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WULFENIA JOURNAL KLAGENFURT, AUSTRIA ISSN:1561-882X

Vol 23, No. 10;Oct 2016

Morphological and functional alterations of human spermatozoa after incubation with two organophosphorus insecticides

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The research is financed by: University of Sri Jayewardenepura

Abstract

Organophosphorus pesticides were reported to impair male reproductive function. Admire and lebaycid are two widely used insecticides in agriculture to combat agricultural pests. It is unclear whether these pesticides impact human spermatozoa. We investigated the effects of admire and lebaycid on morphology and functional aspects of human spermatozoa. Human sperm were incubated with admire and lebaycid at different doses (1.25, 2.5 and 5 µg/mL) and Biggers Whitten Whittingham (BWW; control). Total motility, vitality, plasma membrane integrity, capacitation, acrosome reaction and sperm DNA damage was examined. The results indicated that admire and lebaycid inhibit total motility in a dose-dependent manner. The vitality and plasma membrane integrity decrease significantly in sperm incubated with the highest dose of admire and lebaycid. Similarly, the capacitation and acrosome reaction were impaired only at the high dose. Pesticides induced increased sperm DNA damages with increasing exposure levels. The results suggest a direct action of admire and lebaycid on the different parameters studied suggesting that exposure to these two pesticides may result in detrimental effect on human sperm function.

Keywords: admire, lebaycid, male fertility, reproductive toxicity

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 application 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 in humans (Recio-Vega et al., 2008). It is known that chemical compounds and environmental pollutants binds to receptors and acts either as an agonist or antagonist (Luconi et al., 2001). Organochlorin pesticides tend to bioaccumulate and are capable of disrupting the male hormone signaling pathway (Lemarie et al., 2004).

Chemically organophosphates are esters of phosphoric or thiophosphoric acids and can be toxic to mammals by disrupting nervous transmission (Kavlock et al., 2001). An association between expose to organophosphate insecticides and reproductive disorders such as infertility, birth defect, adverse pregnancy outcomes and perinatal deaths has been shown by several authors (Ratnasooriya et al. 1996). Pesticides are known to delay sperm capacitation, sperm acrosome reaction and induce DNA damages in human spermatozoa (Kudavidanage and Peiris, 2016).

Lebaycid (trade name lebaycid 500; IUPAC: *O*-2,4-dichlorophenyl, *O,O*-diethyl phosphorothioate) and admire (IUPAC: 1H-Imidazol-2-amine, 1-[(6-chloro-3-pyridinyl)methyl]-4,5-dihydro-N-nitro-(105827-78-9) are widely used organophosphorus insecticides in Sri Lanka against agricultural pests. Lebaycid is an insecticide that inhibit the action of acetylcholine esterase, a neurotransmitter breakdown thus resulting in continuous firing at nerve endings while admire mimic the acetylcholine and therefore, will bind to the acetylcholine receptors at the nerve endings thus resulting in inhibiting the nervous transmission (Cox, 2001). Some organophosphate is known to accumulate in biological membrane resulting in oxidative stress for which spermatozoa are sensitive due to lack of cytoplasmic defense mechanism (Saleh and Agrawal, 2002). Excessive production of reactive oxygen species could result in impairment of sperm function. Hence, the present study aimed to determine the effects of lebaycid and admire, two commonly used organophosphates on sperm motility, sperm viability, sperm capacitation, acrosome reaction and DNA damages *in vitro*.

2. Materials and methods

2.1 Semen 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=6) 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%, progressive motility > 60%, 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

2.2 Experimental design

Unformulated admire and lebaycid (purity: 99.0%) was obtained from Haylee's Agriculture Ltd., Colombo, Sri Lanka The doses were selected according to the field recommended dose for the pesticides. 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 two insecticides, same dosage (high dose: 5 µg/mL, mid dose: 2.5 µg/mL and low dose: 1.25 µg/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 80×10^6 spermatozoa/mL) were placed in Eppendorf tubes and desired volume of either BWW or test solution were added (final volume of the tube is equal to 1mL).

2.3 Assessment of total sperm motility

Sperm samples were mixed either with different concentrations (1.25, 2.5, 5 µg/mL) of admire and lebaycid or the control (BWW), and sperm suspensions were incubated in a humidified incubator (Sanyo Electric Co. Ltd, Tokyo, Japan) at 37°C in 5% CO_2 for 4 hr. Subsequently, Percentage motile spermatozoa were estimated according to WHO laboratory manual for semen analysis (WHO, 2010).

2.4 Assessment of sperm viability

The viability of spermatozoa treated with different doses of pesticides (n=6) and the control were assessed using Eosin Y stain technique (WHO, 2010) upon incubation with respective insecticides for 4 hr. 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 using total number of 200 spermatozoa.

2.5 Assessment of sperm capacitation and acrosome reaction

Two hundred µL of BWW 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 suspensions treated with different doses of both admire and lebaycide (1.25, 2.5, 5 µg/mL) or BWW (control) were added to the medium in Petri dishes (n=6). Petri dishes were incubated at 37 °C for 4 hr in 5% CO_2 and 95% O_2 . Subsequently 10 µL of each of each sperm suspensions were transferred on to a warm glass slide at 37 °C and number of hyper activated sperms were recorded. A spermatozoon was considered to be hyper activated if it followed a nonlinear swimming with

vigorous tail movement and marked lateral excursions of head.

Sperm ability to undergo acrosome reaction was determined according to the method described by Guptha et al. (1993). The acrosome reaction was induced by incubating spermatozoa with calcium iornophore A23187 with three doses (1.25, 2.5, 5 µg/mL; n=6/group) of both pesticides (admire and lebaycid) and the control (n=6). After incubation at 37 °C for 4hr in 5% CO_2 incubator, sperm samples were centrifuged and acrosomal status were determined using fluoresceinated *Pisum sativum* agglutinin. Sperm smears were prepared and acrosome reacted spermatozoa were determined under a fluorescent microscope (Olympus Corporation, Japan) with excitation of 488nm. A spermatozoon is considered to be acrosome reacted if initial fusion phase or loss of acrosomal content was observed. In both experiments, 200 spermatozoa were counted.

2.6 Determination of the functional integrity of sperm plasma membrane & DNA damage in spermatozoa

The functional integrity of sperm plasma membrane of treated (admire and lebaycid) and control samples (n=6/group) was assessed using the Hypo Osmotic Swelling (HOS) test described by Jeyendran *et al*. (1984). All spermatozoa showing characteristic swelling changes (curling of tails or swelling of tails) following incubation in the hypoosmotic medium were consider as having functionally normal plasma membranes. At least 200 spermatozoa were assessed per preparation.

Spermatozoa incubated with respective doses of both pesticides and the control (BWW) for 4 h were used (n=6) for assessment of DNA damage. Sperm smears were prepared, air dried and stained with acridine orange for five minutes. Smears were evaluated using a fluorescent microscope (Olympus Corporation, Japan) with excitation of 490 nm. For each treatment, 200 spermatozoa 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.

2.7 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 statistical difference was determined at $P < 0.05$.

3. Results

3.1 Effects on sperm motility and viability

The total motility were significantly inhibited at 1.25, 2.5 and 5 µg/mL doses of admire

respectively by 32.3%, 34.6% and 44.5%. Similarly, lebaycid too inhibited total motility at all dose levels (1.25μg/mL: 33.8%; 2.5 μg/mL: 38.9%; 5μg/mL: 50.9%). The total motility of was decreased than limited value of normal in spermatozoa treated with 2.5, 5 μg/ml of admire and with all doses of lebaycid. Percentage viability as determined by vital staining was decreased at both admire and lebaycid concentration of 5 μg/mL respectively by 38.6% and 46.3% (Figure 1).

3.2 Effects on sperm capacitation and sperm acrosome reaction

Only human sperm incubated *in vitro* with 5 μg/mL of lebaycid significantly inhibited both capacitation and acrosome reaction by 45.3% and by 44% respectively (Figure 2).

3.3 Effects on functional integrity of plasma membrane and DNA damage in spermatozoa

Significant reduction in functional integrity was recorded in spermatozoa treated with 5 μg/mL of both admire and lebaycid respectively by 44.5% and 51.5%. Percentage DNA damaged in spermatozoa treated with both pesticides was increased significantly at all doses levels. The percentages of damages induced by admire were increased by 165.3% with the lowest dose, by 292.1% at the mid dose and by 338.6% at the highest dose. Similar DNA damages were observed with lebaycid treated spermatozoa (1.25μg/mL : 341.6%; 2.5μg/mL : 516.8%; 5μg/mL : 565.3%). See Fig. 3.

4. Discussion

Reproductive effects of organophosphorus pesticides have been reported but limited information is available on admire and lebaycid insecticides. Long term exposure to organophosphorus pesticides could result in impairment of normal sperm functions thus leading to reduction of sperm fertilizing abilities. Hence in the present study we exposed human sperm *in vitro* to different concentrations (1.25, 2.5 & 5μg/mL) of admire and lebaycid. High doses (5μg/mL) of both admire and lebaycid resulted in inhibition of functional integrity, viability, sperm capacitation and acrosome reaction. Both pesticides at all doses induced DNA damages and inhibited total motility. The present study revealed that sperm function was affected at very low doses than recommended field doses of both insecticides. Though sperm were alive after exposing to lower doses (1.25 & 2.5μg/mL) of admire and lebaycid, the total motility was impaired significantly. 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 to decrease the production of ATP thus affecting the motility (Wang et al. 2003). Disulfide bonds in the tail are essential 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 both admire and lebaycid could alter integrity of the sperm head at all dose levels

and hence it is possible that the same alteration could be observed in the disulfide bonds of the tail resulting impaired motility.

Organophosphates have been shown to cause high levels of oxidative damages (Saleh & Agrawal, 2002). Reactive oxygen species could cause membrane damage resulting in continuous decrease in sperm motility and viability after ejaculation (Calamera et al., 2001). Viability is the proportion of live spermatozoa determined by the evaluation of cellular and or membrane integrity and is associated with intact, functional and semipermeable plasma membranes (Mandani *et al*., 2013). Further, reactive oxygen species could result in disruption of DNA and pesticides are known to generate ROS thus producing DNA damage observed in the present study (Choi et al. 2015). Damages to disulfide bonds in the tail could also lead to reduction in capacitation thus inhibiting hyperactivation which, is essential for penetration through the cumulus mass and zona pellucida (Peiris and Moore, 2001 b). Acrosome reaction is essential for successful penetration of the egg zona pellucida. At high dose levels, both admire and lebaycid interfered with acrosome reaction, indicating that both pesticides could interfere with calcium ion influx, thus affecting spontaneously induced human sperm acrosome reaction (Luconi et al., 2001). Sperm plasma membrane 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. Conclusions

Organophosphates are water soluble pesticides, hence can penetrate via skin during formulation and field applications and could result in induction of morphological and functional alterations during epididymal storage of spermatozoa. In conclusion, the present data document that both admire and lebaycid are highly toxic to human spermatozoa. Sperm functions were decreased with increased exposure to admire and lebaycid thus increasing the risk of infertility.

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Figures

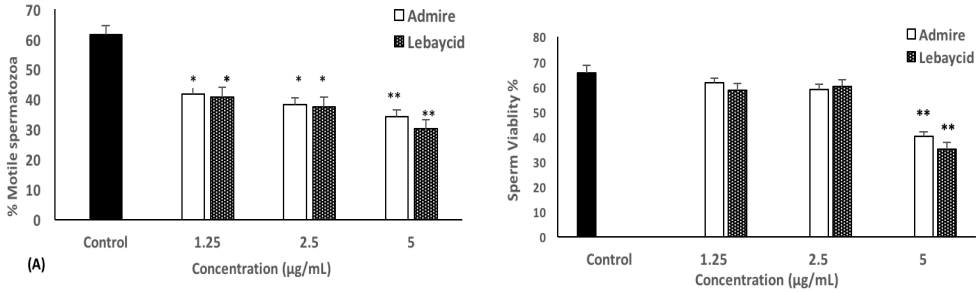

Figure 1: Total human spermatozoa motility (A) and viability (B) after incubation with 3 doses of Admire and lebaycid (1.25 µg/mL; 2.5 µg/mL and 5 µg/mL) or BWW (control). Results are presented as mean ± SE Mean (n=6). *P ≤ 0.05; **P ≤ 0.01 significantly different from control.

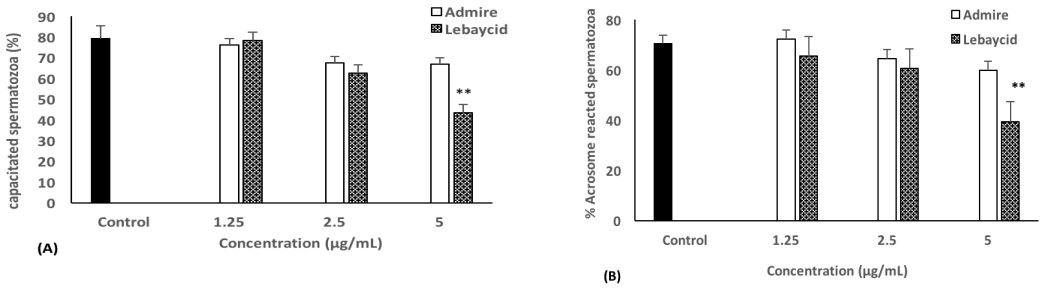

Figure 2: Assessment of sperm capacitation (A) and acrosome reaction (B) after incubation with 1.25 µg/ml; 2.5 µg/ml and 5 µg/ml concentrations of admire, lebaycid and BWW (control). Results are presented as mean ± SE Mean (n=9). **P ≤ 0.01, significantly different from control.

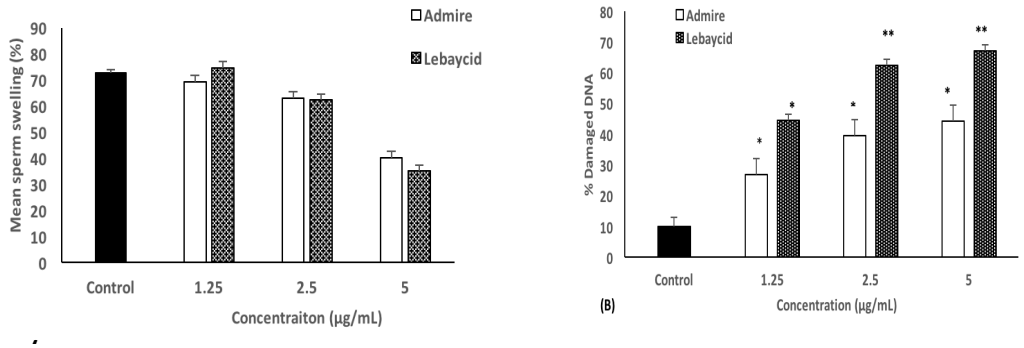

Figure 3: Effects of admire and lebaycid on human sperm DNA damage (A), and functional integrity of plasma membrane (B). Control group was incubated with BWW while treated groups were incubated with 1.25, 2.5 & 5 µg/mL of the pesticides. The data are given as mean ± SE (n=6). Values are statistically significant at *p<0.05, ** p < 0.01.