Detrimental Effect of Atrazine on Testicular Tissue and Sperm quality: Implication for Oxidative stress and Hormonal Alterations

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ABSTRACT

Background: Atrazine (ATR) is used as an agriculture herbicide worldwide. It has been shown that ATR adversely affect the reproductive system in rodents. In this study we aimed to evaluate the impact of chronic exposure to Atrazine (ATR) on male testicular tissue, sperm parameters, serum level of total thiol molecules (TTM) and malodialdehyde (MDA) content of testes.

Methods: To follow-up this study, 72 adult and mature male rats were divided into test and control-sham groups. The animals in test group received the compound at dose levels of 100, 200 and 300 mg/kg, b.w., orally representing the low, medium and high doses of ATR respectively for 12, 24, 48 days. Control-sham group received the corn oil (0.2 ml/day) in the same manner as test groups.

Results: Light microscopic analyses revealed increased thickness of tunica albuginea, atrophied seminiferous tubules, arrested spermatogenesis, decreased leydig cells/ mm^2 of interstitial tissue (2.0±0.7/mm^2 in high dose received rats), vasodilatation and thrombosis. Sperm parameters assays showed that the sperm count (26.50±2.16×10^6 vs control 70.25±1.25**),** viability (21.26±2.58% vs control 90.75±6.23) and motility (12.00±1.58% vs control 90.41±2.12) decreased in ATR-exposed animals in a dose-dependent fashion. Biochemical analyses for TTM and MDA demonstrated that in ATR-exposed animals the serum level of TTM (0.100±0.005 Mol/ml vs control 0.321± 0.002) decreased significantly ($P<0.05$) and by contrast the testicular MDA level (4.053±2.28 nMol/mg vs control 1.75±0.34) elevated in testicular tissues.

Conclusion: the current data provide inclusive histological feature of chronic exposure against ATR in testicular tissue. Moreover, other reproductive-related disorders including abnormalities in spermatogenesis, sperm viability, volume and motility, may attribute to the ATR-induced oxidative stress, which reflected by remarkable alteration in TTM and MDA levels.

Key words: Atrazine, Hormonal Alterations, Oxidative Stress, Spermatogenesis, Sperm Parameters.

INTRODUCTION

Over the last 30 years the methods for both analyzing and treating the fertility-related disorders have become more sophisticated and powerful. Simultaneously, the risk factors for infertility have increased substantially, particularly those resulting from the environmental chemicals. The increase in the number of, and exposure to, physical and chemical agents poses significant problems for human fertility.

ATZ is a member of chloro-s-triazine family herbicide that is mostly used as a weed killer in corn fields. After several years using of ATR, this compound's residues were found in the soil, surface, ground and drinking water (1-4). Besides the soil and water ATR has been detected in animal feed mostly in corns (5).

There are reports indicating that ATR can affect the reproductive system. The indirect influence of ATR on the pituitary–gonadal axis of male and female offspring was studied by the treatment of rat mothers during pregnancy and lactation (6). In the offspring of both sexes a slower maturation of the gonadotrophic system has been documented as the

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modification of 5□-reductase activity was detected in both males and females (7). This enzyme is responsible for testosterone metabolism to its active metabolite 5□-dihydrotestosterone in the anterior pituitary and prostate glands (7,8,9). According to previous reports, ATR induces suppressive effects on gonadal endocrine system's function in both male and female (10-11).

Hence the primary goal of this study was to investigate the influence of ATR on spermatogenesis process, sperm count, motility and mortality in different doses and various periods of exposure time. Additionally the impact of ATR on serum and tissue oxidative defense including; serum level of thiol molecules (TTM) and tissue malodealdehyde content (MDA) was evaluated.

MATERIALS AND METHODS

Animals

Seventy two mature male Wistar rats, 8 weeks olds and weighting 200 ± 20 g were used. The rats were obtained from the Animal Resources Center of faculty of Veterinary Medicine, Urmia University, Iran and they were acclimatized in an environmentally controlled room (temperature, 20-23°C, and 12h light/12h dark). Food and water were given *ad libitum*. In this study all experiments which conducted on animals were in accordance with the guidance of ethical committee for research on laboratory animals of Urmia University.

Experimental design

Following a week acclimation the animals were assigned into 12 groups as control and test groups. Six rats were allocated to each single group. All animals from mentioned groups prior to the starting of experiment and after the latest step of treatment were weighted to evaluate any changes in body weight gain (BWG). Animals in the control group received corn oil (0.2 ml/day) and the rats in test groups were administrated ATR at dose levels of 100 mg /kg, b.w., (low dose: LD), 200 mg/kg,b.w., (medium dose: MD), and 300 mg/kg,b.w., (high dose: HD), orally once a day for 12, 24 and 48 days. The rats were sampled on days 12, 24, 48 after dosing.

Histopathological analyses

On days 12, 24 and 48 following an anesthesia which given with diethyether, the testicular specimens were dissected out and fixed in 10% bouin's fixative for histological investigations and subsequently embedded in paraffin. Sections (5-6 µm) were prepared with rotary microtome (GMbh, Germany) and stained with Iron-Weigert (Pajohesh Asia., Iran) for detection of germinal cells nucleuses in the testis. The prepared samples were studied by multiple magnifications (400X and 1000X). For the quantification of cells and their dimensions, we used 100µm morphometrical lens–device (Olympus Co., Germany) and the dimensions were expressed in µm.

Mononuclear Immune Cells (MIC) count

Eighteen random regions from one cross section were considered in total 200 slides in order to evaluate the number of MICs per one mm^2 of the connective tissue by using 100 squares morphometric lens device (Olympus Co., Germany).

Assessment of epididymal sperms

Epididymides were separated carefully from the testicles under a 20-time magnification provided by a stereo zoom microscope (model TL2, Olympus Co., Tokyo, Japan). The epididymis was divided into 3 segments; head, body and tail. The epididymal tail was trimmed and minced in 5 mL Hams F10 medium (Sigma Co, USA). After 20 minutes the ground epididymal tissue was separated from the released spermatozoa.

Sperm count and motility

The sperm count was performed according to standard hemocytometric lam method using the improved Neubauer (Deep 1/10mm,LABRAT,Germany) chamber (12), in order to reduce the errors into lower than 10%. In brief, after dissecting the epididymal tissue, the sperm samples underwent to swimming-up process and following diluting (1/20) the total sperm count were conducted. A total of 400 spermatozoa from each rat were examined for morphological study.

Sperm viability and morphological abnormalities

Sperm viability (live or dead) was determined from 400 smears of spermatozoa which were stained with eosin-nigrosin staining technique (using 1% Eosin and 5% nigrosin in 3% sodium citrate dehydrate) The sperms with red stained head were considered as dead sperm (sperms with damaged plasma membrane). The sperms with residual cytoplasm were considered as morphologically abnormal sperm content.

Measurement of tissue total thiol molecules (TTM)

Total sulfhydryl levels in the testis tissues were measured as described previously (13). Briefly, 0.2 ml of the previously prepared serum was added to 0.6 ml Tris-EDTA buffer (Tris base 0.25 M, EDTA 20 mM, pH 8.2) and thereafter 40 □l 5.5'-Dithiobis-2-nitrobenzoic acid (10 mM in pure methanol) was added to the 10 ml glass test tube. The final volume of this mixture was made up to 4.0 ml by an extra addition of methanol. After 15 min incubation at room temperature, the samples were centrifuged (Roter-Uni II, BHG, Germany), at 3000 g for 10 min and ultimately the absorbance of the supernatant was measured at 412 nm. The TTM capacity was expressed as nMol per mg of protein in samples. The protein content of the samples was measured according to the Lowry et al., method (14).

Measurement of the malodialdehyde-content of tissue samples

To determine the lipid peroxidation rate in the control and test groups, the MDA content of the testes was measured using the thiobarbituric acid (TBA) reaction as described previously (15). Briefly, 0.2-0.3 g of the samples were homogenized in ice-cooled KCL (150 mM), and then the mixture was centrifuged at 3000 *g* for 10 min; 0.5 ml of the supernatant was mixed with 3 ml phosphoric acid (1% V/V) and then after vortex mixing, 2 ml of 6.7 g L^{-1} TBA was added to the samples. The samples were heated at 100 °C for 45 min, and then chilled in ice. After adding of 3 ml N-butanol, the samples were centrifuged at 3000 *g* for 10 min. The absorbance of supernatant was measured spectrophotometerically (Pharmacia, Novaspec II, Biochrom, England), at 532 nm and the amount calculated according to the simultaneously prepared calibration curve using MDA standards. The amount of MDA was expressed as nMol per mg protein. The protein content of the samples was assessed based of Lowery et al., method (14).

Testosterone measurement

After 12, 24 and 48 days exposure to ATR, the blood samples from corresponding animals were collected directly from the heart and the serum samples separated by centrifugation (Hittech, EBAIII., Japan). The collected serum samples were subjected to hormonal analysis. The testosterone level of serum was measured by using radioimmunoassay method and special kit for rat (WHO/Sigma Asso-RFGC-78/549). The limit of detection (LOD) was 0.12 ng/ml for testosterone. The intra-assay and inter-assay coefficients variances for testosterone was found 4.8 (for 10 times) and 9.9 (for 10 times), respectively.

Statistical analyses

The statistical analyses were performed on numerical data by using two-way ANOVA and using SPSS software version 13.0. All values were expressed as the mean ± SD. $P<0.05$ was considered to be statistically significant.

RESULTS

Histopathological analyses

Histological observations revealed that the tunica albuginea was increased in thickness on day 24 after dosing of ATR. The control group showed normal tunica thickness. Sub-capsular and interstitial connective tissues were manifested with remarkable edema in all of the ATR-exposed rats and the edema was substantiated by the time. After 24 days, a considerable vasodilatation associated with remarkable thrombosis demonstrated in both right and left testicles of the animals in ATR-exposed groups (Figure 1).

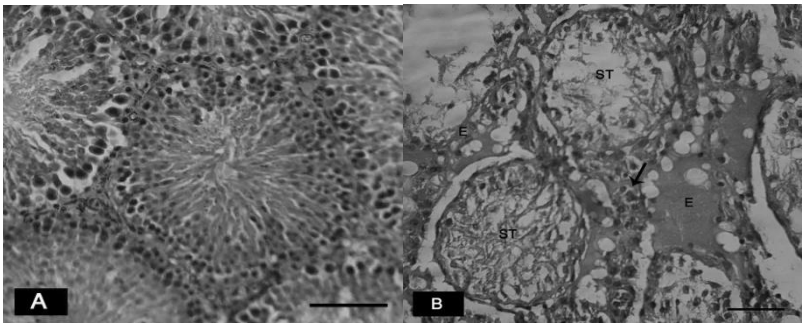

Figure 1. Cross section from testis, (**A**) Control group; no germinal epithelium depletion and interstitial connective tissue edema are detectable in seminiferous tubules, (**B**) High dose atrazine administrated group, the severe edema in interstitial connective tissue (*E*), high infiltration of mono nuclear immune cells in connective tissue (*arrow*) and depleted seminiferous tubules (*ST*). H&E staining technique (100X), *scale bars is 0.2 mm*.

Infiltration of the mono-nuclear immune cells in one mm^2 of the interstitial connective tissue was increased to 37.83±1.16 in group of animals which received ATR at high dose level for 48 days. After day 48, leydig cells were seen with a significant decrease in number per one mm^2 of the interstitial connective tissue (2.00±0.70). These cells were demonstrated with considerable hypertrophy and cytoplasmic granulation in ATR-induced rats. No histological changes were observed in control group. The data for the leydig cells distribution and immune cells infiltration per one mm^2 of the interstitial connective tissue are presented in (table 1).

Table 1. mean average of leydig and mononuclear immune cells distribution in one mm^2 of the interstitial connective tissue in different test and control groups. All data are presented as Mean±SD.

*^{abcd} are representing significant differences (P<0.05) between the tests and control groups in the same column and stars show significant differences between various time periods of study at the same raw. Note; **L.D**: low dose, **M.D**: medium dose and **H.D**: high dose and n=6 for each group*

Groups	Leydig Cells NO/mm^2			Immune cells NO/mm^2		
	12ds	24ds	48ds	12ds	24ds	48ds
Control	10.20±0.44^{a}	9.30±0.75^{a}	10.21±0.75^{a}	19.83±1.94^{a}	18.75±1.35^{a}	19.88±1.85^{a}
L.D, ATR	7.20±0.83^{b}	6.16±0.47^{b}	5.60±0.54^{b}	21.33±1.86^{a}	24.00±1.09^{b*}	26.50±1.04^{b*}
M.D, ATR	5.40±0.89^{c}	4.40±0.89^{c}	3.40±0.84^{c}	23.83±1.72^{b}	27.66±1.21^{c*}	30.83±1.72^{c*}
H.D, ATR	3.40±0.54^{d}	2.40±0.88^{d}	2.00±0.70^{c}	26.50±1.04^{c}	31.66±1.36^{d*}	37.83±1.16^{d*}

Light microscopic analyses showed that, the seminiferous tubules (STs) were severely atrophied following exposure to ATR. Exposure to ATR for 24 days resulted in a decrease in germinal epithelium height to 172.0±1.58µm in approximately 60% of the STs which was significantly lower than control germinal epithelium height (208.20±7.56µm). Moreover tubular depletion was manifested in more than 30% of the STs of ATR-exposed animals after 24 days. This impairment increased in more than 40 % in 48 days ATR-exposed cases (Table 2). No histological changes were observed in control group. In animals which exposed to ATR for 24 days, increased spaces between germinal cells of the STs were revealed. Also the junction between sertoli and germinal cells was disrupted time-dependently (Figure 2).

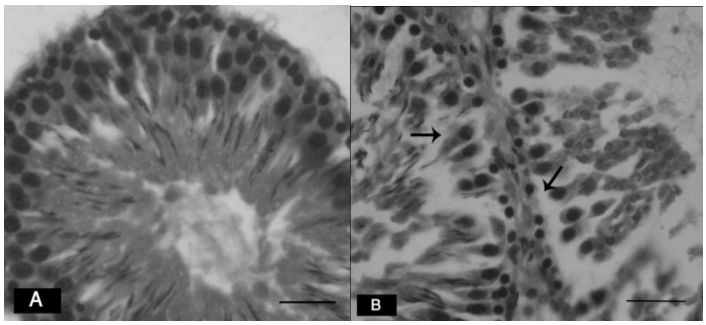

Figure 2. cross section from testis; (A) control group, (B) ATR-induced group; the germinal epithelium dissociation (arrows) are represented. H&E staining technique (400X), scale bar is 0.2 mm.

Table 2. Mean average of germinal epithelium height and percentage of seminiferous tubules with arrested spermatogenesis in different test and control groups. All data are presented in Mean ± SD.

Groups	Germinal Epithelium Height (µm)			Seminiferous Tubules with Spermatogenesis Arrest (%)		
	12ds	24ds	48ds	12ds	24ds	48ds
Control	208.20±7.56^{a}	201.34±8.41^{a}	200.65±5.12^{a}	1.83±0.75^{a}	1.62±0.85^{a}	1.59±0.51^{a}
L.D, ATR	198.7±1.20^{b}	194.20±2.77^{a}	193.60±1.34^{b}	8.66±1.03^{b}	12.00±1.41^{b*}	16.33±1.21^{b*}
M.D, ATR	191.40±1.14^{c}	190.00±1.22^{b}	180.60±2.70^{c*}	21.16±1.47^{c}	26.34±1.21^{c*}	28.50±1.22^{c}
H.D, ATR	174.80±2.58^{d}	172.00±1.58^{c}	170.60±1.94^{d}	24.16±3.18^{d}	31.67±1.36^{d*}	33.50±2.42^{d}

*^{abcd} are representing significant differences (P<0.05) between the tests and control groups in the same column and stars show significant differences between various time periods of study at the same raw. Note; **L.D**: low dose, **M.D**: medium dose and **H.D**: high dose and n=6 for each group*

Sperm count, morphology and viability

Total sperm count decreased by the time in all ATR-exposed animals. The animals, which exposed to high dose of ATR for 48 days, manifested with $26.50\pm2.16\times10^{6}$ sperms that was significantly lower in number in comparison to control cases ($74.83\pm1.16\times10^{6}$). Observations demonstrated that the percentage of morphologically abnormal sperms significantly (P<0.05) increased by the time in ATR-exposed animals. Light microscopic observations by using special staining of eosin-nigrosin showed significantly (P<0.05) decreased sperm viability in the test groups (21.26±2.58% in group of animal that received the high dose of ATR) in comparison to the control rats (91.20±4.20%). The data for sperm count and viability are presented in (table 3).

Table 3. Sperm viability, abnormality and count in the different test and control groups, all data are presented in Mean±SD.

Groups	12ds	24ds	48ds
	Sperm Viability (%)		
Control-sham	91.20±4.20^{a}	92.58±5.21^{a}	90.75±6.23^{a}
L.D, ATR	71.80±1.92^{b}	50.40±0.54^{b*}	29.80±3.34^{b*}
M.D, ATR	54.60±2.07^{c}	44.00±4.30^{c*}	27.00±3.87^{c*}
H.D, ATR	41.20±2.38^{d}	33.00±2.91^{d*}	21.26±2.58^{d*}
	Morphological Abnormalities (%)		
Control-sham	**9.83±1.47^{a}**	**8.85**±1.32^{a}	**8.12**±1.00^{a}

L.D, ATR	**69.16±1.16^{b}**	**71.33±1.75b**	**74.66±1.75^{b*}**
M.D, ATR	**71.50±1.37^{c}**	**74.50±1.64^{b}**	**77.66±1.21^{c*}**
H.D, ATR	**73.83±1.60^{c}**	**77.33±1.33^{c*}**	**83.00±2.59^{d*}**
	Sperm count (NO×10^{6})		
Control-sham	74.83±1.16^{a}	73.81±1.56^{a}	70.25±1.85a
L.D, ATR	65.15±0.75^{b}	60.16±1.47^{b*}	55.16±1.94^{b*}
M.D, ATR	57.17±1.72^{c}	52.50±1.04^{c*}	50.83±0.98^{c*}
H.D, ATR	41.33±1.21^{d}	30.45±1.54^{d*}	26.50±2.16^{d*}
	Sperm motility (%)		
Control-sham	90.60±1.51^{a}	91.60±1.23^{a}	90.41±2.12^{a}
L.D, ATR	74.30±0.67^{b}	44.00±2.34^{b*}	57.60±2.07^{b*}
M.D, ATR	54.00±4.30^{c}	41.80±1.78^{b*}	35.20±3.70^{c*}
H.D, ATR	30.60±0.84 ^{d}	25.60±2.30^{c*}	12.00±1.58^{d*}

*^{abcd} are representing significant differences (P<0.05) between the tests and control groups in the same column and stars show significant differences between various time periods of study at the same raw. Note; **L.D**: low dose, **M.D**: medium dose and **H.D**: high dose and n=6 for each* group

Alterations in serum level of TTM and tissue content of MDA

Biochemical analyses indicated that the rate of total thiol molecules decreased by the time in the ATR-received animals, while it was statistically unchanged in control cases. Accordingly after 48 days, animals in the test groups showed considerable reduction in TTM level (0.10±0.005 vs control 0.32± 0.002 nMol/ml). By contrast, MDA content of the testes elevated in ATR-exposed animals (4.05±2.28 nMol/mg) in comparison to 1.75±0.34 nMol/mg in control group (Figure 3).

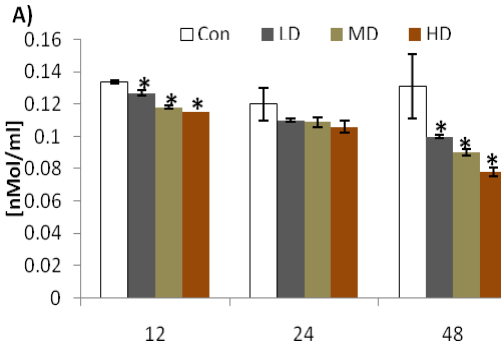

Figure 3. Average of the serum TTM (A) and tissue MDA (B); stars are indicating significant differences between marked groups with each other and with control-sham. All data are presented in mean ± SD, (P<0.05).

Changes in serum level of testosterone

The serum level of testosterone declined dramatically in all ATR-exposed groups (Figure 4). Accordingly animals that exposed against the high dose of ATR for 48 days showed the lowest level of testosterone (1.23±0.08 ng/ml) in comparison to control group (6.24±0.08 ng/ml).

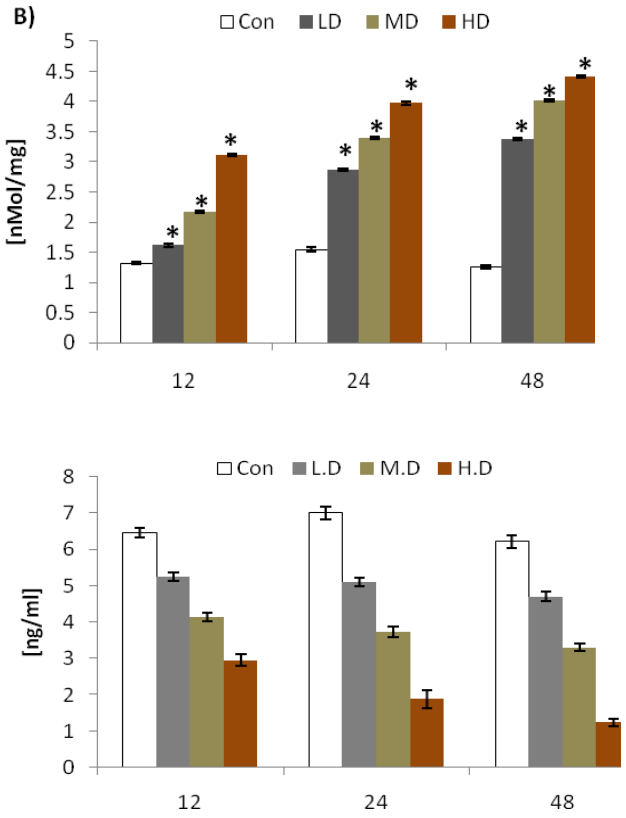

Figure 4. Average of serum testosterone level; there are significant differences between all ATR-induced groups with each other and control group. All data are presented in mean±SD, ($P<0.05$) and n=6 for each group.

DISCUSSION

Present study showed that ATR induces severe detrimental impact on testicular tissue including; remarkable histoplathological changes and biochemical alterations in oxidant system. Moreover ATR-induced pathological effects were substantiated by the time as a severe reduction in semen quality after long time exposure was obtained.

Each year, lots of pesticides are produced industrially and are used in agriculture and forestry, as well as in and around the homes. ATR is used as a worldwide agricultural herbicide and according to previous findings, this compound affects the reproductive organs at sub-cellular levels (17,18). Until now there have been no available data about the exact mechanism of the ATR-induced impairments in spermatogenesis processes. In the present study the adult rats were treated with three different doses of ATR in order to identify the impact of ATR exposure on spermatogenesis, sperm count, viability and oxidative stress. Our light microscopic analyses showed that after 48 days administration of ATR, remarkable STs depletion and germinal epithelium dissociation occurred in more than 30% of the STs. These impairments might be related to blocking and/or inhibiting of the non-specific esterase activity in leydig cells and reduction of endocrine function (19,20). It is well understood that testosterone affects and controls the physiological functions of sertoli cells and histological integrity (19,21), thus we may conclude that following chronic exposure to ATR, the leydig cells are degenerated and reduced in number per one mm^2 of the interstitial tissue. At the same time, their ability for synthesis of testosterone decreased by the time. These destructive processes led to remarkable functional impairment in sertoli cells. The reduction in serum level of testosterone, which was determined following ATR-exposure in this study, confirmed that there is a links between ATR-exposure, leydig and sertoli cells function and integrity.

According to previous reports, ATR can influence the hypothalamic-pituitary-testes axis (22-24). Also some reports indicate that the serum levels of FSH and LH decreases in ATR-induced rats (24, 25). Therefore, it may also explain that ATR not only affects the intra-testicular endocrine functions but also influences on extra testicular endocrine axis. In the case of decreased gonadotrophic hormones, the feed back between leydig cells and upper endocrine controlling center (pituitary gland) disrupts. In line with this impairment sertoli cells and germinal epitheliums integrity affect, respectively.

Our histological observations indicated that 12 days after ATR administration, all test animals exhibited high infiltration of immune cells in interstitial tissue, germinal epithelium degeneration and dissociation following severe inflammation. It is well documented that the over-production of reactive oxygen species (ROS) influences the germinal cells quality, amount of lipid peroxidation, infiltrations of immune cells in testicular tissue and sperms quality in the original semen (26,28). To find the pathogenesis of ATR-induced damages the serum level of TTM was determined. Our findings showed that the chronic exposure to ATR caused remarkable decrease in serum level of thiol molecules, indicating severe ATR-induced oxidative stress. The MDA analyses confirmed the early findings and showed that by the time ATR caused significant lipid peroxidation in testes tissues of test groups.

Once the spermatogenesis process exposes to a considerable degeneration, the cytoplasmic extrusion mechanisms do not act in normal condition and the released spermatozoa from the germinal epithelium carries surplus residual cytoplasm and is considered as immature and functionally defective spermatozoa, which increasing of immature and abnormal spermatozoa content in STs and original semen leads to pathologically elevation of ROS stress (29,31). Previous reports indicated that an excessive increase in ROS generation correlated with the reduction of sperm motility (31,32). The exact mechanism(s) which explain how overproduction of ROS can reduce the sperm motility, remains to be clarified, however an intensive decrease in axonemal protein phosphorylation and inhibition of metabolic enzymes such as G6PDH due to diffusion of free radicals across the membranes may partly explain our findings (31,33,35). Our observations demonstrated that after 12 days the ATR-exposed rats manifested with high percentage of immotile sperms. It may be occurred due to the fact that spermatozoa are particularly susceptible to such stress because their plasma membranes are so enriched with unsaturated fatty acids, particularly decosohexaenoic acid with six double bonds per molecule. In order to exactly identify the rate of sperm plasma membrane damage, we used eosin-nigrosin staining technique and it confirmed the increased number of sperms with defected plasma membrane. Hence, our findings support the hypothesis that ATR-induced damages associate with increased oxidative stress in long time exposure period. Moreover, severe damages in plasma membrane due to ATR exposure may cause immobilization of the sperms.

CONCLUSION

According to our results male reproductive system could be accounted as a target tissue for ATR. Chronic exposure to ATR can cause histological damages on testicular tissue by inducing remarkable inflammation associated with severe oxidative stress. Also it could be considered as a potent toxic compound against sperm quality. Furthermore the ATR capability in altering the testicular endocrine function such as testosterone release may contribute in ATR-induced injuries to leyding and sertoli cells.

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REFERENCES

1. Goh KS, Weaver DJ, Hsu J, Richman SJ, Tran D, Barry TA. ELISA regulatory application: compliance monitoring of simazine and atrazine in California soils. Bull Environ Contam Toxicol. 1993;51(3):333-40.
2. Hrlec G. Ograničenja u primjeni herbicida. Glasnik Zaštite Bilja. 1990;13:252-5.
3. Graham JA. Monitoring groundwater and well water for crop protection chemicals. Anal Chem. 1991;63(11):613A-22A.
4. Gojmerac T, Kartal B, Bilandzic N, Roic B, Rajkovic-Janje R. Seasonal atrazine contamination of drinking water in pig-breeding farm surroundings in agricultural and industrial areas of Croatia. Bull Environ Contam Toxicol. 1996;56(2):225-30.
5. Norris RF, Fong IE. Localization of atrazine in corn (Zea mays), oat (Avena sativa), and kidney bean (Phaseolus vulgaris) leaf cells. Weed Science. 1983:664-71.
6. Kniewald J, Peruzović M, Gojmerac T, Milković K, Kniewald Z. Indirect influence of s-triazines on rat gonadotropic mechanism at early postnatal period. J Steroid Biochem. 1987;27(4-6):1095-100.
7. Kniewald J, Jakominić M, Tomljenović A, Simić B, Romać P, Vranesić D, et al. Disorders of male rat reproductive tract under the influence of atrazine. J Appl Toxicol. 2000;20(1):61-8.
8. Kniewald J, editor. Studies on Neuroendocrine Effects of Atrazine in Male Rats. 1997.
9. Simić B, Kniewald J, Kniewald Z. Effects of atrazine on reproductive performance in the rat. J Appl Toxicol. 1994;14(6):401-4.
10. de Kretser DM, Kerr JB. The cytology of the testis. In: Knobil E, Neill J, editors. The Physiology of Reproduction. 2nd ed. New York: Raven Press; 1994. p. 1177–290.
11. Kniewald J, Osredecki V, Gojmerac T, Zechner V, Kniewald Z. Effect of s-triazine compounds on testosterone metabolism in the rat prostate. J Appl Toxicol. 1995;15(3):215-8.
12. Babić-Gojmerac T, Kniewald Z, Kniewald J. Testosterone metabolism in neuroendocrine organs in male rats under atrazine and deethylatrazine influence. J Steroid Biochem. 1989;33(1):141-6.
13. Organization WH. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed: Cambridge University Press; 1999.
14. Ranjbar A, Khorami S, Safarabadi M, Shahmoradi A, Malekirad AA, Vakilian K, et al. Antioxidant Activity of Iranian Echium amoenum Fisch & C.A. Mey Flower Decoction in Humans: A cross-sectional Before/After Clinical Trial. Evid Based Complement Alternat Med. 2006;3(4):469-73.
15. Lowry Oh, Rosebrough Nj, Farr Al, Randall Rj. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-75.
16. Niehaus WG, Samuelsson B. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem. 1968;6(1):126-30.
17. Bustos-Obregón E, González JR, Espinoza O. Melatonin as protective agent for the cytotoxic effects of diazinon in the spermatogenesis in the earthworm Eisenia foetida. Ital J Anat Embryol. 2005;110(2 Suppl 1):159-65.
18. Chapin RE, Phelps JL, Somkuti SG, Heindel JJ, Burka LT. The interaction of Sertoli and Leydig cells in the testicular toxicity of tri-o-cresyl phosphate. Toxicol Appl Pharmacol. 1990;104(3):483-95.
19. Skinner MK, Fritz IB. Testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell functions. Proc Natl Acad Sci U S A. 1985;82(1):114-8.
20. Ashby J, Tinwell H, Stevens J, Pastoor T, Breckenridge CB. The effects of atrazine on the sexual maturation of female rats. Regul Toxicol Pharmacol. 2002;35(3):468-73.
21. Stoker TE, Guidici DL, Laws SC, Cooper RL. The effects of atrazine metabolites on puberty and thyroid function in the male Wistar rat. Toxicol Sci. 2002;67(2):198-206.
22. Laws SC, Ferrell JM, Stoker TE, Schmid J, Cooper RL. The effects of atrazine on female wistar rats: an evaluation of the protocol for assessing pubertal development and thyroid function. Toxicol Sci. 2000;58(2):366-76.
23. Kalla NR, Kaur S, Ujwal N, Mehta U, Joos H, Frick J. alpha-Glucosidase activity in the rat epididymis under different physiological conditions. Int J Androl. 1997;20(2):92-5.
24. Chapdelaine P, Tremblay RR, Dubé JY. P-Nitrophenol-alpha-D-glucopyranoside as substrate for measurement of maltase activity in human semen. Clin Chem. 1978;24(2):208-11.
25. Marmar JL. The pathophysiology of varicoceles in the light of current molecular and genetic information. Hum Reprod Update. 2001;7(5):461-72.

26. Hsu HS, Chang LS, Chen MT, Wei YH. Decreased blood flow and defective energy metabolism in the varicocele-bearing testicles of rats. Eur Urol. 1994;25(1):71-5.
27. Gomez E, Irvine DS, Aitken RJ. Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. Int J Androl. 1998;21(2):81-94.
28. Gil-Guzman E, Ollero M, Lopez MC, Sharma RK, Alvarez JG, Thomas AJ, et al. Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. Hum Reprod. 2001;16(9):1922-30.
29. Malekinejad H, Mirzakhani N, Razi M, Cheraghi H, Alizadeh A, Dardmeh F. Protective effects of melatonin and Glycyrrhiza glabra extract on ochratoxin A--induced damages on testes in mature rats. Hum Exp Toxicol. 2011;30(2):110-23.
30. Najafi G, Razi M, Hoshyar A, Shahmohamadloo S, Feyzi S, Urmia I. The Effect of Chronic Exposure with Imidacloprid Insecticide on Fertility in Mature Male Rats. International Journal of Fertility and Sterility. 2010;4(1):9-16.
31. Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod. 1997;56(3):602-7.
32. Iwasaki A, Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. Fertil Steril. 1992;57(2):409-16.
33. De Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. J Androl. 1992;13(5):379-86.
34. Kessopoulou E, Tomlinson MJ, Barratt CL, Bolton AE, Cooke ID. Origin of reactive oxygen species in human semen: spermatozoa or leucocytes? J Reprod Fertil. 1992;94(2):463-70.
35. Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. Mol Hum Reprod. 1998;4(5):439-45.