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Research Article

In Vitro Toxicity of Some Pesticides on Goat and Dog Spermatozoa [1] [2]

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Abstract

In vitro toxic effects of selected pesticides (cypermethrin, flumethrin, propoxur, carbaryl, chlorpyrifos, metamidophos) were evaluated on dog and buck fresh spermatozoa using MTT (mitochondrial activity/viability), NeutralRed-NR (lysosomal activity/viability) and CASA (motility parameters/VCL, VSL, VAP, linearity, straightness, wobble) tests. For buck, the most toxic compounds were carbamates followed by organophosphate and pyrethroids. Carbamates, again induced the highest toxicity in dog sperm, followed by pyrethroids. In general, all pesticide treatments were found to increase the hyperactivity of buck spermatozoa except flumethrin; whereas a decrease was present for dog spermatozoa (except carbaryl). A decrease of VCL, VSL and VAP parameters was evident in buck ($P<0.05$), whereas no difference was found in dog sperm ($P>0.05$). Overall, flumethrin was found to induce less effect on motility parameters compared to cypermethrin. As a conclusion, the combination of MTT and CASA along with NR would provide more accurate data for the *in vitro* evaluation of chemicals on spermatozoa; where alternative testing strategies for the ReproTox tests (especially for cosmetic product safety assessment where animal testing is banned) are recommended nowadays with battery strategies and species specific differences would play a key role for understanding the defense mechanisms.

Keywords: *Pesticides, Buck spermatozoa, Dog spermatozoa, In vitro toxicity, MTT, CASA, Neutral red*

Bazı Pestisitlerin Teke ve Köpek Sperması Üzerine *İn Vitro* Toksisiteleri

Özet

Bu çalışmada, bazı pestisitlerin (sipermetrin, flumetrin, propoksur, karbaril, klorprifos, metamidofos) köpek ve teke taze spermaları üzerindeki etkileri, MTT (mitokondriyal etkinlik/canlılık), Nötral Kırmızı-NK (lizozomal etkinlik/canlılık) ve Bilgisayar Destekli Semen Analizi-CASA (motilite parametreleri, VCL, VSL, VAP, lineerlik, doğrusallık, yalpalama) kullanılarak *in vitro* olarak araştırıldı. Teke sperması üzerine canlılık ve motilite bakımından en toksik bileşiğin karbamatlar, ardından organofosfatlar ve piretroitler olduğu; köpek spermasında ise aynı şekilde karbamatların, ardından da piretroitlerin toksik etki gösterdiği tespit edildi. Genel olarak tüm pestisit uygulamalarının teke spermasında hiperaktiviteyi (flumetrin dışında) arttırdığı, köpek spermasında ise azalttığı tespit edildi (karbaril dışında). Tekede VCL, VSL ve VAP parametrelerinde azalma gözlenirken ($P<0.05$), köpek spermasında anlamlı bir fark gözlenmedi ($P>0.05$). Piretroitler arasında flumetrinin, motilite parametreleri üzerine etkisi sipermetrine göre daha az bulundu. Sonuç olarak üreme toksisite testlerinde (özellikle kozmetik ürünlerin toksisite testlerinde hayvan deneylerinin yasaklandığı göz önünde bulundurularak) alternatif testler arasında bulunan ve batarya testleri içerisinde yer alan *in vitro* sperm toksisite araştırmalarında; MTT ve CASA ile birlikte NK'nın birlikte kullanılmasının kimyasalların sperma üzerine etkilerinin değerlendirilmesinde daha etkili olacağı ve türe özgü farklılıkların kimyasalların sperma canlılık ve motilite parametrelerini farklı şekil ve düzeyde etkileyeceği, dolayısıyla spermada türe özgü ksenobiyotik moleküler savunma mekanizmalarının araştırılmasının gerekliliği gösterilmiştir.

Anahtar sözcükler: *Pestisitler, Teke sperması, Köpek sperması, İn vitro toksisite, MTT, CASA, Nötral kırmızı*

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INTRODUCTION

Male infertility due to impaired semen quality is related to a variety of possible causes including genetic abnormalities, lifestyle (smoking, alcohol, clothing etc), diseases *(Brucella, Chlamydia)*, condition *(varicocele, hematocele* and *torsion)*, environment and occupation (hormone disrupters-xenoestrogens, anti-androgens, toxic substances-lead etc.) [1]. The physiological mechanisms that ultimately lead to healthy sperm production is complex; where a disturbance can take place at different periods in a lifetime and differ for each species [2]. Along with the increased concern on endocrine disrupting compounds, mainly due to their effects on triggering morphological and functional abnormalities in reproduction systems, screening chemicals for male infertility raised an increasing interest. Since March of 2013, European cosmetic products cannot be tested on animal for reproductive toxicity (the 7th amendment to the European Union's Cosmetics Directive). Non-animal *in vitro* alternative tests for the assessment of the effects of chemicals on reproductive system have been developed and many of them are validated. However, these models are often not fully reflective of physiological processes and need to be complemented with additional tests to accurately illustrate the overall reproductive function. Innovative non animal testing strategies are sought from regulatory authorities for rapid and accurate detection strategies [3].

Sperm motility is considered as one of the most important parameters in evaluating the fertilizing ability of sperm in the human or other mammal species where motility parameters directly affect sperm penetration of mucus or oocyte vestments [4]. The conventional, manual method of sperm count and assessment of motility under the optical microscope was found to be fraught with inevitable subjective [5]. Computer-assisted sperm analysis (CASA) has been developed as a sensitive and reliable method in the quantification of deficiencies in sperm motility pattern and, together with assessment of sperm concentration and viability for the evaluation of the fertilizing ability of an ejaculate [4].

The aim of this study was to evaluate the *in vitro* effects of pesticides *(carbaryl, chlorpyrifos, cypermethrin, flumethrin, methamidophos* and *propoxur)* on dog and goat spermatozoa using MTT and Neutral Red tests for viability and CASA system for the motility. In particular, the combination of viability assays and comparative investigation of species specific differences for the evaluation of xenobiotic exposure on spermatozoa *in vitro* has never been performed. Even though the combination MTT and CASA analysis as an objective, simple, inexpensive and efficient method for the screening of xenobiotics in spermatozoa was introduced previously, species specific differences are expected to provide an important aspect for the evaluation of these chemicals.

MATERIAL and METHODS

Animal experiments were conducted according to ethical principles and this study was approved by the animal ethics committee in Ankara University (2015-21-230).

Semen samples were collected from Angora buck (4 years of age) under uniform feeding and housing conditions in Ankara University Faculty of Veterinary Medicine Research Farm, where they are proven to be free from any general or genital diseases. Ejaculate was collected with the aid of an electro ejaculator (Ruakura®, New Zealand). Animals were strained and topical lidocaine was used to reduce the pain caused by rectal smooth muscle spasm or *intra pelvic nerve* stimulation, then after probe was inserted into *rectum*. Ejaculate from buck were directly immersed in a water bath at 35.5°C until it could be assessed for total and progressive motility as well as sperm concentration. Then samples at 35.5°C were cooled down to 24°C for laboratory transfer.

Semen samples from the known healthy fertile German shepherd male dog (2 years of age and sired a litter previously) were collected by digital manipulation. The first and third fraction were collected in two separate pre-warmed plastic tubes. The volume of 1st fraction, 2nd fraction and 3rd fraction with a graduated collection tubes and pH were determined with stripes. Only the second sperm-rich fraction of ejaculate collected was used for the experimental design.

Semen was evaluated macroscopically for volume, color and pH; microscopically for motility, concentration, viability and morphology. Total semen volume was determined from the graded collection tube soon after collection and the concentration was determined using an Accucell photometer. Spermatozoa were primarily analyzed by CASA and only ejaculates with a minimum concentration of 3×10^9 spermatozoa/mL and 75% progressively motile cells were used for dilution to a final concentration of 3.6×10^6/mL spermatozoa. For the dilutions a Tris-based extender (30.7 g of Tris, 16.4 g of citric acid, 12.6 g of fructose and 1000 mL of distilled water at a pH of 6.8 with no cryoprotectant) was used.

For cytotoxicity studies, collected sperm in Tris buffer were transferred to 96-well plates at 3.6×10^6 sperm/mL per well (100 µL per well). Pesticides *(carbaryl, chlorpyrifos, cypermethrin, flumethrin, methamidophos* and *propoxur)* were dissolved in Tris buffer (only for *flumethrin* 500 µL DMSO and 500 µL Tris) at 1000 µg/µL concentration. Working solutions at log conc. first, then half conc. were prepared (166.67-0.0000167 µg/µL per well). Viability was measured using MTT and Neutral Red Assays; wherein MTT, metabolic activity of the cells are evaluated by the conversion of a yellow tetrazolium salt to purple formazan particles by mitochondrial succinate dehydrogenase of

intact mitochondria of living cells and in Neutral Red, viable cells incorporate and bind the supravital neutral red dye in viable lysosomes. MTT assay was performed according to our previous study on sperm [6] modified from Mosmann [7] and neutral red assay was performed according to Repetto et al.[8]. Viability assays were both then quantified using a micro plate reader (SpectraMax i3x-Multimode Detection Platform, Molecular Devices, Sunnyvale, CA, USA) at 540 nm. Cytotoxicity values, expressed as percentage, was calculated with regard to the untreated cell control (containing only the vehicle buffer), which was set to 100% viability and the dead cell control (containing Triton-X) which was set to 0% viability. A plot of % cytotoxicity versus sample concentrations was used to calculate the concentration which was then evaluated to calculate 50% cytotoxicity defining IC_{50} value.

For motility studies, collected sperm in Tris buffer were transferred to 96-well plates at 3.6×10^6 sperm/mL per well (100 µL per well). Pesticides were dissolved in Tris buffer (only for *flumethrin* 500 µL DMSO and 500 µL Tris) and prepared in the stock concentrations to be applied at IC_{50} doses in 20 µL IC_{50}, $IC_{50}/2$, $IC_{50}/4$ and $IC_{50}/8$ concentrations were applied for both sperms. The results were recorded at 0 and 4th hour following the exposure.

A 5 µL sperm suspension was placed on preheated siliconized slides and covered with 22 × 22 mm^2 coverslips to achieve a calculated depth of 20 psm. Sperm movement was recorded using a 100 frame/s camera (Basler, 782 × 582 resolution) attached to a microscope (600×, Nikon eclipse 50I, SCA, Barcelona, Spain) with a phase-contrast objective (10 × 10 magnification) and connected to a computerized motion analysis system, the Sperm Class Analyzer (SCA, Microptics®, Spain).

The sperm motility characteristics were determined using a 10× objective microscope lens at 37°C. The SCA acquisition parameters were set with 5 µm^2 <particle area <80 µm^2; progressivity >8% of STR; Circular <50% LIN; Vap points = 5; frame rate = 100/s; total captured images = 180 for dog and 10 µm^2 <particle area <80 µm^2; progressivity >75% of STR; circular <50% LIN; Vap points = 5; frame rate = 100/s; total captured images = 180 for goat. Each recorded field consisted of a mean of five replicates, each one analyzing from 100 to 300 sperm tracks and minimum average path at 50 µm/s and 40 µm/s; with >50% and >55% progressive motility were accepted for dog and goat sperm, respectively. For 5 µL of each sample, at least 200 up to 300 spermatozoa in 5 different areas were evaluated.

Measured data were plotted against the corresponding inhibition values using NCSS 2007, resulting in the inhibition curves as regression analysis, selected by the highest coefficient of determination (R^2); where IC_{50} (half maximal inhibitory concentration) values were calculated by interpolation of experimental data. Data were checked for parametric test assumptions by Kolmogrov-Smirnov test and Levene test to determine the homogeneity. Statistical significance between the pesticides and the animal species were determined by one-way ANOVA followed by posthoc analysis. A "P" value of <0.05 was defined as statistically significant.

RESULTS

IC_{50} values for *methamidophos, chlorpyrifos, carbaryl, propoxur, flumethrin* and *cypermethrine* using MTT assays are as follows in µg/mL; 104.62; 3.61; 23.98; 15.14; 67.89; 1.71 for dog sperm and 45.47; 11.36; 25.01; 1.47; 114.47; 1.58 for buck sperm. IC_{50} values was found to be lower in Neutral Red assay (NR) compared to MTT in general. IC_{50} values for the tested pesticides using NR (except *flumethrin*, where IC_{50} cannot be calculated) were as follows in µg/mL; 33.16; 3.80; 20.47; 58.53; ND; 0.79 for dog and 19.45; 2.51; 25.40; 2.42; ND; 0.39 for buck *(Table 1)*. IC_{50} values were found to be lower in buck ($P<0.05$) except *carbaryl* by Neutral Red and MTT assays and *chlorpyrifos* and *flumethrin* by MTT assay. For buck, the most toxic compounds were carbamates followed by organophosphate and pyrethroids. *Carbamates*, again induced the highest toxicity in dog sperm, followed by pyrethroids then organophosphates.

All pesticide treatments at $IC_{50}/2$ concentrations were found to increase the hyperactivity of buck spermatozoa except *flumethrin* (no change was present). On the contrary

Table 1. *IC_{50} values for the tested pesticides on dog and buck sperm*

Chemical Family	Main Mechanism of Action	Drug Name	IC_{50}			
			MTT		Neutral Red	
			Dog	Buck	Dog	Buck
Organophosphates	Acetylcholine esterase inhibitors (Irreversible)	*Methamidophos*	104.62	45.47	33.16	19.45
		Chlorpyrifos	3.61	11.36	3.80	2.51
Carbamates	Acetylcholine esterase inhibitors (Reversible)	*Carbaryl*	23.98	25.01	20.47	25.40
		Propoxur	15.14	1.47	58.53	2.42
Pyrethroids	Sodium channel modulators	*Flumethrin*	67.89	114.47	ND	ND
		Cypermethrin	1.71	1.58	0.79	0.39

Table 2. *Effects on the kinetic parameters (VCL, VSL and VAP) of pesticides on buck and dog sperm (percent control)*

% of Control±SD (Total = Slow + Medium + Rapid)			Curvilinear Velocity (VCL)		Straightline Velocity (VSL)		Average Path Velocity (VAP)	
			Buck	Dog	Buck	Dog	Buck	Dog
Organophosphates	*Methamidophos*	IC_{50}	117.10±26.56	89.01±6.18	85.24±12.72	92.73±8.38	101.84±29.13	97.57±6.38
		$IC_{50}/2$	111.60±21.63	97.07±7.79	105.62±22.69	104.39±47.78	112.63±22.86	120.08±21.35
		$IC_{50}/4$	137.04±26.56	103.14±10.42	130.89±28.13	79.03±20.62	134.80±27.36	84.95±5.05
		$IC_{50}/8$	152.70±29.59	71.41±9.65	148.83±31.98	58.48±6.59	151.84±30.82	68.00±9.56
	Chlorpyrifos	IC_{50}	40.52±7.46	39.43±3.95	48.42±0.99	33.58±1.07	42.37±1.65	35.01±2.11
		$IC_{50}/2$	103.08±11.67	68.06±3.49	57.71±0.86	61.50±3.58	86.66±22.60	62.27±3.66
		$IC_{50}/4$	74.99±8.49	95.51±4.32	70.26±1.05	80.78±15.82	64.06±1.92	83.76±8.54
		$IC_{50}/8$	49.46±5.60	59.12±3.32	42.73±0.64	51.19±14.21	37.63±1.13	55.64±13.23
Carbamates	*Carbaryl*	IC_{50}	103.81±17.53	99.36±6.90	92.88±2.69	130.41±11.69	96.71±8.58	112.39±7.35
		$IC_{50}/2$	131.65±14.12	75.81±6.79	75.02±1.42	114.68±15.40	141.94±7.45	83.18±17.03
		$IC_{50}/4$	82.34±8.83	72.04±6.35	64.77±1.23	146.46±11.86	66.79±3.50	88.62±4.20
		$IC_{50}/8$	188.42±20.21	70.46±5.80	193.10±3.66	138.96±17.01	188.08±9.87	88.47±10.35
	Propoxur	IC_{50}	40.92±9.66	75.00±5.21	35.85±15.48	78.22±7.07	37.32±6.47	87.42±5.72
		$IC_{50}/2$	50.58±6.62	80.39±5.43	53.50±8.99	100.94±38.97	60.00±14.43	95.69±15.24
		$IC_{50}/4$	57.51±7.53	62.38±4.40	58.81±9.89	107.42±22.86	60.83±4.49	86.94±26.90
		$IC_{50}/8$	105.02±13.75	71.52±4.08	136.18±22.89	101.50±12.67	126.87±9.36	93.43±25.99
Pyrethroids	*Cypermethrin*	IC_{50}	41.99±8.09	92.19±14.18	45.28±3.03	78.15±11.93	41.23±4.36	91.75±18.76
		$IC_{50}/2$	99.46±11.58	100.38±18.46	80.68±2.43	86.63±26.42	79.63±4.62	91.44±27.76
		$IC_{50}/4$	48.59±5.66	62.78±15.04	58.21±1.76	99.32±30.60	50.85±2.95	85.26±11.34
		$IC_{50}/8$	63.22±7.36	62.77±12.25	88.93±2.68	85.73±14.33	73.76±4.28	74.93±23.66
	Flumethrin	IC_{50}	37.34±2.46	107.64±10.78	31.16±9.92	97.79±3.11	34.32±5.68	103.00±6.21
		$IC_{50}/2$	67.06±3.60	81.54±11.41	84.35±16.23	89.85±15.25	74.54±7.51	85.26±14.75
		$IC_{50}/4$	52.77±2.84	86.19±12.76	70.11±13.49	124.32±10.36	52.21±5.26	104.42±8.54
		$IC_{50}/8$	59.85±3.22	95.90±15.87	83.25±16.02	103.99±28.81	67.67±6.81	113.95±11.25

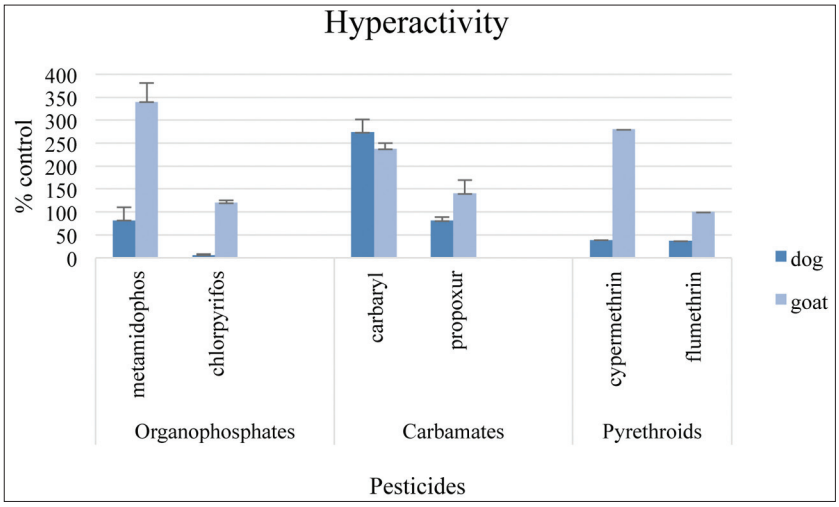

Fig 1. Effects on the hyperactivity of pesticides on buck and dog sperm (percent control)

a decrease was present for dog spermatozoa (except *carbaryl)*. For pyrethroids *(cypermethrin* and *flumethrin)* this decrease in hyperactivity in dog for both drugs were found as 39.09 and 37.27% compared to untreated control, whereas the most decrease was observed for *chlorpyrifos* treatment with a value of 6.37% *(Fig. 1)*. A decrease of VCL, VSL and VAP parameters was evident in buck ($P<0.05$), whereas no difference was found in dog sperm ($P<0.05$). Overall, *flumethrin* was found to induce less effect on motility parameters compared to *cypermethrin (Table 2)*.

Table 3. *Effects on the kinetic parameters (LIN, STR and WOB) of pesticides on buck and dog sperm (percent control)*

% of Control±SD (Total = Slow + Medium + Rapid)			Linearity		Straightness		Wobble	
			Buck	Dog	Buck	Dog	Buck	Dog
Organophosphates	*Methamidophos*	IC_{50}	72.79±1.43	104.37±4.95	83.70±1.43	95.26±4.65	86.97±2.14	110.01±0.87
		$IC_{50}/2$	94.64±5.69	107.75±22.51	93.78±5.69	87.14±7.96	100.92±3.51	124.15±2.33
		$IC_{50}/4$	94.85±5.70	76.76±7.29	96.73±5.70	93.25±14.59	97.97±3.62	82.65±4.15
		$IC_{50}/8$	96.92±5.83	82.05±25.21	97.44±5.83	86.21±24.73	99.56±3.65	95.56±8.53
	Chlorpyrifos	IC_{50}	119.49±1.42	85.32±12.83	114.29±1.04	96.13±5.46	104.56±3.65	89.11±8.06
		$IC_{50}/2$	55.99±0.79	90.53±48.99	66.60±0.57	99.00±10.63	84.07±3.64	91.82±15.94
		$IC_{50}/4$	94.27±8.33	84.73±17.51	109.77±0.95	96.67±20.21	85.82±3.72	88.01±20.21
		$IC_{50}/8$	87.07±1.23	86.75±6.41	114.29±0.99	92.22±16.64	76.24±3.30	94.45±11.45
Carbamates	*Carbaryl*	IC_{50}	89.48±17.53	130.41±6.19	96.04±2.69	115.36±5.63	93.16±1.25	113.51±0.90
		$IC_{50}/2$	56.98±14.12	114.68±29.93	52.85±1.42	104.57±11.56	107.82±1.34	110.12±2.13
		$IC_{50}/4$	78.78±8.83	146.46±18.49	97.20±1.23	119.12±27.08	81.31±6.01	123.45±5.67
		$IC_{50}/8$	102.61±20.21	138.96±10.83	103.18±3.66	110.72±28.95	99.94±1.24	126.02±15.38
	Propoxur	IC_{50}	87.62±2.85	104.50±4.96	96.08±18.57	89.69±4.37	91.20±14.93	116.98±0.93
		$IC_{50}/2$	105.77±2.41	125.80±26.31	89.17±13.34	105.74±9.09	118.61±4.92	119.46±2.38
		$IC_{50}/4$	102.26±2.33	172.52±19.14	96.68±16.45	123.85±22.14	105.77±4.39	139.87±7.18
		$IC_{50}/8$	129.67±2.96	142.18±11.48	107.34±19.61	108.89±17.29	120.80±5.01	131.10±20.27
Pyrethroids	*Cypermethrin*	IC_{50}	107.83±2.62	84.93±5.62	109.81±3.76	85.38±3.64	98.20±10.85	99.88±3.49
		$IC_{50}/2$	81.12±1.49	86.46±20.49	101.32±12.90	94.96±6.80	80.06±1.27	91.4±6.88
		$IC_{50}/4$	117.90±2.17	158.49±13.70	112.92±3.23	116.76±15.60	104.30±10.65	136.29±20.18
		$IC_{50}/8$	138.06±2.54	136.84±79.03	118.12±9.38	114.68±35.17	116.50±1.85	119.81±32.09
	Flumethrin	IC_{50}	82.83±10.06	91.02±13.69	89.40±17.44	95.16±5.41	92.26±3.98	96.03±8.68
		$IC_{50}/2$	126.88±5.95	110.39±3.73	115.27±10.63	105.63±11.23	110.88±3.85	104.94±19.63
		$IC_{50}/4$	133.74±6.27	144.51±38.84	136.32±12.57	119.34±26.35	98.64±5.42	121.58±21.40
		$IC_{50}/8$	140.60±6.59	108.64±17.80	125.31±11.56	91.48±27.39	112.74±8.91	119.25±13.20

For organophosphate drugs (chlorpyrifos and methamidophos), a decrease in VCL, VSL, VAP parameters for both drugs in buck was observed, whereas this decrease was present for only chlorpyrifos in dogs ($P<0.05$). Propoxur were found to decrease VCL, VSL and VAP values where no difference was found for carbaryl ($P>0.05$). Even though the minor changes, in general, the linearity, straightness and wobble values were found to have decreased in buck for all treatments except chlorpyrifos. Meanwhile this difference were insignificant in most of them and did not exert dose correlation *(Table 3)*.

DISCUSSION

The species specific differences in sperm morphology including spermatozoon membrane structural composition, ion channel distribution and oxidative damage are the key response elements for the toxic effects of xenobiotics on spermatozoa. Phospholipids, especially phosphatidylcholine and phosphatidylethanolamine, compose the majority of lipid fraction of the sperm cell membranes, functioning as a natural barrier for chemical and physical stress defining the functional characteristics of the sperm [9]. Long-chain polyunsaturated fatty acids (PUFAs) in spermatozoa influence the membrane fluidity [10] can lead to reduced membrane fluidity and a functional defect in sperm-oocyte fusion and fertilization [11]. As PUFAs and docosahexaenoic acid (DHA) are expected to effect the sperm viability, motility and the ability of spermatozoa to survive cryogenic storage and restores the protection [9,10], species specific differences might play an important role in the xenobiotic transport to sperm; meanwhile the alteration in the PUFAs composition could be the common base of different degenerative processes [12]. The goat sperm plasma membrane was found to be particularly rich in ether lipids phosphatidylcholine and phosphatidylethanolamine [13] and yet dog semen also contained great amounts of PUFA [12]. This lipid structure of the spermatozoa membrane also effects the the absorption of the chemical into the cell which is also determined by partitioning between aqueous and lipid phases. The octanol/water partition coefficient (log K_{ow}), defining the ratio of the concentration of the chemical in octanol to the concentration of water, is important in the absorption

of drugs. Hydrophobic drugs with high octanol/water partition coefficient are preferentially distributed to the lipid bilayers of cells [14]. In the current study *cypermethrin* having the highest Log K_{ow}, (6.6) were found to exert the lowest IC_{50} in both viability assays; however, this relation (Log K_{ow} of the tested pesticides are 5.9 for *flumethrin*, -1.74 for *methamidophos*, 4.7 for *chlorpyrifos*, 1.45 for *propoxur* and 2.36 for carbaryl) was not present for the rest of the drugs.

The distribution of the ion channels in different species could also implicate the toxicity differences of the tested pesticides. Amiloride sensitive Na^+ channels contribute to the regulation of resting sperm membrane potential [15]; whereas voltage-dependent Na^+ channels are required for the regulation of mature sperm function with an important role in the initial capacitation steps for the hyperpolarization before the acrosome reaction [16]. After capacitation, the sperm cell hyperpolarizes with an increase in K^+ permeability and blocking of the epithelial sodium channel. This effect is similar to the mechanism of action of pyrethroids. In the current study, this *in vitro* effect might have induced a potency on the transmembrane ion channels; where an increase in the overall hyperactivity is evident. On the other hand, the sperm activates the oocyte by causing either a single or series of Ca^{2+} oscillations and T-type Ca^{2+} channels are the key components in male reproduction, such as in the acrosome reaction and sperm motility [17]. The influence on the voltage independent Ca^{2+} influx, especially from the extracellular environment such like the conditions in our experiment are utilized during fertilization and might initiate a false acrosome reaction making the cells more hyperactive.

Acrosome contains hydrolytic enzymes like acrosin, hyaluronidase and many other hydrolases and esterases including acetylcholine esterase and acetylcholine transferase [18]. Also several reports suggest that nACh receptors (α7 nicotinic acetylcholine receptor) are present in mammalian sperm which is involved in the zona pellucida-induced acrosome reaction along with increased intracellular calcium levels [19]. As well known, organophosphorus compounds (OPs) and *carbamates* bind to an active site of acetylcholinesterase (AChE) and inhibit the functionality of this enzyme. In the current study, OPs increased the hyperactivity in buck and decrease in dog; where *carbamates* increased hyperactivity in both species; which might also be attributed to the species specific differences in the nACh receptor expression.

Another key factor; free-radical induced oxidative damage to mitochondrial membrane lowers the production of ATP ultimately affecting the motility and it has deleterious effects on sperm plasma membrane and DNA damage [20]. In the study by Zalata et al.[21] *in vitro* effects of *cypermethrin* on human spermatozoa were investigated; where *cypermethrin* was demonstrated to preferentially get localized in the hydrophobic core of the membrane, increasing the lipid packing and decreasing the membrane fluidity. It was concluded that, *cypermethrin* produced oxidative stress by generating reactive oxygen species (ROS) and reducing the antioxidant defenses. Although high concentrations of ROS cause sperm pathologies resulting in a loss of sperm motility and viability, low concentrations play an important role in capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion [20]. This might explain current study findings; where *cypermethrin* was found to be highly toxic compared to other pesticides *in vitro*. In buck, the toxic effect of *cypermethrin* in the current study, was more evident with an increased hyperactivity and a possible loss of ATP and death compared to dog.

Since motility of the spermatozoon depends on the energy expense produced in mitochondria [21]; MTT results in the current study, indicating the mitochondrial function, with lower IC_{50} values reveal that pesticides altered the mitochondrial functions, with an over increase of hyperactivity leading to cell death. MTT method, is efficient in processing a large number of specimens and therefore may be a powerful tool for preliminary screening of toxic compounds in spermatozoa. Neutral red assay, for the lysosomal activity, could also be used as a supportive quantitative-colorimetric method for the viability assays along with MTT and not only for the microscopic analysis for the acrosome reaction [22]. As CASA variables unfolds the important indicators for the fertilization capability of spermatozoa such as motility; it is expected to provide as prognostic tool for *in vitro* toxicity assays along with the viability quantitative such as MTT and NR for the potential screening of xenobiotics *in vitro*. The combination of these protocols would provide cheap, repeatable results where many samples are screened in a very short time. Even though these *in vitro* models represent only a very simplified picture of reality which encompasses only a small part of the complex reproductive cycle and has its own limitations; it is required especially for the reproductive toxicity battery assays for the cosmetic products testing where *in vivo* studies are no longer allowed. Researchers should also include the species specific differences for their decisions using these simplified *in vitro* sperm toxicity assays.

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