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EPRA International Journal of

Multidisciplinary Research

Volume: 2 Issue: 9 September 2016

Published By :
EPRA Journals

EPRA International Journal of Multidisciplinary Research (IJMR) ISSN (Online): 2455-3662

SJIF Impact Factor: 3.395 (Morocco) Volume: 2 Issue: 9 September 2016

EXPOSURE OF JUDO 40 ALTERS DNA INTEGRITY AND SPERM FUNCTION OF RAT AND HUMAN SPERMATOZOA

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ABSTRACT

Judo 40 is a widely used organophosphorus pesticide in agriculture and information on male reproductive effects is limited. Hence, the present study has evaluated the effects of Judo 40 human sperm function in vitro. Human sperm were incubated with equivalent doses of 0 (conttol; cornoil) 20 and 50 mg/kg Judo 40 for 5 min, 15 min and 30 min and percentage motility and vitality was evaluated. Rats were monitored for water intake and subsequent to autopsy, organ body weights, sperm count, sperm motility, sperm proteins, pH of seminal vesicle fluid, sperm vitality, and sperm DNA damage was examined. Water intake of rats were significantly reduced at both dose levels on post-treatment day 1 but returned to normal levels from day 2. Result showed significant declining of sperm motility at both doses (20 mg/kg: by 37.24%; 50 mg/kg by 51.24%). pH of seminal vesicle fluid was decreased at both dose levels. The sperm count and organ body weights remain unaltered following the treatment. Similarly, DNA damages were significantly increased at low dose by 95.9% and at high dose by 51.31%. Human sperm motility was significantly reduced at low dose by 48.8% after 15 min and by 69.6% after 30 min incubation. With the high dose motility was significantly impaired by 78.3%, 78.8% and 83.4% after 5 min, 15 min and 30 min incubation respectively. However, human sperm vitality was significantly decreased only in high dose by 48.8% after 15 min incubation and by 52.1% after 30 min incubation. The observations suggested that exposure to Judo 40 may result in detrimental effects on both rat and human sperm function.

KEYWORDS: *Judo 40, organophosphate, reproductive toxicant, sperm function*

1. INTRODUCTION

Due to increasing demand of food production and corresponding increase of use of pesticides, there has been a substantial increase in the number of pesticides in the environment (Sharma et al. 2005). Several 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).

Organophosphate (OP) pesticides are esters of phosphoric and thiophosphoric acids and exert their toxic effects mainly by inhibiting phosphorylate of acetylcholinesterase enzyme, causing accumulation of acetylcholine in synapses (Colovic et al. 2013). Many studies have identified association between expose to OP and reproductive disorders such as infertility, birth defect, adverse pregnancy outcomes and perinatal deaths (Peiris and Moore 2001a, Peiris and Moore 2001b, Ratnasooriya et al. 1996). Organophosphates

believed to modify reproductive function by reducing brain acetylecholinesterases activity and then influencing the gonads (Perry *et al.*, 2011) 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).

Judo 40 is the commercial formulation of Chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloro-2-pyridil) phosphorothioate that is marketed as an emusifiable concentrate containing 400g /L of active ingredient. Judo 40 is an organophosphate insecticide, widely used in Sri Lanka because of its wide spectrum insecticide property. The toxic mechanism of Judo 40 is by its active ingredient chlorpyrifos, which metabolize to chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol and acts as an acetylcholinesterase (AChE) inhibitor, and as a delayed neurotoxic agent (Mahajna *et al.*, 1997).

Chlorpyrifos (CPF) is highly toxic to many animals including copepods, amphipods (Zafar et al. 2011), fish (Brandit et al. 2015), earthworms (Muangphra et al. 2015) and mammals (El-Tawil 2014). Although no reproductive toxicity reported at higher doses of acute exposure (Mandal and Das 2011), chronic exposure resulted in decreased semen quality and increased abnormal sperm morphology (El-Bendary et al. 2014). Occupational exposure of males during formulation and farmers during applications of Judo 40 could result in reproductive toxicity.

2. OBJECTIVES

2.1 To investigate the effects of acute doses of Judo 40 on epididymal structure, epididymal sperm parameters and fertilizing ability of sperm in male rats after oral exposure to the pesticide.

2.2 To study effects of rat equivalent doses of Judo 40 on human spermatozoa.

3. MATERIALS AND METHODS

3.1 TEST MATERIAL

Test material, Judo 40 (commercial product of chlorpyrifos; concentration 400g/L) was obtained from Lankem Ceylon Ltd., Colombo 10. Since Judo 40 is soluble in corn oil, was used as the control.

3.2 EXPERIMENTAL DESIGN

Lethal dose 50 value of Judo 40 was predetermined using 30 male rats were randomly divided in to 5 groups (n = 6/group). Rats were orally administered either with 1 ml of 2, 20,100, 200 mg/kg Judo 40 or corn oil (control) and number of deaths in each group was recorded for two weeks. Similar to previous records, acute oral LD 50 value was found to lies between 20 mg/kg and 100 mg/kg. Hence 20 mg / kg and 50 mg/kg doses (calculated for active ingredient) was used for further studies. Twenty seven male rats were randomly divided in to 3 groups (n=9). Rats in groups 1 and 2 received 20 mg/kg and 50 mg/kg Judo 40 and the rats in the control group received corn oil only. The doses were administered twice a day between 8.00 am – 9.00 am and 15.00 pm – 16.00 pm for 2 alternative days.

3.3TOXICOLOGICAL OBSERVATIONS

Animals were observed daily between 11.00 a.m. and 12 noon for any overt signs of toxicity (diarrhoea, salivation, lachrymation, tremors, ataxia, loss of fur, change of fur colour, postural abnormalities or behavioural changes), stress (fur erection and exophthalmia), aversive behaviours, during the treatment period. Water intake was determined on the post treatment day 1, 3 and day 7.

3.4 BODY AND ORGAN WEIGHTS

Animals were weighed and the body weight was recorded daily up to post treatment day 3 using an animal balance (MP 6000, Chyo YMC and Corporation Ltd. Japan). Animals were sacrificed on day 3, and the right testes and epididymis, seminal vesicles and prostrate glands were excised and weights were recorded.

3.5 SPERM DENSITY AND MOTILITY

Animals were sacrificed and reproductive organs were used for experimental protocols as appropriately. The left cauda epididymis was used for sperm motility and right cauda epididymis was used for sperm counts and morphology. Cauda epididymis was sectioned out and to determine sperm motility, the cauda epididymis were nicked in few site examined with in 5 minute after their isolation from epididymis. The counting of both motile and immotile sperms was done under a phase contrast microscope (Nikon Eclips E600) at 40x magnification. Calculated results were expressed as percentage motility.

Right epididymis was diluted in 1:20 with physiological saline (0.9% NaCl) solution in a petri dish and the dispersion of sperm into medium. Sperm suspension was pipetted very gently 20 times and placed in a haemocytometer and total number of the sperm head counted under a Nikon microscope (Nikon Eclips E600) at 40x magnification. Each sample was counted thrice and mean value was taken for calculation.

3.6 DETERMINATION OF DNA DAMAGE IN SPERMATOZOA

Sperm smears from cauda region were prepared on pre-cleaned microscopic slides and air dried for 5 minutes. Smears were fixed in Carnoy's solution for at least 3h. Subsequently, slides were washed in distilled water and air-dried. Slides were stained with acridine orange for 5min. Smears were evaluated using a fluorescent microscope (Olympus Corporation, Japan) with excitation of 490 nm. Two 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 (Peiris, 1998).

3.7 DETERMINATION OF SPERM PROTEIN

Proteins were extracted from 3X 10^6 sperms in 10% SDS sample buffer medium. Samples were centrifuged and supernatant was used for running 10% polyacrylamide gel. The gel was subsequently stained with Commmassie blue stain and the differences among the bands positioned and the widths were determined.

3.8 pH OF SEMINAL VESICLE FLUID

Seminal vesicles were removed and crushed and brought up to 5 ml by adding distilled water. pH was measured using a pH meter (HM 30V, TOA Electronics Ltd. Japan).

3.9 EFFECTS OF JUDO 40 ON HUMAN SPERM *IN VITRO*

Semen samples were collected from healthy male (age 20 - 28 years) donors 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 donors with normal parameters (sperm concentration > 40 x 10^6 spermatozoa/ml, total sperm motility > 50%, normal sperm morphology > 50%) were included in this study. Equivalent doses for humans were calculated for 20 mg/kg and 50 mg/kg doses (3.2 mg/kg and 8 mg/kg respectively). Fresh semen samples were diluted with isotonic saline (0.9% NaCl, w/v) to obtain final sperm concentration of 40 x 10^6 spermatozoa/ml. Subsequently samples were incubated either with pesticide at concentrations of 20 and 50 mg/kg Judo 40 in corn oil. Incubations were done in 1ml of final volume for 5, 15 and 30 min at 37 °C in an incubator (Sanyo Electric Co. Ltd., Tokyo, Japan) in 5% CO_2 and 95% O_2 atmosphere. Percentage motile spermatozoa (WHO, 2010) were estimated (by counting approximately 100 cells) for each concentration at the each time points by a single observation under phase contrast optics (X 400; Olympus Corporation, Japan). Percentage motility was calculated as follows: Percentage motility= (Total number of cells - number of immotile sperm/ Total number of cells) X 100. Total number of cells = 100.

The viability of spermatozoa of Judo 40 treated (n=9) and control samples following 5, 15, 30 min of incubation were assessed using Nigrosin-Eosin double stain technique (WHO, 2010). All spermatozoa showing any 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.

4. 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 $P < 0.05$.

5. RESULTS

5.1 GENERAL TOXICITY

Acute poisoning symptoms such as salivation, difficulty in breathing, tremor, convulsions, rigid posture, lacrimation, piloerection, exopthalmia, reddening around the nose and mouth were observed. Symptoms appeared with the second fraction of the dosing and climaxed with the last fraction of the dosing. Symptoms subsidized on the 1st day post-treatment and fully disappeared on the 2nd day post-treatment. Diarrhea was observed in both treated groups, most severely with the high dose and moderately with the low dose. Defecation returned to normal in animals treated with low dose of chlorpyrifos within day 3 post-treatment but in the animals treated with 50 mg/kg of chlorpyrifos took longer to recover. No mortality was observed among any of the treated rats. The severity of symptoms was dose dependent.

5.2 WATER CONSUMPTION

Water intake of rats treated with both 20 mg/kg and 50 mg/kg of chlorpyrifos showed a significant ($P<0.01$) reduction compared to the control on post treatment day one. However, on post treatment days 3 and 7 the water intake was comparable to that of the control group (Figure I).

5.3 ORGAN WEIGHT DETERMINATION

No significant changes of weights in epididymis, testis, prostate or seminal vesicles were observed when compared to the control (Table I).

5.4 EFFECTS ON SPERM COUNTS

Both high and low doses of Judo 40 failed to cast any impairments in the number of sperm in the cauda epididymal spermatozoa number when compared to the control. Results were summarized in Table I.

5.5 EFFECTS ON SPERM MOTILITY

In contrast to sperm concentration, there was a significant ($p<0.05$) reduction in cauda sperm motility at both dose levels (Table I). In the high dose the motility was reduced by 37.24% (control: 93.67±0.97 vs. treatment 58.78±10.74) and in the

low dose by 51.72% (control: 93.67±0.97 vs. treatment 45.22±6.78).

5.6 EFFECTS ON DNA DAMAGE IN SPERMATOZOA

Percentage of DNA damaged spermatozoa count was increased at both doses. The percentages of damages were significantly ($p<0.05$) increased by 95.9% with low dose (control: 5.11±1.1; treatment: 10.01±1.36) and significantly increased ($p<0.01$) by 513.11% (control: 5.11±1.1; treatment: 31.33±2.65). Results are summarized in Table I.

5.7 SPERM PROTEIN ANALYSIS

Vertical gel electrophoresis carried out for sperm protein analysis showed no alterations in the protein level and any dose level.

5.8 pH Of Seminal Vesicle Fluid

There was a significant decrease ($p<0.01$) in the pH of the seminal vesicle fluids compared to the control values. See Table 1.

5.9 EFFECTS OF JUDO 40 ON HUMAN SPERM *IN VITRO*

Human sperm incubated with Judo 40 in vitro elicited significant ($p<0.05$) effects on sperm motility at all time points (by 78.3%: 5 min; 78.8%: 15 min and 83.4%: 30 min) with 8 mg/kg (rat equivalent dose to 50 mg/kg) and only at 15 min (by 48.8%) and 30 min (by 69.6%) incubation with 3.2 mg/kg (rat equivalent dose is 20 mg/kg) dose level (Figure 2). Similarly, sperm viability was reduced only at 15 min (48.8%) and at 30 min (52.1%) incubation with the high dose. Results are summarized in Figure 3.

6. DISCUSSION

Results obtained from the present study indicated that Judo 40 could exert significant effect on both rat and human sperm *in vitro*. Similar results were observed with chlorinated hydrocarbon (Pflieger-Bruss & Schill 2010) and benzene metabolites (Mandani *et al.* 2013). The present study demonstrates that Judo 40 exhibited general toxicity signs, which can be denoted by general behavior due to reduction of acetylcholine esterase enzyme. However, oorganophosphorus insecticides can be detoxified in mammals through enzymatic hydrolysis thus diminishing the symptoms and body weight reduction at post treatment day 3 and day7. (Colovic et al. 2013).

The water intake of rats was significantly reduced on post treatment day 1. Similar thirst inhibition was also observed with methoamidophos (Peiris et al. 1995) and with monochrotophos (Ratnasooriya et al. 1996). Permeability changes in hypothalamic osmoreceptors or sodium receptors that regulate water levels of the body. The permeability changes of the membranes develop within a period of 10 h (thus leading to observed (Danziger and Zeidel, 2014) reduction of water consumption on post-treatment day 1 in the present study. The thirst is under the control of hypothalamus. In rats, the area concerned with thirst is the lateral hypothalamus, posterior to the feeding center. Further, in the renin-angiotensin II mediated pathway of inducing thirst, angiotensin acts on the subfornical organ, a specialized receptor area in the diencephalon to stimulate the neural areas of the thirst region. The links from the subfornical organs to the neural areas are cholinergic (de Lima et al. 2013). Cholinergic neurons are mediated by acetylcholine and excess accumulation of the neurotransmitter at nerve endings may block its own action (Colovic et al. 2013) thus resulting in a deceased in drinking frequency.

In the present study a significant reduction of sperm motility was observed in both low (20 mg/kg) and high (50 mg/kg) doses of Judo 40 treated rat and human sperms. Sperm motility is considered to be one of the most sensitive detectors for sperm cytotoxic effects. Changes in mitochondrial membrane potential by different pesticides can result (Pant et al. 2014) and uncoupling of oxidative phosphorylation could reduce energy produced for motility (Betancourt *et al.*, 2006) thus resulting in reduction in sperm motility. Similarly, 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). Moreover, disulfide bonds formed in the tail are important to maintain rigidity that is essential for progressive motility and oxidation of disulfide bonds could result in loss of sperm motility (Buffon et al. 2012). Acridine orange test in the present study revealed that Judo 40 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. Diminution of sperm protein has been shown to compromise both fertility and sperm motion (Choi et al. 2015). In the present study, although the motility of sperms was affected no change in the banding pattern was observed. Hence, it is clear that sperm motility was not mediated through alterations of sperm proteins.

Typically pH value of seminal vesicle fluid is closer to neutral, which can be attributed mainly to cations such as calcium and magnesium ions present in the fluid (Chowdhury & Joy 2007). Any alterations to ionic gradients could result in deviation from normal pH values (Alavi & Cosson 2006) observed in the present study. Moreover, the pH and ionic concentration of seminal vesicle plasma play a key role in sperm motility (Zhou et al. 2015). Hence, the retardation in sperm motility observed in the present study can be attributed to low pH observed in the seminal vesicle fluid.

Reactive oxygen species are known to damage cellular membrane bound polyunsaturated fatty acids. Since spermatozoa are rich with polyunsaturated fatty acids, they may be highly

susceptible for reactive oxygen species damages thus resulting in loss of membrane integrality and cellular function. The overall effect of membrane damage might be responsible for continuous decrease in sperm motility and viability after ejaculation (Calamera *et al.*, 2001). Organophosphorus compounds have been shown to cause oxidative damages (Sharma *et al.*, 2005) and resulting in reduction of sperm motility, and viability. The Vitality or viability is the proportion of live spermatozoa determined by the evaluation of cellular and or membrane integrity (Rao, 2006). Viability of sperm is associated with intact, functional and semipermeable plasma membranes (Mandani *et al.*, 2013). Hence, reduced viability observed with human spermatozoa in this study could be due to changes in sperm plasma membrane (Eberhard *et al.* 2010).

Some organophosphate compounds are known to incorporate residues of pesticides metabolites in to the DNA by indirect alkylation, thus resulting in genetic damage. If the disulfide bonds formed during the maturation process are disrupted, it results in single strand DNA thus increasing the permeability of the AO stain (Agrawal & Said 2003). The disruption 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 organophosphates are known to generate ROS thus producing DNA damage observed in the present study (Choi et al. 2015).

7. CONCLUSION

From the results of the present study, it can be concluded that both rat and human sperm functions were decreased with exposure of Judo 40. High concentration of Judo 40 altered both rat and human sperm motility and altered human sperm vitality. Further it induced DNA damages. Henall results of the study and natural human biological responses such as degradation and clearance of chemical, it could be recommended that tested higher dosage (200 µg/mL) and above highest dosage may cause the risk of human health and toxicity may increase with exposure time. Therefore, acephate can be considered as a reproductive toxicant and may carry a risk to human health.

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APPENDIX

Table I. Effects of Judo 40 organ weights, sperm parameters of rats and pH of seminal vesicle fluid

Parameters	Control (Corn oil)	Treatment (Judo 40)	
		20 mg/kg	50 mg/kg
Organ weights			
Kidney	1.025±0.033	1.037±0.037	0.97±0.025
Liver	11.02±0.024	10.80±0.502	10.56±0.475
Adrenal	0.033±0.001	0.034±0.037	0.036±0.003
Spleen	0.65±0.044	0.59±0.049	0.63±0.048
Epididymis	0.53±0.017	0.57±0.029	0.54±0.032
Testes	1.29±0.008	1.26±0.028	1.27±0.032
Prostrate	0.26±0.014	0.23±0.035	0.26±0.015
Seminal Vesicle	0.64±0.034	0.60±0.022	0.46±0.003
Rat sperm Parameters			
Sperm count (10^6/g)	1514.144±139.8	1502.6±144.53	1583±155.7
Cauda sperm motility (%)	93.67±0.97	59.78±10.74**	45.22±6.78
DNA damage in sperm	5.11±1.11	10.01±1.36*	31.33±2.65**
pH of seminal vesicle fluid	6.15±0.085	5.89±0.048**	5.85±0.045**

The data are given as mean ± S.EM (n = 9). Values are statistically significant at ** $p < 0.01$. Control group was given Corn oil while treated group was given 20 mg/kg & 50 mg.kg chlorpyrifos. The data was analyzed by parametric method-ANOVA.

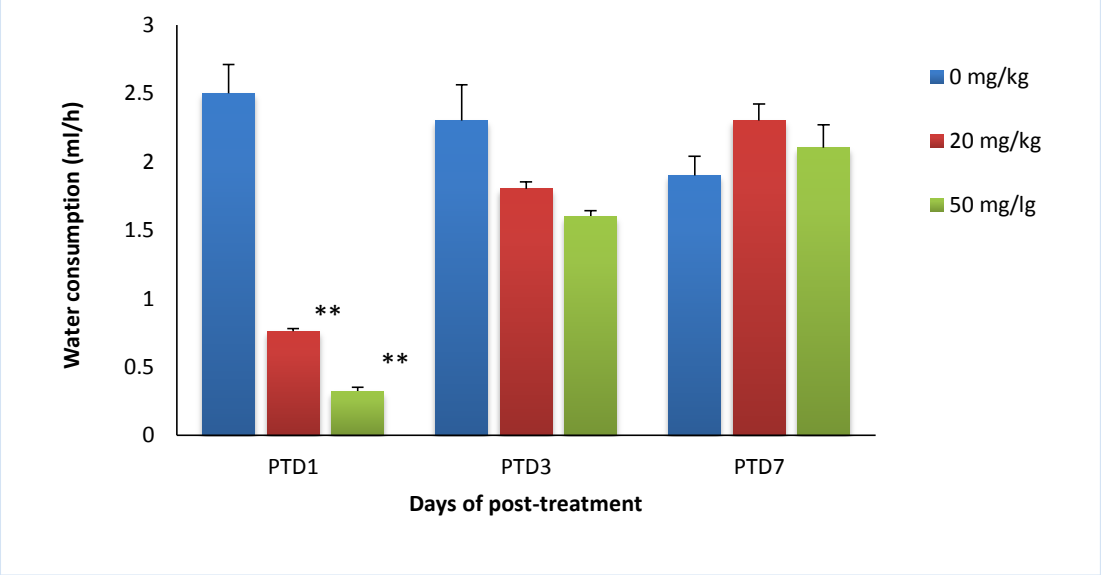

Figure 1: Water consumption at post-treatment day (PTD) 1, 3 and 7 with different doses of Judo 40 (20 mg/kg and 50 mg/kg) and the control (corn oil). Values represent mean ± SME Mean (n=9). **P ≤ 0.01 significantly different from control.

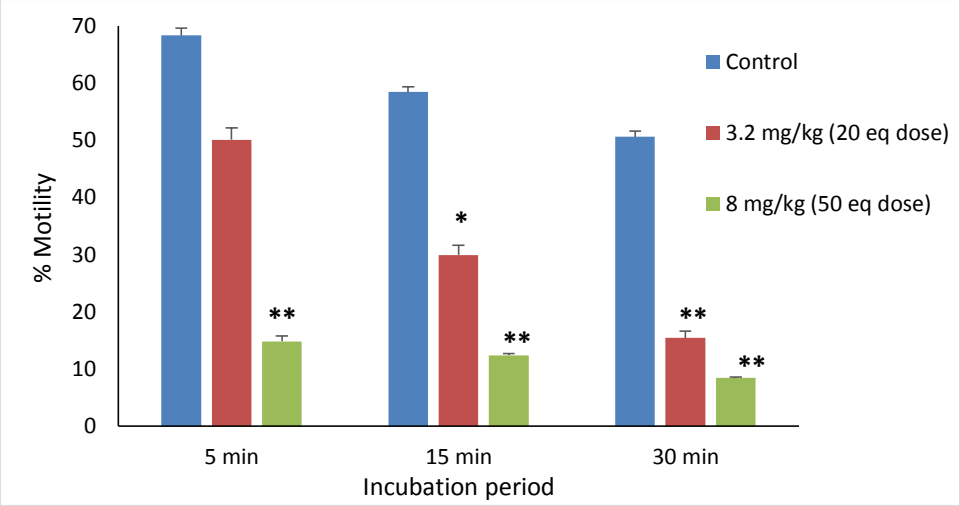

Figure 2: Percentage of human spermatozoa motility after 5 min, 15 min and 30 min of incubation with 2 doses of Judo 40 (3.2 mg/kg; equivalent dose of 20 mg/kg and 8 mg/kg; equivalent dose of 50 mg/kg) or Corn oil (control). Results are presented as mean ± SE Mean (n=9). *P ≤ 0.05, **P ≤ 0.001 significantly different from control.

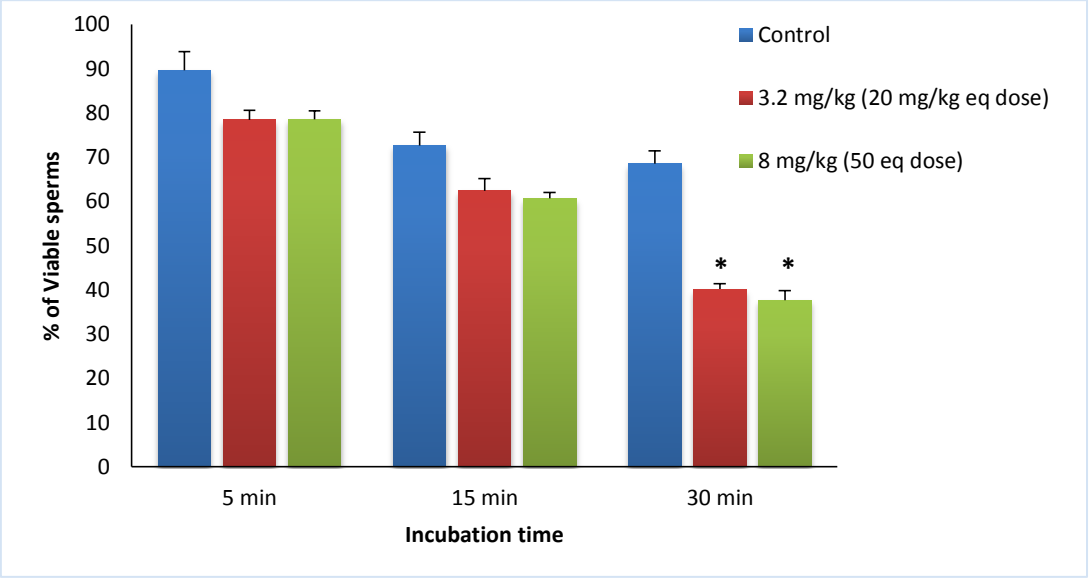

Figure 3: Percentage of living human spermatozoa after 5 min, 15 min and 3o min of incubation with 3.2 mg/kg (rat equivalent dose to 20 mg/kg) and 8 mg/kg (rat equivalent dose to 50 mg/kg) concentrations of Judo 40 and control (Corn oil). Results are presented as mean ± SE Mean (n=9). *P ≤ 0.05, significantly different from control.