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IN VITRO EFFECTS OF SELECTED TRICHOTHECENES ON THE RABBIT SPERMATOZOA MOTILITY BEHAVIOR – A COMPARATIVE STUDY*

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Summary: *This study was designed to describe and compare the time- and dose-dependent in vitro effects of selected trichothecenes (deoxynivalenol-DON, zearalenone-ZEA and T-2 toxin) on the motility behavior of rabbit spermatozoa. The rabbit semen was diluted in PBS supplemented with different concentrations (1, 5, 10, 50, 100 µmol/L) of DON, ZEA or T-2 while the Control carried no mycotoxin. At culture times of 0h, 2h, 4h and 8h, the spermatozoa motility was assessed using the computer-aided sperm analysis (CASA) with the help of the IDENT stain and fluorescent illumination. The motility assessment revealed different behavior patterns, specific and unique to each of the studied mycotoxins. DON exhibited the ability to temporarily increase the sperm motility, followed by its rapid decline at later stages of the experiment (P<0.001). ZEA proved to act as a highly toxic substance on the spermatozoa, causing a rapid decline of the motion and resulting in a fast and complete sperm motility inhibition (P<0.001). Lastly, T-2 revealed to be highly detrimental to the sperm activity even at small concentrations (P<0.001). Our data suggest that further experiments are needed due to the lack of evidence emphasizing the toxinogenic effects of trichothecenes on male reproductive capacity.*

Key words: *mycotoxins, trichothecenes, deoxynivalenol, zearalenone, T-2 toxin, spermatozoa, motility, rabbits.*

INTRODUCTION

Fungi produce diverse secondary metabolites which may provide the fungus with an ecological advantage in certain environments. Fungal secondary metabolites include plant growth regulators, pharmaceutically useful compounds, pigments, and mycotoxins (Keller and Turner, 2005). Mycotoxins may accumulate in infected crop plants and, following ingestion, lead to the occurrence of diseases (mycotoxicoses) in animals and humans (Ueno, 1977). Trichothecenes are a major class of mycotoxins, causing a significant economic impact on cereal and grain crops every year (Foroud and Eudes, 2009).

Trichothecenes are a family of over 200 toxins primarily produced by the *Fusarium* species. Four types of trichothecenes have been identified, among which type A and B are the most prevalent and notable due to their toxicity in mammals. The major type A trichothecenes include T-2 toxin (T-2) and HT-2 toxin (HT-2) (Hussein and Brasel, 2001). Type A trichothecenes are highly toxic, T-2 has been reported to be approximately ten times more toxic than deoxynivalenol (DON) (Sudakin, 2003). DON belongs to more phytotoxic type B trichothecenes, produced primarily by *F. culmorum* and *F. graminearum* (Pestka, 2010). Other mycotoxins, such as zearelenone (ZEA) – a powerful endocrine disruptor, fumonisins, moniliformin and butenolide are also produced by *Fusarium* species (Zinedine et al., 2007).

Early toxicity studies showed that trichothecenes have the ability to inhibit eukaryotic protein synthesis, particularly by preventing peptide bond formation at the peptidyl transferase center of the 60S ribosomal subunit. Such inhibition affects polypeptide chain initiation or elongation, although polypeptide chain termination may also be ceased (Cundliffe et al., 1974). Trichothecenes were later shown to interfere with mitochondrial protein synthesis

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(Pace et al., 1988) and protein sulfhydryl groups (McCormick et al., 2011). The activity of trichothecenes has been correlated with oxidative damage due to overgeneration of free radicals (Ponts et al., 2009).

Semen quality may be affected by age, stress, hormonal status, nutrition, as well as numerous environmental factors, such as toxins (Giwercman and Bonde, 1998). Ewuola and Egbunike (2010a) suggested that exposure of breeding male rabbits to feed contaminated with *Fusarium* mycotoxins could decrease testicular sperm reserves and sperm production. Further research revealed that trichothecenes may delay puberty, impair spermatogenesis and semen quality, and induce embryo mortality (Ewuola and Egbunike, 2010b). Mycotoxins are potential disruptors of semen biochemical equilibrium by causing lipid peroxidation, altering enzymatic activity and seminal plasma protein concentration (Yousef et al., 2006). The disturbance of reproductive homeostasis may be translated into a decline in physical and chemical parameters of semen, such as pH, ejaculate volume, sperm concentration, motility and viability (Garcia-Tomas et al., 2006).

The knowledge concerning interactions between mycotoxins and reproductive functions in animals is scarce. It has been previously suggested that trichothecenes affect various aspects of male reproductive function, such as sperm quality and quantity (Sprando et al., 2005). Therefore, the aim of our study was to characterize and compare the time- and dose-dependent *in vitro* effects of selected trichothecenes (DON, ZEA, T-2) on the motility behavior of rabbit spermatozoa.

MATERIAL AND METHODS

Ten male rabbits (New Zealand white broiler line) were used in the experiment. The animals were 4 months old, with a weight of 4.0±0.2 kg and kept at an experimental farm of the Animal Production Research Centre Nitra, Slovak Republic. The rabbits were housed in a partially air-conditioned rabbit house under a photoperiod of 16L:8D (a minimum light intensity of 80 lux), kept in individual cages and fed with a commercial diet. Water was provided *ad libidum*. The air temperature of 20-24 °C and relative humidity of 65% were maintained in the rabbit house. Institutional and national guidelines on the care and use of animals were followed, and all the experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic (no. 3398/11-221/3) and Ethics Committee.

One ejaculate was collected from each rabbit on a regular collection schedule (once a week for three consecutive weeks) using an artificial vagina. Immediately upon collection, the sperm concentration and motility were assessed in each ejaculate. Individual ejaculates were mixed together in order to acquire a pooled sample of rabbit semen.
The semen sample was diluted in Dulbecco's PBS supplemented with different concentrations (1, 5, 10, 50, 100 μmol/L) of DON, ZEA or T-2 using a dilution ratio of 1:20. The Control group carried no mycotoxin supplementation. The samples were cultured at 25 °C.

At culture times of 0h, 2h, 4h and 8h, the spermatozoa motility (percentage of motile spermatozoa; motility > 5 μm/s; %) was assessed using the computer-aided sperm analysis (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). In order to avoid false positive results, the samples were stained using the IDENT stain, a DNA-specific dye based on Hoechst bisbenzimide (Hamilton-Thorne Biosciences) and analyzed under fluorescent illumination. The system was set up as follows: frame rate – 30 at 60 Hz, dark field; minimum contrast - 50; static head size - 0.28-4.30; static head intensity - 0.12-2.92; static elongation - 8-97; minimum cell size - 7 pixels; default cell intensity – 70, magnification – 1.75, illumination intensity - 2198. Ten μL of each sample were placed into the Makler counting chamber (depth 10 μm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. 10 microscopic fields were subjected to each analysis in order to include at least 300 cells.
All the data were subjected to statistical analysis using the GraphPad Prism program (a 3.02 version for Windows, GraphPad Software incorporated, San Diego, California, USA, http://www.graphpad.com/). The results are quoted as the arithmetic mean ± standard error of mean (SEM). The comparative analysis was carried out by a one-way ANOVA with the Dunnett's post test. The level of significance for the analysis was set at * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

RESULTS

The changes in the motility patterns of rabbit spermatozoa exposed to selected trichothecenes are displayed in Table 1. The immediate motility assessment (Time 0h) revealed different behavior patterns, specific and unique to each of the studied mycotoxins. DON supplementation resulted in an improvement of sperm motility, with significant differences particularly in case of high DON concentrations when compared to the Control ($P<0.001$ with respect to 100 μmol/L DON, $P<0.05$ in case of 10-50 μmol/L DON). On the other hand, ZEA revealed its instant highly toxic effects on male reproductive cells, by significantly decreasing the sperm motility, especially in relation to the highest concentrations administered to the culture ($P<0.001$; 10-100 μmol/L ZEA). Similar instant toxic effects

were observed in the instance of high T-2 concentrations (P<0.001; 50-100 μmol/L T-2), however with less aggressive effects when compared to ZEA.

After 2h, it was revealed that all trichothecenes had detrimental effects on the rabbit sperm motion. All concentrations of DON and T-2 led to a significant inhibition of the motility of rabbit male gametes when compared to the Control (P<0.001), and these alterations remained notable and significant during the following assessment at Time 4h.

Meanwhile, 50 and 100 μmol/L ZEA led to a complete sperm motility inhibition after 2h, while 5 and 10 μmol/L ZEA significantly decreased the motion of rabbit spermatozoa when compared to the Control (P<0.001). On the other hand, the lowest ZEA concentration administered to the sperm culture (1 μmol/L ZEA) had no impact on the spermatozoa motility neither after 2h nor after 4h of *in vitro* culture.

The final assessment at Time 8h revealed that the concentration range of 10-100 μmol/L of all selected mycotoxins exhibited long-term toxic effects on the motility behavior of rabbit spermatozoa (P<0.001 in case of 100 μmol/L DON, 50 and 100 μmol/L ZEA and T2; P<0.05 with respect to 10 and 50 μmol/L DON, 10 μmol/L ZEA and 10 μmol/L T-2). High concentrations of DON and ZEA led to an absolute cease of sperm motility, while T-2 significantly decreased the motion without inhibiting the functional activity of rabbit spermatozoa completely as compared to DON and ZEA. At the same time it was recorded that 1-5 μmol/L of all trichothecenes had no impact on the motion activity of rabbit spermatozoa in comparison with the Control.

Table 1. Spermatozoa motility (%) following treatment with selected trichothecenes (Mean ± SEM)

Groups	Motility 0h	Motility 2h	Motility 4h	Motility 8h
Control	54.23±2.44	48.09±3.66	17.66±5.04	6.77±0.98
100 μmol/L DON	68.88±3.56***	4.00±0.98***	2.09±0.78***	0.00±0.00***
50 μmol/L DON	65.21±4.32*	10.22±2.01***	5.77±1.03***	2.66±0.76*
10 μmol/L DON	63.56±1.34*	17.56±1.00***	6.45±1.22***	2.76±0.54*
5 μmol/L DON	57.33±2.22	18.45±2.03***	9.79±2.00***	3.78±0.87
1 μmol/L DON	56.87±3.44	30.54±5.09***	5.55±0.34***	7.65±0.97
100 μmol/L ZEA	1.24±0.10***	0.00±0.00***	0.00±0.00***	0.00±0.00***
50 μmol/L ZEA	7.55±0.65***	0.00±0.00***	0.00±0.00***	0.00±0.00***
10 μmol/L ZEA	36.34±3.65***	4.88±0.99***	4.32±0.98***	2.43±0.45*
5 μmol/L ZEA	52.77±6.02	5.32±1.02***	4.33±0.88***	3.58±0.55
1 μmol/L ZEA	53.55±4.76	40.44±4.32	19.90±2.67	5.67±0.74
100 μmol/L T-2	40.55±2.78***	9.09±1.01***	4.65±0.69***	1.11±0.23***
50 μmol/L T-2	40.09±1.99***	6.08±0.89***	3.99±0.93***	1.57±0.33***
10 μmol/L T-2	45.56±1.99	8.65±0.75***	4.02±0.51***	2.22±0.55*
5 μmol/L T-2	51.22±3.90	17.95±3.01***	6.06±0.56***	3.45±0.45
1 μmol/L T-2	50.67±5.09	20.12±2.01***	6.67±1.00***	3.99±0.41

* P<0.05; ** P<0.01; *** P<0.001.

DISCUSSION

Mycotoxins are well-known contaminants of the food chain. Their global occurrence is widely regarded as an important risk factor for human and animal health, as up to 25% of the world crop production may be contaminated with mycotoxins (Oswald and Comera, 1998). As such, the economic impact of mycotoxins includes increased mortality, increased veterinary care costs, reduced livestock production as well as disposal of contaminated foods and feeds (Hussein and Brasel, 2001).

Trichothecenes are of great concern due to their occurrence in toxicologically relevant concentrations in grains, having a direct impact on the health and productivity of farm animals (Doll and Danicke, 2004). These mycotoxins are commonly found on cereals such as wheat, barley, oats, rye, and maize, and less often in rice grown in Europe, America and Asia (Pestka, 2010). Nevertheless, there are limited data about their potential toxic effects after

common dietary exposure, which is why further research shedding more light on their mechanism of action is highly required.

This study was therefore focused on the evaluation of the possible impacts of three selected trichothecenes – DON, ZEA and T-2 on the motility behavior of rabbit spermatozoa. The selection of the trichothecenes was based particularly on their high abundance in the food chain as well as their notable adverse effects on the biological system. At the same time, the spermatozoa motility is an important prerequisite for their distribution in the female reproductive system, followed by an effective passage through the cervix and penetration into the ovum (Elia et al., 2011). Numerous studies on animals (Massanyi et al., 2008; Lukac et al. 2011) and humans (Björndahl, 2010; Eliasson, 2010) have emphasized the importance of sperm motility assessment in order to determine or predict the fertilizing capacity in males, to evaluate the possible impact of environmental factors on male fertility, as well as to assess the reproductive performance affected by a disease.

Among the three mycotoxins selected for our experiment, ZEA has been partially studied before on boar and stallion sperm. Previous *in vitro* studies showed that estrogenic mycotoxins including ZEA, alpha- and beta-zearalenol affected the fertilization ability of boar sperm because of their negative effect on viability, motility and spontaneous acrosome reaction (Tsakmakidis et al., 2006; Benzoni et al., 2008). Other reports emphasize the interference of ZEA and its derivatives on the spermatogenesis process related to a reduction in testosterone synthesis by Leydig mouse cells due to down-regulation of P450scc and 3 -HSD-1 (Yang et al., 2007). Since testosterone supports spermatogenesis, sperm maturation and sexual function, the disruption of testosterone biosynthesis can significantly contribute to male reproductive dysfunction. On the other hand, Filannino et al. (2011) revealed that alpha-zearalenol was the only ZEA derivative able to decrease the percentage of motile stallion spermatozoa, induce changes in motility characteristics of the remaining motile sperm consistent with hyperactivation, and induce the acrosome reaction. ZEA and beta-zearalenol induced only some of the motility parameters characteristic of hyperactivation and, of the two, only beta-zearalenol induced the acrosome reaction. Nevertheless the authors conclude that estrogenic mycotoxins have the ability to induce a premature stage of reproductive physiology and are therefore likely to inhibit the fertilizing potential of affected sperm.

There are very limited data discussing the effects of DON on male fertility. Medveďová et al. (2012) evaluated short-term effects of 10, 100, 1000 ng/mL DON on rabbit spermatozoa characteristics. Their results indicated no significant differences between the Control and experimental groups after the evaluation of the total and progressively motile spermatozoa, average path distance and velocity as well as amplitude of lateral head displacement. Sprando et al. (2005) observed a statistically significant, dose- related decrease in homogenization resistant testicular spermatid counts, spermatid numbers, absolute cauda epididymal sperm numbers and cauda epididymal sperm numbers per gram of cauda epididymis following 5.0 mg/kg b.w. DON administration to male rats. Furthermore, sperm tail abnormalities (broken tails) were significantly higher. Serum follicle-stimulating hormone and luteinizing hormone concentrations were increased in a dose dependent manner groups receiving treatment of 2.5 mg/kg and 5.0 mg/kg b.w. DON, while serum testosterone concentrations were decreased in a dose-related manner across all experimental groups.

To date, only *in vivo* information is available on the behavior of T-2 toxin in male reproduction. Yang et al. (2010) evaluated the effects of T-2 toxin on semen quality, fertility and serum testosterone concentration in mice exposed to intraperitoneal injection of 0, 5, 10 or 15 mg/kg b.w. T-2. The results showed that the number of abnormal spermatozoa increased significantly and a significant decrease in spermatozoa with integrated acrosome was observed in males treated with T-2 toxin at all doses. The amount of live spermatozoa decreased significantly in mice treated with 10 and 15 mg/kg b.w. T-2. The testicular and cauda epididymal sperm counts, efficiency of sperm production and serum testosterone concentration were significantly reduced in mice treated with T-2 toxin at all doses in a dose-dependent manner. Moreover, Yang et al. (2016) revealed that T-2 significantly suppressed hCG-induced testosterone secretion, in correlation with a decrease in the level of transcription of enzymes crucial for steroidogenesis, such as 3β-HSD-1, P450scc, and StAR.

CONCLUSION

Our study allows a preliminary definition of the *in vitro* toxic effect of selected trichothecenes on rabbit spermatozoa. Each mycotoxin affected the sperm motion behavior in a specific manner. DON may have the ability to temporarily increase the sperm motility, followed by its rapid decline at later stages of the *in vitro* culture. On the other hand, ZEA proves to act as a highly toxic risk factor on the sperm activity, causing a rapid decline of the motion parameters and resulting in a fast and complete motility inhibition. Lastly, T-2 revealed to be highly detrimental to the sperm behavior even at small concentrations. The results of this study may serve as an important foundation for more complex studies to ascertain the specific mechanisms of trichothecenes activity affecting male reproductive cells.

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