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A characterization of the antioxidant enzyme activity and reproductive toxicity in male rats following sub-chronic exposure to areca nut extracts

Pei-Fen Wu[a], Tai-An Chiang[b], Min-Tzu Chen[b], Chi-Pin Lee[c], Ping-Ho Chen[d,e], Albert Min-Shan Ko[e], Kai-Jen Yang[f], Po-Ya Chang[g], Der-Shin Ke[h], Ying-Chin Ko[d,e,g,*]

[a] Department of Occupational Safety and Hygiene, Tajen University, Pingtung, Taiwan
[b] Department of Medical Technology, College of Medicine and Life Science, Chung-Hwa University of Medical Technology, Tainan, Taiwan
[c] Graduate Institute of Medicine, Kaohsiung Medical University, Taiwan
[d] Division of Environmental Health and Occupational Medicine, National Health Research Institutes, Kaohsiung, Taiwan
[e] Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[f] Graduate Institute of Environmental Management, Tajen University, Pingtung, Taiwan
[g] Department of Public Health, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[h] Division of Neurology Medicine, Department of Internal Medicine, Chi-Mei Medical, Center, Tainan, Taiwan

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ABSTRACT

In the present study, areca nut extracts (ANE) administered to male rats by gavage at a dose of 100 mg/kg/day for a period of 15, 30, or 45 days resulted in signs of reproductive toxicity. ANE administration resulted in a significant decline (30–57% in epididymal sperm count and 27–61% in sperm motility) as well as substantial abnormalities in sperm morphology. Significant variances in activities of antioxidant enzymes were also observed. Malondialdehyde (MDA) levels, which represent the level of lipid peroxidation, increased by 16–188% and levels of sialic acid decreased by 2–46% compared with that in controls. These results indicate that ANE induced spermatogenic damage, as indicated by a decrease in sperm counts and sperm motility as well as the activity of antioxidant enzymes, an increase in sperm abnormalities, and alterations in sialic acid and MDA levels. Such effects reflect that ANE administration resulted in reactive oxygen species (ROS)-induced oxidative stress in the testis, cauda epididymis, and sperm of male rats.

1. Introduction

Areca quid is a substance that is widespread in world (approximately 600 million users). Mostly concentrated in Southeast Asia, migrant communities, and originates from these regions [1]. Betel chewing is the fourth most commonly use psychoactive substances (after tobacco, alcohol, and caffeine) [2]. The practice is widespread in Taiwan, with approximately 2 million people habitually chewing areca quid. The prevalence of use in the general population was found to be 9.8% in men and 1.6% in women, with a greater occurrence in less educated older men, blue collar workers, and individuals who regularly smoke or drink alcohol [3]. In recent years, the prevalence of betel quid substance use has increased 14.3% in men, and adolescents have become the high-risk group for chewing areca quid [4]. There is sufficient evidence in humans for the carcinogenicity of betel quid with/without tobacco [5]. Our epidemiological studies have also revealed that betel quid chewing during pregnancy has adverse outcomes, such as a lower male to female sex ratio at birth, decreased birth weight, and shorter birth length among aborigines in Taiwan [6]. Our previous study identified the global impact of arecoline on gene transcription in oral fibroblast cells through high-density microarray analysis and quantitative real-time reverse transcription PCR in normal human gingival fibroblasts [7]. Arecoline was also demonstrated to reduce sperm counts and motility in male rats [8], and caused micronuclei and cytokinesis in Chinese hamster ovary cells [9]. Areca nuts were verified to inhibit male sexual behavior and induce shape abnormalities of sperm heads in mice [10]. Arecoline, the major alkaloid component of the betel nut, has been known to cause cytotoxicity and genotoxicity in mammalian cells *in vivo* as well as *in vitro*, and even contributes to carcinogenicity.

In Taiwan, the two major types of areca quid are: (1) Laohwa quid, which is a mixture of tender areca nut, inflorescence of Piper betel Linn. (IPB), and red lime paste, and (2) "betel quid," a mixture of tender areca nut, Piper betel leaf (PBL), and white lime paste [11]. Under alkaline conditions of pH ≥9.5, areca quid

* Corresponding author at: Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Taiwan, No. 100 Shih-Chuan 1st Road, Kaohsiung 807, Taiwan. Tel.: +886 7 3114418; fax: +886 7 3162725.
E-mail address: ycko@kmu.edu.tw (Y.-C. Ko).

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extract (ANE) and lime generate reactive oxygen species (ROS), such as hydroxyl radicals ($HO^{\bullet}$) [5,11,12]. When catalyzed by metal ions, such as Cu^{2+} and Fe^{2+}, $HO^{\bullet}$ yields increase and 8-hydrox-2-deoxyguansine (8-OHdG) is formed *in vitro*, resulting in possible oxidative DNA damage [5,11]. Moreover, the testicular tissue and sperm plasma membrane, which is rich in polyunsaturated fatty acids, are highly susceptible to ROS damage [13]. Malondialdehyde (MDA) is an end-product of lipid peroxidation that indicates the level of lipid peroxidation [14,15]. Enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as well as non-enzymatic antioxidant components are related to ROS detoxification [5,14].

The generated ROS from chewing betel quid may activate cellular antioxidant defense mechanisms and exhaust antioxidant capacity to adapt to the elevated level of peroxides in testicular tissue and spermatozoa. Therefore, the present study evaluated the effects of ANE on antioxidant enzyme activity (MDA levels, SOD and CAT activities, and correlations between these parameters), and sialic acid levels, which is involved in maintaining the sperm surface charge, in various segments of the testis and epididymis of Sprague–Dawley (SD) rats. The effect of ANE on epididymal sperm count and sperm motility was also determined in order to assess male reproductive toxicity.

2. Materials and methods

2.1. Animals and experimental design

SD male albino rats (150–165 g), which were 4 weeks old, were purchased from the National Laboratory Animal Center (NLAC), Taipei. The quarantine and acclimation period was set at 14 days and all animals were 6 weeks old at the start of male treatment. No signs in clinical observations or negative findings in body weight were detected during the quarantine and acclimation period. A total number of 30 male pups were randomly divided into three groups and ten animals were maintained in each group (four for water-treatment and six for ANE-treated at an equal volume). ANE was administered through a gastric tube at a dose of 100 mg/kg body weight for 15, 30, or 45 days, while the corresponding control groups received sterile water.

Animals were housed in polypropylene cages with clean chip bedding and maintained in an air-conditioned animal house (temperature of $21 \pm 2\,^{\circ}C$ and relative humidity of $50 \pm 10\%$), under a well-regulated light:dark (12 h:12 h) schedule. The rats were maintained on a standard pellet diet (Purina LabDiet 5001; PMI Nutrition International, Inc., Brentwood, MO) and water ad libitum. At the end of the study, the terminal body weight of each animal was recorded. The testis and cauda epididymis were dissected and weighed. Sperm counts, motility, and morphology as well as antioxidant enzyme activity and levels of sialic acid and MDA were calculated. The animals used in this study were treated and sacrificed in accordance with the Guide for the Care and Use of Laboratory Animals by the National Laboratory Animal Center [16].

2.2. Reagents and ANE preparation

All reagents were of analytical grade, and solutions were prepared with double-distilled water. Fresh areca nuts (AN) were purchased from local commercial stores in Tainan, Taiwan. AN was weighed, crushed, and incubated at 37 °C for 2 h. Fibers were removed with two layers of gauze and 90 μm filters. The aqueous ANE was collected and condensed to powder form, and then stored at −20 °C as a stock solution of 0.25 g/ml. Dosing solutions were prepared by dissolving the appropriate amount of the test substance in distilled water.

2.3. Necropsy and collection of tissues

All animals were weighed and sacrificed by Zoletil 50 (Virbac, France), 24 h after the last treatment, following previously described protocols and ethical procedures. The testes and epididymis were dissected and weighed.

2.4. Collection of epididymal sperm

Epididymal sperm were collected by cutting the cauda epididymis and perfusing the cauda with 37 °C (0.9%) normal saline. The epididymal perfusate was centrifuged at $225 \times g$ for 10 min. The pellet was re-suspended in 1.0 ml of normal saline. An aliquot of sperm suspension was used for the examination of sperm counts, motility, and morphology.

2.5. Epididymal sperm counts and sperm motility

Epididymal sperm counts and evaluation of the motility of epididymal sperm were performed using the methods of Joshi et al. [17] and the WHO manual for semen analysis [18]. In brief, sperm count and motility were visually determined in a counting chamber by microscopy. The sperm count was calculated in 10 fields of the counting chamber and expressed as 10^6 per sperm dilution. Sperm motility was determined by counting both motile and non-motile sperms in at least 10 separate and randomly selected fields. These results were expressed as percent motility. The count was repeated three times for each sample to minimize error.

2.6. Epididymal sperm morphology

A drop of sperm suspension was smeared onto a slide to obtain a uniform smear. After the smears were air dried and stained with Liu's stain (Handsel Technologies, Inc., Taipei, Taiwan), the slides were washed in water and air dried again. The smears were examined at a magnification of ×1000 under a light microscope for observation of abnormalities.

2.7. Preparation of homogenate

A fraction of the right testicle and epididymis of each animal was collected for biochemical assays and homogenates were prepared using the method of Linares et al. [19]. After perfusion in 0.9% saline, testis and epididymis were homogenized in 0.2 M sodium phosphate pH 6.25 buffer (1: 20, w/v) in a Potter–Elvehjem homogenizer fitted with a Teflon pestle. Homogenates were centrifuged at $10{,}000 \times g$ for 1 h and the supernatants were obtained. The supernatants were stored at −20 °C and utilized for biochemical analyses.

2.8. Biochemical studies

2.8.1. Superoxide dismutase (SOD)

Superoxide dismutase activity was assayed using the methods of Marklund and Marklund [20], modified from Ramasarma and Rao [21]. The standard solution of SOD was prepared (Cu, Zn-SOD from bovine erythrocytes, Sigma chemical Co., USA). A 100 μl portion of the homogenate was added in 2.7 ml of 50 mM Tris–HCl buffer containing 1 mM EDTA (pH 8.2) and 200 μl of 0.4 mM pyrogallol in a quartz tube. The mixture was measured immediately on a Jasco Spectrophotometer at 325 nm as the increase in absorbance against a blank at 5 s intervals for 1 min. SOD activity was expressed as U/mg protein.

2.8.2. Catalase (CAT)

Catalase activity was assayed using the method of Aebi [22]. A 90 μl aliquot of the homogenate was added to 1.9 ml of phos-

phate buffer (0.05 M, pH 7.0), 1 ml of 30 mM hydrogen peroxide, and 10 μl of Triton X-100 (1%). The mixture was immediately measured at 240 nm on a Jasco Spectrophotometer and the decrease in absorbance against a blank was recorded at 15 s interval for 1 min. Catalase activity was expressed as K/mg protein.

2.8.3. Lipid peroxidation

The method described by Davey et al. [23] was used to determine the content of MDA, the breakdown products of lipid peroxidation, with a thiobarbituric acid reactive substance (TBA) assay. Briefly, 0.2 ml of the homogenate was added to 3 ml of 1% H_3PO_4 in Pyrex tubes. Tetraethoxypropane(TEP) working standard solutions were prepared in gradient concentrations (0.825, 1.65, 3.3, and 6.6 nM/0.2 ml) with TEP stock (8.26 mM). A 0.2 ml aliquot of each working standard solution was added in the Pyrex tubes, and TEP was replaced with the same volume of ethanol in the blank tube. In each tube, 0.8 ml of KCl and 1 ml of TBA solution (42 mM) were added. The mixtures were vortexed, heated for 45 min in boiling water, and cooled in tap water. The mixtures were then prepared by addition 4 ml of butanol, vortexed for 20 s, and centrifuged at 1000 × *g* for 20 min. The supernatant absorbance was measured on a spectrophotometer at 532 nm and the level of MDA was obtained by comparison with the absorbance of standard solutions.

2.8.4. Sialic acid

Sialic acid was assayed by the method of Aminoff [24], modified from Saradha and Mathur [13]. Briefly, 40 μl of the homogenate was added to 250 μl of periodate reagent (25 mM periodic acid in 0.125N H_2SO_4, pH 1.2) in polypropylene tubes, and incubated at 37 °C in a water bath for 30 min. Sodium arsenite (2% solution of sodium arsenite in 0.5N HCl) was used for an excess of periodate. When the yellow color of liberated iodine began to fade after 1–2 min, 2 ml of thiobarbituric acid (0.1 M solution of 2-thiobarbituric acid in water, pH adjusted to 9.0 with NaOH) was added. The tubes were heated for 7.5 min in boiling water, cooled in ice water, and shaken with 5 ml of an acid butanol mixture (butan-1-ol containing 5% (v/v) 12N HCl). After vortexing and rapid centrifugation, the color intensity in the butanol phase was measured on a spectrophotometer at 549 nm. The levels of sialic acid were calculated based on the previous standard curve, and expressed as μg/mg protein.

2.9. Statistical analyses

All quantitative data were expressed as the mean ± S.D. for animals per group and statistically analyzed for homogeneity of variance. When homogeneity of variance was indicated, one-way ANOVA among the experimental groups and unpaired Student's *t*-test was applied between each exposed group and the concurrent control. If significant inter-group variations were found, then the Dunnett multiple comparison test was applied. When the variance was not homogenous the Kruskal–Wallis procedure and the Mann–Whitney *U* test were utilized for nonparametric analysis. Correlation coefficients were tested for trends of antioxidant enzyme activities over the time-course. Statistical differences were considered at $p < 0.05$.

3. Results and discussion

Male rats exposed to ANE for a period 15, 30, and 45 days exhibited various signs of reproductive toxicity. The present study was conducted to evaluate time-course effects of ANE, and demonstrated that ANE induces oxidative stress in the testis, epididymal cauda, and sperm of male rats. The *in vivo* reproductive toxicity of ANE was determined, instead of focusing on a single toxicant, since ANE is comprised of a complicated composition of betel-quid chewing in Taiwanese consumers. We examined the oxidant enzyme activities and peroxidative injury resulting from ANE treatment in male rats. This study is the first to indicate the possibility that the generated ROS from ANE might reduce sperm counts, and sperm motility, affect the antioxidant enzyme activity and increase sperm abnormalities.

3.1. Body and organ weights

Administration of ANE did not result in significant changes in body weight of rats at any experimental dose when compared to the corresponding groups of control animals. The weights of testis and epididymis also were not significantly different at all doses of ANE treatment compared to the corresponding control groups (Table 1).

3.2. Sperm examinations

3.2.1. Sperm count and motility analysis

The number of sperm in the caudal epididymis was significantly decreased in animals administered with ANE at 100 mg/kg for 15, 30, or 45 days compared to the corresponding control groups ($p < 0.01$) (Table 2). Moreover, the sperm count was significantly decreased for the ANE-exposed groups from 15 to 45 days (the correlation coefficient ($r = -0.75$, $p < 0.01$)), but the sperm count did not exhibit significant changes in control groups. Progressive sperm motility decreased significantly in the 100 mg/kg dosage of ANE-treated rats at 30 or 45 days compared to the corresponding group of control rats (Table 2). Moreover, sperm motility was strongly negatively correlated with treated days in ANE-exposed rats ($r = -0.72$, $p < 0.01$), but there was no significant correlation with the control groups.

3.3. Sperm morphology analysis

Morphological examinations were performed on epididymal sperm of both ANE-treated and corresponding control groups. Morphologically abnormal sperm were observed in ANE-treated rats at

Table 1
Comparison of tissue weight between male rats exposed to areca nut extracts (ANE) at 100 mg/kg/d and unexposed controls.

Treatment	0 days	15 days	30 days	45 days
ANE (*N* = 6)				
Body weight (g)	213.75 ± 12.51[a]	330.0 ± 14.49	409.71 ± 14.2	466.67 ± 28.40
Testes (g)		2.89 ± 0.19	3.10 ± 0.35	2.90 ± 0.22
Epididymis (g)		0.67 ± 0.06	0.94 ± 0.08	1.02 ± 0.05
Controls (*N* = 4)				
Body weight (g)	216.29 ± 13.52	342.50 ± 9.57	417.4 ± 9.04	466.25 ± 19.31
Testes (g)		2.76 ± 0.13	2.91 ± 0.26	2.88 ± 0.08
Epididymis (g)		0.67 ± 0.04	0.87 ± 0.04	1.07 ± 0.09

N: number of male rats.
[a] Mean ± standard deviation.

Table 2
Comparison of sperm count and motility between male rats exposed to areca nut extracts (ANE) at 100 mg/kg/d and unexposed controls.

Treatment	15 days	30 days	45 days
ANE ($N=6$)			
Sperm count (10^6)	54.33 ± 9.11[a,**]	48.00 ± 4.65[**]	33.17 ± 9.77[**,aa]
Sperm motility (%)	67.25 ± 6.25	61.68 ± 19.26[*]	34.55 ± 10.20[**,aa]
Controls ($N=4$)			
Sperm count (10^6)	77.33 ± 4.16	78.25 ± 5.38	77.50 ± 4.80
Sperm motility (%)	91.80 ± 2.29	91.93 ± 3.03	89.63 ± 5.03

N: number of male rats.
[a] Mean ± standard deviation.
[*] $p<0.05$ as compared with control group.
[**] $p<0.01$ as compared with control group.
[aa] $p<0.01$ as compared with treated for 15 days.

45 days (coiled tails) and 30 days (sticky sperms), but no sperm abnormalities were indicated at 15 days (Fig. 1).

In the present study, ANE administration resulted in a significant decline in epididymal sperm counts and sperm motility, and there were substantial abnormalities in the sperm morphology. Saradha and Mathur indicated that a decreased density of epididymal sperm could be correlated with testicular spermatogenic arrest and fragment of sertoli cells [13]. This may be a result of androgen deprivation accelerating sperm transition, and thus, affecting the process of epididymal sperm maturation. Rajeswary et al. indicated that impaired testis leydig cells could not normally secrete testosterone, resulting in the decline of sperm counts [25]. In addition, CHO-*K1* cells treated with ANE resulted in DNA damage and the alteration of sperm density [9,26]. However, the body weights as well as testis and epididymis weights of animals treated with ANE did not exhibit any significant change compared to the corresponding group of control animals. The shorter period of administration in our study may account for the lack of observed effects on these organ weights. The present study demonstrated that sub-chronic ANE administration at 100 mg/kg/day dosage reduces epididymal sperm number and sperm motility, which indicates an interference with spermatogenesis.

3.4. Antioxidant enzyme activity in tissues of testis and epididymis

The activities of SOD in the testis of ANE-treated animals at 15 and 30 days were significantly higher than that of the corresponding controls. However, there was a significant decrease in the animals treated with ANE at 45 days compared to the corresponding control animals ($p<0.01$). The activities of CAT in testis of ANE-treated animals at 15, 30, and 45 days were significantly higher than that of the corresponding controls (Table 3). The activities of SOD were negatively correlated with ANE-treated days ($r=-0.95$, $p<0.01$). Conversely, the activities of CAT were positive correlated with ANE-treated days ($r=0.97$, $p<0.01$). However, the activities of SOD and CAT did not exhibit any significant changes in

Table 3
Comparison of SOD and catalase activity in tissue of male rats exposed to areca nut extracts (ANE) at 100 mg/kg/d and unexposed controls.

Treatment	15 days	30 days	45 days
ANE ($N=6$)			
Testes			
SOD (Unit/mg protein)	6.43 ± 0.26[a,**]	5.32 ± 0.41[**,aa]	2.23 ± 0.36[**,aa]
Catalase (nmol/min/mg protein)	3.31 ± 0.38[*]	4.80 ± 0.53[**,aa]	6.95 ± 0.27[**,aa]
Cauda			
SOD (Unit/mg protein)	2.90 ± 0.58[**]	2.44 ± 0.28[**]	1.66 ± 0.30[**,aa]
Catalase (nmol/min/mg protein)	1.48 ± 0.24[**]	3.55 ± 0.28[aa]	5.68 ± 0.47[**,aa]
Controls ($N=4$)			
Testes			
SOD (Unit/mg protein)	3.60 ± 0.33	3.44 ± 0.31	3.39 ± 0.35
Catalase (nmol/min/mg protein)	2.68 ± 0.41	2.68 ± 0.39	2.18 ± 0.24
Cauda			
SOD (Unit/mg protein)	5.62 ± 0.27	5.69 ± 0.33	5.33 ± 0.23
Catalase (nmol/min/mg protein)	3.33 ± 0.28	4.00 ± 0.42	3.31 ± 0.23

N: number of male rat.
[a] Mean ± standard deviation.
[*] $p<0.05$ as compared with control group.
[**] $p<0.01$ as compared with control group.
[aa] $p<0.01$ as compared with treated for 15 days.

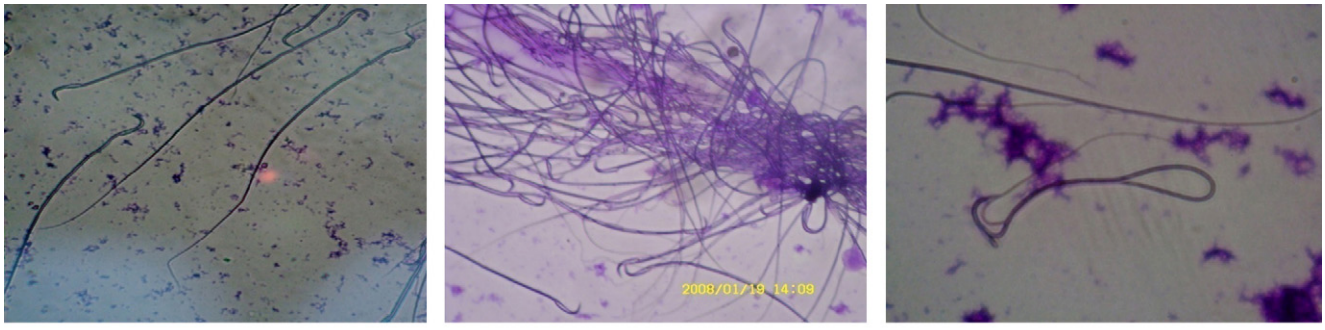

Fig. 1. Photomicrographs (1000× magnification) of morphologically normal sperm from control rats (a), sticky sperm from ANE-treated rats after 30 days of exposure (b), and coiled tails from ANE-treated rats after 45 days of exposure (c).

Table 4
Comparison of MDA and sialic acid in tissue and sperm between male rats exposed to areca nut extracts (ANE) at 100 mg/kg/d and unexposed controls.

Treatment	1 5 days	30 days	45 days
ANE (N = 6)			
Testes			
MDA (nmol/ml tissue)	12.13 ± 0.52**,a	13.96 ± 0.35**,aa	15.63 ± 0.41**,aa
Sialic acid (μg/mg protein)	3.49 ± 0.18**	3.79 ± 0.45**	3.88 ± 0.15**
Cauda			
MDA (nmol/ml tissue)	12.42 ± 2.11**	21.21 ± 0.69**,aa	23.39 ± 1.04**,aa
Sialic acid (μg/mg protein)	3.30 ± 0.18	3.14 ± 0.24**	3.23 ± 0.13**
Sperm			
MDA (nmol/ml tissue)	1.22 ± 0.11**	1.48 ± 0.04**,aa	3.04 ± 0.26**,aa
Controls (N = 4)			
Testes			
MDA (nmol/ml tissue)	8.91 ± 0.90	8.97 ± 0.54	12.83 ± 0.34aa
Sialic acid (μg/mg protein)	3.93 ± 0.03	5.18 ± 0.23aa	7.18 ± 0.23aa
Cauda			
MDA (nmol/ml tissue)	4.32 ± 1.28	7.71 ± 0.58aa	8.82 ± 0.10aa
Sialic acid (μg/mg protein)	3.36 ± 0.27	3.85 ± 0.22	5.27 ± 0.32aa
Sperm			
MDA (nmol/ml tissue)	0.96 ± 0.10	1.28 ± 0.09aa	2.33 ± 0.07aa

N: number of male rat.
[a] Mean ± standard deviation.
** p <0.01 as compared with control group.
aa p <0.01 as compared with treated for 15 days.

the control groups. Similar results were observed for the activities of SOD and CAT in the cauda epididymis (Table 3).

Antioxidants are the frontline defense mechanism against damage from free radicals [27]. SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, which is converted into hydrogen monoxide and molecular oxygen by catalase [13,28]. In the present study, the activity of SOD increased significantly in animals treated with ANE for 15 days, and conversely, decreased significantly in animals treated with ANE for 45 days compared to the corresponding controls. In contrast, the activity of CAT exhibited an opposite effect in ANE treated animals. An increase in SOD activity of SOD may be stimulated by excess ROS to eliminate superoxide radicals. The decrease in SOD amounts of SOD over time with ANE treatment reflects the elimination of superoxide radicals and the excessive generation of hydrogen peroxide to stimulate an increase in CAT activity.

3.5. Lipid peroxidation in testis, epididymis and sperm

The levels of MDA in ANE-treated animals at 15, 30, and 45 days were found to increase significantly in the testis, cauda epididymis, and sperm compared to the corresponding controls (p < 0.01) (Table 4). Moreover, the levels of MDA were positively correlated with all treatment days in both groups (all p < 0.01).

The lipid peroxidation of unsaturated fatty acids in sperm membranes is one of the most important effects from ROS-induced cell damage [14], and might impair sperm motility [13,14]. Chitra et al. observed that increased levels of lipid peroxidation caused the reduction of sperm count and viability [29]. MDA is an end-product of lipid peroxidation, and thus, MDA production reflects the peroxidation of polyunsaturated phospholipids [14,15]. In our study, MDA levels in the testis, epididymis, and sperm of ANE-treated animals increased in comparison to the corresponding controls and exhibited a positive correlation over time. A negative correlation between MDA activity, and sperm motility or concentration was also observed (data not shown). In addition to the aforementioned results, our study identified that the accessory sex organs of the ANE-treated animals were affected by excessive ROS. However, MDA levels of the control animals over the time-course were similar with the ANE-treated animals. Many reports indicate that there were age-related increases in MDA levels of the reproductive system in experimental animals as well as human males [30,31].

3.6. Sialic acid in testis and cauda epididymis

Except for the cauda epididymis of the 15 day treatment group, the levels of sialic acid of ANE-treated animals were found to decrease significantly in testis and the cauda epididymis when compared to the corresponding controls (p < 0.01) (Table 4). Moreover, the levels of sialic acid were positive correlated over time in the control groups (r = 0.98 for testis and r = 0.93 for cauda), but did not exhibit significant changes in cauda tissue of the ANE-treated rats.

Sialic acid has also been reported to act as a potent hydrogen peroxide scavenger by imparting defense against oxidative damage [13]. The amounts of sialic acid are related to the maturation of spermatozoa in epididymis and the maintenance of membrane structural integrity [32]. The decreased levels of sialic acid may reduce sperm count and motility, and decrease the capacity for sperm to execute the acrosomal reaction and fertilize [13,14]. In the present study, levels of sialic acid decreased in testis and cauda epididymis of ANE-treated rats in comparison to the controls. Furthermore, the levels of sialic acid increased along with aging in the control groups, which was not observed in ANE-treated groups. These results suggest that ANE administration may reduce levels of sialic acid, resulting in impaired sperm function.

4. Conclusions

In conclusion, the present study indicates that ANE induced spermatogenic damage, affecting sperm counts, sperm motility, the activity of antioxidant enzyme, and levels of sialic acid and MDA. These effects demonstrated that ANE administration caused the excessive generation of ROS related oxidative stress in the testis and cauda epididymis. This dose level in present study is estimated by human daily exposure. Thus, the results reflect ANE may affect fertility in human males in long-term exposure, and provide health education on the harmful effects of substance use. Further identification and characterization of an appropriate model to elucidate

ANE-induced effects reproductive pathogenesis should be a high future priority.

Conflict of interest statement

No potential conflicts of interest were disclosed.

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