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IS ATRAZINE A POTENTIAL RISK ON MAMMALIAN DIVERSITY?

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Modern agricultural practices usually include the intensive use of a number of conventional pesticides, which can act as endocrine disrupting compounds (EDC) and for this reason may adversely affect species diversity. The aim of this study was to evaluate the impact of EDC herbicide atrazine (ATR) on the mammalian reproductive ability. For this purpose, effects of atrazine on morphological and morphometrical characteristics of Leydig cells of Wistar rats testes were evaluated. Animals were treated from 23rd to 53rd postnatal day (PND) with atrazine in doses of 50 mg/kg of body weight (bw) and of 200 mg/kg bw. Our results have shown that both doses have caused a decrease in body and testicular weight in a dose-responsive manner. Also, both of these treatments caused an increase in interstitial space volume of testes and change in number, nucleocytoplasmic ratio and degree of vacualisation of Leydig cells. In this study we have shown that atrazine affects the structure and number of Leydig cells in a way that it can decrease the reproductive capability of rats, as well as other male mammals which is a serious and growing threat to mammalian biodiversity regarding the fact that the herbicide atrazine is excessively used in non-EU countries, as well as in the United States.

Key words: atrazine, endocrine disrupting chemicals-EDCs, Leydig cells, mammalian reproduction, mammalian biodiversity

INTRODUCTION

Biodiversity (biological diversity) in relation with the effects of endocrine disrupting chemicals (EDC) is a particularly important subject of multidisciplinary environmental research.

The biggest driver of terrestrial biodiversity loss in the past 50 years has been habitat conversion, in large part due to conversion of natural and semi-natural landscapes to agricultural fields which greatly contributed to the disappearance of natural habitats. In highly fragmented landscapes, as agricultural fields usually are, populations occupy small habitat fragments and

become more isolated, further reducing effective population size and threatening the viability of a given population (Hanski, 2002). Habitat fragmentation and its destruction reduce the population size of the species which in turn causes the reduction of genetic diversity in small populations and decrease of the evolutionary potential of species (Van Dyke, 2003). Modern agricultural practices, including the intensive use of conventional pesticides, have been broadly linked to declines in biodiversity in agro-ecosystems. The intensive use of pesticides in agro-ecosystems for controlling insects, plant pathogens and weeds can influence the biodiversity of many species for the reason that high percentage of applied pesticides reach and affect nontarget organisms and spread to ecosystems, adjacent to agricultural lands, as chemical pollutants (Secretariat of the Convention on Biological Diversity, 2008). Thus, terrestrial, but also aquatic biodiversity within and around agricultural fields, has also been strongly influenced by agricultural practices (Tilman, 1999; Tilman *et al.* 2002). On this way, pesticides, a number of which can act as endocrine disrupting compounds (EDCs), cause pollution of natural ecosystems and can adversely affect species diversity, ecosystem stability, food chains, energy flow, nutrient cycling, genetics of organisms and physical resources (Pimentel and Edwards, 1982). Having the potential negative effect on animal organisms, especially on the reproductive system, EDCs can have a great impact on mammalian populations. For these reasons, the relation between biodiversity and reproductive effects of EDCs is a very important subject of multidisciplinary environmental research (Exon, 1984; La Rocca and Mantovani, 2006; Hayes *et al.* 2002a; Crews and McLachlan, 2006).

The term EDC is commonly used to describe environmental agents that alter the endocrine system, causing adverse effects at the level of the organism, progeny, population, or subpopulation. ESCs have hormonal activity *in vivo*, and can therefore interact and perturb normal physiological functions (US EPA, 1998). Also, EDCs are known to mimic the actions of sex steroid hormones and to cause serious dysfunctions of reproductive organs through disruption of endocrine homeostasis (McLachlan, 2001). Studying of EDCs effects on reproduction and development is of great interest, since disturbances of these processes can lead to reduction of the population size, reduction of genetic diversity and evolutionary potential of species.

Chlorotriazine herbicide atrazine (IUPAC: 6-chloro-N_2-ethyl-N_4-isopropyl-1,3,5-triazine-2,4-diamine), as an EDC, is excessively used on crop fields to control broadleaf weeds in the production of corn, sugar cane and sorghum (US EPA, 2001). Due to its heavy use, the toxicological profile of this herbicide has been investigated over the years. To date, toxicity of atrazine found in both wildlife and laboratory conditions was primarily related to morphological and physiological endocrine and reproductive endpoints (Stoker *et al.* 2000; Friedman, 2002; Jooste *et al.* 2005; Solomon *et al.* 2008). The aim of this study was to evaluate the impact of atrazine on the number and structure of Leydig cells, which produce the male sex hormone testosterone essential for the maintenance of spermatogenesis and secondary sex characteristics and determines the male reproductive capacity. Potential adverse impact of atrazine on Leydig cells can lead to the reduction of the population size and disappearances of some species

and in that way influence the species diversity. Our studies were conducted on laboratory rats, as the most widely studied mammalian experimental model.

MATERIAL AND METHODS

Animals

The study of atrazine effect on testicles was performed on male Wistar rats subjected to treatment from 23rd to 53rd postnatal day (PND). This particular period of ontogeny (PND 23 to 53) was chosen according to the EPA (Environmental Protection Agency, USA) protocol for the assessment of the impact of endocrine-disrupting compounds on male rats (Stoker *at al.* 2000). During the experiment, animals were kept in conditions of controlled light (14h light, 10h dark) and temperature (23^{o}C ± 2^{o}C). They were fed standard food for rats and drank *at libitum*.

The investigation was made with the permission of the Ethical Committee on Animal Experiments of the University of Novi Sad.

Experimental procedure

On 22. PND animals were divided into three groups. Each group consisted of 10 rats with similar initial body weights. The first and second group were receiving atrazine *per os* technical atrazine solution (98% purity, a gift from professor Sanja Lazić, Institute for Environmental and Plant Protection, Faculty of Agriculture, University of Novi Sad, Serbia) in edible olive oil in a dose of 50 mg/kg bw and 200 mg/kg bw respectively, administered daily between 08.00 and 09.00 AM. The third group was the control which was given the corresponding amount of olive oil only (placebo treatment). Atrazine treated animals, as well as control animals, were sacrificed by decapitation on 53 PND, after daily treatments. Testicles were taken for histological examination. Animal body and testicular mass of both atrazine treated groups and control group, were weighed.

Light-microscopy

After sacrificing the animals, the testicles were removed, fixed in Bouin's solution, embedded in paraffin and sectioned on Reichert's rotation microtome into serial 5 μm thick slices. For histological analysis and quantitative estimation of Leidyg cells and intratesticular tissue the histochemical staining method with hematoxylin and eosine was used.

Stereological analysis

The testicle sections of rats from each of the experimental groups were analyzed using a multipurpose stereological grid M42 placed in the ocular of a Reichert's light microscope under total magnification of x400 and x1000. The point counting method (Weibel *et al.* 1966) was performed on 100 fields of vision per testis for the estimation of the volume density of testicular interstitium. Also, volume density of cytoplasm (Vvc) and nucleus (Vvn) of 100 Leydig cells per testicle were determined and used to calculate the nucleocytoplasmic volume ratio (Nv=Vvn/Vvc) of these cells.

The data which were obtained by examining particular stereological parameters in the control and atrazine treated animals was statistically analyzed by analysis of variance (followed by Duncan's test) and Kruskal-Wallis test. *p*-values less than 0.05 were considered significant.

RESULTS

Effect of atrazine on body and testicles weight

Daily treatment with both doses of atrazine, 50 mg/kg bw and 200 mg/kg bw, significantly influenced body mass of animals. The treatment with 50 mg/kg bw resulted in a statistically significant ($p<0.05$) reduction in body weight compared to control animals. The treatment with 200 mg/kg bw also resulted in a statistically significant ($p<0.001$) reduction in body weight compared to control animals (Table 1).

Also, treatment with atrazine in both doses, 50 mg/kg bw and 200 mg/kg bw, caused a statistically significant reduction in testicular weight compared to control animals (both at $p<0.05$), whereas a significant difference was not found between treatment with low and high dose of atrazine (Table 1).

Table 1. Body and testes weight of rats treated with atrazine in doses of 50 mg/kg bw (ATR50) and 200 mg/kg bw (ATR 200)(Mean value ±SE)

	Control group	ATR 50	ATR 200
Body weight [g]	163 ±16.82	163±16.82	128.8±11.63
Testis weight [mg]	765.13±88.06	656.83±77.73	625.46±75.35

Histological and stereological analysis of testicular interstitium and Leydig cells

Histological analysis showed that extensive widening of the interstitial space of testicles was the most prominent morphological change in the testicles of rats treated with low or high, doses of atrazine. However, these changes were more prominent in the low dose treatment (Fig. 1, Fig 2).

Stereological analysis, also, demonstrated an increase in volume density of interstitial spaces for both treatments with atrazine, but this increase was not statistically significant compared to the control and also to both atrazine treatments (Fig. 3).

Histological analysis has showed that treatment with both doses of atrazine (50 mg/kg bw and 200 mg/kg bw) resulted in changes of Leydig cell number and morphology (Fig. 2, Fig.4). Leydig cells were easily recognized with their deeply stained eosinophilic cytoplasm and prominent nuclei by hematoxilin – eosine (HE) dying technique. Lower dose of atrazine slightly increased the number of Leydig cells and induced much more irregularity in their distribution and arrangement compared to control animals (Fig. 4).

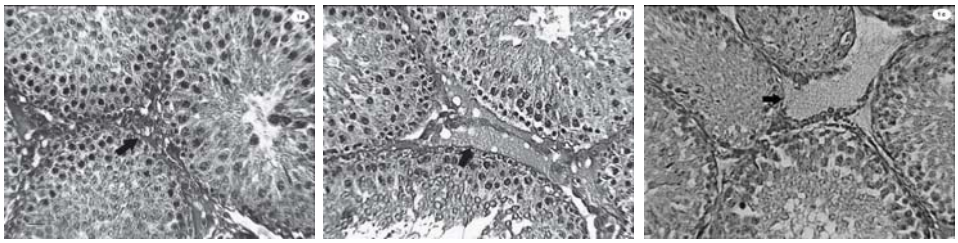

Figure 1. Rat testicle. Leydig cells and interstitial connective tissue (arrows). Bouin, HE.
a) Control animals, b) Animals treated with 50 mg/kg bw of atrazine, c) Animals treated with 200 mg/kg bw of atrazine. All figures are of same magnification. Scale bar, 50 μm

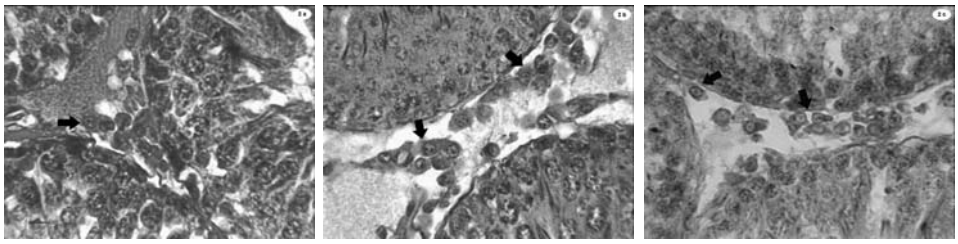

Figure 2. Rat testicle. Leydig cells (arrows). Bouin, HE.
a) Control animals, b) Animals treated with 50 mg/kg bw of atrazine, c) Animals treated with 200 mg/kg bw of atrazine. All figures are of same magnification. Scale bar, 15 μm

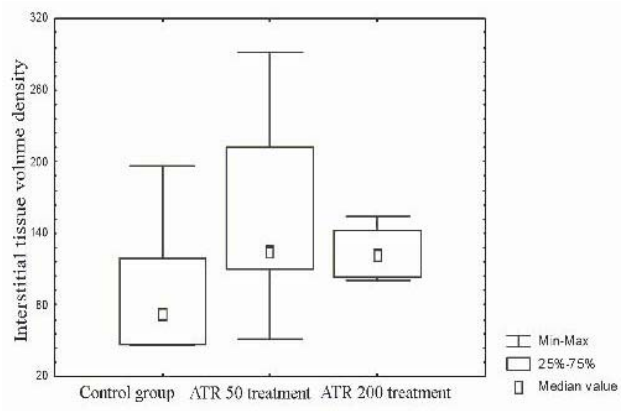

Figure 3. Interstitial tissue (spaces) volume density (mm^0) of testicles of control animals and animals treated with atrazine. Control group - placebo treatment, ATR 50 – 50 mg/kg bw, ATR 200 – 200 mg/kg bw

In the control group, these cells were usually round, arranged in a more or less regular triangular formation (Fig. 4a), while in animals treated with atrazine,

Leydig cells were arranged rather in the form of smaller or larger islands (Fig. 4b) or in the form of stripes (Fig. 4c). Treatment with 50 mg/kg bw of atrazine, also, led to a slight increase in the volume of Leydig cells compared to control animals (Fig. 2a-c), and in an increase of its nucleocytoplasmic volume ratio, but this increase was not statistically significant compared to control animals (Fig. 5).

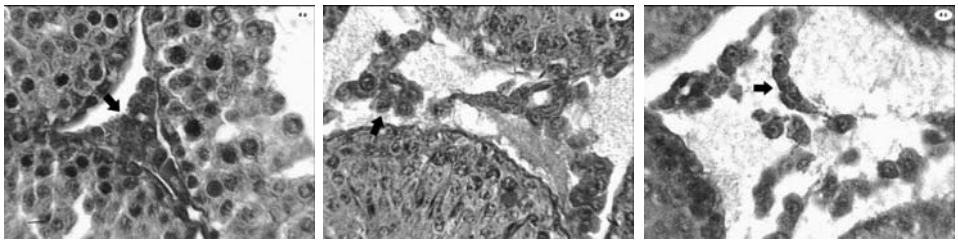

Figure 4. Rat testicle. Grouping of Leydig cells in interstitial connective tissue (arrows). Bouin, HE
a) Control animals; b) and c) Animals treated with 50 mg/kg bw of atrazine. All figures are of same magnification. All figures are of same magnification. Scale bar, 15 μm

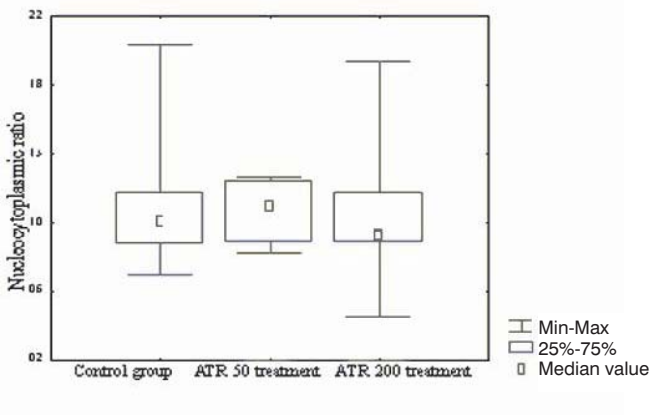

Figure 5. Nucleocytoplasmic volume ratio of Leydig cells in testicles of control animals. Control group - placebo treatment, ATR 50 – 50 mg/kg bw, ATR 200 – 200 mg/kg bw

Leydig cells are characterized by irregular shape and well expressed nucleus and nucleolus. Unlike the control, in the cytoplasm of many of these cells a larger number of vacuoles is well evident reflecting, probably, the presence of dilated smooth endoplasmic reticulum (SER) (Fig. 6a).

Treatment of rats with 200 mg/kg bw of atrazine resulted in somewhat different morphological changes of Leydig cells than those observed in the testicles of rats treated with lower dose. Namely, this treatment led to a reduction in the number of Leydig cells compared to the control animals (Fig. 7a-b).

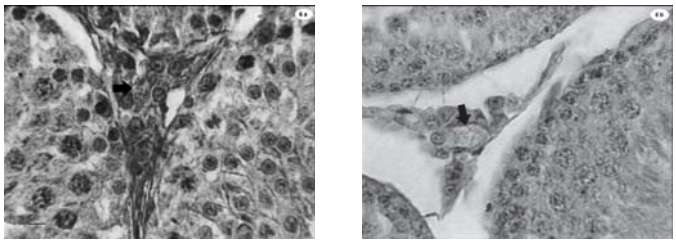

Figure 6. Rat testicle. Leydig cells with vacuolated cytplasm (arrows). Bouin, HE.
a), b) Animals tested with 50 mg/kg bw of atrazine; c) Animals tested with 200 mg/kg bw of atrazine. All figures are of same magnification. Scale bar, 15 μm

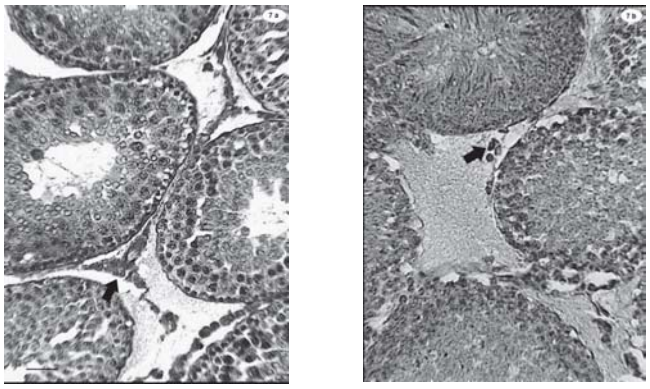

Figure 7. Rat testicle. Leydig cells in interstitial connective tissue (arrows). Bouin, HE
a) Control animal; b) Animal treated with 200 mg/kg bw of atrazine. All figures are of same magnification. Scale bar, 50 μm

In some parts of the testicles their number was drastically reduced (Fig. 8a-b). However, this treatment induces not only a decrease of Leydig cells number, but also change of their arrangement in testicular interstitium compared to the control animals. Thus, they are more elongated and can be seen mostly in isolated groups of only few cells in the widened interstitial spaces, on a distance from the seminiferous tubules (Fig. 2c, Fig. 9, Fig. 10).

In 200 mg/kg bw treatment, as well as in treatment with 50 mg/ kg bw of atrazine, Leydig cell cytoplasm was vacuolated but the diameter of their vacuoles and number of visualized cells was much higher than in the treatment with the lower dose of atrazine (Fig. 6b).

Nucleocytoplasmic volume ratio of Leydig cells in this treatment is decreased compared to control animals, but this decrease was not statistically significant (Fig. 2, Fig.5).

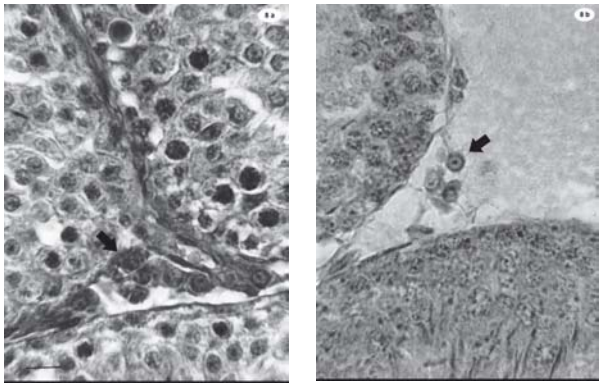

Figure 8. Rat testicle. Leydig cells (arrows). Bouin, HE.
a) Control animal; b) Animal treated with 200 mg/kg bw of atrazine. All figures are of same magnification. Scale bar, 15 μm

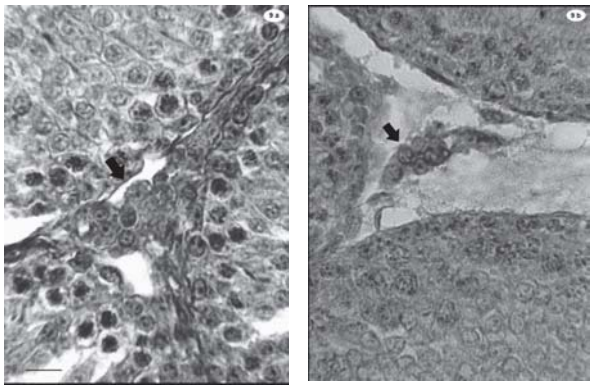

Figure 9. Rat testicle. Leydig cells (arrows). Bouin, HE.
a) Control animal; b) Animal treated with 200 mg/kg bw of atrazine. All figures are of same magnification. Scale bar, 15 μm

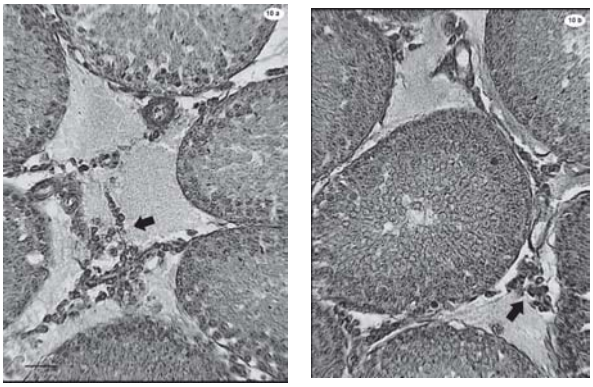

Figure 10. Rat testicles.Leydig cells (arrows). Bouin, HE.
a), b) Animals treated with 200 mg/kg bw of atrazine. All figures are of same magnification. Scale bar, 15 μm

DISCUSSION

Peroral treatment of peripubertal male Wistar rats with atrazine in doses of 50 and 200 mg/ kg bw respectively, conducted in the experiment, showed a dose dependent effect of ATR on the body and testicles weight, the number and

structure of Leydig cells and, also, on the volume of interstitial space which provided an additional insight on ATR male reproductive effects. Our findings present that application of a lower dose of atrazine has resulted in significant reduction in testicular and body weight and in a significant increase in the volume of interstitial space compared to control animals. Also, a slight increase in the number and nucleocytoplasmic volume ratio of Leydig cells, as well as prominent vacuolization of these cells were observed. A higher dose of atrazine caused a statistically significant decrease in body and testes weight, and in a decrease in the number of Leydig cells and reduction in their nucleocytoplasmic volume ratio compared to control animals. In addition, Leydig cells showed much more relevant morphological changes. These cells had a more pronounced cytoplasmic vacuolization when compared to control animals and to animals treated with the lower dose of ATR. Also, an increase of interstitial space volume was present, but not significant compared to the control group.

Since the late 1950s, ATR is probably the most widely used pesticide in the world (Short and Colborn, 1999, Kiely *et al.* 2004). As a result, ATR is one of the most common contaminants of ground and surface water (Gerecke *et al.* 2002) where it can persist for several years after application (Goldman, 1994). However, toxicity of atrazine found in both wildlife and laboratory conditions was primarily related to the morphology and physiology of reproductive organs. Atrazine, even at low concentrations, has a detrimental effect on reproduction in amphibians (Hayes *et al.,* 2002b; 2003), and at high doses in mammals (Babić-Gojmerac *et al.,* 1989; Stoker *et al.,* 2000).

Our results are consistent with the results which indicate that in addition to the atrazine impact on spermatogenesis *(*Carlsen *et al.,* 1992*;* Šimić *et al.,* 1994*;* Kniewald *et al*. 2000), it also has an impact on steroidogenesis. Disturbance of steroidogenesis by atrazine has been reported in mammals (Babić-Gojmerac *et al.* 1989; Kniewald *et al.* 1995; Sanderson *et al.* 2000, 2001) and reptiles (Crain *et al.* 1997). Atrazine may interfere with male hormone regulation and activity possibly through the activation of aromatization which can result in a reduction in androgen levels (as androgens are the substrate for aromatase) (Sanderson *et al.,* 2000; Hayes *et al*., 2002a; 2002b). Stocker *et al.* (2000) report a reduction in intratesticular testosterone level and increase in serum estrone and estradiol levels, after exposure of rats to 200 mg/kg bw of atrazine. Atrazine has effects on estrogenic activity, but it also has been reported to inhibit androgen receptor function *in vitro* (Danzo, 1997). Also, oral treatment with atrazine in a dose of 100 or 200 mg/kg suppressed the pulsatile release of GnRH (Cooper *et al.*, 1996) and reduced serum luteinizing hormone (LH) level (Stoker *et al.*, 2000) which can decrease Ledyg cells testosterone production. On the other hand, the application of atrazine *in vivo* and *in vitro* causes a decrease in activity of 5α-reductase, an enzyme responsible for conversion of testosterone to the 5α-dihidrotestosterone in the adenohypophysis and prostate (Kniewald *et al.* 1995). Atrazine in a dose of 100 mg/kg/day induces pubertal delay in male and female rats (Laws *et al*., 2000; Stoker *et al*., 2000; Trentacoste *et al*., 2001). However, mode and mechanisms of action of ATR on pubertal timing is unknown and deserve further investigation.

Intraperitoneally applied atrazine in a dose that is 12.5 and 25 times less than the LD_{50} dose (which value for rats is 1500 and 2000 mg/kg bw) causes disintegration and vacuolization of Leydig cells in the testes of Fisher rats (Kniewald *et al*., 2000). We observed the same phenomena in Leydig cells after treatment with both doses of atrazine which proves the harmful impact of ATR on steroidogenesis. According to some authors, cytoplasmic vacuoles are formed under various conditions in tissues and in cultured cells. This process probably occurs through several different pathways involving different cell organelles (Henics and Wheatley, 1999., Isobe *et al*., 2003, Abrami *et al*., 1998), such the endoplasmic reticulum (ER), lysosomes, and mitochondria (Papini *et al*., 1994; Wong *et al*., 1995; Dal Canto and Gurney, 1995; Abrami *et al*., 1998). The diversity of cytoplasmic vacuoles, with regard to their structural origin and underlying mechanisms by which respective types of vacuoles are formed, has not yet been investigated in detail. In the field of cell pathology, cell deteriorations characterized by cytoplasmic vacuolization are called vacuolar degeneration. We believe that vacuolization of Leydig cells in our experiment was due to cytotoxicity of atrazine and probably resulted in decreased Leydig cell steroidogenic activity. This hypothesis can be supported with results of the first examination of atrazine effects at the proteomic level in human cells which revealed a total of 22 differentially expressed identified proteins, predominantly involved in transcription processes, stress regulation and structural components and indicate that atrazine treatment seemed to decrease the activity of these cells (Lasserre *et al*., 2009). They found that atrazine has effects on the expression of several proteins located in different cell compartments and is involved in various processes such as oxidative stress, DNA damages and shape of the cell, gene expression regulation or spermatogenesis. These findings may contribute a lot to understanding of the harmful effects of atrazine on Leydig cells in our experiment, but also of molecular mechanisms on general impacts of atrazine. If so, decrease of body and testes weight of atrazine-treated animals observed in our experiment, like those already recorded in similar experiments (Kniewald *et al.,* 2000; Laws *et al.,* 2000; Stoker *et al.,* 2000; 2002) could be explained by the same mechanisms. In addition, normal testis weight varies only modestly within a given test species and this relatively low inter animal variability suggests that decrease of absolute testis weight should be a precise indicator of gonadal injury (Schwetz *et al.* 1980; Blazak *et al.* 1985).

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ATRAZIN KAO POTENCIJALNI RIZIK ZA BIODIVERZITET SISARA

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SADRŽAJ

Savremena poljoprivredna praksa podrazumeva intenzivno korišćenje pesticida koji mogu da deluju kao supstance koje remete rad endokrinog sistema (endokrini disruptori - EDC) i u tom smislu mogu negativno da utiču na diverzitet vrsta. Cilj ovog rada je bio da se proceni efekat herbicida atrazina, koji deluje kao endokrini disruptor, na reproduktivnu sposobnost sisara. Za ovu svrhu, procenjen je efekat atrazina na morfološke i morfometrijske karakteristike Lajdigovih ćelija testisa pacova soja Wistar. Životinje su tretirane atrazinom u koncentracijama od 50 mg/kg telesne mase (bw) i od 200 mg/kg telesne mase od 23. do 53. postnatalnog dana (PND). Naši rezultati pokazuju da su obe doze izazvale doza - zavisno smanjenje u telesnoj masi i masi testisa. Oba tretmana su izazvala povećanje intersticijalnog prostora testisa i promenu u broju, nukleocitoplazmatskom odnosu i stepenu vakuolizacije Lajdigovih ćelija. U ovoj studiji je pokazano da herbicid atrazin utiče na strukturu i broj Lajdigovih ćelija na način koji može da dovede do smanjenja reproduktivne sposobnosti kod pacova kao i mužjaka drugih sisarskih vrsta što predstavlja ozbiljnu pretnju biodiverzitetu sisara budući da se herbicid atrazin koristi u SAD i evropskim državama koje nisu članice Evropske unije.