Computer Assisted Semen Analysis of Epididymal Spermatozoa after an Interperitoneal Administration of Diazinon and Cadmium

Maria Adamkovicova[1], Robert Toman[2], Michal Cabaj[2], Svatoslav Hluchy[2], Peter Massanyi[1], Norbert Lukac[1], Monika Martiniaková[3]

[1]*Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, 94976 Nitra, Tr. A. Hlinku 2, Slovakia*
[2]*Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, 94976 Nitra, Tr. A. Hlinku 2, Slovakia*
[3]*Constantine The Philosopher University, Faculty of Natural Sciences, 94974 Nitra, Tr. A. Hlinku 1, Slovakia*

Abstract
The effects of cadmium and diazinon administration on rat sperm motility parameters were performed using a Computer Assisted Semen Analyzer (CASA) system. CASA was made on semen samples collected 36 h following an intraperitoneal injection of physiological solution (control group), diazinon (20 mg/kg body wt), cadmium (2 mg/kg body wt), separately and in combination. Sperm kinematic measurements revealed significant decline in the percentage of motile spermatozoa ($P<0.05$), significant higher amplitude of lateral head displacement (ALH) ($P<0.01$) with significant decline in beat cross frequency (BCF) ($P<0.001$) after the diazinon administration. Cadmium-treated males exhibited significantly lower percentage of motile spermatozoa ($P<0.01$) and percentage of spermatozoa with progressive motility ($P<0.001$) with significant decrease in straightness (STR) and BCF ($P<0.05$). Simultaneous exposure to cadmium and diazinon led to significant increase in ALH ($P<0.05$) and significant decrease in BCF ($P<0.001$). Cadmium and diazinon exerted deleterious effect inducing spermatozoa motility alterations which could be subsequently negatively related to male fertility.

Keywords: cadmium, computer assisted semen analysis, diazinon, rat, spermatozoa motility

1. Introduction

Exposure to environmental pollutants is suggested to be one of the culprits to reproductive problems worldwide. Studies of the effects of environmental agents on semen quality in animals and in man have largely focused on organic toxicants and heavy metals with potential endocrine disrupting activity [1,2]. Given the adverse effects of endocrine disrupting chemicals exposure on laboratory animals, negative response to pollutants on human fecundity are extrapolated [3]. Although it is known that environmental contaminants are responsible for a range of noxious effects on various health aspects, little information is available on their possible combined effect on male reproductive function. Simultaneous exposure of humans to a number of chemicals may produce toxicity which is different than that seen if the exposure to these compounds were sequential. Scientific studies have found that exposure to multiple chemicals can have additive, synergistic and cumulative effects in humans [4]. Diazinon is a thionophosphorus organophosphate pesticide [O,O-Diethyl O-(2-isopropyl 6-methyl 4-pyrimidinyl phosphorothioate] commonly used in agriculture and urban pest control [5]. Despite its low persistence in the environment, it is a nonspecific insecticide and highly toxic to animals

*Corresponding author: Maria Adamkovicova, +42137 641 4466, Maria.Adamkovicova@uniag.sk

and humans [6], exposure to diazinon induces several neurological and endocrine alterations [7]. The primary action of diazinon is the inhibition of acetylcholinesterase activity by phosphorylation of the serine hydroxyl group in the substrate-binding domain of the enzyme which results in accumulation of acetylcholine and associated neurotoxicity [6,8]. Organophosphorus pesticides are related to male reproductive effects, diazinon can impact several organs and tissues including a decrease in genital weights, reduced sperm motility and viability, and increased sperm morphological abnormalities [5,9-12].

Cadmium is a highly toxic heavy metal occurring in the environment naturally or through anthropogenic activities and is known to have numerous undesirable affects on health in both animals and humans, targeting the kidneys, liver and vascular system in particular [13,14]. Cadmium has been recognized as an endocrine disruptor because of adverse effects on the reproductive system leading to the inhibition of steroidogenesis and spermatogenesis and ability to bind to androgen and estrogen receptors [2,3]. Several studies have illustrated that the testis is exceedingly sensitive to cadmium toxicity, likely due to disruption of the blood–testis barrier via specific signal transduction pathways and signaling molecules [15]. Exposure to cadmium has been reported to induce testicular and epididymal damage [16,17] and may contribute to male infertility by reducing sperm quality in both humans and rodents [2,18,19].

Current health research generally tests the effects of exposure to only one chemical substance at a time. This standard approach makes it difficult to come to a conclusion about the interactions of different chemicals. One way of determining the toxic potency of exposure to the mixture in humans is to carryout laboratory tests in experimental animals [4]. Therefore, the present study was designed to determine the combined effects of cadmium and diazinon after an intraperitoneal administration on the rat spermatozoa motility parameters measured by the CASA system.

2. Materials and methods

Animals

Forty mature, 4 months old male rats of the Wistar strain (weighing approximately 410 g) were randomly assigned into four groups of ten animals. The males were housed individually in plastic cages under constant temperature (20-22°C), humidity (55±10%), and 12/12 h cycle of light and darkness with *ad libitum* access to food (feed mixture M3, Machal, Czech Republic) and drinking water. All experiments were conducted in accordance with accepted standards of animal care in accredited laboratory (SK PC 50004, SUA Nitra).

Experimental Design

Rats in the group A were injected with a single dose (20 mg/kg body wt) of diazinon (Sigma-Aldrich, USA, purity 99%) intraperitoneally in physiological solution, rats of the group B were injected a single intraperitoneal dose (2 mg/kg body wt) of cadmium in the form of $CdCl_2$ (Reachem, Slovak Republic, purity 96%) in physiological solution, and rats in the C group were given a mixture of cadmium (2 mg/kg body wt) and diazinon (20 mg/kg body wt) intraperitoneally in physiological solution. The fourth group served as a control, received only physiological solution.

Semen collection, sperm motility analysis

The effects of cadmium and diazinon administration on the rat spermatozoa motility parameters were performed using a computer-assisted semen analysis - SpermVision™ CASA System (MiniTüb, Tiefenbach, Germany) with Olympus BX 51 phase microscope (Olympus, Japan). CASA was made on semen samples collected 36 h following an intraperitoneal administration of physiological solution (control group), cadmium (2 mg/kg body wt), diazinon (20 mg/kg body wt) separately and in combination. Sperm samples isolated from dissected epididymis at time of sacrifice were diluted with physiological solution (10 µl) and pipetted into a Makler Counting Chamber (depth 10µm, Sefi–Medical Instruments, Germany). In the samples, the percentage of motile spermatozoa, the percentage of progressive motility, DAP (distance average path, µm), DCL (distance curved line, µm), DSL (distance straight line, µm), VAP (velocity average path, µm/s), VCL (velocity curved line, µm/s), VSL (velocity straight line, µm/s), STR (straightness, VSL/VAP, %), LIN (linearity, VSL/VCL, %), WOB (wobble, VAP/VCL, %), ALH (amplitude of lateral head displacement,

μm), and BCF (beat cross frequency, Hz) were evaluated.

Statistical analysis

Statistical analysis was performed using the statistical software program Statgraphic Centurion XV and a probability of P = 0.05 was considered to be the minimum level of significance. Differences between individual ejaculate traits and sperm parameters per male were studied by performing a one-way analysis of variance (ANOVA) with Scheffe's *post hoc* test.

3. Results and discussion

During the past decades, the quality and fertility potential of sperm have decreased dramatically. Sperm motility has a high correlation with fertility and is an early and sensitive endpoint for evaluating chemical effects on male fertility [20]. The efficacy of CASA has been demonstrated for use with a variety of species in assessing male reproductive quality as well as the impact of various treatments on sperm motility; CASA allows an objective assessment of different cell characteristics: motion, velocity, and morphology [21].

The summary statistics for the sperm motility parameters are shown in Table 1.

Table 1. Summary statistics of sperm motility parameters

GROUP	Control	A	B	C
PARAMETERS	*Mean ± SD*	*Mean ± SD*	*Mean ± SD*	*Mean ± SD*
Motility %	50.31±9.15	36.09±16.67*	24.47±18.36 **	39.50±21.68
Progressive Motility %	27.69±7.34	21.38±10.09	10.16±9.73***	18.82±13.99
DAP (μm)	21.95±3,29	22.87±4.75	23.03±6.53	21.17±3.65
DCL (μm)	33.91±4.98	36.12±8.65	37.77±11.42	33.44±5.24
DSL (μm)	17.65±2.15	18.58±4.36	16.39±4.16	16.02±2.96
VAP (μm/s)	52.73±9.25	57.52±11.90	56.75±16.76	52.93±9.45
VCL (μm/s)	81.10±13.88	89.36±21.18	92.76±30.83	83.02±13.12
VSL (μm/s)	42.37±5.92	46.97±11.18	40.57±10.58	40.15±7.67
STR (%)	0.81±0.04	0.81±0.08	0.74±0.08*	0.77±0.04
LIN (%)	0.53±0.04	0.55±0.09	0.49±0.12	0.50±0.04
WOB (%)	0.65±0.03	0.66±0.06	0.64±0.11	0.65±0.03
ALH (μm)	4.52±1.26	6.76±1.46 **	5.57±1.06	5.70±0.70*
BCF (Hz)	22.37±2.71	13.84±2.83***	19.81±1.46*	17.42±1.66***

*P < 0.05, **P < 0.01, ***P < 0.001; SD - standard deviation; DAP - distance average path, DCL - distance curved line, DSL - distance straight line, VAP - velocity average path, VCL - velocity curved line, VSL - velocity straight line, STR - straightness, LIN - linearity, WOB - wobble, ALH - amplitude of lateral head displacement, BCF - beat cross frequency

Recent studies have shown that diazinon exposure could have a negative impact on reproductive performance of males in association with the changes in testosterone metabolic pattern, altering the activity of the direct hypothalamus–pituitary–gonadal neural pathway or a direct influence of diazinon in testes [7]. Diazinon has been found to affect sperm quality, such as viability, motility, and morphology by having detrimental effects on sperm chromatin structure, including DNA damage [5,22]. Sperm kinematic measurements revealed significant decrease in the percentage of motile spermatozoa (P<0.05), significant increase in ALH (P<0.01) with highly significant decline in BCF (P<0.001) of epididymal spermatozoa after the diazinon administration. The ALH is calculated from the amplitudes of the lateral deviations of sperm head about the axis of progression [23]. It is a valuable measurement, as this is one of the parameters affecting the outcome of *in vitro* fertilization and sperm penetration ability [21], and with BCF are indicators of sperm vigor [24]. The results of this study corroborate previous findings on reproductive toxic effects of diazinon [5,7,12,20] resulting in a decrease in the fertility levels of humans and animals [25].

Data from several sources have identified the toxic influence of cadmium on male reproductive

system [16], that causes reduction in testis and epididymis weight, testicular degeneration with seminiferous tubule damage [17], disruption of spermatogenesis [15], increased incidence of dead and abnormal sperm [26], decline in sperm concentration and sperm motility [2] by incorporation into sperm chromatin of the developing spermatozoa [14,27]. Our results obtained by motion analysis depict a significant decline in the percentage of motile spermatozoa ($P<0.01$), percentage of spermatozoa with progressive motility ($P<0.001$), and significant decrease in STR and BCF ($P<0.05$). These findings confirm the positive relationship between cadmium levels and asthenozoospermia, supporting the hypothesis that environmental cadmium exposures may contribute significantly to reduced sperm motility [2,18]. The decrease in sperm motility may be explained by cadmium effects on microtubules and sperm mitochondrial function [28]. The other parameters did not show significant changes compared to the controls. This is in accordance with some previous observations, which showed that cadmium toxic effect on spermatozoa distance and velocity parameters become significant with the passage of time [19], because when absorbed, heavy metals combine with the body's biomolecules as proteins and enzymes to form stable biotoxic compounds [13].
Simultaneous exposure to two toxicants cadmium and diazinon led to significant increase in ALH ($P<0.05$) and highly significant decline in BCF ($P<0.001$). The total sperm motility and progressive motility were inhibited insignificantly. The other movement parameters showed only minor alterations with no significant differences. Contrary to expectations, the results of this study did not show a greater impact on parameters of sperm motility after a simultaneous cadmium-diazinon exposure; a cumulative effect was not confirmed. Animals exposed to the mixture exhibited a better health status compared to those exposed to single chemicals at the same nominal toxic dose [29]. A possible explanation might be competitive interactions between heavy metal and pesticide toxicity. Very little was found in the literature on the question of toxic effects of heavy metals and pesticides mixture on the male reproduction functions. Semen samples exposed with cadmium, lead, chlorpyrifos, endosulfan have negatively affected the spermatozoa plasma membrane integrity, mitochondrial membrane potential and fertilization competence because of spermatozoa movement dysfunction [30]. Significantly decreased total motility, progressive forward motility, VSL, VAP have confirmed earlier statement that heavy metals and organophosphates act as testicular toxicants disrupting normal function of the reproductive system [17]. Although the acute intraperitoneal administration has confirmed that these chemicals caused spermatozoa movement dysfunctions, there is a need to investigate the toxic effect after peroral administration of such composites typical for long-term low-level environmental exposure.

Acknowledgements

This work has been supported by the grant from the Cultural and Educational Grant Agency of Ministry of Education, Science, Research and Sport of the Slovak Republic (KEGA) project No. 025UKF-4/2012.

4. Conclusions

The present study was conducted to evaluate the reproductive toxicity of environmental pollutants diazinon and cadmium on the rat sperm motility patterns analyzed with a CASA system. Sperm kinematic measurements revealed a significant decline in sperm motility, and sperm motion variables, including progressive motility, STR and BCF in relation to single cadmium administration. Diazinon negatively influenced sperm motility, decreasing percentage of motile spermatozoa and BCF. In spite of decreased sperm motion, ALH values were significantly increased in diazinon and cadmium-diazinon treated group. Although simultaneous exposure to cadmium and diazinon led to highly significant decrease in BCF, the results did not show a greater impact on parameters of sperm motility after a simultaneous cadmium-diazinon exposure; a cumulative effect was not confirmed. Cadmium and diazinon were shown to deteriorate sperm motility parameters which could be subsequently negatively related to male fertility. Therefore, future work will involve examining the relationship among cadmium and diazinon after long-term low-level exposure to reveal the mechanism of these effects on the sperm development and function.

References

1. Skakkebaek, N.E., Jorgensen, N., Main K.M., Rajpert-De Meyts, E., Leffers, H., Andersson, A.M., Juul, A., Carlsen, E., KrogMortensen, G., Kold, Jensen, T., Toppari, J., Is human fecundity declining?, Int J Androl, 2006, 29, 2-11
2. Benoff, S., Hauser, R., Marmar, J.L., Hurley, I.R., Napolitano, B., Centola, G.M. Cadmium concentrations in blood and seminal plasma: correlations with sperm number and motility in three male populations (infertility patients, artificial insemination donors, and unselected volunteers), Mol Med, 2009, 15, 248-62
3. Yeung, B.H.Y., Wan, H.T., Law, A.Y.S., Wong, Ch.K.C., Endocrine disrupting chemicals, Multiple effects on testicular signaling and spermatogenesis, Spermatogenesis, 2011, 1, 231-239
4. Neal, R.A. Evaluation of Mixtures: Laboratory Tests, In: Methods for Assessing the Effects of Mixtures of Chemicals, Vouk. V.B., Butler, G.C., Upton, A.C., Parke, D.V., Asher, S.C., Scope, 1987, 543-553.
5. Pina Guzman, B., Solis-Heredia, M.J., Quintanilla-Vega, B. Diazinon alters sperm chromatin structure in mice by phosphorylating nuclear protamines, Toxicol Appl Pharmacol, 2005, 202, 189-198
6. Colovic, M., Krstic, D., Petrovic, S., Leskovac, A., Joksic, G., Savic, J., Franko, M., Trebse, P., Vasic, V., Toxic effects of diazinon and its photodegradation products, Toxicol Lett, 2010, 193, 9-18
7. ElMazoudy, R.H., Attia, A.A. Endocrine-disrupting and cytotoxic potential of anticholinesterase insecticide, diazinon in reproductive toxicity of male mice, J Hazard Mater**,** 2012, 209-210, 111-120
8. Fulton, M.H., Key, P.B., Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects, Environ Toxicol Chem, 2001, 20, 37-45
9. Abd El-Aziz, M.I., Sahlab, A.M., Abd El-Khalik, M., Influence of diazinon and deltamethrine on reproductive organs and fertility of male rats, Dtsch Tierarztl Wochenschr, 1994, 101, 230-232
10. Swan, S.H., Kruse, R.L., Liu, F., Barr, D.B., Drobnis, E.Z., Redmon, J.B., Wang, Ch., Brazil, Ch., Overstreet, J.W., Semen quality in relation to biomarkers of pesticide exposure, Environ Health Perspect, 2003, 111, 1478-1484
11. Fattahi, F., Parivar, K., Jorsaraei, S.G.A., Moghadamnia, A.A., The effects of diazinon on testosterone, FSH and LH levels and testicular tissue in mice, Iranian J Reprod Med, 2009, 7, 59-64
12. Zidan, N.E.H.A., Evaluation of the reproductive toxicity of chlorpyrifos methyl, diazinon and profenofos pesticides in male rats, Int J Pharmacol, 2009, 5, 51-57
13. Duruibe, J.O., Ogwuegbu, M.O.C., Egwurugwu, J.N., Heavy metal pollution and human biotoxic effects, Int J Phys Sci, 2007, 2, 112-118
14. Thompson, J., Bannigan, J., Cadmium: toxic effects on the reproductive system and the embryo, Reprod Toxicol, 2008, 25, 304-315
15. Siu, E.R., Mruk, D.D., Porto, C.S., Yan Cheng, C., Cadmium-induced testicular injury, Toxicol Appl Pharmacol, 2009, 3, 240-249
16. De Souza Predes, F., Diamante, M.A., Dolder, H., Testis response to low doses of cadmium in Wistar rats, Inter J Experim Pathol, 2010, 91, 125-131
17. Toman, R., Adamkovicova, M., Hluchy, S., Cabaj, M., Golian, J., Quantitative analysis of the rat testes after an acute cadmium and diazinon administration, Scientific Papers: Animal Science and Biotechnologies, 2011, 44, 188-191
18. Xu, L.C., Wang, S.Y., Yang, X.F., Wang, X.R., Effects of cadmium on rat sperm motility evaluated with computer assisted sperm analysis, Biomed Environ Sci, 2001, 14, 312-317
19. Roychoudhury, S., Massanyi, P., Bulla, J., Choudhury, M.D., Lukac, N., Filipejova, T., Trandzik, J., Toman, R., Almasiova, V., Cadmium toxicity at low concentration on rabbit spermatozoa motility, morphology and membrane integrity in vitro, J Environ Sci Health A Tox Hazard Subst Environ Eng, 2010, 45, 1374-1383
20. Lifeng, T., Shoulin, W., Junmin, J., Xuezhao, S., Yannan, L., Qianli, W., Longsheng, C. Effects of fenvalerate exposure on semen quality among occupational workers, Contraception, 2006, 73, 92-96
21. Verstegen, J., Iguer-Ouada, M., Onclin, K. Computer assisted semen analyzers in andrology research and veterinary practice, Theriogenology, 2002, 57, 149-179
22. Sanchez-Pena, L.C., Reyes, B.E., Lopez-Carrillo, L., Recio, R., Moran-Martinez, J., Cebrián, M.E., Quintanilla-Vega, B., Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers, Toxicol Appl Pharmacol 2004, 196, 108-113
23. Mukhopadhyay, D., Varghese, A.C., Nandi, P., Banerjee, S.K., Bhattacharyya, A.K., CASA-based sperm kinematics of environmental risk factor-exposed human semen samples designated as normozoospermic in conventional analysis, Andrologia, 2010, 42, 242-246
24. Duty, S.M., Calafat, A.M., Silva, M.J., Brock, J.W., Ryan, L., Chen, Z., Overstreet, J., Hauser, R., The relationship between environmental exposure to phthalates and computer-aided sperm analysis motion parameters, J Androl, 2004, 25, 293-302
25. Joshi, S.C., Sharma, P., Male reproductive toxicity of organophosphorous compounds: a review., Toxicol Environ Chem, 2011, 93, 1486-1507
26. El-Demerdash, F.M., Yousef, M.I., Kedwany, F.S., Baghdadi, H.H., Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: protective

role of vitamin E and beta-carotene., Food Chem Toxicol, 2004, 42**,** 1563-1571
27. Bennetts, L.E., Aitken, R.J., A comparative study of oxidative DNA damage in mammalian spermatozoa, Mol Reprod Dev, 2005, 71, 77-87
28. Oliveira, H., Spano, M., Santos, C., de Lourdes Pereira, M., Adverse effects of cadmium exposure on mouse sperm, Reprod Toxicol, 2009, 28, 550-555
29. Dondero, F.**,** Banni, M.**,** Negri, A.**,** Boatti, L., Dagnino, A., Viarengo, A., Interactions of a pesticide/heavy metal mixture in marine bivalves: a transcriptomic assessment, BMC Genomics, 2011, 12, 195
30. Selvaraju, S., Nandi, S., Gupta, P.S., Ravindra, J.P., Effects of heavy metals and pesticides on buffalo (Bubalus bubalis) spermatozoa functions in vitro, Reprod Domest Anim, 2011, 46, 807-813