference was observed between NAC treatment group and control group. Meanwhile, compared with NaF treated group, the sperm count and sperm quality showed no significant difference in the NaF+ NAC group, but the result showed improving trend in the sperm count and sperm quality. These results implied that NaF affected testicular spermatogenic function and decreased sperm count and sperm quality, and NAC could improve sperm quality parameters.

3.3. Fluoride content in testis

Fluoride content in rat testis of each group was shown in Fig. 1. Testicular fluoride content in the NaF treated group and NAC+ NaF group was significantly increased compared with the control group ($P < 0.01$). Meanwhile, fluoride content in NAC group was similar to those of the control group ($P > 0.57$). Fluoride content in NAC+ NaF group was lower slightly than NaF group, the difference was not significant ($P > 0.15$). This obvious accumulation of fluoride suggested that fluoride enter the testicular tissue, and animal models of sub-chronic fluorosis in rats were successfully established.

3.4. Effects of NAC on serum testosterone level of NaF-treated rats

As seen in Fig. 2, administration of NaF significantly lowered serum testosterone levels compared with the control group ($P < 0.01$). Through NAC pretreatment, serum testosterone levels slightly increased relative to the NaF treated group with non-significant difference ($P > 0.55$), but still lower than control group significantly ($P < 0.01$).

3.5. Oxidative stress of serum

As we all know, oxidative stress occurs in a cellular system when the production of free radical moieties exceeds the antioxidant capacity of that system. The common reactive oxygen species (ROS) including superoxide anion free radicals (O^{2-}), hydroxy free radical ($^{\bullet}OH$), lipid free radical, hydrogen peroxide (H_20_2), Singlet oxygen (O_2), ozone (O_3) and so on. MDA is the product of lipid oxidation induced by ROS. SOD and CAT are enzymes to counteract oxidative stress. SOD could catalyze O^{2-} and generate H_20_2. CAT could catalyze H_20_2 and generate H_20 and O_2. Excessive generation of ROS could elevate MDA content and expend SOD and CAT in vivo. Therefore, MDA, SOD and CAT could be used as parameters of assessing oxidative stress. Effects of NAC on serum MDA content and SOD, CAT activity in experimental NaF-treated rats were shown in Table 3. As was shown, compared with the control group, NAC could increase SOD, CAT activity and decrease MDA content significantly ($P < 0.05$), exposure of NaF could elicit elevation of MDA content and decrease of SOD, CAT activity significantly in serum ($P < 0.05$). Although, through NAC treatment, MDA content decreased significantly ($P < 0.05$), the activities of SOD and CAT were not significantly increased relative to the NaF treated group ($P > 0.15$). However, the above oxidative stress parameters in NAC+ NaF group did not show significant improvement compared to the control group ($P > 0.05$). These results indicated that oxidative stress occurred in blood exposed to fluorine for 7W and NAC had certain anti-oxidative stress effects.

Table 3
Serum MDA content (nmol/ml) and SOD, CAT activity (U/ml) in male rats from four groups ($n = 15$; mean ± SD).

Groups	MDA	SOD	CAT
Control	8.00 ± 0.93	19.60 ± 1.50	14.51 ± 1.30
NAC	5.33 ± 1.32**	22.60 ± 1.38*	17.96 ± 1.42*
NaF	10.67 ± 0.98**	16.87 ± 3.90*	11.86 ± 2.10*
NaF+ NAC	8.48 ± 1.31#	20.53 ± 1.90	12.72 ± 2.10

* $P < 0.05$, compared with the control group.
** $P < 0.01$, compared with the control group.
\# $P < 0.05$, the NaF+ NAC group versus the NaF treated group.

3.6. Histopathology evaluation of testes

Representative hematoxylin and eosin-stained light photo micrographs of seminiferous tubules in fluorosis rats were shown in Fig. 3. As was shown, the seminiferous tubules of the control group [Fig. 3(A)], was full in shape and tubule wall was smooth with non-crimp. The epithelial cells of seminiferous tubule were about 5 ~ 8 layers, consisted of spermatogenic cells and sertoli cells. The arrangement of basal epithelial cells was tight and orderly, spermatogonia with a large nucleus and deep stain were along with the periphery of the seminiferous tubules. Spermatogenic cells arranged in turn towards inside, a large number of mature sperms were in tubule space. The seminiferous tubules structure of NAC treatment group [Fig. 3(B)] was normal and similar to that of the control group, normal leydig cells could be seen in interstitial tissue [Fig. 3(B1), arrow]. The shape of seminiferous tubules wall in NaF treatment group was irregular and collapsed with interstitial thickening [Fig. 3(C1), arrow]. Serious degeneration of seminiferous

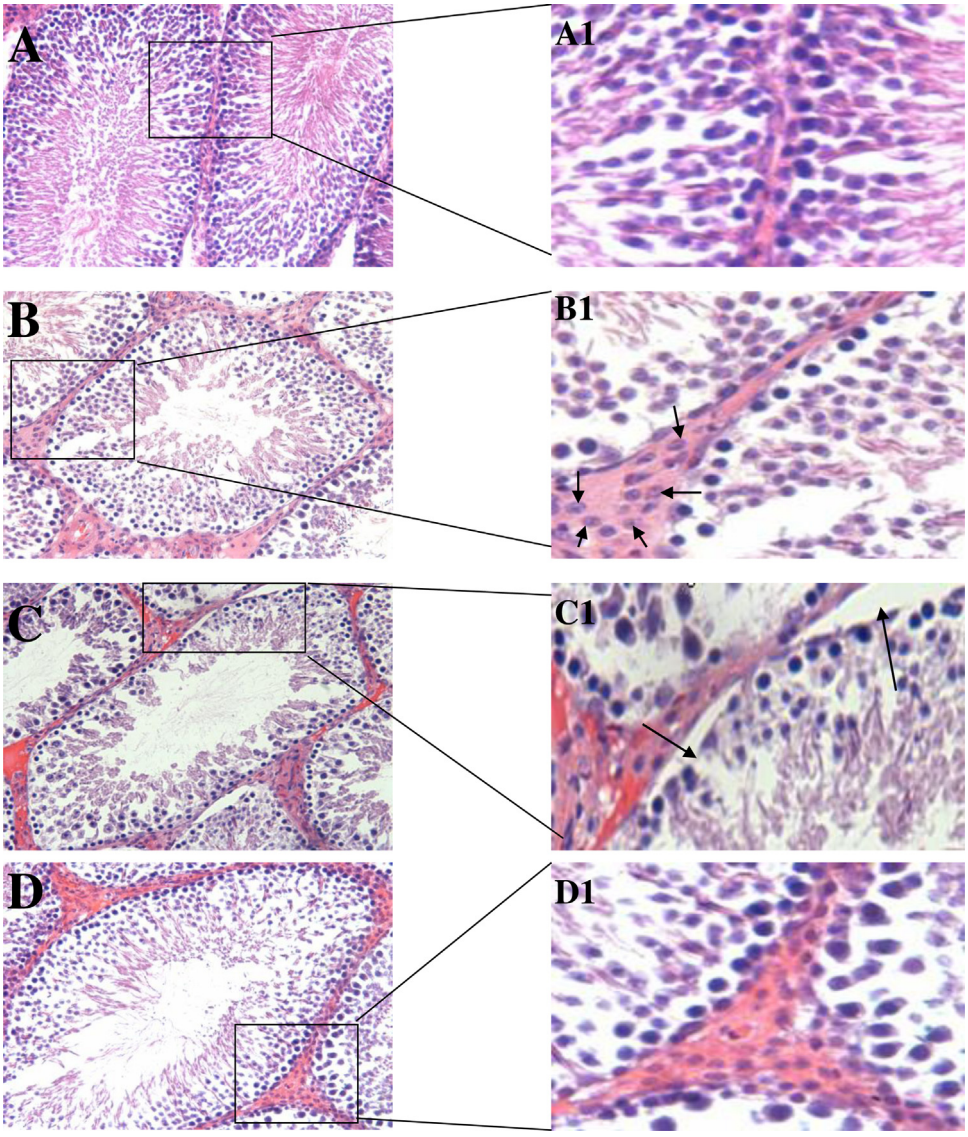

Fig. 3. Representative hematoxylin and eosin-stained light photo micrographs of seminiferous tubules in the male rats from the control (A), NAC treatment (B), NaF treatment (C) and NAC+ NaF (D) groups. Right Fig. (A1, B1, C1 and D1) is an amplified view of Fig. in the black box. All figures have been magnified ×200.

epithelium and the depletion of sperm cells were observed. Cell gap grew bigger than control, the layers of cells significantly reduced, almost 2 ~ 3 layers. Spermatogonia aligned loosely and disorderly, counts of mature sperm in tube decreased [Fig. 3(C)]. The seminiferous tubules structure of NAC plus NaF group was fairly well. The layers of cells were about 4 ~ 5 layers and cells arranged well. However, sperm counts were lower than control group [Fig. 3(D)].

3.7. *The localization and expression levels of 8-OHdG in rat testis by immunohistochemistry*

The cross section of a testis contains many different seminiferous tubules in different stages (stages I to XIV), As shown in Fig. 4, saline control group was taken as an example, we classified the seminiferous tubules of the mature rat testes in saline control group into five groups(stages I–III, IV–V, VII–VIII, X–XII, XIII–XIV) according to the method of Dym & Clermont (1970). It showed that, At stage VII–VIII seminiferous tubules stained with hematoxylin and eosin in each treatment group (Fig. 5a–d), spermatogenic cells arranged regularly and its morphologic character was clear (elongating spermatids in the basilar part of seminiferous epithelium were moved close to tubule space, cytoplasm was stripped away from the spermatids in elongating process and thus hyperchromatic residual plasmids were formed near the tubule space) [26]. Consequently, we sorted out the high power fields of stage VII-VIII seminiferous tubule from seminiferous tubules immunostained with anti-8-OHdG antibody in each treatment group [Fig. 5(A–D)] and analyzed the localization of 8-OHdG. 8-OHdG is an important biomarker for assessing DNA damage, first, we investigated the expression levels and localization of 8-OHdG in the testis specimens by means of immunohistochemistry. The visible immune staining intensity of 8-OHdG in the control group and NAC treated group [Fig. 5(A,B)] was both lower than NaF treated group and NaF+ NAC group [Fig. 5(C,D)]. As was shown, in stage VII–VIII seminiferous tubules, the late pachytene spermatocytes in control group and NAC treatment group [Fig. 5(A1,B1), arrow] showed no positive reaction, so did the spermatids [Fig. 5(A1,B1), arrowhead]. The late pachytene spermatocytes [Fig. 5(C1), arrow] and the spermatids [Fig. 5(C1), arrowhead] in NaF treatment group both showed a strong positive reaction. Meanwhile, the late pachytene spermatocytes [Fig. 5(D1), arrow] in NaF+ NAC group showed a positive but slightly weaker reaction than the late pachytene spermato-

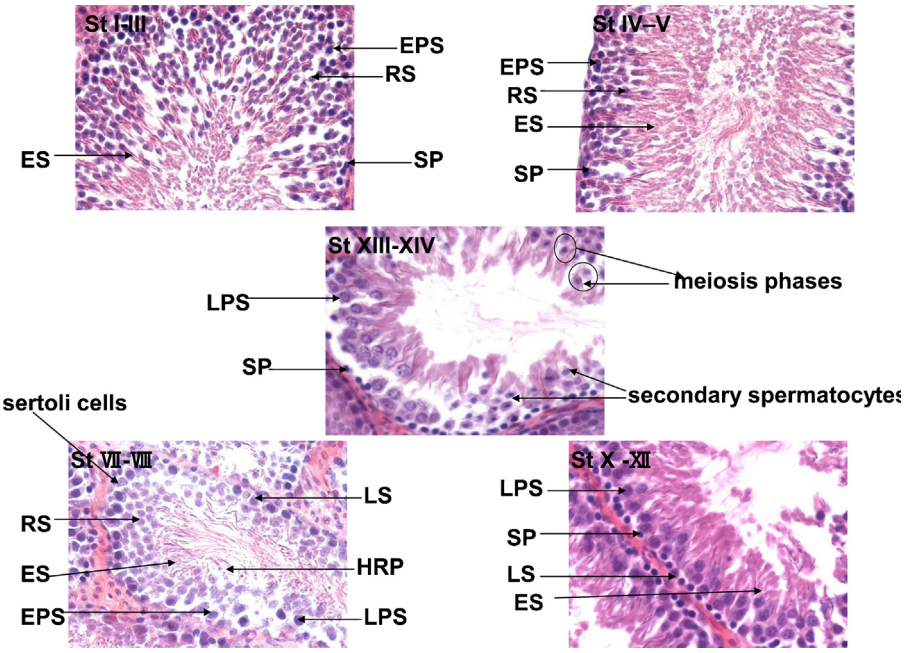

Fig. 4. Representative light photo micrographs of seminiferous tubule sections at the 14 stages of the rat seminiferous epithelium in the saline control group. Images were from testis tissue fixed by 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. The seminiferous tubules (stages I–XIV) were classified into five categories (stages I–III, IV–V, VII–VIII, X–XII, XIII–XIV) according to the method of Dym & Clermont (1970). At stages I–III, the seminiferous epithelium consisted of spermatogonia (SP), early pachytene spermatocytes (EPS), round spermatids (RS) and elongating spermatids (ES), besides, ES were dispersedly localized in epithelial cells of each layer. At stages IV–V, the structure was similar to I–III except that ES became more and move towards the tubular space. At stages VII–VIII, some SP became leptotene spermatocytes (LS) and some EPS became late pachytene spermatocytes (LPS), ES in the basilar part of seminiferous epithelium were moved close to tubule space, cytoplasm was stripped away from the spermatids in elongating process and thus hyperchromatic residual plasmids (HRP) were formed near the tubule space. At stages X–XII, the cell size of LPS increased, RS could not be seen. At stages XIII–XIV, the primary spermatocytes were divided into two secondary spermatocytes, and meiosis phases could be seen. All figures have been magnified ×400.

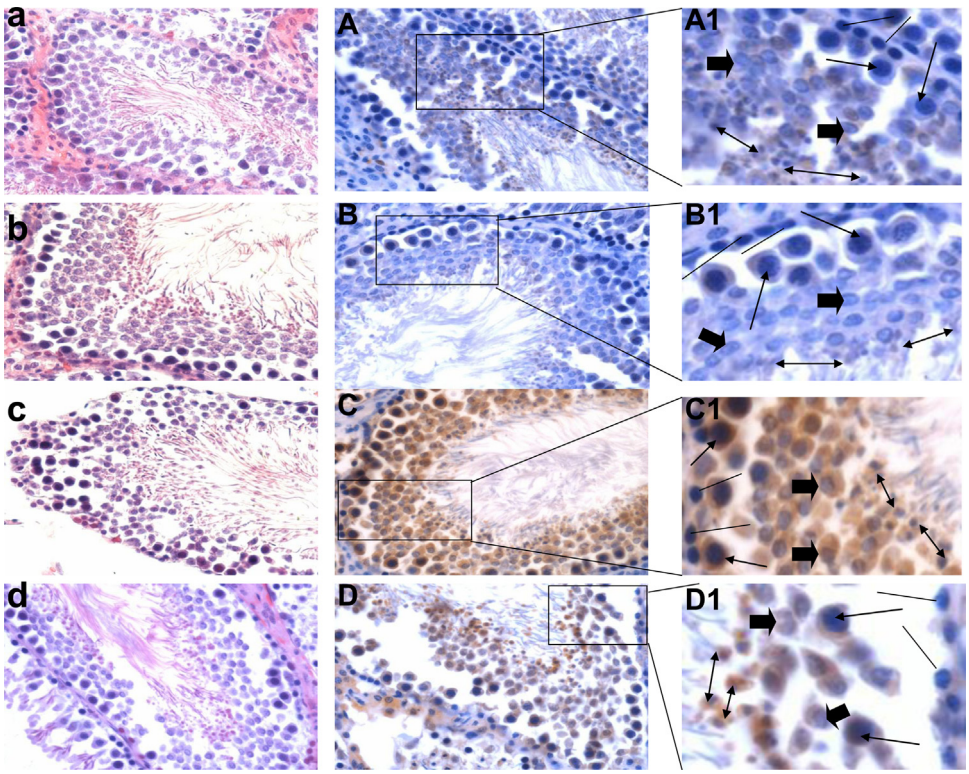

Fig. 5. Immunohistochemical localization of 8-OHdG in rat testes. Representative light photo micrographs of stages VII–VIII seminiferous tubule sections stained with hematoxylin and eosin in the control (a), NAC treatment (b), NaF treatment (c) and NAC+ NaF (d) groups. The high power fields of stage VII–VIII seminiferous tubule immunostained with anti-8-OHdG antibody in each treatment group [Fig. 5(A–D)]. Immunostaining sections in control group (A), NAC treatment group (B), NaF treatment group (C) and NAC+ NaF group (D) labeled using antibodies for 8-OHdG (brown–yellow). The merged image shows that 8-OHdG is located in cytoplasm. The pictures (A1, B1, C1 and D1) are an amplified view of Fig in the black box. (line: spermatogonia, arrow: late pachytene spermatocytes, arrowhead: spermatids, double arrow: hyperchromatic residual plasmids) All figures have been magnified ×400.

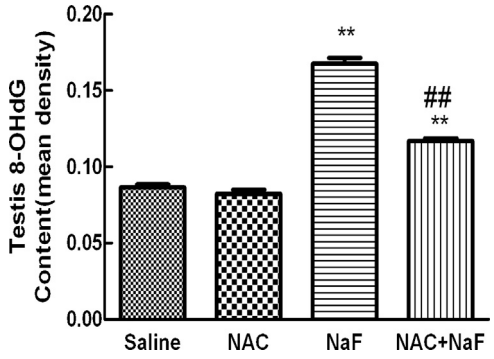

Fig. 6. Immunohistochemical expression level of 8-OHdG in rat testes. The mean optical density of immunostaining sections that express 8-OHdG could reflect 8-OHdG content in testes tissue. Values represented mean ± SD. Significant level was at 0.05. $^{**}P < 0.01$, compared with control group. $^{\#\#}P < 0.01$, compared between rats exposed to NaF and NaF+ NAC.

cytes in NaF treatment group [Fig. 5(C1), arrow], and the spermatids [Fig. 5(D1), arrowhead] showed a very weak or no positive reaction. The reactive substance mainly distributed in cytoplasm, the positive performance of the seminiferous tubules appeared as diffuse cytoplasmic brown–yellow or brown particles. Spermatogonia did not show a positive reaction in all treatment groups [Fig. 5(A1,B1,C1,D1, line)]. One other thing to note is that hyperchromatic residual plasmids in NaF treatment group and NaF+ NAC group [Fig. 5(C1,D1), double arrow] showed strong positive reaction. Though meanwhile, residual plasmids in control group and NAC treatment group [Fig. 5(A1,B1), double arrow] showed a slight positive reaction.

The expression levels of 8-OHdG was shown in Fig. 6. The testis 8-OHdG content was expressed as average optical density of high power fields with immunoreactive 8-OHdG. NaF exposure and NaF plus NAC exposure both induced a significant increase in the expression levels of 8-OHdG ($P < 0.01$) in rat testes compared with the control group, 8-OHdG content in NAC group was similar to control group ($P > 0.28$). Compared with NaF treatment group, the expression levels of 8-OHdG in NaF+ NAC group showed a significant decrease ($P < 0.01$). These results suggested NaF lead to increase of 8-OHdG in rat testes, and NAC administration alleviated the upregulation of 8-OHdG considerably.

3.8. Correlation analysis between oxidative stress and DNA damage

The expression levels of 8-OHdG in testis were positively correlated with serum MDA content ($P < 0.001$, $r = 0.707$) (Fig. 7). We found that the higher MDA content in serum was, the higher the expression of 8-OHdG in testis was. The testicular 8-OHdG content was inversely correlated with serum SOD activity ($P < 0.01$, $r = -0.619$) (Fig. 8) and CAT activity ($P < 0.01$, $r = -0.638$) (Fig. 9). Moreover, SOD, CAT and MDA are the typical parameters of oxidative stress. 8-OHdG is an important biomarker for assessing DNA damage. The data suggested that DNA damage in testis was positively correlated with oxidative stress induced by excessive fluoride.

4. Discussion

4.1. The major route of human exposure to fluoride in China

Fluoride widely distributes on earth as the essential microelement in human body, which plays an important role in regulating bone growth, development and remodeling. Excessive fluoride may enter to the residents who live in high fluorine environment through water, food and air, and drinking water intake is the main way, which induced dental fluorosis and crippling fluorosis as the main characteristics of endemic fluorosis diseases. In China, previously according to the epidemic range and characters, endemic fluorosis diseases could be divided into three types which consisted of drinking water type fluorosis wards, burning coal pollution-type fluorosis wards and drinking brick-tea type fluorosis wards. Among this drinking-water type endemic fluorosis was the most destructive endemic and has the largest number of sufferers in China, especially in north China. Although the defluoridation projects has got the remarkable effects on the improvement of endemic fluorosis diseases, however, some researches revealed that the atmospheric emissions of fluorine from coal combustion by power and heat generation in China were large in recent years [27,28], and the fluorine in the atmosphere transported to the surface water or groundwater by the rain. Because there was no piped water in some rural areas of China, the residents still drank shallow contaminated groundwater in which fluoride concentration was over the regulated criteria 1.0 mg/L. Excessive fluoride exposure could cause damage to non-skeletal tissue. Fluoride-induced male reproductive damage has been reported by multiple studies. The present study was aimed to explore the mechanism of male reproductive damage induced by NaF, and determine whether NAC could resist the reproductive damage induced by fluorosis. The gastrointestinal tract is a major route of human exposure to fluoride. Therefore, intragastric administration was chosen as the fluoride exposure mode in this study. Here were the explanations of the questions about the dose of fluoride exposure and NAC intervention, median lethal dose (LD50) of sodium fluoride through orally administration in rats is 172 mg/kg [29], the exposure dose in subchronic toxicity experiment is usually 1/20–1/5 of LD50, therefore, 25 mg/kg/day was chosen as fluoride exposure dose. According to the clinical administration dose of NAC as expectorants and relevant studies [30,31], we chose 150 mg/kg/day as NAC intervention dose.

4.2. Sub-chronic fluorosis caused oxidative stress and DNA damage

Some researches revealed that there was a close connection between fluoride-induced male testicular toxicity and oxidative stress [25,32]. In the present study, exposure of NaF elicited elevation of MDA content and decrease of SOD, CAT activity significantly in serum, which indicated fluoride exposure caused enhanced oxidative stress to the rats. This finding is consistent with those of previous studies [5,12,13]. Oxidative stress leading to the generation of ROS in metabolizing cells could attack DNA base guanine and induce the hydroxylation of guanine at C-8, finally forming 8-oxo-G lesions namely 8-OHdG. Under the normal body repairing mechanism, 8-hydroxyl-guanine-DNA glycosidase (hOGG1) could excise 8-OHdG from the double-stranded DNA and add normal guanine bases, the removed 8-OHdG was moved into the blood and excreted through urine [33]. Therefore, 8-OHdG in serum, urine or tissue was routinely used as a biomarker for DNA damage and carcinogenesis. Previous animal experiment data showed that NaF exhibited genotoxic activity in bone marrow, liver and kidney cells by the comet assay [34–36]. Nevertheless, some rodent studies suggested NaF did not result in systemic genotoxic effect in multiple organs, including liver and kidney [37], which had contradiction between these researches. In this study, fluoride exposure induced the high expression levels of 8-OHdG in rat testis, which implied fluoride exposure caused DNA damage in male reproductive system. This experiment result was consistent with previous studies [38,39], though the methods of detecting DNA damage were different. We could clearly observed the accurate position of DNA damage in the seminiferous tubules by the localization of 8-OHdG, spermatogonia

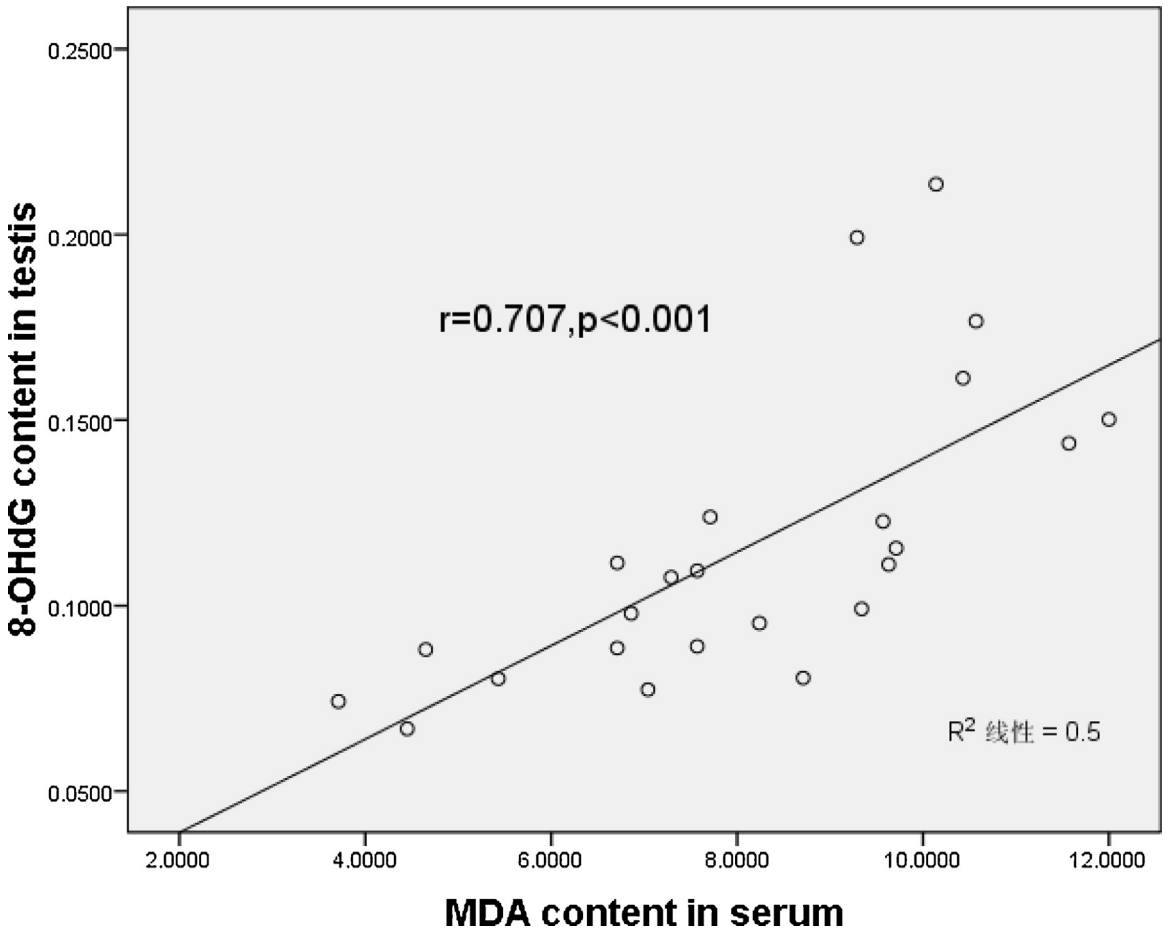

Fig. 7. Correlation between the expression levels of 8-OHdG in testis and serum MDA content. Testis 8-OHdG content and serum MDA content in 24 rats: $P < 0.001$, $r = 0.707$ by line correlation analysis.

did not show a positive reaction and the late pachytene spermatocytes showed a strong positive reaction, which was similar to the previous report [14]. The probable reason was that DNA was mainly replicated in spermatocytes not in spermatogonia and easily attacked by ROS in replicating process. Besides, present study suggested that DNA damage in testis was positively correlated with oxidative stress induced by excessive fluoride. Taking all those affecting factors into account, we could come to the conclusion that fluorosis caused DNA damage by the mechanism of oxidative stress in male reproductive system.

4.3. Excessive fluoride could induce male reproductive damage

In the research, we found that the animal models of sub-chronic fluorosis in rats were successfully established. After 7 weeks fluoride exposure, the rat sperm quality and serum testosterone level were decreased significantly, which were consistent with previous research results [3,5,40]. In addition, NaF exposure decreased the body weight, testis and epididymis wet weight significantly, but the organ coefficient of testis and epididymis did not show significant change. The experimental results were mostly consistent with previous existing literature which demonstrated that no significant difference in body weight and major organ coefficient was observed after 24 mg NaF/L treatment in drinking water for 8 weeks [2]. Whereas, the research results were completely at odds with another material which demonstrated that sodium fluoride treatment at 20 mg/kg/day for 29 days by oral gavage led to significant decrease in the relative wet weight of the testis, prostate, and seminal vesicle without alteration in the body weight gain [32]. The most probable cause of the differences was that fluoride exposure modes and times were different in our experiment from previous studies. In our study, qualitative analysis of testicular histology suggested that fluorosis induce shriveled seminiferous tubule and the diminution of mature sperm in the lumen of the seminiferous tubule. This was in conformity with Ghosh's report [32] which elucidated that fluoride treatment was associated with a reduction in mature sperm in the lumen of the seminiferous tubule, except the different results in Ghosh's report that fluoride treatment induced dilated seminiferous tubule. Besides, it was reported that spermatogonia and primary spermatocytes were normal in appearance but there was widespread degeneration of the other spermatogenic cells in the seminiferous epithelium in NaF-treated rats [12]. In addition to that, population-based data indicated that a fluoride exposure of 3–27 mg/day induces a subclinical reproductive effect [8]. However, some research suggested that exposure to NaF at the dose of 25 ppm does not adversely affect testis structure or spermatogenesis in the rat [41]. Our research results proved that oral exposure to 25 mg/kg/day NaF for 7 weeks could induce male reproductive damage.

4.4. NAC pretreatment reduced fluoride-induced oxidative stress and DNA damage

Based on the literature information, substances which antagonized the toxicity of fluoride were summarized as follows. Some researches showed that vitamins C and E [42], resveratrol [43], arjunolic acid (AA) [44] and epigallocatechin gallate (EGCG) [45] could protect endometrium, brain and hepatocytes against the

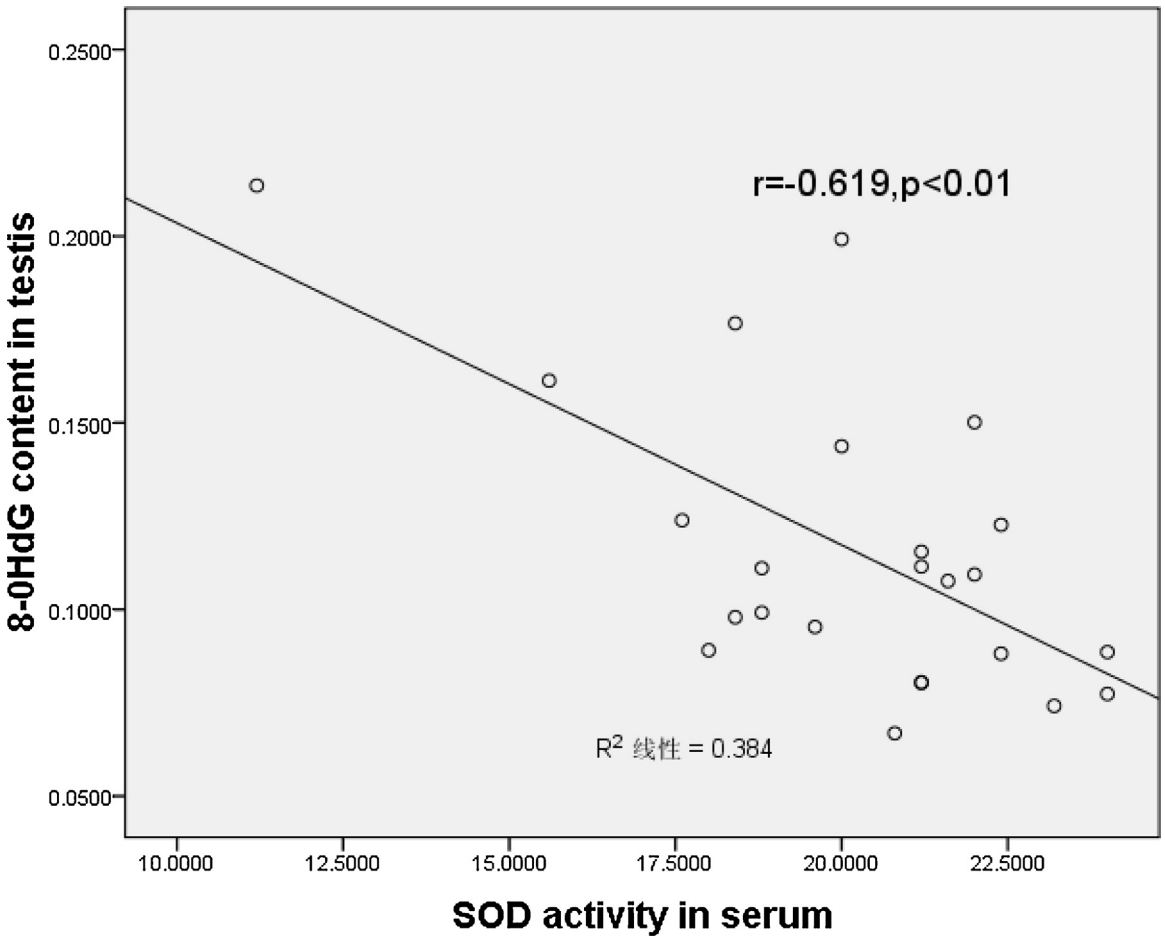

Fig. 8. Correlation between the expression levels of 8-OHdG in testis and serum SOD activity. Testis 8-OHdG content and serum SOD activity in 24 rats: $P < 0.01$, $r = -0.619$ by line correlation analysis.

fluoride-induced damage in rats. Moreover, it could be seen that these antidotes exhibited the role of resisting fluorosis mainly through the mechanism of inhibiting oxidative stress and DNA damage. Some animal researches showed that NAC could prevent postischaemic reperfusion injury in hepatic tissue, CCl4 -induced liver damage and brain intoxication induced by propionic acid (PPA) by reducing DNA damage and oxidative stress [46–48]. Moreover recent human studies implied that NAC could attenuate pesticide-induced DNA damage in human peripheral blood mononuclear cells, and age-related increases in genetic damage in human T lymphocytes, in vitro and ex vivo [49,50]. Thus, we hypothesized that NAC may act as an antidote of fluorosis through the same mechanism of reducing oxidative stress and DNA damage. To test this hypothesis, NAC was used as an intervention factor in our study and the results indicated that NAC played a critical role in the antagonism against oxidative stress induced by fluorosis, which was in agreement with previous studies that demonstrated NAC pre-treatment ameliorated fluoride-induced oxidative stress in hepatocytes [51]. In addition to animal tests, our study in vitro also showed that NAC restored the reduced cell viability and excessive oxidative stress in NaF-treated sertoli cells [52]. In our study, NAC could significantly decrease the expression levels of 8-OHdG in testicular tissue, which is a typical biomarker of the oxidative DNA damage. This suggested that our hypothesis is valid. Taken together, a conclusion that NAC resisted oxidative stress and genotoxicity induced by fluorosis in testes could be drawn, which was in consistent with previous research results that elucidated NAC pre-treatment significantly reduced fluoride-induced genotoxicity in mouse bone marrow cells [53].

4.5. NAC pretreatment protected male reproductive function against fluorosis

After using interference factors NAC, the representative parameters which indicated the male rat reproduction function improved a lot. Although some indicators did not show statistically significant improvements, there was still a tendency to improve in male reproductive function, including the increase of body weight, testis and epididymis weight, sperm count, sperm motility, serum testosterone levels and the decrease of sperm malformation. The treatment of NAC improved the structure of seminiferous tubule in testicular tissue compared with the NaF group, but did not improve that compared with the control group. We could see that NAC relieve reproductive damage induced by fluorosis to some extent but could not completely inhibit the toxic effects of fluoride.

In summary, this study partly demonstrates that NaF exposure induces male reproductive damage by the mechanism of oxidative stress and oxidative DNA damage. Furthermore, our study also implies that the intervention of NAC might be an essential role in inhibiting the male reproductive damage caused by sub-chronic fluorosis and NAC might act as an antidote against fluoride-induced male reproductive toxicity. However, as in response to fluorosis, except the mechanism of inhibiting oxidative stress and DNA damage, the specific signal pathways which are probably regulated by

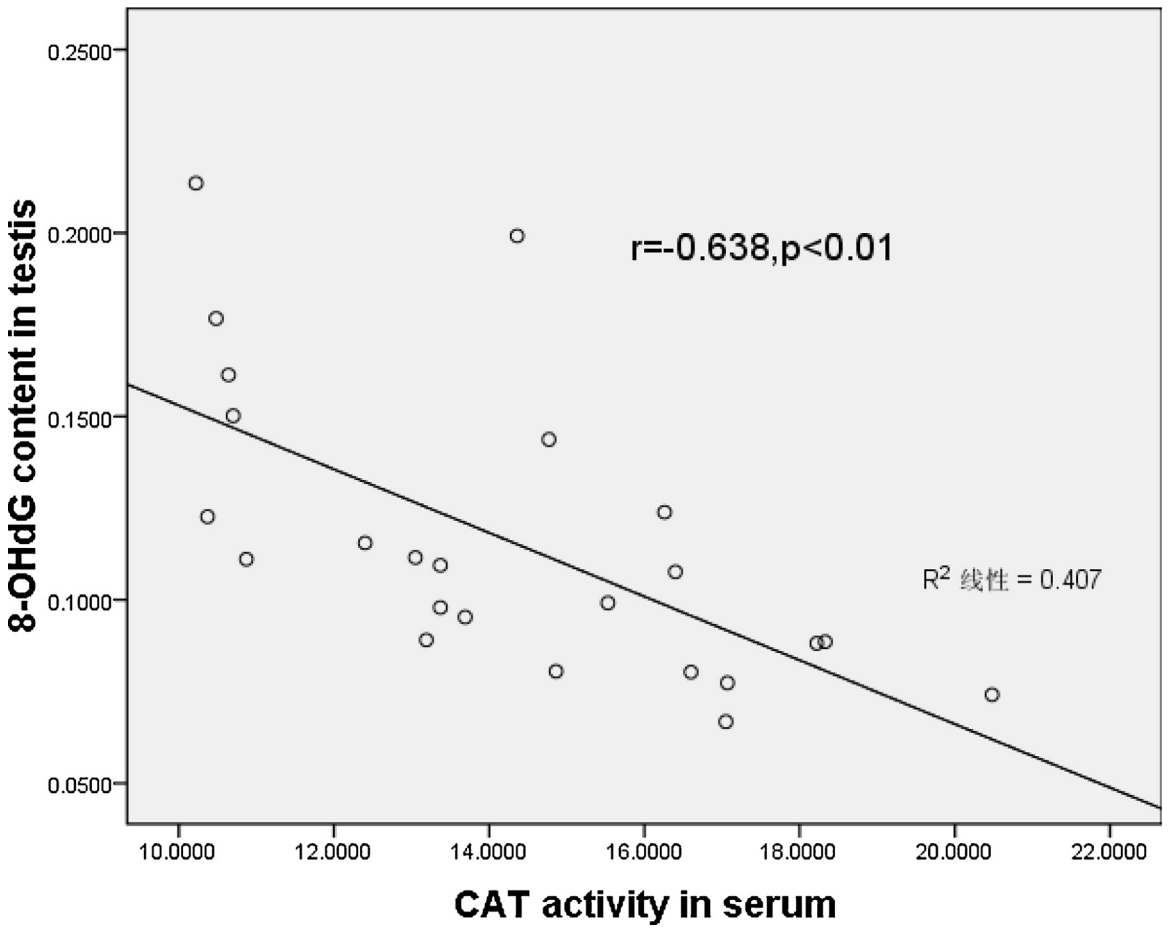

Fig. 9. Correlation between the expression levels of 8-OHdG in testis and serum CAT activity. Testis 8-OHdG content and serum CAT activity in 24 rats: $P < 0.01$, $r = -0.638$ by line correlation analysis.

NAC are still unknown. Our further study will explore the special signal transduction pathways.

Funding

This work was funded by a grant from scientific research key project of Education Department of Henan province, China (grant no.13A330735). The contents are solely the responsibility of the authors, and do not necessarily represent the official views of Education Department of Henan province.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors wish to acknowledge Professor Yue Ba, Jingyuan Zhu and Huizhen Zhang for critical discussion about this research.

References

[1] A.R. Gupta, S. Dey, M. Saini, D. Swarup, Toxic Effect of Fluoride on Biochemical Parameters and Collagen Metabolism in Osseous and Non-Osseous Tissues of Rats, in: Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 2015, pp. 1–6.
[2] Z. Lu, S. Wang, Z. Sun, R. Niu, J. Wang, In vivo influence of sodium fluoride on sperm chemotaxis in male mice, Arch. Toxicol. 88 (2014) 533–539.
[3] P.S. Reddy, T. Pushpalatha, P.S. Reddy, Suppression of male reproduction in rats after exposure to sodium fluoride during early stages of development, Naturwissenschaften 94 (2007) 607–611.
[4] T. Pushpalatha, M. Srinivas, P.S. Reddy, Exposure to high fluoride concentration in drinking water will affect spermatogenesis and steroidogenesis in male albino rats, Biometals 18 (2005) 207–212.
[5] C. Huang, R. Niu, J. Wang, Toxic effects of sodium fluoride on reproductive function in male mice, Fluoride 40 (2007) 162–168.
[6] G. hua Song, R.L. Wang, Z.Y. Chen, B. Zhang, H.L. Wang, M.L. Liu, J.P. Gao, X.Y. Yan, Toxic effects of sodium fluoride on cell proliferation and apoptosis of Leydig cells from young mice, J. Physiol. Biochem. 70 (2014) 761–768.
[7] P.N. Dimcevici, C. Bălălău, M. Bârcă, I. Ion, D. Baconi, C. Baston, P.V. Băran, Testicular histopathological changes following sodium fluoride administration in mice, Rom. J. Morphol. Embryol. 54 (2012) 1019–1024.
[8] D. Ortiz-Pérez, M. Rodríguez-Martínez, F. Martínez, V.H. c. Borja-Aburto, J. Castelo, J.I. Grimaldo, E. de la Cruz, L. Carrizales, F. Díaz-Barriga, Fluoride-induced disruption of reproductive hormones in men, Environ. Res. 93 (2003) 20–30.
[9] S.C. Freni, Exposure to high fluoride concentrations in drinking water is associated with decreased birth rates, J. Toxicol. Environ. Health Part A Curr. Issues 42 (1994) 109–121.
[10] J.D. Meeker, M.G. Rossano, B. Protas, M.P. Diamond, E. Puscheck, D. Daly, N. Paneth, J.J. Wirth, Cadmium, lead, and other metals in relation to semen quality: human evidence for molybdenum as a male reproductive toxicant, Environ. Health Perspect. 116 (2008) 1473–1479.
[11] F. Marchetti, B. Eskenazi, R.H. Weldon, G. Li, L. Zhang, S.M. Rappaport, T.E. Schmid, C. Xing, E. Kurtovich, A.J. Wyrobek, Occupational exposure to benzene and chromosomal structural aberrations in the sperm of Chinese men, Environ. Health Perspect. 120 (2012) 229.
[12] M. Öncü, A. Kocak, E. Karaoz, H. Darici, E. Savik, F. Gultekin, Effect of long-term fluoride exposure on lipid peroxidation and histology of testes in first-and second-generation rats, Biol. Trace Elem. Res. 118 (2007) 260–268.
[13] Z. Sun, R. Niu, B. Wang, Z. Jiao, J. Wang, J. Zhang, S. Wang, J. Wang, Fluoride-induced apoptosis and gene expression profiling in mice sperm in vivo, Arch. Toxicol. 85 (2011) 1441–1452.
[14] Y. Sakai, M. Aminaka, A. Takata, Y. Kudou, H. Yamauchi, Y. Aizawa, H. Sakagami, Oxidative stress in mature rat testis and its developmental changes, Dev. Growth Differ. 52 (2010) 657–663.
[15] D. Nakae, H. Akai, H. Kishida, O. Kusuoka, M. Tsutsumi, Y. Konishi, Age and organ dependent spontaneous generation of nuclear

8-hydroxydeoxyguanosine in male Fischer 344 rats, Lab. Invest. 80 (2000) 249–261.
[16] K.B. Kim, B.M. Lee, Oxidative stress to DNA, protein, and antioxidant enzymes (superoxide dismutase and catalase) in rats treated with benzo (a) pyrene, Cancer Lett. 113 (1997) 205–212.
[17] M. Ríos-Silva, X. Trujillo, B. Trujillo-Hernández, E. Sánchez-Pastor, Z. Urzúa, E. Mancilla, M. Huerta, Effect of chronic administration of forskolin on glycemia and oxidative stress in rats with and without experimental diabetes, Int. J. Med. Sci. 11 (2014) 448.
[18] A. Jo Watanabe, T. Ohse, H. Nishimatsu, M. Takahashi, Y. Ikeda, T. Wada, J. Shirakawa, R. Nagai, T. Miyata, T. Nagano, Glyoxalase I reduces glycative and oxidative stress and prevents age-related endothelial dysfunction through modulation of endothelial nitric oxide synthase phosphorylation, Aging cell 13 (2014) 519–528.
[19] S. Kobayashi, T. Myoren, S. Oda, M. Inari, H. Ishiguchi, W. Murakami, M. Fukuda, T. Tanaka, S. Okuda, T. Nao, Urinary 8-hydroxy-2′-deoxyguanosine as a novel biomarker of inflammatory activity in patients with cardiac sarcoidosis, Int. J. Cardiol. 190 (2015) 319–328.
[20] S.P. Yoon, Apurinic/apyrimidinic endonuclease immunoreactivity in germ cells of experimental varicocele-induced rat testes, Acta Histochem. 115 (2013) 887–892.
[21] T. Ishikawa, H. Fujioka, T. Ishimura, A. Takenaka, M. Fujisawa, Increased testicular 8-hydroxy-2′-deoxyguanosine in patients with varicocele, BJU Int. 100 (2007) 863–866.
[22] A. Meister, Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy, Pharmacol. Ther. 51 (1991) 155–194.
[23] Y. Suzuki, G. Ichihara, S.M. Sahabudeen, A. Kato, T. Yamaguchi, K. Imanaka-Yoshida, T. Yoshida, Y. Yamada, S. Ichihara, Rats with metabolic syndrome resist the protective effects of *N*-acetyl L-cystein against impaired spermatogenesis induced by high-phosphorus/zinc-free diet, Exp. Toxicol. Pathol. 65 (2013) 1173–1182.
[24] Y.L. Ji, H. Wang, C. Zhang, Y. Zhang, M. Zhao, Y.H. Chen, D.X. Xu, *N*-acetylcysteine protects against cadmium-induced germ cell apoptosis by inhibiting endoplasmic reticulum stress in testes, Asian J. Androl. 15 (2013) 290–296.
[25] J.A. Izquierdo-Vega, M. Sánchez-Gutiérrez, L.M. Del Razo, Decreased in vitro fertility in male rats exposed to fluoride-induced oxidative stress damage and mitochondrial transmembrane potential loss, Toxicol. Appl. Pharm. 230 (2008) 352–357.
[26] X. He, Y. Zhang, J. Zhang, Evaluation of seminiferous epithelium stage in rat testis by PAS staining, Chin. J.f Zool. 39 (2003) 50–52.
[27] J. Chen, G. Liu, Y. Kang, B. Wu, R. Sun, C. Zhou, D. Wu, Atmospheric emissions of F, As, Se, Hg, and Sb from coal-fired power and heat generation in China, Chemosphere 90 (2013) 1925–1932.
[28] K. Luo, L. Xu, R. Li, L. Xiang, Fluorine emission from combustion of steam coal of north China plate and northwest China, Chin. Sci. Bull. 47 (2002) 1346–1350.
[29] M. Koskinen-Kainulainen, H. Luoma, J. Tuomisto, The LD50, excretion and serum and bone levels of F after a high single F and F + Mg dose in rats with findings on cardiac Ca and Mg, Magnes. Trace Elem. 9 (1989) 15–27.
[30] B.H. Harvey, C. Joubert, J.L. du Preez, M. Berk, Effect of chronic *N*-acetyl cysteine administration on oxidative status in the presence and absence of induced oxidative stress in rat striatum, Neurochem. Res. 33 (2008) 508–517.
[31] G. Bazigos, D. Alexandropoulos, T. Kotsis, A. Pantopoulou, N. Tragotsalou, M. Kanakis, D. Perrea, V. Smyrniotis, A. Kondi-Pafiti, Study of the influence of anti-inflammatory and antioxidative substances on rat kidneys in ischaemia-reperfusion of the superior mesenteric artery, Hellenic J. Surg. 85 (2013) 261–268.
[32] D. Ghosh, S. Das, R. Maiti, D. Jana, U. Das, Testicular toxicity in sodium fluoride treated rats: association with oxidative stress, Reprod. Toxicol. 16 (2002) 385–390.
[33] S. Boiteux, J.P. Radicella, The human OGG1 gene: structure, functions, and its implication in the process of carcinogenesis, Arch. Biochem. Biophys. 377 (2000) 1–8.
[34] S. Podder, A. Chattopadhyay, S. Bhattacharya, Reduction in fluoride-induced genotoxicity in mouse bone marrow cells after substituting high fluoride-containing water with safe drinking water, J. Appl. Toxicol. 31 (2011) 703–705.
[35] G.H. Song, F.B. Huang, J.P. Gao, M.L. Liu, W.B. Pang, W. bin Li, X.Y. Yan, M.J. Huo, X. Yang, Effects of fluoride on DNA damage and caspase-mediated apoptosis in the liver of rats, Biol. Trace. Elem. Res. (2015) 1–10.
[36] G.H. Song, J.P. Gao, C.F. Wang, C.Y. Chen, X.Y. Yan, M. Guo, Y. Wang, F.B. Huang, Sodium fluoride induces apoptosis in the kidney of rats through caspase-mediated pathways and DNA damage, J. Physiol. Biochem. 70 (2014) 857–868.
[37] A. de Lima Leite, J.F.S. Junior, F.M. Levy, A.G. Maria, M. da Silva Fernandes, D.M.F. Salvadori, D.A. Ribeiro, M.A.R. Buzalaf, Absence of DNA damage in multiple organs (blood liver, kidney, thyroid gland and urinary bladder) after acute fluoride exposure in rats, Hum. Exp. Toxicol. 26 (2007) 435–440.
[38] J. Manivannan, S. Sinha, M. Ghosh, A. Mukherjee, Evaluation of multi-endpoint assay to detect genotoxicity and oxidative stress in mice exposed to sodium fluoride, Mutat. Res./Genet. Toxicol. Environ. Mutagen. 751 (2013) 59–65.
[39] Z. Sun, R. Niu, B. Wang, J. Wang, Altered sperm chromatin structure in mice exposed to sodium fluoride through drinking water, Environ. Toxicol. 29 (2014) 690–696.
[40] J. Zhang, C. Liang, J. Ma, R. Niu, J. Wang, Effects of sodium fluoride and sulfur dioxide on sperm motility and serum testosterone in male rats, Fluoride 39 (2006) 126–131.
[41] R. Sprando, T. Collins, T. Black, N. Olejnik, J. Rorie, Testing the potential of sodium fluoride to affect spermatogenesis: a morphometric study, Food Chem. Toxicol. 36 (1998) 1117–1124.
[42] M. Guney, B. Oral, H. Demirin, N. Karahan, T. Mungan, N. Delibas, Protective effects of vitamins C and E against endometrial damage and oxidative stress in fluoride intoxication, Clin. Exp. Pharmacol. Physiol. 34 (2007) 467–474.
[43] S. Pal, C. Sarkar, Protective effect of resveratrol on fluoride induced alteration in protein and nucleic acid metabolism, DNA damage and biogenic amines in rat brain, Environ. Toxicol. Pharmacol. 38 (2014) 684–699.
[44] J. Ghosh, J. Das, P. Manna, P.C. Sil, Cytoprotective effect of arjunolic acid in response to sodium fluoride mediated oxidative stress and cell death via necrotic pathway, Toxicol. In Vitro 22 (2008) 1918–1926.
[45] S. Thangapandiyan, S. Miltonprabu, Epigallocatechin gallate effectively ameliorates fluoride-induced oxidative stress and DNA damage in the liver of rats, Can. J. Physiol. Pharmacol. 91 (2013) 528–537.
[46] M.S. Keles, N. Demirci, A. Yildirim, S.S. Atamanalp, K. Altinkaynak, Protective effects of *N*-acetylcysteine and Ginkgo biloba extract on ischaemia-reperfusion-induced hepatic DNA damage in rats, Clin. Exp. Med. 8 (2008) 193–198.
[47] H. Aksit, A. Bildik, Determination of DNA damage in experimental liver intoxication and role of *N*-Acetyl cysteine, Cell Biochem. Biophys. 70 (2014) 1119–1125.
[48] A.M. Aldbass, R.S. Bhat, A. El-Ansary, Protective and therapeutic potency of N-acetyl-cysteine on propionic acid-induced biochemical autistic features in rats, J. Neuroinflammation 10 (2013) 42.
[49] T. Ahmed, R. Pathak, M. Mustafa, R. Kar, A.K. Tripathi, R.S. Ahmed, B. Banerjee, Ameliorating effect of *N*-acetylcysteine and curcumin on pesticide-induced oxidative DNA damage in human peripheral blood mononuclear cells, Environ. Monit. Assess. 179 (2011) 293–299.
[50] S. Marthandan, P. Hyland, G. Pawelec, Y. Barnett, An investigation of the effects of the antioxidants, ebselen or N-acetyl cysteine on human peripheral blood mononuclear cells and T cells, Immun. Ageing 10 (2013).
[51] K. Pawłowska-Góral, E. Kurzeja, M. Stec, N-acetylcysteine protects against fluoride-induced oxidative damage in primary rat hepatocytes, Toxicol. In Vitro 27 (2013) 2279–2282.
[52] Y. Yang, H. Huang, D. Feng, W. Liu, X. Cheng, Y. Ba, L. Cui, Effects. of *N*-acetylcysteine on fluoride-induced endoplasmic reticulum stress in sertoli cells, Wei Sheng Yan Jiu (J. Hyg. Res.) 43 (2014) 805–808, 813.
[53] S. Podder, A. Chattopadhyay, S. Bhattacharya, M.R. Ray, A. Chakraborty, Fluoride-induced genotoxicity in mouse bone marrow cells: effect of buthionine sulfoximine and *N*-acetyl-L-cysteine, J. Appl. Toxicol. 31 (2011) 618–625.