Atrazine Induced Morphological Alterations in Spermatocytes of Goat in vitro

R.K. Sharma, P.K. Chauhan and A. Fulia

The presence of pesticide residues in food, in soils and sediments as well as in run-off water are ubiquitous environmental toxicants that pose a risk to human health. Aim of the present investigation was to study the effect of different doses of atrazine on spermatocytes of Capra hircus in vitro. Small pieces (approximately 1 mm³) of testicular tissue were divided into one experimental and one control group; the experimental group was treated with atrazine (1 × 10⁻³, 1.0 and 100 nmol mL⁻¹) and exposed for different durations. Pycnosis, chromolysis and vacuoles of varied sizes and shapes were frequently observed in spermatocytes due to the atrazine exposure. All the degenerating changes increased as the exposure duration was increased from 1 to 4 and 4 to 8 h. The relative frequency of atretic spermatocytes was 30, 38 and 44% after 1, 4 and 8 h, respectively at 1 × 10⁻³ nmol mL⁻¹ atrazine concentration. Ultra-structurally, shrinkage in cytoplasm of zygotene primary spermatocytes was distinct and prominent, chromolysis, ruptured membrane of turgid mitochondria and Golgi bodies were scattered in the spermatocyte cytoplasm.

Key words: Atrazine, spermatocytes, histomorphology, ultrastructure, Capra hircus

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INTRODUCTION

The presence of pesticide residues in food, in soils and sediments as well as in run-off water are ubiquitous environmental toxicants that pose a risk to human health (Bain and LeBlanc, 1996; Bain et al., 1997). A wide variety of biocides have been reported that depict toxicity to target arthropods as well as to other vertebrates and mammals (Das et al., 2006; Koc et al., 2009; Joseph and Raj, 2011). Acute toxicity of endosulphan have been observed in Japanese quails by Prakash et al. (2009). Nano-molar concentration of endosulphan induced alterations in fine morphology of goat spermatozogonia (Sharma et al., 2010). It has been reported that exposure to certain chlorotriazine herbicides (i.e., Atrazine, Simazine, or Cyanazine) induce a persistent estrous condition in certain strains of rats (i.e., Sprague Dawley but not Fischer 344) (Eldridge et al., 1994). Reproductive health is very sensitive to environmental conditions (physical, biological, behavioral and social). Due to the variations in environmental characteristics, the relative effects of various pesticides vary in different parts of the world or even within the same country (Kaiser, 2005). Exposure of sub-lethal concentrations of these biocides induced alterations in reproductive efficiency in mammals (Ray et al., 1991; Chapin et al., 1990). DDT, organotin compounds, various azole fungicides and large number of 2-chloro-a-triazine herbicides alters the steroidogenic activities in animals (Sanderson and Van Dan Berg, 2003). In an earlier study, it has been observed that there was increased risk of genital malformation in the children whose parents were exposed with pesticides during their occupation (Weidner et al., 1998). Atrazine induced degeneration in seminiferous tubules of goat in vitro (Sharma and Chauhan, 2009). Triazine herbicides are used to control the weeds with broadleaf and grasses in the agricultural fields all over the world (Tchoumwoa et al., 2000). Atrazine exposure has increased the number of intersex frogs (Hayes et al., 2002a, b, 2003; Carr et al., 2003) and affect normal gonadal development (Taverna-Mendoza et al., 2002a, b). Both the female (Ashby et al., 2002; Rayner et al., 2004) and male (Kniewald et al., 2000; Feldman and Krishan, 1995; Trentacoste et al., 2001) reproductive systems have been affected due to the atrazine exposure in rats. Keeping in view the hazardous effect of atrazine on male fertility, the effect of atrazine on morphology and ultrastructure of spermatocytes of goat Capra hircus has been analyzed in vitro.

MATERIALS AND METHODS

The mature goat (Capra hircus) testis was procured from the slaughter houses around Kurukshetra (29º6’ N, 76º5’ E) and Delhi (28º38’ N, 77º12’ E). The material was brought to the Reproductive Physiology Laboratory, Department of Zoology, Kurukshetra University Kurukshetra at 4ºC in normal saline during year 2009.

**Testicular tissue culture:** After washing with the normal saline the testis was encapsulated and cut into small pieces (approximately 1 mm³) for culture. After washing three times with TCM-199, small pieces of testicular tissue were immediately placed on nucleopore filter and floated on medium. The medium was prepared by mixing TCM-199 and antibiotics (200 unit penicillin 100 U mL⁻¹ and streptomycin 100 g mL⁻¹). The tissues were divided into two groups (one experimental group and one control group). Experimental group was treated with different concentrations of atrazine (1×10⁻³, 1.0 and 100 nmol mL⁻¹) and was further divided into three subgroups (1), (2) and (3) exposed for 1, 4 and 8 h, respectively at temperature 39ºC, humidity 95% and 5% CO₂ concentration and the control was run simultaneously.

![](image)

**Atrazine:**
- **IUPAC name:** 6-chloro-N-(ethyl-N’-isopropyl)-1, 3, 5-triazine-2, 4-diamine
- **Chemical formula:** C₇H₆ClN₃
- **Molecular mass:** 215.7

Harvesting of testicular tissues was carried out after the specified duration and tissues from all the experimental and control groups were processed for histomorphological studies using the standard techniques given by Pearse (1968).

**Statistical analysis:** During the present investigation the under mentioned statistical formulas were used for statistical analysis of the data according to Zar (1984).
• Mean, standard deviation and standard error
• Student “t” test
• Chi-square test

Transmission electron microscopy: Simultaneously harvested testicular tissue from atrazine concentration 1.0 mmol mL⁻¹ was fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer saline (pH 7.2 to 7.4) at 4°C for 24 h. Transmission Electron Microscopy has been conducted by using the method of Zamboni (1976). Ultrathin sections were examined and photographed under electron microscope model installed CM-10 Philips at All India Institute of Medical Sciences, New Delhi.

RESULTS

Histologically, the testis was comprised of seminiferous tubules and interstitium. A large number of seminiferous tubules were packed in the loose connective tissue. Seminiferous tubule of sexually mature goat was characterized by the presence of a series of well-defined cellular associations and stages. In control group, the seminiferous tubule revealed the normal arrangement of different types of cells. All the somatic and germ cells present in the testicular tissue exhibited no change in general contour. Spermatocytes possessed large nucleus and were lying on the basal lamina. Nucleus appeared spherical to elongate in shape. Spermatocytes were clearly visible by light microscope and they were the largest germ cells present in the intermediate part of the seminiferous epithelium (Fig. 1a). Three doses of atrazine (1×10⁻³, 1.0 and 100 mmol mL⁻¹) induced morphological alterations in spermatocytes. After the atrazine exposure, vacuoles of varied sizes and shapes were frequently observed in spermatocytes. Pyknosis and chromolysis were observed and all these changes were increased as the exposure duration increased from 1 to 4 and 8 h. The diameter of spermatocytes were declined from 10.51±0.2052 μm in control to 9.925±0.2520 μm after 1 h, 8.70±0.25 μm after 4 h and 8.30±0.2 μm after 8 h of exposure duration respectively (Table 1, 2 and 3). All the variations recorded were statistically significant at (p<0.05). Decline in nuclear diameter of spermatocytes was from 7.10±0.2395 μm in control group to 7.345±0.188 μm after 1 h, 6.74±0.268 μm after 4 h and 6.11±0.180 μm after 8 h, respectively (Table 1, 2 and 3). The atretic spermatocytes were 36%, 38% and 44% after 1, 4 and 8 h, respectively at 1×10⁻³ mmol mL⁻¹ atrazine concentration (Table 4). Chi-square values were analyzed and 0.831, 1.13 and 1.52 values were obtained after 1, 4 and 8 h of exposure durations respectively, (χ² = 0.05) (Table 5).

As the concentration of atrazine was enhanced to 1.0 mmol mL⁻¹, vacuolization, fragmentation, pyknosis and chromolysis were further enhanced (Fig. 1b). Hyalinisation in spermatocytes was also observed. These changes were increased with increasing dose and exposure duration. At atrazine dose level 1.0 mmol mL⁻¹, there was decline in spermatocyte cell diameter and nuclear diameter. Spermatocytes cell diameter was declined from 10.51±0.2052 μm in control to 9.245±0.3048 μm, 8.24±0.20 μm and 8.00±0.215 μm after 1, 4 and 8 h of exposure duration respectively. All the variations recorded were statistically significant (p<0.05) (Table 1-3). At the same dose level of atrazine, there was decline in nuclear diameter of spermatocytes from 7.10±0.2395 μm in control group to 6.920±0.221 μm, 6.10±0.241 μm and 5.80±0.209 μm after 1, 4 and 8 h of exposure durations respectively. The values were statistically significant at 4 and 8 h (p<0.05) (Table 1-3). The nuclear diameter of atretic spermatocyte was declined from 7.525±0.1969 μm in control to 7.115±0.295 μm, 6.27±0.223 μm and 5.52±0.205 μm after 1, 4 and 8 h of exposure duration, respectively (Table 1-3). The values at 1 and 8 h were statistically significant. Atretic spermatocytes at atrazine dose level 1.0 mmol mL⁻¹ were 40, 46 and 58% after 1, 4 and 8 h respectively (Table 4). Chi-square values were recorded and 3.78, 3.47 and 6.827 after 1, 4 and 8 h of exposure duration, respectively and 3.78 and 6.827 are statistically significant (χ² = 0.05) (Table 5).

As the atrazine concentration further elevated to 100 mmol mL⁻¹, there were further increases in atretogenic changes in spermatocytes. Hyalinisation, chromolysis and large vacuoles in cytoplasm were clearly observed. The spermatocyte diameter declined from 10.51±0.2052 μm in control to 7.915±0.2972 μm after 1 h, 7.5±0.211 μm after 4 h and 7.14±0.225 μm after exposure of 8 h. All the values were statistically significant at (p<0.05) (Table 1-3). Nuclear diameter of spermatocytes was declined from 7.10±0.2395 μm in control to 6.33±0.2649 after 1 h, 5.30±0.217 μm after 4 h and 4.87±0.225 μm after 8 h of exposure. All the variations recorded were statistically significant (Table 1-3). There was also decline in nuclear diameter of atretic spermatocytes from 7.525±0.1969 μm in control to 6.84±0.191 μm after 1 h, 4.55±0.141 μm after 4 h and 3.82±0.148 μm after 8 h. All the variations recorded were statistically significant (p<0.05) (Table 1-3). The spermatocytes at advanced stage of atresia were observed and the atretic spermatocytes were 48, 58 and 66% after 1, 4 and 8 h of exposure durations respectively (Table 4). Chi-square values were analyzed and 7.42, 6.98 and 11.56 values were obtained. Chi-square values were statistically significant (χ² = 0.05) (Table 5).
Fig. 1(a-f): (a) Light micrograph of control testicular tissue depicting a portion of seminiferous tubule with normal arrangement of different germ cells and somatic cells and also revealing large sized Leydig cell with round shape nucleus present in interstitial tissue (X 1000), (b) Light micrograph of testicular tissue treated with atrazine exhibiting vacuolization, condensation and pycnosis in spermatocyte. (X 1000), (c) Transmission electron micrograph of control testicular tissue showing a magnified view of spermatocyte (Spc) and round spermatid (Spd). Higher level of condensation was observed in the nucleoplasm of spermatocyte and nucleus is granulated (X 4000), (d) Transmission electron micrograph of testicular tissue treated with atrazine (1.0 nmol mL$^{-1}$) for 1 h showing degeneration of almost half of the nuclear membrane (arrow) in spermatocytes (Spc). Cytoplasm was vacuolated (V). Chromolysis and condensation (Cd) in nuclear material were noticed. Intracellular vesicles (v) were also prominent (X 3500), (e) Transmission electron micrograph of testicular tissue treated with atrazine (1.0 nmol mL$^{-1}$) for 4 h displaying increase in number of necrotic spermatocytes (Spc). Condensed chromatin (Cd), Chromolysis (Ch), intranuclear vesicles (v) and cytoplasmic vacuolization (V), ruptured nuclear membrane and pinching off of chromatin material, Lipid droplets were also noticed (X 3500) and (f) Transmission electron micrograph of testicular tissue culture treated with atrazine (1.0 nmol mL$^{-1}$) for 8 h exhibiting increased vacuolization, condensation nuclear material in spermatocytes. Chromolysis (Ch), disrupted intercellular junction and ruptured Golgi vesicles were noticed in the heterogeneous cytoplasm (X 3500)
Table 1: Effect of different concentrations of atrazine on spermatoocytes after 1 h of exposure duration

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Concentration (10^-4 mmol mL^-1)</th>
<th>Concentration (1.0 mmol mL^-1)</th>
<th>Concentration (100 mmol mL^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of spermatoocyte</td>
<td>10.51±0.2052</td>
<td>9.925±0.252*</td>
<td>9.245±0.348*</td>
<td>7.915±0.2972*</td>
</tr>
<tr>
<td>Diameter of nucleus</td>
<td>7.10±0.2305</td>
<td>7.345±0.188</td>
<td>6.920±0.221</td>
<td>5.630±0.2649*</td>
</tr>
<tr>
<td>Atretic cell nuclear diameter</td>
<td>7.25±0.1969</td>
<td>7.340±0.185</td>
<td>7.115±0.295*</td>
<td>6.840±0.191*</td>
</tr>
</tbody>
</table>

*Statistically significant (p<0.05)

Table 2: Effect of different concentrations of atrazine on spermatoocytes after 4 h of exposure duration

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Concentration (10^-4 mmol mL^-1)</th>
<th>Concentration (1.0 mmol mL^-1)</th>
<th>Concentration (100 mmol mL^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of spermatoocyte</td>
<td>9.77±0.1969</td>
<td>8.70±0.255*</td>
<td>8.24±0.200*</td>
<td>7.50±0.211*</td>
</tr>
<tr>
<td>Diameter of nucleus</td>
<td>6.91±0.2926</td>
<td>6.74±0.208*</td>
<td>6.10±0.241*</td>
<td>5.30±0.217*</td>
</tr>
<tr>
<td>Atretic cell nuclear diameter</td>
<td>6.70±0.2626</td>
<td>6.47±0.185</td>
<td>6.27±0.221</td>
<td>4.55±0.141*</td>
</tr>
</tbody>
</table>

*Statistically significant (p<0.05)

Table 3: Effect of different concentrations of atrazine on spermatoocytes after 8 h of exposure duration

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Concentration (10^-4 mmol mL^-1)</th>
<th>Concentration (1.0 mmol mL^-1)</th>
<th>Concentration (100 mmol mL^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of spermatoocyte</td>
<td>8.90±0.2580</td>
<td>8.30±0.200*</td>
<td>8.00±0.215*</td>
<td>7.14±0.225*</td>
</tr>
<tr>
<td>Diameter of nucleus</td>
<td>6.31±0.2050</td>
<td>6.11±0.180*</td>
<td>5.8±0.209*</td>
<td>4.87±0.225*</td>
</tr>
<tr>
<td>Atretic cell nuclear diameter</td>
<td>6.25±0.1902</td>
<td>6.06±0.224</td>
<td>5.52±0.205*</td>
<td>3.82±0.148*</td>
</tr>
</tbody>
</table>

*Statistically significant (p<0.05)

Table 4: Percentage of atretic spermatoocytes observed after different exposure durations of atrazine

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Concentration (10^-4 mmol mL^-1)</th>
<th>Concentration (1.0 mmol mL^-1)</th>
<th>Concentration (100 mmol mL^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h Atretic cells</td>
<td>22%</td>
<td>30%</td>
<td>40%</td>
<td>48%</td>
</tr>
<tr>
<td>4 h Atretic cells</td>
<td>28%</td>
<td>38%</td>
<td>46%</td>
<td>58%</td>
</tr>
<tr>
<td>8 h Atretic cells</td>
<td>32%</td>
<td>44%</td>
<td>58%</td>
<td>68%</td>
</tr>
</tbody>
</table>

Table 5: The comparison of number of atretic spermatoocytes between control (1.4 and 8 h) and different concentrations of atrazine (10^-4, 1.0 and 100 nmol mL^-1) showing Chi-square values

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Concentration (10^-4 mmol mL^-1)</th>
<th>Concentration (1.0 mmol mL^-1)</th>
<th>Concentration (100 mmol mL^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>0.831</td>
<td>3.78*</td>
<td>7.42*</td>
</tr>
<tr>
<td>4 h</td>
<td>1.13</td>
<td>3.47*</td>
<td>6.98*</td>
</tr>
<tr>
<td>8 h</td>
<td>1.527</td>
<td>6.827*</td>
<td>11.563*</td>
</tr>
</tbody>
</table>

*Statistically significant difference (χ² = 0.05)

Transmission electron microscopy: In the cycle of seminiferous epithelium, spermatoocytes occupied the next layer to that of the spermatogonia. By the transmission electron microscopy, spermatoocytes at different stages of meiosis were noticed. Condensed clumps of chromatin material were observed within the nucleus. Nucleus of spermatoocyte was granulated and almost round in control group (Fig. 1c). In the cytoplasm, large number of round to oval mitochondria with lamellar cisternae was scattered. Round mitochondria and endoplasmic reticulum was also noticed in the spermatoocytes and they were more frequently encountered.

After the exposure of atrazine (1.0 nmol mL^-1) concentration, degeneration in fine morphology of spermatoocytes was noticed. Small vacuoles were observed in cytoplasm of pachytene primary spermatoocytes and diplotene spermatoocytes after 1 h of exposure duration. Nuclear membrane was degenerated at some places. Almost half of the degenerated nuclear membrane at one end was clearly visible in spermatoocytes. Shrinkage in cytoplasm of zygotene primary spermatoocytes was observed due to the atrazine exposure. Chromolysis, condensation in chromatin material in nucleus was noticed. Ruptured membrane of turgid mitochondria and Golgi bodies were observed in the spermatoocyte cytoplasm (Fig. 1d). As the exposure duration enhanced from 1 to 4 h, number of necrotic spermatoocytes increased. These degenerated changes were increased after 4 h of exposure duration. Vacuolization was enhanced in cytoplasm of the spermatoocytes. Vacuoles of larger sizes were observed as compared with 1 h of exposure duration. Highly condensed chromatin material was observed in the spermatoocytes exposed with atrazine concentration 1.0 nmol mL^-1. Chromosomal margination was noticed. Very large vacuoles were observed in the nucleus of the spermatoocyte. Pinching of chromatin material into the large vacuoles present in the nucleoplasm was also observed. Nuclear membrane was degenerated at certain places. Vacuoles of various sizes and shapes were observed in the cytoplasm. Swollen round mitochondria were noticed due to the atrazine exposure. Debris of ruptured mitochondria and Golgi body was noticed in cytoplasm around the nucleus. Intercellular junction between different cells was affected (Fig. 1e).
After 8 h of exposure duration of atrazine (1.0 nmol mL\(^{-1}\)), nuclear membrane was degenerated in spermatocytes. Cytoplasm was filled with vacuoles. As the exposure duration further enhanced to 8 h, atrazine treatment (1.0 nmol mL\(^{-1}\)) showed more advanced alterations. Margined chromatin along the nuclear membrane was observed. Vacuoles were enlarged in size. Apoptotic spermatocytes were observed after 8 h of exposure duration. Nuclear membrane was ruptured at more places. Large abnormal vacuoles were noticed in the nucleoplasm of spermatocytes. Chromolysis and chromatin condensation was observed due to atrazine exposure. Apoptotic zygotene spermatocytes were also observed frequently. Due to atrazine treatment, abnormal cytoplasm was noticed. Swollen and ruptured mitochondria were observed in cytoplasm. Golgi vesicles in cytoplasm of spermatocytes were found. Intercellular bridges were lost due to the atrazine treatment. Nucleoplasm was degenerated. These degenerated changes due to atrazine (1.0 nmol mL\(^{-1}\)) treatment were enhanced in the time dependant manner. Pinching of nuclear material was also observed. Degenerated nuclear membranes were noticed (Fig. 1f).

**DISCUSSION**

During the present investigation, in vitro evaluation of different doses of atrazine on mature goat (*Capra hircus*) testis was studied. Three dose levels, 1×10\(^{-5}\), 1.0 and 100 nmol mL\(^{-1}\) of atrazine were used for 1, 4 and 8 h of exposure in vitro on the goat testicular tissue. The results of the experimental study revealed that atrazine affected the testicular histomorphology and induced severe degeneration in spermatocytes. The fine details of the cellular damage induced by atrazine have been analyzed using transmission electron microscopy. The ultrastructural changes induced by pesticides include vacuolization, clumping of nuclear material, shrinkage of cytoplasmic and nuclear membrane, swollen and ruptured mitochondria, abnormal vesicles and multilayered bodies. The observations of the present histological study demonstrated that atrazine induced vacuolization in the spermatogenic cells, strongly support the histopathological study by Moustafa *et al.* (2007) wherein they have studied the effect of pesticide profenofos, on the nuclei of the spermatogonia and reported appearance of vacuoles and these vacuoles included dense and coalesced materials possibly resulted from destruction and degeneration of some organelles. The present findings that atrazine induced degeneration in nuclear material are in consistent with the earlier findings of Nehez *et al.* (1982) that pesticide induced alteration in nuclear material. The present study indicates that atrazine induced atretic changes in germ and in somatic cells which possibly will lead to impair the male fertility. Histological analysis of testicular tissue of goat from treated group showed the cell disorganization and formation of cell clusters with spermatocytes. Similar changes have been observed by Eldridge *et al.* (1999) wherein atrazine was shown to adversely affect reproductive tissues in the rat. The results of the present investigation demonstrated that atrazine induced pyonosis in the spermatocyte nuclei. Pyonosis was also reported by Almansour (2009) due to the exposure of lead in bird spermatocytes. These ultrastructural changes induced by the atrazine support the findings of earlier researchers who have observed the similar set of variations triggered by different endocrine disrupters (Barchielli *et al.*, 1982, Mangelsdorf *et al.*, 2003). According to Bremer *et al.* (2005), in the normal testis, these three major cell types have been shown to be sensitive to chemical insult. In addition, the epididymis, prostate gland and seminal vesicles, as well as hormone-receptor interactions and hormone production by the endocrine glands are altered by reproductive toxicants (Bremer *et al.*, 2005). The present study demonstrated that atrazine was able to enhance apoptosis at low doses and at higher doses it led to the cellular necrosis in the testis. The results of the present study strongly advocate the findings of Liu *et al.* (2006) who observed with the help of fluorescent and transmission electron microscopy that the atrazine incubated cells displayed a series of morphological changes, including condensation of the nuclear material, margination of chromatin material to form dense granular caps and formation of apoptotic bodies were noticed. Endocrine disrupting compounds may alter the germ cell attachment which leads to sloughing off different types of cells present in the seminiferous tubules. Exposure of endocrine disrupting chemical also disturbed the apical cytoskeletal transport or induced microtubule dependent transport defects Foster *et al.* (1984). All these changes resulted into the germ cell loss and the disruption of the seminiferous epithelium.

**CONCLUSION**

Nano-molar concentration of atrazine induced increase in atretic percentage of spermatocytes and also induced increased in alteration in fine morphology of spermatocytes leading to decrease in fertility of goat *Capra hircus*. The results will be of great value in assessing the health hazards of atrazine to domestic animals and can provide important clues about the damage induced by Nano-molar concentrations of atrazine in reproductive potential of mammals.
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