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Abstract

Tebufenozoideis a widely used insecticide in agriculture specially against lepidopteron, but their effects in the male reproductive tract are scarcely known. Hence, thepresent study has evaluated the effects of tebufenozoidehuman sperm function *in vitro*.Human sperm were incubated with equivalent doses of 0 (control; BWW medium), 1.25 (low dose), 2.5 (mid dose), 5 (high dose) µg/ml and total motility and sperm viability was evaluated. Similarly, sperm capacitation, acrosome reaction and sperm DNA damage was examined.Result showed significant declineingof total spermmotility atall doses(1.25µg/ml: by 33.7%;2.5µg/ml by 36.4%; 5 µg/ml: 36.3%) and sperm viability was reduced significantly only at the highest dose by 46.2%. DNA damages were significantly increased by 164.8% with 1.25µg/ml dose, by 326.9% with 2.5 µg/ml dose and by 357.9% with 5µg/ml dose.Percentage of capacitated spermatozoa were affected at all dose levels (by 20.6%: low dose; 45.5%: mid dose and 58.0%: high dose), but acrosome reaction was impaired only at the highest dose (36.6%). The observations suggest that exposure to tebufenozoideand its accumulation in the seminal plasma mayincrease the risk of infertility.

KEYWORDS: Tebufenozoide, growth hormone regulator, reproductive toxicant, sperm function

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1. Introduction

Use of environmental chemicals have increased as a consequence of efforts to meet increase food demand. Pesticides are potentially hazards to individuals involved in manufacture, formulation and applications in the field (Sankoh et al., 2016). Due to increase use of pesticides, there has been a substantial increase in the number of pesticides in the environment (Sharma et al. 2005). Studies have shown that increase usage of man-made chemicals result in destructive reproductive function in animals(Peiris et al. 1995 and Peiris-John & Wickremasinghe, 2008)and in humans (Skakkebaek et al., 2006).It is known that chemical compounds binds to receptors and acts either as an agonist or antagonist (Luconi et al., 2001) and could induce DNA damages in human spermatozoa (Kudavidanage and Peiris, 2016).

Tebufenozoide(trade name mimic 20F)is a widely used insecticides in Sri Lanka. It is an insecticide which, acts as a hormone that induces metamorphosis of insect larvae (Dharmadasa et al., 2008).An association between expose to insecticides and reproductive disorders such as infertility, birth defect, adverse pregnancy outcomes and perinatal deaths has been shown by several authors (Peiris and Moore 2001a, Peiris and Moore 2001b, Ratnasooriya et al. 1996).Pesticides are known to influence the gonads and to cause abnormal semen parameters with decreased sperm concentration (Perry et al., 2007), decreased sperm volume and decreased sperm counts (Recio-Vega *et al.*, 2008).Once spermatozoa are produced in the testes, they undergo maturation in the epididymis. Upon ejaculation, in the female reproductive tract, spermatozoa undergo a process known as capacitation. To penetrate the egg zona pelucida, the sperm releases its enzyme via a process known as acrosome reaction. Any disturbances in any of these processes will impair sperm function.

Although effects of these pesticides on other animals were recorded the effects on human is limited. Hence the present aimed to determine the effects of tebufenozoideon sperm motility, sperm vitality sperm capacity and acrosome reaction *in vitro*.

2. Materials and methods

2.1 Test material

Unformulated tebufenozoide(mimic 20F, purity: 99.0%) was obtained from Haylee's Agriculture Ltd., Colombo, Sri Lanka.

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2.2Semen collection and preparation

Semen samples were collected from healthy male (age 20 - 28 years) donors from University of Sri Jayewardenepura, Sri Lanka in a sterile specimen vial. Prior to semen collection donors were given an information sheet and a consent form seeking their willingness to participate in the study. All participating subjects were asked to abstain from any sexual activity for 3 to 5 days before semen collection. After liquefaction at 37°C for 30 min, semen quality parameters were measured (n=9) according to World Health Organization guidelines (WHO, 2010. Only ejaculates from healthy volunteers with normal parameters (sperm concentration > 40 x 10^6 spermatozoa/ml, total sperm motility > 50%, normal sperm morphology > 50%) were included in this study. Ethical approval was obtained by Ethics Review Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura, Nugegoda, Sri Lanka

2.3Experimental design

The doses were selected according to the field recommended dosefor tebufenozoide. The LD_{50} values of field recommended doses were selected the highest dose levels while one half of and one fourth of the LD_{50} values were taken as mid and the lowest dose levels respectively. However, to compare the toxicity of tebufenozoide, the same dosage (low dose: 1.25µg/ml, mid dose: 2.5µg/ml and high dose: 5µg/ml) were used for the study. All the insecticides are readily dissolved in water and hence,Biggers Whitten Whittingham (BWW) media was taken as the control. Sperm suspensions (fixed to 40 × 10^6 spermatozoa/ml) were placed in Eppendorf tubes and desired volume of either isotonic saline or test solution were added (final volume of the tube is equal to 1ml).

2.4Assessment of total sperm motility

Samples were mixed with different concentrations (1.25µg/ml, 2.5µg/ml, 5µg/ml) of tebufenozoideor BWW (control) and sperm suspensions were then incubated in a humidified incubator (Sanyo Electric Co. Ltd, Japan) at 37°C in 5% $CO_2$for 4h. Subsequently,total percentage motile spermatozoa (WHO, 2010) were estimated (by counting approximately 100 cells)under Olympus microscope with phase contrast optics (X 400; Olympus Corporation, Japan).

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2.5 Assessment of sperm viability

The viability of spermatozoa of treated with different doses of tebufenozoide (n=6) and the control were assessed using Eosin Y stain technique (WHO, 2010) upon incubation for 4 h. Spermatozoa with red or pink colouration in their head regions was classified as 'dead' and those appearing white or light pink were classified as 'live' and percentage vitality was calculated. Percentage vitality = (total number of cells - number stained/ total number of cells) X 100. Total number of cells = 100.

2.6Assessment of sperm capacitation

Two hundred µl ofBWW medium was placed at the center of a glass Petri dish and covered with liquid paraffin oil (BDH Chemical Ltd., Poole, UK). Subsequently, 100 µl of sperm suspension was added to the BWW medium in the petri dish and mixed well. Either 100 µl of different doses of tebufenozoide (50, 100, 200 µg/ml) or BWW (control) were added to the BWW medium in a Petri dish (n=6). These were incubated at 37 °C for 3h in 5% CO_2 and 95% O_2. Subsequently 10 µl of each of these sperm suspensions was transferred on to a warm glass slide at 37 °C and was examined using phase contrast microscope (Olympus Corporation, Japan) at 100 magnifications. Then the number of hyper activated sperms and totally immotile sperms were recorded (at least 100 spermatozoa per suspension). A spermatozoon was considered to be hyper activated if it followed a nonlinear swimming with vigorous tail movement and marked lateral excursions of head.

2.7Assessment of acrosome reaction

Sperm ability to undergo acrosome reaction was determined according to the method described by Guptha et al. (1993). Briefly, the acrosome reaction was induced by incubating spermatozoa with calcium iornophore A23187with three doses of tebufenozoide and the control (n=6). After 3 h incubation at 37 °C for 3h in 5% CO_2 incubator, sperm samples were centrifuged and acrosomal status were determined using fluoresceinated *Pisum sativum* agglutinin. Sperm smears were prepared by placing 10ul of each sperm suspension on cleaned microscopic slides and acrosome reacted spermatozoa were determined under a fluorescent microscope (Olympus Corporation, Japan) with excitation of 490 nm by counting 100 spermatozoa in 3 microscopic fields.

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A spermatozoon is considered to be acrosome reacted if initial fusion phase or loss of acrosomal content was observed.

2.8 Determination of DNA damage in spermatozoa

Spermatozoa incubated with respective doses of tebufenozoide and the control (BWW) for 1 h were used (n=6) for assessment of DNA damage. Sperm smears were prepared on cleaned microscopic slides for both treated and control samples and air dried for 5 minutes. The smears were fixed in Carnoy's solution for at least 3h. Slides were washed with distilled water, air-dried and stained with acridine orange for five minutes. Smears were evaluated with the aid of fluorescent microscope (Olympus Corporation, Japan) with excitation of 490 nm. Hundred sperm from each staining protocol were scored and graded. All sperm exhibiting yellow to red colour was scored as denatured DNA and sperm exhibiting green colour was scored as normal DNA(Tajeda et al. 1994).

2.9 Statistical analysis

Statistical analysis was performed with Two-way analysis of variance (ANOVA) using Minitab software package (Minitab Co., USA) for the main effect of pesticides. Where a significant treatment effect was found, differences among individual group means were tested by" Tukey 95% ".The data are expressed as mean ± SD. The *p* value was set to $P<0.05$.

3 Results

3.1 Effects on sperm motility

According to figure 1, tebufenozoidesignificantly ($P<0.01$) decreased the motility at all dose levels respectively by 33.7%, 36.4% and 46.3% at 1.25, 2.5 and 5 µg/ml.

3.2 Sperm viability

Though sperm viability was decreased (figure 2) in spermatozoa treated with 2.5 and 5 µg/ml doses of tebufenozoide, a significant ($P<0.05$) reduction of sperm viability (by 46.2 %)was recorded only in the highest dose.

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3.3 Effects on DNA damage in spermatozoa

Percentage of DNA damaged spermatozoa count was increased at all tebufenozoide treated doses. The percentages of damages were significantly ($p<0.05$) increased by 164.8% with the lowest dose, by 326.9% and significantly increased ($p<0.01$) at the mid dose and by 357.9% at the highest dose.Results are summarized in Table I.

3.4 Effects on sperm capacitation and sperm acrosome reaction

Human sperm incubated *in vitro*with tebufenozoideelicited significant ($p<0.05$) effects on sperm capacitation at all dose levels (by 20.6%: low dose; 45.5%: mid dose and 58.0%: high dose). Acrosome reactionwas impaired only at the highest dose (36.6%). Results are summarized in Table 1.

4 Discussion

Results obtained from the present study indicated that tebufenozoidecould exert significant effect on human sperm *in vitro*. Similar results were observed with chlorinated hydrocarbon (Pflieger-Bruss&Schill 2010), methoamidophos (Peiris et al., 1995) andJudo 40 (Kudavidanage and Peiris, 2016). In the present study, sperm motility was reduced in all dose levelsof tebufenozoide treated human sperms. Changes in mitochondrial membrane potential by different pesticides can result (Pant et al. 2014) and oxidative stress induced by organophosphorus pesticides (Lukaszewicz-Hussain 2010) could damage mitochondrial membrane todecrease the production of ATP thus affecting the motility (Wang et al. 2003).Disulfide bonds in the tail are vital for rigidity of spermatozoa tail that is essential for progressive motility and oxidation of disulfide bonds could result in loss of sperm motility (Buffon et al. 2012). Further, acridine orange test revealed that tebufenozoide could alter integrity of the sperm head at both dose levels and hence it is possible that the same alteration could be observed in the disulfide bonds of the tail resulting impaired motility.

Reactive oxygen species could cause membrane damage resulting in continuous decrease in sperm motility and vitality after ejaculation (Calamera et al., 2001).Tebufenozoide have been shown to cause oxidative damages (Carter et al., 2001) resulting in reduction of sperm motility, and viability.The Viability is the proportion of live spermatozoa determined by the evaluation of cellular and or membrane integrity

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(Rao, 2006). Vitality of spermis associated with intact, functional and semipermeableplasma membranes (Mandani *et al.*, 2013). Disruption of DNA occurs mainly though the oxidation of disulfide bonds. It has been reported that high levels of ROS mediate the oxidation of disulfide bonds, which are commonly observed in the spermatozoa of infertile men (Aitken and Krausz 2001). Furthermore, it has been shown that pesticides are known to generate ROS thus producing DNA damage observed in the present study (Choi et al. 2015). Damage to disulfide bonds in the tail could also lead to reduction in capacitation, which observed in the present study. Hyperactivation is in an indication of sperm completion of capacitation and is essential for penetration through the cumulus mass and zona pellucida (Peiris and Moore, 2001a). Acrosome reaction is essential for successful penetration of the zona pellucida (Flesch and Gadella, 2000) and tebufenozoide interfered with acrosome reaction. Tebufenozoide could alter calcium ion concentrations, thus affecting spontaneously induced human spermatozoa acrosome reaction (Luconi et al., 2001). Plasma membrane of sperm contains high levels of poly-unsaturated fatty acids. which make spermatozoa susceptible to oxidative damage (Gravance et al. 2003). Hence alteration observed in the acrosome reacted spermatozoa probably due to membrane changes exerted by the insecticide (Aitken and Krauz, 2001).

5 Conclusion

From the results, it can be concluded that human sperm functions were decreased with exposure of tebufenozoide. With longer exposure totebufenozoide, the risk of infertility may increase.

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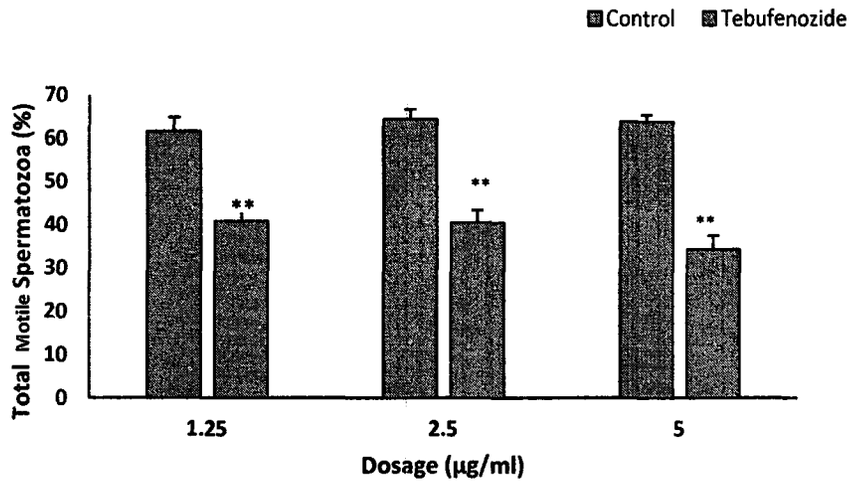

Figure 1: The effects of different doses of tebufenozoide (1.25 µg/ml; 2.5 µg/ml and 5 µg/ml) or BWW (control) on sperm motility. Results are presented as mean ± SE Mean (n=6). **P ≤ 0.01 significantly different from control.

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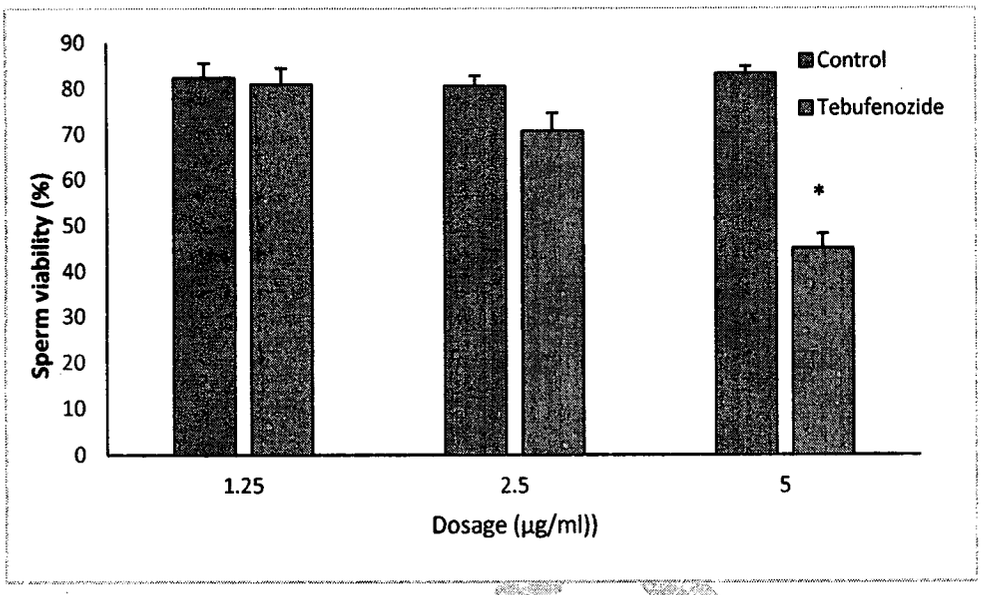

Figure 2:Effects of different concentrations of tebufenozoide (1.25 µg/ml, 2.5 µg/ml and 5 µg/ml)andBWW (control). Results are presented as mean ± SE Mean (n=9). *P ≤ 0.05, significantly different from control.

Table I. Effects of tebufenozoide on human sperm DNA damage, capacitation and acrosome reaction

Parameters	**Control (BWW)**	**Treatment (µg/ml) (Tebufenozoide)**		
		1.25	**2.5**	**5**
DNA damaged spermatozoa (%)	14.5±0.03	38.4±0.04*	61.9±0.25**	80.9±1.15**
Capacitated spermatozoa (%)	68.6±0.94	54.5±0.65*	37.4±0.89**	28.8±1.0**
Acrosome reacted spermatozoa (%)	80.9±0.02	70.8±0.56	71.6±0.47	51.3±0.91**

The data are given as mean ± SEM (n=6). Values are statistically significant at *p<0.05, ** p < 0.01. Control group was treated with BWW while treated groupsincluded 1.25, 2.5 & 5 µg/ml of tebufenozoide.