



## Sublethal effects of tricyclazole fungicide in male mice, the structure of the testis and testosterone levels studies

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### Abstract

Tricyclazole is one of widely-used fungicides to control Blast disease in agriculture. It is thought to affect the mammalian reproduction system. Therefore, we studied its impact on testes tissue and testosterone levels in mice. In an experimental-analytical study, 32 mice were randomly divided into four equal groups including control, sham and experimental 1 and 2. The animals in experimental groups 1 and 2 intraperitoneally received doses, respectively, of 0.5 and 1.5 mg/kg BW animal the tricyclazole for two weeks. The control animals did not any receiving toxin and sham receiving distilled water. The animals of all groups were ether anesthetized and killed. Sections of testes were prepared and various spermatogenic and Leydig cell were detected. Levels Testosterone was measured using radioimmunoassay. Data were analyzed using of one-way ANOVA. The levels of Testosterone, number of Leydig cells and the diameter of seminiferous tubules in experimental groups 1 and 2 significantly increased compared to controls. In parallel, the number of spermatogonia, spermatocytes and spermatids were significantly reduced compared to their counterparts in control group. The results indicated that tricyclazole might be able to damage testes and disturb Testosterone biosynthesis thereby affecting spermatogenesis.

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## Introduction

A variety of synthetic compounds applied in urbanized, industrial and agricultural regions are delivered to the environment that contaminates ecosystems in particular water ecosystems. Pesticides have agricultural origin used to eradicate plant diseases and pest and increase products (Jurasko and Sanjuán, 2011; Fattahi *et. al*, 2009). However, these compounds affect our health by entering our living environment and contaminating the food that we consume (Torres *et. al*, 1996, Tang *et. al*, 2012). Tricyclazole [(5-methyl-1, 2, 4 triazole 9, 3, 4-b) benzotiazole] belongs to triazoles and is one of the common fungicides used to control blast disease in Iran specially in Mazandaran province. High residual quantities of this toxin might remain in soil and water for months and years (Padovani *et. al*, 2006). World Health Organization (WHO) has classified tricyclazole within pesticides with range of moderate damage. This toxin and its metabolites exert little damage on mammals but have serious effect on aquatics (WHO, 2005; Jeong *et. al*, 2012).

The levels of damage pesticides make on human body depend on contact route, dose, biological changes, accumulation of metabolites and cellular organization (Das Gupta *et. al*, 2010). These toxins are converted to active metabolites in the liver and excreted via kidneys, but their residues aggregate in various organs including reproductive tract where they can cause damages to tissues. In this line, some studies point to the effect of triazoles on male reproductive cells and increased abnormalities in them (Li *et. al*, 2012; Goetz *et. al*, 2009). Some investigators blame reactive oxygen species (ROS) and free radicals as well as increased lipid peroxidation due to triazole metabolism as main mechanisms of cell/tissue damages in liver, testes and ovary (Bagchi *et. al*, 1995). These toxins results in increased weight of testes, prostate and liver beside reduced rate of pregnancy and indicators of fertility. Triazoles also increase Testosterone levels within the testes but reduce its metabolism in the liver (Goetz *et. al*, 2007; Hester *et. al*, 2012).

Triazoles may cause inhibition of gene expression, chromosomal damage, and malformed differentiation, blockage of mitosis in the embryo and reduction of DNA synthesis (Hermsen *et. al*, 2012; Tully *et. al*, 2006). The tricyclazole toxin disturbs production of body compounds and increases production of triglycerides, cholesterol, glucose and lactate in the blood. Increased levels of Alanine aminotransferase and aspartate aminotransferase beside reduced levels of alkaline phosphatase have been reported as other unwanted effects of the toxin (Sancho *et. al*, 2009). Although the mechanism of tricyclazole is not well known, but reports indicate that this and similar compounds can target various organs including reproductive tract. We therefore design this study to examine the effect of tricyclazole on the structure of testes and levels of blood Testosterone.

## Materials and methods

### Animals

In this experimental-analytical study was carried out using 32 male laboratory NMRI mice weighing an average 35±5g and aged between 10 to 12 weeks in Research Lab in Islamic Azad University Ayatollah Amoli Branch. The animals were obtained from Pasteur Institute in Amol, Mazandaran. One week before we began experiments, the animals were kept in special cages with 12-hour light and 12-hour dark at 23±2 °C with food and water supplied. All animal-related protocols were approved by the Ethical Committee of Babol University of Medical Science.

### Preparation and injection of toxin

Tricyclazole, a commercial formulation (95% active ingredient), was obtained from Fortune Company of china. Various dilution of the toxin was prepared and injected to animals using insulin syringes. The animals were randomly divided to three groups with equal numbers: control group, experimental groups 1 and 2. We injected 0.5 and 1.5 mg/kg tricyclazole, respectively, to experimental groups 1 and 2 for two weeks (5 consecutive days with two resting days). The injections were done intra-peritoneally. The controls did not receive any toxin but sham receiving distilled

water. The animals were anesthetized using ether and sacrificed 35 days after the last injection.

#### Preparation of tissue sections

To examine changes in tissues, the testes were extracted, rinsed and dried and then fixed in 10% formalin after weighing them. The middle region of the testes was selected and consecutively sectioned to 5- $\mu$ m sections. All sections were stained using hematoxylin and eosin. We then used these sections to count number of spermatogonia, spermatocytes, spermatids and Leydig cells per unit area under light microscope and using an eye piece. For each section, the diameter of testes and seminiferous tubules were measured using a micrometer and eyepiece graticule (Fattahi *et. al*, 2012). Three regions of each section were randomly selected with overall 100 fields examined for each section.

#### Hormone assay

Blood was collected from auxiliary vessels and centrifuged at 3000 rpm for 15 minutes to separate

their serum. Radioimmunoassay and Spectria Kit (The Netherland) were applied to measure serum levels of testosterone (Fattahi *et. al*, 2012).

#### Statistical analysis

The data collected from our experiments were analyzed in SPSS version 20 and using One-way ANOVA and Tukey's HSD.  $P < 0.05$  were considered statistically significant.

## Results

#### Testes weight and diameter

The relative weight of the testes in two experimental groups 1 and 2 were, respectively,  $0.262 \pm 0.002$  g and  $0.275 \pm 0.003$  g that in significantly increased compared to control group ( $0.223 \pm 0.002$  g) ( $P < 0.0491$ ; Table 1). Testes diameter in experimental groups 1 and 2 was, respectively,  $7.39 \pm 0.354$  and  $6.95 \pm 0.361$  mm. this figure was reduced compared to control group  $7.67 \pm 0.341$  mm, but the difference was not significant. There were no significant differences between control and sham groups (Table 1).

**Table 1.** The effect of tricyclazole on testes, spermatogenic cells and seminiferous tubules (Mean $\pm$ SE) in different 150 animal groups.

Mice exposure	control	sham	Experimental 1 (0.5 mg/kg)	Experimental 2 (1.5 mg/kg)	P-Value
Relative testes weight(gr)	$0.223 \pm 0.002^{cb}$	$0.225 \pm 0.004^{cb}$	$0.262 \pm 0.002^b$	$0.275 \pm 0.003^a$	$P < 0.0491$
Diameter of testes(mm)	$7.67 \pm 0.341^a$	$7.59 \pm 0.324^a$	$7.39 \pm 0.354^a$	$6.95 \pm 0.361^a$	-
spermatogonia( $\mu$ m <sup>2</sup> )	$8.72 \pm 0.5^a$	$8.65 \pm 0.45^a$	$8.12 \pm 0.365^b$	$7.52 \pm 0.38^{bc}$	$P < 0.001$
spermatocytes( $\mu$ m <sup>2</sup> )	$15.45 \pm 0.87^a$	$15.39 \pm 0.77^a$	$14.25 \pm 0.66^b$	$14.08 \pm 0.71^c$	$P < 0.001$
spermatid( $\mu$ m <sup>2</sup> )	$9.63 \pm 0.35^a$	$9.58 \pm 0.30^a$	$8.95 \pm 0.4^b$	$8.54 \pm 0.38^{bc}$	$P < 0.001$
Diameter of seminiferous tubules( $\mu$ m)	$125.38 \pm 5.76^c$	$127.23 \pm 5.26^c$	$131.63 \pm 3.67^b$	$134.37 \pm 3.45^a$	$P < 0.001$

Same letters indicate no significant differences between mean groups, and different letters is significant at the 0.05 level.

#### Diameter of seminiferous tubules

The diameters in experimental groups 1 and 2 were, respectively,  $131.63 \pm 3.67$   $\mu$ m and  $134.37 \pm 3.45$   $\mu$ m. These figures show significant increase compared to control group which was  $125.38 \pm 5.76$   $\mu$ m. The controls and sham groups did not shown significant differences ( $P < 0.001$ ; Table 1).

#### Number of spermatogonia and spermatocytes

The number of spermatogonia in experimental groups

1 and 2 per unit area were, respectively,  $8.12 \pm 0.365$  and  $7.52 \pm 0.38$  which show significant reduction compared to  $8.72 \pm 0.5$  of the control group ( $P < 0.001$ ). The two experimental groups did not shown significant differences. Moreover, microscopic examination of the sections showed that the number of spermatocytes within experimental groups 1 and 2 were, respectively,  $14.25 \pm 0.66$  and  $14.08 \pm 0.71$ , that were reduced significantly compared to  $15.45 \pm 0.87$  of controls ( $P < 0.001$ ). Also the number of

spermatocytes in experimental group 2 was significantly lower than that in experimental group 1 (fig.1)( $P<0.049$ ).

#### Number of spermatids and Leydig cells

Comparison of sections prepared from different experimental groups showed that there exists a significant difference between experimental and

control groups. Spermatid number in experimental groups 1 and 2 were, respectively,  $8.95\pm 0.4$  and  $8.54\pm 0.38$ , both of which were significantly decreased compared to  $9.63\pm 0.35$  of the control group ( $P<0.001$ ). Finally, we observed significant increase in Leydig cell number between experimental groups 1 ( $36.25\pm 1.37$ ) and 2 ( $37.03\pm 1.95$ ) on one hand, and controls ( $34.44\pm 1.65$ ) ( $P<0.001$ ; Table 2).

**Table 2.** The effect of tricyclazole on number of Leydig cells and testosterone levels (Mean $\pm$ SE) in different animal groups.

Mice exposure	control	sham	Experimental <sub>1</sub> (0.5 mg/kg)	Experimental <sub>2</sub> (1.5 mg/kg)	P-Value
Leydig cells	$34.44\pm 1.65^c$	$34.62\pm 1.73^c$	$36.25\pm 1.37^b$	$37.03\pm 1.95^a$	$P<0.001$
Testosterone levels(Nmol/L)	$1.05\pm 0.15^b$	$1.12\pm 0.17^b$	$1.64\pm 0.1^a$	$2.02\pm 0.12^a$	$P<0.001$

Same letters indicate no significant differences between mean groups, and different letters is significant at the 0.05 level.

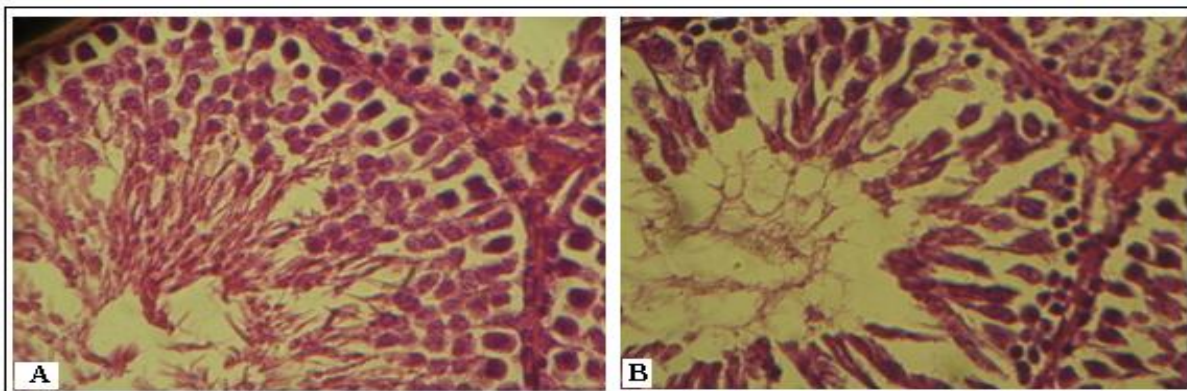
#### Testosterone levels

The concentrations of testosterone in experimental groups 1 ( $1.64\pm 0.1$ ) and 2 ( $2.02\pm 0.12$ ) increased in comparison to controls ( $1.05\pm 0.15$ ). This increase was statistically significant ( $P<0.001$ ; Table 2). No difference significant observed between control and sham.

#### Discussion

In this study, significant reduction was detected in the number of spermatogenic cells in experimental groups compared to controls. Our findings also indicate in significantly reduced testes relative weight and diameter. Our results show that Leydig cell

number, the diameter of seminiferous tubules and testosterone levels have significantly increased in experimental groups in comparison to controls. Experimental groups 2 which received higher levels of the toxin showed greater differences compared to experimental group 1. These data match with those reports made by those that claim triazoles cause testes atrophy (Watermann *et. al*, 2013). It has to be born in mind that such changes are not limited to testes and can affect many other tissues including ovary, seminal vesicle, prostate and liver causing reduction in their weight (Watermann *et. al*, 2013; Skolness *et. al*, 2013).



**Fig. 1.** cross section from mice testes in control (A) and tricyclazole (B) groups, staining with H&E (40X). Treated mice with tricyclazole show decrease in the number of spermatogenic lines in the seminiferous tubule.

Amongst other factors that cause reduction in testes weight and diameter, the reaction of such toxins with cellular macromolecules, generation of free radicals and increased peroxidation can be listed (Bagchi *et. al* 1995). Various studies have been carried out on the effect of triazoles on testes and in particular on the levels of testosterone that indicate progressive disturbances in testes and in spermatogenesis (Goetz *et. al*, 2007 ). Such disturbances directly correlate with increase in toxin dose and duration of exposure to toxins. In our study, increased dose of tricyclazole caused destructive effects on testes, and, destruction of various cell types at high doses of the toxin imply that the effect of triazoles such as tricyclazole can be dose-dependent. So, their high dose utilities could damage the reproductive system. Indeed, the increased levels of testosterone support this notion. Some investigations indicate that triazoles affect germinal cells in the testes and eventually caused reduced number of sperms in animals such as laboratory animals (Li *et. al*, 2012). Results of our study suggest that, with slowed rate spermatogenesis, cell types particularly in the germinal layer, undergo changes in favor of reduced sperm numbers. On the other hand, tricyclazole induced testosterone levels of testosterone a determinant hormone in initiation and maintenance of spermatogenesis (Walker, 2009). Therefore, impaired spermatogenesis and reduced number of sperms are not unexpected. Also, reduction in testes weight and diameter occur probably due to reduction in the number of gametes, as shown in our study.

Our data imply that increased dose of tricyclazole can cause further reduction in the number of spermatogenic cells and other parameters relevant to spermatogenesis. It appears that triazoles cause reduction in germinal cell number by inhibiting mitosis and inducing cell death (Duan *et. al*, 2013). Since these germinal cells are crucial for spermatogenesis, reduction in their number would inevitably affect various cell types including spermatocytes and ultimately reduce number of sperms.

Increased diameter of seminiferous tubules is one of the observations we made in the current study. Some pesticides cause changes in the diameter of these tubules by loosening connective tissues and smooth muscles around them. Similarly, our data imply that the increased diameter might be a indication of tubules undergoing destruction (Dutta and Meijer, 2003). Also in this study, the levels of serum testosterone were shown to be significantly higher than those of control. This hormone is secreted by Leydig cells. The number of Leydig cells which increased upon injection of the toxin in our study. Therefore, levels of the hormone were expected to rise in the serum by its secretion from Leydig cells. Triazoles can reportedly affect production, transfer and metabolism of androgens (Sancho *et. al*, 2009; Selim *et. al*, 2013 ). Tricyclazole can also inhibit aromatase that converts testosterone to estradiol (Vinggaard *et. al*, 2000). Therefore tricyclazole just like other members of triazoles can inhibit testosterone to be changed to estradiol and so increase its serum levels.

Testosterone levels rise with increased synthesis of steroids and Leydig cells beside inhibition of aromatase. Although, increase in testosterone levels in the blood stream cannot be considered an ultimate reason for increased number of Leydig cells, but our data indicate a direct correlation between increased testosterone levels and increased number of Leydig cells. This supports the notion that increase in testosterone levels is an indication for increased Leydig cells. In this study, the number of germinal cells, spermatocytes and spermatids were significantly reduced. Since tricyclazole caused reduction in germinal cells and subsequently sperm production, it can be concluded that tissues in the body might be particularly prone to such toxins.

### Conclusion

Our data show that tricyclazole can disturb spermatogenesis via two pathways: it does so either by directly affecting testes and causing cells to be atrophic and die, and or, inflicting disturbances in secretion of sexual hormones including testosterone.

Results of our study and others indicate that chemical toxins such as tricyclazole probably affect human fertility by affecting testes or testosterone biosynthesis driving individuals toward an inevitable infertility. Therefore, the consumption of such compounds must be carried out with care and under certain safety regulations.

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