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REPRODUCTIVE TOXICITY OF *BACILLUS THURINGIENSIS* BIO-PESTICIDE IN MALE ALBINO RATS

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ABSTRACT

The present study is a trial to investigate the toxic effects of the bio-pesticide *Bacillus thuringiensis* (Bt) on male reproductive system of rats. Rats received dietary doses each approximately equivalent to 1/10 or 1/100 of the LD_{50} value of the Bt bio-pesticide (Agerin) for 90 consecutive days. Sex organs weight, semen picture, concentrations of the hormones [i.e., testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH)], and histopathological changes in testes were the criteria used to evaluate the reproductive toxicity on the treated rats. Bt at the higher dose caused a significant decrease in the weight of testes and seminal vesicles as compared with control. Both Bt doses decreased sperm count associated with an increase in the morphologically abnormal spermatozoa; however sperm motility was significantly decreased in treated rats with the higher dose only. The concentration of serum testosterone was significantly reduced in both treated groups; however LH and FSH levels were significantly reduced in treated rats with the higher dose. Histopathological examination of Agerin-treated male rat's testes revealed that both Bt doses caused testicular degeneration in a dose-dependent manner. In conclusion, Agerin may decreases fertility in adult male rats by affecting the concentrations of pituitary gonadotrophins, testosterone and thus subsequent spermatogenic impairment.

INTRODUCTION

A number of environmental pollutants are now known to alter endocrine physiology without acting as classic toxicants. Hence, a new field of inquiry has emerged within the discipline of environmental toxicology, the study of endocrine disrupting chemicals (EDCs) **(Johnson *et al* 2000).** The possibility that environmental exposure to chemicals might affect human reproduction is not new. However, the hypothesis that environmental chemicals acting as EDCs could be causative agents of changes in population-based, reproductive health trends is relatively recent. Much of the interest in the male reproductive system has stemmed from a hypothesis proposed by **Sharpe and Skakkebaek (1993)** that agents interfere with normal development of the reproductive system via an endocrine mechanism could plausibly be related to increases noted in human male reproductive disorders over a number of years. In particular, a link was made between developmental events that could result in decreased sperm count/quality and increased incidences of testicular cancer, testicular maldescent (cryptorchidism), and male reproductive tract malformations, such as hypospadias. These could be expressions of one underlying entity, the testicular dysgenesis syndrome **(Skakkebaek *et al* 2001)**, which could result from disruption of gonadal development during fetal life. Pesticides represent one of the better studied groups of EDCs. Experimental data show that a number of chemicals can disrupt development of the male reproductive tract via endocrine mechanisms **(WHO, 2002).**

One of the promising alternatives to the conventional pesticides is the use of the microbial insecticide Bt which is a ubiquitous gram-positive soil bacterium, and produces insecticidal crystal proteins (ICPs) which have active against certain insect species among the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, Mallophaga, nematodes, mites, and protozoa **(Schnepf *et al* 1998)**. So far, Bt has been

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extensively used as bio-pesticide to control lots of crop pests **(Hofte and Whiteley, 1989; Schnepf *et al* 1998)**. Bt toxin is produced by *Bacillus thuringiensis* in an inactive form (protoxin), which is transformed to its active form (delta-endotoxin) in the guts of certain insects. The active toxin binds to receptors in the gut, killing the insect **(Lorence *et al* 1995)**. Bt bio-pesticides have the advantage of being much more specific than broad spectrum synthetic chemical insecticides and are thus considered environmentally friendly insect control agents. Bt usage in operational control programs has resulted in a remarkable safety record unparalleled by synthetic chemical insecticides. Despite this excellent record, some recent papers have expressed concern about the production of *Bacillus cereus* enterotoxins by Bt isolates which cause food poisoning **(Siegel, 2001)**.

The purpose of this study, therefore, is to evaluate the reproductive toxicity of Bt bio-pesticide which is recommended universally and in Egypt to control various economic pests. Sex organs weights, semen picture, concentrations of certain sex steroidal hormones, and testes histopathology were investigated in treated male albino rats.

MATERIALS AND METHODS

1. Bio-pesticide used

Agerin *(Bacillus thuringiensis)* 6.5% W.P. was purchased from Biogro International Company, Egypt.

2. Test animals

Adult male albino rats (Sprague-Dawely), *Rattus norvegicus* var. albinus, weighting 200-230 gm were purchased from the Biological Products & Vaccines Holding Company, Helwan Farm, Cairo. All animals were in good health. Rats were kept under the laboratory conditions of 25 ± 5ºC and 65±5% R.H., two weeks as an acclimatization period. They were housed in metal cages (35 x 25 x 20 cm) and maintained on *ad libitum* diet and water.

3. Acute toxicity

An acute oral toxicity study was performed in accordance with the Organization of Economic Cooperation and Development (OECD) guidelines (**OECD, 2001**). Twenty five adult (seven to eight-weeks-old) male rats with a body weight ranging from 190 to 210 g were used in this study. All the rats were randomly divided into five groups, and each group contains five males. Agerin powder preparation was suspended in distilled water, and administered orally at doses of 375, 750, 1500, and 3000 mg/kg bw by oral gavage to single male rats, while control group received distilled water only. All the tested rats were observed shortly after dosing, and then each rat was observed daily for a period of 14 consecutive days.

4. Experimental design

Rats were divided randomly to three groups, each of ten rats. The first and second groups received diet containing Agerin at doses approximately equivalent to 1/10 and 1/100 of the LD_{50} value, respectively. The third group received pesticide-free diet and was considered as a control. Feeding administration lasted for 90 successive days.

5. Toxicated diet preparation

To calculate the quantities of Bt required to be mixed with diet, preliminary experiment was performed to determine the quantity of the diet normally consumed by each rat per day. Five male rats were caged individually, starved and each was offered adequate weighed quantity of diet. After 24 hr, the residual quantity of diet was thoroughly collected and weighed. Average weight of diet consumed daily by each rat was calculated. Determination of individual feed consumption was determined periodically according to ages and body weights of the tested animals during the experimental period. The desired quantity of Bt was diluted in a proper volume of distilled water and mixed thoroughly with diet. The mixture was allowed to dry at room temperature and then kept in a deep freezer till being used. This mixture was considered as a fresh treated diet for three days; afterwards, new mixtures were periodically prepared by the same manner. The ratio of Bt bio-pesticide/diet in the mixture was calculated on the basis that toxicated diet ingested by an animal/24 hr, should carry the required quantity of Bt that is nearly equivalent to the desired dose (i.e., 1/100 or 1/10 LD_{50}). Variations of body weights of animals through the experimental period were taken in consideration.

6. Parameters studied

Clinical observations to detect moribund or dead rats and abnormal behavior and/or appearance were conducted at least twice daily throughout the study. At the end of the experimental period (90 consecutive days of dietary bio-pesticide treatment), rats were sacrificed by decapitation,

blood samples were collected in non-heparinized tubes, left till clotting occurred and centrifuged at 4000 rpm for 10 minutes. The obtained sera were kept frozen till being used for hormonal assay that include the determination of testosterone, LH and FSH levels. Genital organs (i.e., testes, epididymis, seminal vesicles and prostate glands) from each sacrificed rats were dissected out, trimmed of excess fat and weighed. Epididymal spermatozoa were examined. Testes were prepared and kept for histopathological examination. The various parameters were performed by the following methods**:-**

6.1. Sperm analysis

Epididymis was excised and weighed from each rat. The right cauda epididymis was used for sperm count and left one for sperm motility and sperm morphology analysis according to the method described by **Jeong *et al* (2005).**

6.2. Hormonal assay

Circulating levels of testosterone, LH and FSH were determined in serum of male rats by Elecsys Analyzer, D-Vi-S, using kits of Roche Diagnostics GmbH, D-68298, Mannheim, Germany.

6.3. Histopathological examination of testes

Testes were fixed in Bouin's solution and processed by dehydration in different concentrations of alcohol, cleared with xylol and embedded in paraffin blocks, then sectioned at 4 µ thicknesses. The paraffin sections were stained by haematoxylin and eosin **Lilie and Fullmen (1976)** and then histopathological examination was carried out microscopically.

7. Statistical analysis

Statistical significance of differences was determined using the program SPSS 12 (SPSS, USA) by performing one-way ANOVA with post hoc comparisons between the control group and each of the treated group followed by Duncan's multiple comparison tests. A p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

1. Acute toxicity

Results of the acute oral toxicity of the tested bio-pesticide are recorded in **Table (1)**. The oral LD_{50} value of Agerin to male albino rats is greater than 3000 mg/kg body weight. The obtained results are in agreement with those found by **Zidan (2005)** who reported that the acute oral LD_{50} for Bt (Xentari®: 13.3%) on rats was greater than 3000 mg/kg BW. **Kamrin, (1997)** demonstrated that the LD_{50} is greater than 5000 mg/kg for the Bt product Javelin in rats and greater than 13,000 mg/kg in rats exposed to the product Thuricide. **Peng *et al* (2007)** stated that the acute oral LD_{50} for GM *B. thuringiensis* powder preparation in subacute test was greater than 5000 mg/kg BW. All acute studies showed that Bt formulations might be safe for rats, rabbits and humans **(Joung and Cote, 2000)**.

Table 1. Acute oral toxicity of Agerin to male albino rats

pesticide	LD_{50} (mg/kg b.w.)	Confidence limits
Agerin	> 3000	-

2. Clinical symptoms and mortality during the test period

Adults male albino rats fed on contaminated rations with two doses i.e., 1/10 or 1/100 LD_{50} (LD_{50} value was considered equal to 3000 mg/kg body weight) of Agerin for 90 consecutive days were daily examined physically and clinically. Results showed that no mortalities were occurred throughout the experimental period. On the other hand, some treated rats with the high dose of Bt exhibited slower reaction in the beginning of administration. With the extension of administration, they gradually manifested rigidity, move "freezing" and flexed posture.

3. Relative weights of genital organs

Overall, relative weights of genital organs were among of the criteria used to evaluate the reproductive toxicity of the Bt bio-pesticides to male rats. Data pertaining to the impact of Agerin on weights of male genital organs are shown in **Table (2)**.

Perusal of these results clearly exhibited that Bt at 1/10 LD_{50} level significantly decreased testes and seminal vesicle weights representing 62.97 and 63.09 % of control, respectively. Otherwise the effect was not significant at 1/100 LD_{50} level. On the other hand, there were no significant differences in weight of epididymis and prostate glands at the end of the experimental period among the treated groups as compared with control.

Table 2. Relative weights of genital organs of treated male rats with Agerin for 90 consecutive days

Treatment	Approximate dose (mg/kg)	Mean relative weight (gm organ/100 gm b.w)							
		Testes		Epididymis		Seminal vesicles		Prostate gland	
		Mean	% of control	Mean	% of control	Mean	% of control	Mean	% of control
Agerin	1/100 LD_{50}	3.92[a]	88.84	0.68[a]	107.94	0.75[a]	89.28	0.69[a]	97.19
	1/10 LD_{50}	2.79[b]	62.97	0.60[a]	95.24	0.53[b]	63.09	0.67[a]	94.37
Control	-	4.43[a]	100	0.67[a]	100	0.84[a]	100	0.73[a]	100

- Each value represents mean of ten replicates.
- Values across each column having the same superscript letter (s) were not significantly different ($p< 0.05$).

Useful information on male reproductive capacity of laboratory animals can be obtained by measuring weights and the volume of testis, prostate, seminal vesicles, epididymis and coagulating glands (**Doul *et al* 1980)**. The weights of testes and accessory sex organs are known to be reliable indices of testicular androgen production **(Price and Williams-Ashman, 1961; Rind *et al* 1963). El-Hamady *et al* (2006)** found that testes weights of male rats treated with daily dietary doses each equivalent to 1/10 or 1/100 LD_{50} of Bt (Xentari®: 13.3%) for 60 days were significantly reduced with the high dose only. **Salama *et al* (2008)** indicated that testes weights of male Japanese quail birds treated with daily single oral doses of Bt (Xentari®: 13.3%) for 30 days were significantly reduced with the high dose only; however weights of epididymis and prostate gland were not affected.

4. Effect on spermatozoal morphology and viability

The effect of daily dietary administration of Bt (at 1/10 and 1/100 LD_{50}) for 90 consecutive days on the sperm motility, count and abnormality of treated rats are given in **Table (3)**. The percentages of sperm motility were significantly decreased at the high dose (i.e., 47.88 % as compared with control). Also, sperm count was significantly decreased representing 81.60 and 36.31% of control at 1/100 and 1/10 LD_{50} levels, respectively. Total sperm abnormalities were significantly increased at 1/100 and 1/10 LD_{50} levels recording 324.46 and 602.57% of control rats, respectively. Generally, the most pronounced malformations were bent tail, coiled tail and protoplasmic droplets. The abnormalities appeared as bent tail, constitute the highest percentages of the total deformities.

Sperm morphology is considered as a better discriminator between fertile and infertile males than sperm concentration **(Guzick *et al* 2001).** Sperm morphology and motility could also be useful markers of toxic damage even in the absence of any effect on fertility. Also the decrease in sperm density in the epididymis is an indicator of reduced spermatogenesis owing to the toxicity of any agent **(Poon *et al* 2004).** Spermatogenesis is controlled by two main regulatory processes, i.e., endocrine regulation via the gonadotropin hormones and local regulation via inter-cellular communications **(Holdcraft and Braun, 2004).** The obtained results are in accordance with those found by **Zidan (2005)** who revealed that treated male rats with dietary daily doses (i.e. 1/10 LD_{50} and 1/100 LD_{50}) of Bt (Xentari®: 13.3%) for 60 consecutive days decreased sperm motility and percentages of living sperm associated with an increase in the percentage of total sperm abnormalities. The hypotheses that xenoestrogens and related endocrine disruptors play a role in increasing male reproductive problems is primarily based on the decreased sperm counts observed by **Carlsen *et al* (1992)** and **Eisenbrand *et al* (1998).** Mean sperm counts of healthy men had declined from $113*10^6$/ml to $66*10^6$/ml within 50 years between 1938 and 1991 due to environmental pollution, and seminal volume decreased also in a 1992 meta-analysis, based on 61 articles **(Carlsen *et al* 1992)**.

5. Hormonal status

Data concerning the impact of Agerin on serum testosterone, LH and FSH levels are shown in **Table (4)**. The results quite indicate that, Agerin caused significant decrease in testosterone levels

Table 3. Effect of Agerin on epididymal sperm characters in treated male rats for 90 consecutive days

Treatment	Approximate dose (mg/kg)	Sperm motility (%)		Sperm count (*10^6/ml)		Sperm abnormality (%)	
		Mean	% of control	Mean	% of control	Mean	% of control
Agerin	1/100 LD_{50}	75^a	93.75	58^b	81.60	6.23^b	324.46
	1/10 LD_{50}	38.3^b	47.88	26.7^c	36.31	11.57^a	602.57
Control	-	82^a	100	75.3^a	100	2.85^c	100

- Each value represents mean of ten replicates.
- Values across each column having the same superscript letter (s) were not significantly different ($p< 0.05$).

Table 4. Concentration of certain serum hormones in treated male rats with Agerin for 90 consecutive days

Treatment	Approximate dose (mg/kg)	Testostrone (ng/ml)		LH (mIU/ml)		FSH (mIU/ml)	
		Mean	% of control	Mean	% of control	Mean	% of control
Agerin	1/100 LD_{50}	4.82^b	79.89	3.26^a	97.57	1.12^{ab}	90.65
	1/10 LD_{50}	3.84^c	63.76	1.94^b	57.91	0.94^{bc}	76.29
Control	-	6.05^a	100	3.52^a	100	1.15^a	100

- Each value represents mean of ten replicates.
- Values across each column having the same superscript letter (s) were not significantly different ($p< 0.05$).

of male rats representing 79.89 and 63.76% of control at 1/100 and 1/10 LD_{50} levels, respectively. In addition, serum LH and FSH levels were significantly decreased in Agerin-treated rats with the high dose only (i.e., 57.91 and 76.29% of control, respectively).

Testosterone is the principal male hormone produced by the interstitial Leydig cells of the male testes and in smaller amount either by the adrenals or the female ovaries. Thus, the testes are responsible for the synthesis of the male sex hormones, or androgens, and for the production of spermatozoa. The most important androgen, both in terms of potency and the amount secreted in testes is the steroidal compound, testosterone, a powerful anabolic hormone. It is vital for the development of secondary sexual characteristics in males and is essential for spermatogenesis **(Guyton, 1991; Mycek *et al* 1997)**. Testosterone is secreted as mentioned earlier by the leydig cells of the testis under the influence of luteinizing hormone (LH). **Krause (1977)** reported that the decreased testosterone levels might be due to a direct damage of leydig cells or to a lowered stimulation of these cells by LH. Disorders of male genital function (hypogonadism) are manifested by a decrease in plasma testosterone level. Hypogonadism may occur with defective seminiferous tubules function or defective leydig cell function and this leads to infertility through decreased production of spermatozoa, but masculinization is usually normal. Defective leydig cell function also results in failure of testosterone dependent functions including spermatogenesis. Sertoli cells, the major epithelial component of the seminiferous epithelium, are essential for the control of spermatogenesis, by supplying nutrients which ensure germ cell proliferation and differentiation, and by

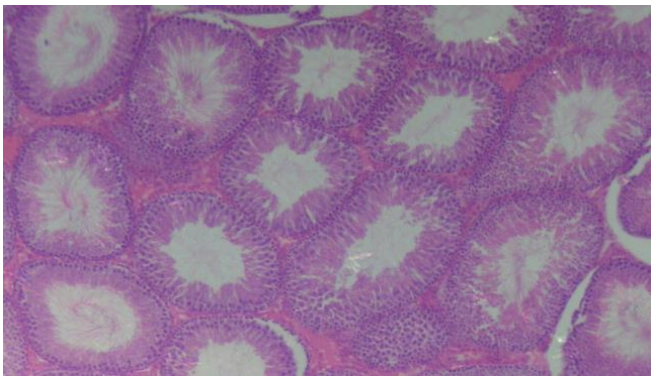

Fig.1. A photomicrograph of a section of control male rat's testes showing normal structure (H & E, x 40)

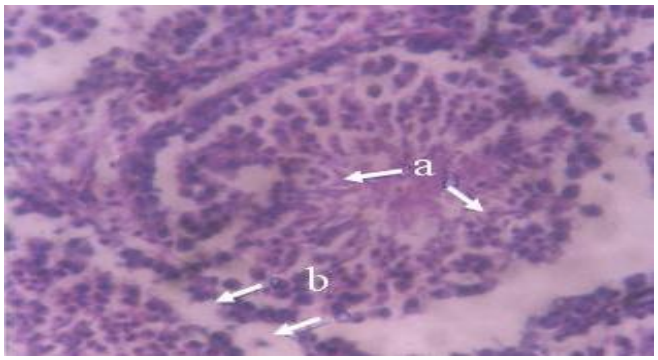

Fig. 2. A photomicrograph of a section of Agerin-treated male rat's testes at 1/100 LD_{50} level showing (a) mild stage of testicular degeneration and (b) testicular edema (H & E, x 10).

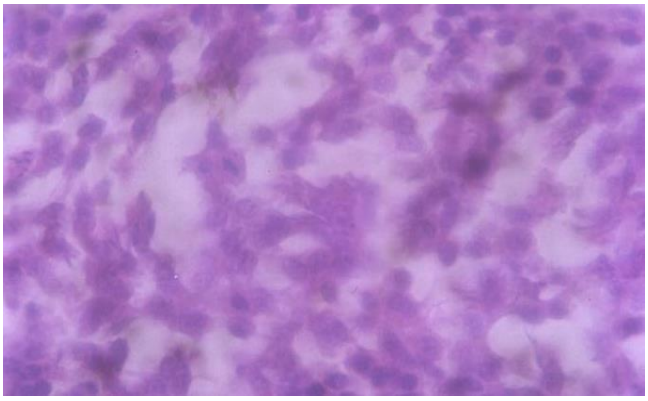

Fig. 3. A photomicrograph of a section of Agerin-treated male rat's testes at 1/10 LD_{50} level showing moderate testicular degeneration (H & E, x 10).

responding to endocrine stimuli **(Saunders, 2003)**. Many sertoli cell functions are regulated by follicle-stimulating hormone (FSH) **(Simoni *et al* 1999)**. The depression in testosterone levels might be due to testicular degeneration which was proved histopathologically. **El-Hamady *et al* (2006)** found that testosterone levels of treated male rats with daily dietary doses each equivalent to 1/10 or 1/100 LD_{50} of Bt (Xentari®: 13.3%) for 30 days were significantly reduced with the high dose only. **Salama *et al* (2008)** found that testosterone levels of treated male Japanese quail birds with daily single oral doses of Bt (Xentari®: 13.3%) for 30 days were significantly reduced in the high dose (1/10 LD_{50}); while the low dose of this bio-pesticide (1/100 LD_{50}) showed no effects. There are several reports demonstrate that pesticides decreased testosterone levels in treated rats with different doses, the effects were always accompanied by defects in gonads, and suppress LH and FSH levels **(Abd El-Aziz *et al* 1994; Abd El-Khalek *et al* 1999 and Kang *et al* 2004).** Suppression of gonadotrophins might have caused decrease in sperm density in testes (**Sinha *et al* 1995).**

6. Histological examination

No histopathological changes were observed in the specimens collected from the control rat's testes which exhibited a normal testicular structure **Fig. (1)**. Only one photomicrograph was selected to represent the damage of testes at each dose. The low dose caused testicular edema with mild stage of testicular degeneration as shown in **Fig. (2)**, while the high dose caused moderate testicular degeneration with decrease in the number of spermatogenic cells and complete absence of spermatids and spermatozoa **Fig. (3)**.

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