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Mycotoxins in the Food Chain Research Group

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**INVESTIGATION OF THE CYTOTOXIC EFFECT OF T-2
MYCOTOXIN *IN VITRO* AND *IN VIVO* EXPERIMENTS**

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1. RESEARCH BACKGROUND AND OBJECTIVE

Beside the sudden population growth, the increasingly shrinking cultivated land has become a major issue of today. Not only a quantitative problem arises in the food supply but the qualitative factors also play an increasingly influential role. We constantly hear a variety of food scandals, an important consequence of which, in addition to the economic delict, can damage people's health. So today's still current and important matter is feed quality, and through this the problem of food quality. To solve it, the European Union has created very strict food quality and food safety standards, and its compliance is mandatory for all Member States, and it refers to the quality of export products as well. The significance of mycotoxins is outstanding in the group of food contaminants of natural origin. In addition to the human health effects, their economic impact on animal husbandry and on crop husbandry is remarkable (Kovács, 2001). The fate of food is strictly controlled and monitored even in Hungary. This task is performed by the NÉBIH (National Food Chain Safety Office) according to the Hungarian Act XLVI of 2008 on the food chain and the official supervision (<https://net.jogtar.hu/jogszabaly?docid=a0800046.tv>).

Mycotoxins are secondary metabolites of microscopic fungi, of which occurrence is to be expected in the food chain in almost all circumstances. To the group of field moulds belong the *Fusarium* species, of which the important toxins in respect of human and veterinary medicine are the ZEA (or F-2 toxin), the trichothecenes (T-2, HT-2, nivalenol [NIV], deoxinivalenol [DON], diacetoxyscirpenol [DAS], fusarenon-X [FX]), and the group B of fumonisins (FB) (Streit et al., 2012). These are molecules with no antigens effect and with high resistance to environmental influences. During kitchen preparation, treatment and processing of food and preparation

of feed toxins do not become harmless; in some cases their toxicity might decrease only marginally. Their chemical structure varies greatly; consequently their effect on the body can also be different (Kovács et al., 1995). According to a 2016 study conducted in Hungary, analyzing grain-based feed for pigs, a certain degree of deoxynivalenol (DON), of zearalenone (ZEA) and of T-2 mycotoxin contamination was detected in all samples. This shows that the presence of Fusarium toxins in the food chain is a problem in Hungary too (Tima et al., 2016).

The global warming phenomenon reinforces the problem of mycotoxin, as it creates favorable conditions for the growth of fungi and for the formation of toxins in countries where there has been no danger before (Mesterházy, 2006). The whole grain products are an important source, because the toxins, just like chemicals, accumulate in the shell of nuts (Rafai and Bata, 1998). Food of animal origin constitutes an indirect source, especially the offals (liver, kidney) and the blood (Kovács, 2001).

Disorders in reproduction biology may be attributable to environmental toxic effects. While the toxic effect on reproduction is well known in females, our knowledge about the effects and mechanism of action of mycotoxins regarding male reproduction and sperm production is insufficient (Alm et al., 2002).

My objectives of the research work were as follows:

1. Determination of the cell-damaging effects *in vivo*: studying the T-2 toxin's spermatogenesis affecting effect, as well as determining its effect on male animals' other reproductive parameters in rabbit, in case of one short-time and high-dose and two chronic and low-dose exposure.
2. Testing the applicability of pig lymphocytes in defining the cytotoxic effects through MTT (Methyl Thiazole Tetrazolium) tests.
3. The detection of cytotoxic effect *in vitro*, in MTT test: determination of the dose and exposure period dependent, collectively and individually prevailing cytotoxic effect of T-2 and the HT-2.

2. MATERIALS AND METHODS

2.1. Reproductive toxicity studies in male rabbits

During the *in vivo* experiments sperm cells were selected as models, because they multiply fast and they are very sensitive to toxic effects. (Schardein et al., 1985; Christian and Hoberman, 1996). Rabbit is an excellent model animal; it is small, it is easy to handle, it is prolific. (Foote and Carney, 2000).

Three experiments were performed in rabbits:

- 1. experiment: high-dose (4 mg/animal/day), short-period (3 days) T-2 exposure;
- 2. experiment: low-dose (0.05, 0.1, and 0.2 mg/animal/day), long-period (65 days) T-2 exposure, where the purified toxin was introduced into the body by gavage;
- 3. experiment: low-dose (0.33 and 0.66 mg/feed kg), long-period (65 days) T-2 exposure, feed mixed with fungal culture.

2.1.1. Experimental animals, their location and their feeding

In all experiments, in-breeding, 9-month-old, trained to semen-collection, weighing about 4050-4500g, Pannon Fehér (*Pannon White*) male rabbits (n=24) were involved. We designed the groups of animals that were placed individually and between controlled climatic conditions. The animals consumed commercial foodstuff and water *ad libitum*.

We checked the mycotoxin content of foodstuff contaminated with T-2 fungal culture and toxin with the help of LC-MS (mass spectroscopy with liquid chromatography) device measurement. The control foodstuff did not contain detectable quantity of T-2.

2.1.2. Production of fungal culture and cleaned T-2 toxin

The production of T-2 toxin was based on the method of Fodor et al. (2006). T-2 mycotoxin with a purity of 96% was produced from the fungal culture.

2.1.3. The method and the period of T-2 exposure

2.1.3.1st High-dose (4 mg/animal/day) sub-acute exposure (1. experiment)

The experimental group (n=12) received cleaned T-2 in suspension and by gavage for 3 days. The control group (n=12) received toxin-free suspension for 3 days.

We measured the individual feed intake daily, while the bodyweight of the animals on the 0th, 17th, 29th, 36th, 43rd and 51st day. We set up a control group with limited food intake (n=12), in which the amount of feedstuff was set in respect of the refusal rate. The health status of animals was checked 3 times a day.

2.1.3.2nd Low-dose (0.05, 0.1, and 0.2 mg/animal/day), long-period (65 days) toxin exposure (2. experiment)

The experimental group (n=10) received cleaned T-2 in suspension and by gavage. The control group (n=10) received toxin-free suspension for 65 days. The individual feed intake was measured daily, the bodyweight of animals was measured weekly, and their health status was measured daily.

2.1.3.3rd Low-dose (0.33 and 0.66 mg/feed kg), long-period (65 days) toxin exposure (3. experiment)

On the basis of the second experiment, we added T-2 with fungal culture to the feedstuff in such a way as the feedstuff would contain the two lower T-2 concentrations of the second experiment. The control group (n=10) received

toxin-free feedstuff for 65 days. The individual feed intake was measured daily, the bodyweight of animals was measured weekly, and their health status was measured daily.

2.1.4. Sampling, semen testing methods

To determine the late effect of the sub-acute toxicosis, on the 48th day following the termination of the 3-day toxin exposure (1. experiment), for the examination of the effects of the chronic exposure on the 65th day of the experimental period (2. and 3. experiment), semen sample was collected by plastic sleeve. Following the autopsy we weighed the testicular weight and samples were taken for histological examination.

Under the semen tests the following parameters were checked:

- semen pH;
- semen concentration;
- semen movement (motility);
- morphological and acrosomal disorders;
- to check the function of the accessory sex glands the citric acid, zinc and fructose concentrate of the seminal plasma were determined.

2.1.5. Statistical analysis

While analyzing the data, I conducted single- and multifactorial variance analysis, t-test, and LSD post hoc test using SPSS 10.0 (2002) software package.

2.2. The examination of the cytotoxic effect of T-2 and HT-2 toxins using MTT method

The major advantages of *in vitro* tests are that it is not needed to maintain experimental livestock, and that equal experimental conditions can be set, and this will enable the easy repeatability. The method we use is a fast,

precise, reliable quantitative measuring procedure, in which a big number of experimental settings can be applied in one step. *In vitro*, in MTT test, the dose and exposure period dependent cytotoxic effect of T-2, HT-2 and T-2 + HT-2 was examined using pig lymphocytes.

The MTT test was conducted with smaller modifications and on the basis of the method of Mwanza et al. (2009).

The chemicals used in the *in vitro* experiments, including T-2 and HT-2 mycotoxin standards, were purchased from the Hungarian office of Sigma-Aldrich.

2.2.1st The main steps of the MTT test

2.1.1. Isolation of lymphocytes

The blood sample was taken from Landrace Large White F1 genotype young pigs (4-month-old, 24 kg individuals), through *vena cava cranialis*. From the blood-RPMI (Roswell Park Memorial Institute) mixture, using Histopaque 1077 liquid, the lymphocyte layer became removable. I produced the cell culture, and then I placed it on a 96-well plate. I incubated the cells used in the experiments under specified conditions.

2.1.2. Cell counts based on paint bond

Using Neubauer cell counting chamber, I determined the rate of normal cells using trypan-blue stain:

$$\text{viability \%} = (\text{unstained cells/all cells}) \times 100$$

2.1.3. Methyl thiazol tetrazolium (MTT) method

I placed the given solution concentration of the examined cleaned toxin on the 96-well ELISA (enzyme-linked immunosorbent assay) slide appropriate to the experimental design. For The reading time points I prepared 1-1 slides. I incubated the slides under specific conditions for 6 and for 24 hours (1.

experiment), as well as for 6, 12 and 24 hours (2. experiment). Following the specific incubation time I determined the cytotoxicity adding MTT solution to it. The living cells convert the MTT into formazan. The optical density value is directly proportional to the number of living cells.

I measured the optical density (OD) on the ELISA reader, at 540 and 620 nm.

$$\text{viability\%} = (\text{ODM}/\text{ODN}) \times 100$$

(Where ODM stands for mycotoxin-treated, while ODN stands for mycotoxin-non-treated, i.e. OD value of control samples.)

In two series of experiments I tested the two mycotoxins separately and I tested their combination:

1. experiment: the T-2 was tested in concentration of 0.5; 0.1; 0.05; 0.01 and 0.001 μM , the HT-2 was tested in concentration of 1.0; 0.5; 0.2; 0.1 and 0.05 μM , and their combination was tested as well, for 6- and 24-hour experimental periods.

2. experiment: the T-2 was tested in concentration of 1.0; 0.5; 0.1; 0.05; 0.01 and 0.001 μM , the HT-2 was tested in concentration of 2.0; 1.0; 0.5; 0.2; 0.1 and 0.05 μM , and their combination was tested as well, for 6-, 12- and 24-hour experimental periods.

The viability of the control cells was taken as theoretical 100%, and comparing to this I calculated the viability % of treated cells.

2.2.2nd Statistical analysis

When assessing the basic data resulting from experience, I applied multifactorial variance analysis (Multiway ANOVA), as well as LSD (Least Significant Difference) and Tukey-type „post hoc” test ($P \leq 0,05$), using SPSS for Windows 19 (2010) software. I determined the expected value of the combination of the two toxins according to the method of Šegvić Klarić et al. (2012).

3. RESULTS

3.1. The effect of T-2 toxin on the semen of male rabbits

3.1.1. The effect of sub-acute toxicosis (1. experiment)

During tests 5 animals from the toxin-intaking group died: 1-1 animals died on the 2nd and 3rd, two on the 4th day, one other died on the 35th day. The symptoms of the animals were: clearly visible nutmeg design in the liver, with expressed centrolobular fatty infiltration, pale kidneys with brighter areas of from pinhead-size to pea-size, bloody stomach contents, punctures on the stomach mucosa, ulcers, faded heart muscle, blooded lungs. In the animal died on the 35th day we saw pathological emaciation. The symptoms of the acute toxicosis were: depression, decreased feed intake and emaciation. In the control group and in the limited feed intake group there were no deaths, the animals were healthy, they did not show pathological symptoms.

In case of various species the T-2 develops acute toxicosis at 0.06-10 mg/kg body weight exposure. The effect appears on non-specific symptoms like weight loss, feed refusal, dermatitis, vomit (cat, dog, pig and duck), diarrhea, bleeding and epidermal necrosis in the stomach and in the intestines, in the bone marrow, in the spleen, in the testicles and in the ovary (SCF, 2001). We should take into account the rabbits' relatively higher sensitivity to T-2 mycotoxin, which is indicated by a relatively low (1.1 mg/kg body weight) LD₅₀ value (causes death to the 50% of the laboratory animals within 24 hours) (Wannemacher and Wiener, 1997). This high degree of sensibility, due to the caecotrophy phenomenon, can be attributed to the repeated intake of the toxin. (Fekete et al., 1989b). The SCF (2002) determined the tTDI value (temporary tolerable daily intake) in case of the T-2 and the HT-2 together in 0.06 µg/kg body weight/day quantity. We applied nearly 15 times the TDI value of T-2 toxin that is close to the LD₅₀ value. We did not

experience specific effect, feed refusal and complete lack of appetite (anorexia) were overall detectable. The lack of typical necrotic lesions on the oral membrane is due to the toxin intake method, because we introduced the toxin directly into the stomach through a sonde. We saw erosions and ulcers on the surface of the intestinal and stomach mucosa of dead animals, which were previously described as immediate cytotoxic effects of the toxin (Fekete et al., 1989a). The pathological changes of the liver, kidney, heart and lungs as well as the feed refusal were previously described in male rabbits (Niyó et al., 1988a; Glávits et al., 1989; Sándor and Ványi, 1990), pigs and turkeys (Sundstøl and Pettesson, 2004) by several authors.

Even after the first T-2 toxin intake the feed intake of the animals decreased dramatically (to 27% of the control group, $P < 0.05$); within one day of the first toxin intake, the daily feed intake of the control group was 2-22 g. On the 2nd-3rd days, with the exception of one animal, the food intake went below 10g. From the 3rd day and following the withdrawal of the toxin, the feed intake of the rabbits did not change, it remained the value of below 10 g; its increase started after the toxin intake and in individually changing periods, between the 3rd and the 10th day. During the two weeks after the toxin feeding the individuals of the experimental group consumed significantly less feed than the control group. On the first week the average consumption of the treated group was 40 g while that of the control group was 150 g; on the second week this ratio was 106 g and 160 g. In the next two weeks their food intake was lower than that of the control group, but the difference was not significant. The consumption of the control group was about 150-160 g during the whole period. The consumption of the limited group, after the first two weeks of the limitation, did not show significant difference (ranged between 156 g and 167 g on average) compared to the experimental and the control group.

There was significant difference in the body weight of the animals of the experimental and of the control group only on the 17th (4027 g and 4557 g) and on the 29th (4001 g and 4579 g) day of the experiment; the experimental group reached 88% of the control group's body weight. Following the 29th day the body weight gain of the experimental group showed slow increase, but it did not reach the values of the control group by the end of the experimental period. A slight reduction of body weight loss was observable in the limited consumption group on the 10th day, but after that there was no significant difference compared to the control group.

The trichothecenes change the serotonin activity of the central nervous system. Serotonin receptors play an important role in regulating appetite, thus the increase of the serotonin concentration causes decrease of appetite and causes feed refusal (Smith, 1992). According to literature data, the lowest T-2 toxin concentrate in rabbits that does not cause feed refusal is 0.01 mg/kg body weight (Fekete and Huszenicza, 1993). This toxin level however reduces the activity of the immune system, changes the hepatic function, changes the glomerular function of the kidneys, and changes the activity of the ovary (Szilágyi et al., 1994).

The toxin exposure did not have any effects on semen pH value (it ranged between 6.9 and 7.2, in both groups). No toxin effect was detectable in the quantity of semen (1ml avg. in all groups) and in the concentration of the spermatozoa (245-263 x 10⁶/ml). In the toxin treated group the motility of the spermatozoa reduced (53%) compared to the control group (65%), but the difference was not significant (P>0.05). The ratio of semen with abnormal morphology increased (P<0.05) in the toxin-treated group and in the limited consumption group. The control group had an avg. 89% of semen with normal morphology, the treated group had 58%, while the limited consumption group had 59%. The most common morphological

abnormalities: tail abnormalities (60%), cytoplasmic droplet retention, acrosome deficiency, or changed shaped head.

The high-dose toxin intake caused decrease (from avg. 12.8 to 2.7 mg/ml) in concentration of seminal plasma citric acid ($P < 0.05$), and it did not cause significant difference in concentration of other components.

It is accepted that even the normal ejaculation contains semen with abnormal morphology in low percent. The ejaculation's high ratio of semen with abnormal morphology indicates decreased fertility (Kruger et al., 1988; Gillan et al., 2008; Parkinson, 2009). Our test results show that even 48 days after termination of intake, the high concentration of T-2 toxin exposure causes reduced motility and increases the ratio of semen with abnormal morphology. This can be considered as immediate cytotoxic effect, which shows the toxic effect of trichothecenes in case of cells which multiply rapidly. The toxin's inhibitory effect of protein synthesis (due to its ribosome binding) and the cell membrane dysfunction (WHO, 1990) can cause cytotoxic effect. In the background of the prolonged toxin effect observed by us too, there may stand the different cell types' diverse sensitivity to toxin, what Sprando et al. (2005) described in case of DON. It revealed that the early-stage spermatocytes responded to DON the most sensitively. In our experiment the feedstuff limitation negatively affected the semen's morphology. Serious feedstuff limitation and weight loss (50-60% of the controls) strongly worsen reproduction in rats. However based on the results of Chapin et al. (1993), the Sprague-Dawley rats are able to tolerate feedstuff limitation and 10-30% weight loss compared to control group and these animals are able to do it for 17 weeks without influence on their productiveness. Feedstuff limitation can cause reproductive disorders in rabbits too (Assane et al., 1995; Brecchia et al., 2006). In our experiment feedstuff limitation caused low (2-4%) weight loss, which hardly explains the

decrease of ratio of normal-morphology semen. This is the only parameter examined by us that shows the negative effect of foodstuff limitation.

The reason behind decreased motility experienced in the toxin-treated group can be the impaired epididymal function that has influence on the sperm maturation process (Yeung, 1995; Parkinson, 2009). Any change in the composition of seminal plasma, such as the reduction in the concentration of citric acid as environmental conditions' deviation from optimal, can cause decrease in semen motility (Cooper and Yeung, 2000).

It is known that the blood-testis barrier is extremely selective and protects the organ from most toxic materials (Steinberger and Klinefelter, 1993). It is not proved whether the trichothecenes pass through the blood-testis barrier or not. According to another explanation, the T-2 has immediate effect on pituitary gland or on inhibit production of Sertolli cells, which was proved in case of DON by Sprando et al. (2005). The function disorder of Sertolli cells can cause increase the number of abnormal-function semen.

3.1.2. Effect of low-dose, long-term, introduced-through-sonde T-2 toxin (2. experiment)

The toxin exposure did not have significant effect on body weight. The animals did not show clinical symptoms related to toxin effect.

The T-2 toxin's 0.1 and 0.2 mg/animal dose reduced significantly the foodstuff intake on the first week to 63% (avg. 98 g/day) and to 47% (avg. 73 g/day) compared to the control group (avg. 155 g/day). During the employment of the lowest dose (0.05 mg/animal) periodic food refusal occurred on the second week ($P < 0.05$; avg. 98 g/day compared to the 141 g/day consumption), while on the third week significant difference was measurable only in case of the highest concentration (avg. 83 g/day food intake compared to the 139 g/day intake). It indicates adaption to the toxin that following the second week the food intake increased in the toxin-treated

group and from the 4th week there was no significant difference among the groups. Among the examined semen quality parameters there was no significant difference among the groups, with the exception of the ratio of the cytoplasmic-droplet remains, which in case of the highest T-2 dose, compared to the control group (avg. 3), increased by 3.2 times (avg. 9.6). The tendency of the data shows that toxin intake influences semen motility negatively, however significant difference was not measurable among the groups. The ratio of the abnormal morphology cells did not show significant difference among the groups. At the daily 0.1 mg/animal exposure a slight hyperplasia of the Leydig cells was measurable, while in case of the highest (0.2 mg/animal/day) exposure group in addition increased proliferative activity was measurable. In the latter case the testicles were slightly hyperemic.

The histological findings of our 2nd experiment suggested the possibility of the Leydig cells' malfunction. The male reproductive processes are highly testosterone dependent. Testosterone regulates the spermatogenesis, the sperm maturation, the seminal plasma production and other sexual processes (De Kretser and Kerr, 1994). So the possible malfunction of the Leydig cells could as well have caused the maturation malfunction of the semen that resulted cells containing cytoplasmic droplet remains in higher ratio. Zhang et al. (2016) *in vitro* showed that T-2 harms Leydig cells isolated from mice and that in the background of the harmful effect there stands the toxin-induced oxidative stress. Yang and et al. (2015b) treating Leydig cells also isolated from mice with T-2 toxin proved that the toxin reduces significantly the hCG-induced testosterone production.

In mice and in rats the T-2 toxin's NOAEL (no observable adverse effect level) value in chronic exposure was determined 0.23 and 0.5 mg/kg body weight/day value (SCF, 2001). According to the SCF the stated tTDI (0.06 µg/kg body weight/day) value reduces the development of the effects of

chronic, sub-chronic and reproductive toxicity. The applied 3 concentration meant an exposure higher than TDI, but substantially lower (0.01; 0.02 and 0.05 mg/kg body weight/day) than NOAEL stated in mice and rats. Counting with the average feed intake this represents 0.3, 0.6 and 1.3 mg/feed kg contamination, from which the two lowest [0.3 and 0.6ppm (mg/kg)] usually occurs even in practical conditions (BIOMIN, 2018).

3.1.3. Chronic effect of low-dose, mixed-into-feedstuff T-2 toxin (3. experiment)

The results of the 2nd experiment showed that the 0.05 mg/animal dose did not cause, while the 0.1 mg/animal exposure caused slight changes of the examined parameters. So we chose these two parameters to determine whether this low toxin exposure causes feed refusal or not if the fungal culture containing the toxin is mixed into the feedstuff. Some deviation in foodstuff intake was observable, this was shown even in the control group, and it was not attributable to toxin effect. None of the examined semen quality parameters showed significant difference between the toxin-treated and the control groups. The toxin concentration equal to the purified toxin treatment mixed into the feedstuff together with fungal culture (3rd experiment) did not cause reduction in food intake, did not worsen the measured semen quality parameters significantly, and did not cause histological pathological lesions. The reason may be the purified, dissolved in ethanol-water mixture, and introduced through fungal culture T-2's different absorption and toxicokinetics. The mycotoxins can form complexes together with the nutrients present in feedstuff (Meca et al., 2012b). In case of DON they showed the diverse biological activity between the clear toxin introduced through sonde as liquid and the toxin consumed through feedstuff as natural contamination (Meca et al., 2012a).

3.2. *In vitro* examination of the T-2's and the HT-2's separate and collective cytotoxic effect depending on time and concentration

I chose the toxin concentrations applied in the experiment on the basis of the data in the literature regarding T-2 and HT-2 mycotoxin's cytotoxic examination. During their examinations made on two human cell lines, Königs et al. (2009) stated higher cytotoxic effect in case of T-2 (IC_{50} value 0.2-0.5 μ M, inhibitor concentration, that inhibits the enzyme activity by 50%), that in case of its metabolites, namely in case of HT-2 and neosolaniol (IC_{50} value 0.7-3.0 μ M). Similar results were reported by Nielsen et al. (2009), who stated the value of the IC_{50} in case of T-2 in 4.4-10.8 nmol/l, while in case of HT-2 in 7.5-55.8 nmol/l. Regarding pig lymphocytes there were no available data. In our previous examination (Mwanza et al., 2009), when examining the separate and collective cytotoxic effect of FB1 and OTA (ochratoxin-A) with *in vitro* MTT test, we confirmed that pig lymphocytes are highly sensitive to the cytopathic effect of mycotoxins and that are practically well adapted at testing cytotoxicity and mycotoxin combinations.

3.2.1. Results of the first experiment

(T-2 in 0.5; 0.1; 0.05; 0.01 and 0.001 μ M concentration, HT-2 in 1.0; 0.5; 0.2; 0.1 and 0.05 μ M concentration, as well as their combination after 6 and 24 hours)

During the examinations both of the toxins showed dose-dependent effect. Following the 6-hour incubation the increase of the T-2 and the HT-2 concentration provoked 17% decrease in cells' viability. After 24 hours the viability of the cells was significantly lower than the 6-hour values in every concentration with the exception of T-2 with 0.5 μ M concentration, and of HT-2 with 0.05 and 1.0 μ M concentration. Following the 24-hour incubation the increase of the T-2 toxin concentration from 0.001 μ M to 0.1 μ M caused

14% decrease in the viability of cells, further decrease due to the increase of toxin concentration (to 0.5 μM) was not observable. Employing HT-2 mycotoxin the highest viability decrease (22%) was measurable at 0.2 μM . Significant ($P < 0.001$) interaction was observable in concentration x treatment and concentration x time connection, while in time x treatment connection the interaction could not be verifiable ($P = 0.084$).

I calculated the expected value of the interaction with T-2 and HT-2 under the methods of Šegvić Klarić et al. (2012), and then I compared it to the measured value. Following the 6-hour incubation and in case of all concentrations I measured higher cell viability than the calculated value, with the exception of the lowest concentration where the difference was not significant. If we compare the toxins' combined and individual effect, in the same concentration and incubation period, it was clearly visible that the combination of the two toxins consistently caused less cell survival than as an individual contaminant following the 6-hour incubation period. This tendency was not observable after the 24-hours incubation period, where in case of concentration category 2 the two toxins' combined effect and in category 4 and 5 the individual effect of T-2 and HT-2 caused higher cell survival, i.e. it was less cytotoxic.

In summation, it can be said that however the two toxins' combined cytotoxic effect was stronger than that of the toxins' individually, it was significantly below the calculated cytotoxicity, i.e. they do not have additive effect in relation to each other.

3.2.2. Results of the second experiment

(T-2 in 1.0; 0.5; 0.1; 0.05; 0.01 and 0.001 μ M concentration, HT-2 in 2.0; 1.0; 0.5; 0.2; 0.1 and 0.05 μ M concentration, as well as their combination after 6, 12 and 24 hours)

Even during this experiment both of the toxins showed dose-dependent cytotoxicity. Following the 6-hour incubation period the concentration increase of T-2 mycotoxin from 0.001 to 0.5 μ M caused more than 16% decrease in cell viability characteristics. The further concentration increase did not show significant difference in the results. In case of HT-2 the lowest viability value (12-13% decrease) was at 0.5 μ M, which did not show significant difference at the further concentration increase. Following the termination of the two further incubation period, the tendency at T-2 was still observable compared to the 6-hour data, but the highest viability decrease here was only of 12-13%. The strongest effect, summarizing the appointments and doses, was shown by the 0.5 and 1.0 μ M T-2 concentration, among which there was no significant measurable difference. The HT-2's cytopathic effect in case of 12 and 24 hours occurred slightly (about 10% cell survival decrease), significant difference was shown only by the lowest concentration compared to the higher ones. Observing the three periods together it is clearly seen that the T-2 toxin caused the major damage in its 0.5-1.0 μ M concentration; compared to the 0.001 μ M it results in 12-16% lower cell survival %. In case of HT-2 the strongest effect was measured following the 12-hour period and at 1.0 μ M concentration, but the cytopathogenicity here only showed 14% difference compared to the lowest HT-2 exposure HT-2 (0.05 μ M).

The measured values of the interaction of T-2 and HT-2 resulted in higher cell viability compared to the calculated values ($P < 0.05$). The results of the second *in vitro* experiment confirmed the experienced in the first experiment.

T-2 is rapidly absorbed from the gut, it distributes throughout the body, excretes relatively rapidly and with slight accumulation. We have little information on the effect of HT-2, on the basis of the available data the toxic effects of T-2 and HT-2 can be considered similar in strength (Joint FAO/WHO Expert Committee on Food Additives, 2001).

Numerous studies on T-2's metabolites (HT-2, T-2 triol and T-2 tetraol) have reported lower cytotoxic effects, assuming the natural detoxifying effect of the body (Babich and Borenfreund, 1991). Since the metabolism of T-2 to HT-2 takes place quickly *in vivo*, the harmful effects caused by T-2 can partly be attributable to the effect of HT-2.

The T-2 and HT-2 concentrations employed in our experiments showed almost the same cell survival data (between 55 and 80%), within the identical categories there was no significant difference in the damage level caused by T-2 and HT-2. Taking into account the fact that per category the concentration of HT-2 was always higher than that of T-2 toxin, our study proved the previous results, according to which the HT-2 has lower toxicity than T-2. Nielsen et al. (2009) tested in eight human cell lines the cytotoxicity of the two toxins. They determined the IC_{50} value in case of T-2 in 4.4–10.8 nmol/l, while in case of HT-2 in 7.5–55.8 nmol/l concentration. In human epithelial cells of the small intestine they detected nearly quadruple toxicity for T-2 than to HT-2 (IC_{50} value T-2: 6.4 nM, HT-2: 24 nM) (Nielsen et al., 2009). Königs et al. (2009) used two primer cell cultures, and they found T-2's metabolites less toxic than that of the intact toxin (IC_{50} value T-2: 0.2-0.5 μ M, HT-2: 0.7-3.0 μ M). In our experiment in case of T-2 and with similar concentration range we did not reach the IC_{50} value, 0.5 μ M T-2 exposure provoked 60-70% viability, and in case of HT-2 the highest dose (2 μ M) provoked only about 40% cell death (60% viability). This may indicate lower sensibility of pig lymphocytes to toxin compared to the cell lines used in the studies relied.

4. CONCLUSIONS

In rabbits the decrease in sperm motility, the increase of cells having abnormal morphology and containing cytoplasm droplet remains, as well as the decrease of the plasma's citric acid concentration even on the 48th day following a 3-day acute toxicosis indicate prolonged effect of the high-dose T-2 toxin intake. All these changes individually or collectively can cause reduced fertilizing ability in male animals.

An adult male rabbit is able to tolerate a 0.33 and 0.66 ppm (i.e. 0.05, and 0.1 mg/animal/day) T-2 exposure without gaining any damage in the semen quality parameters and without feed refusal. The 0.2 mg/animal/day exposure provoked feed refusal, highly increased the ratio of the cytoplasmic droplet (which indicates disturbances in semen maturation process) and caused detectable lesions in the testicles. Based on these for the examined parameters the NOAEL value in adult male rabbits is <0.1 mg/animal/day (<0.02 mg/kg body weight/day).

Employing lymphocytes isolated from healthy pigs in MTT test we identified the exposition-time and concentration-dependent effect of T-2 and HT-2. HT-2 in 2-50 times higher concentration shows similar level of cytotoxicity as T-2 mycotoxin. Employing the two toxins together, we consistently observed lower cell survival compared it to the individual effects, following the 6-hour incubation period. Since the measured cytotoxicity, as the combined effect of the two toxins, was significantly lower than the expected (calculated) cytotoxicity, the effects of the two toxins are not additive.

5. NEW RESEARCH RESULTS

1. The 3-day-long and high-dose (4 mg/animal) T-2 mycotoxin exposure has long-term (48 days after the termination of toxin intake) negative effects on male rabbits' semen morphology.
2. In male rabbits with respect to feedstuff refusal and sperm motility and morphology, the NOAEL value related to T-2 mycotoxin is <0.1 mg/animal/day (<0.02 mg/kg body weight/day).
3. I proved the T-2 and HT-2 mycotoxin's exposure period and dose-dependent cytotoxic effect using MTT test on lymphocytes isolated from pigs.
4. In case of pig lymphocytes the HT-2 mycotoxin is able to perform similar T-2 toxin cytotoxic effect in 2-50 times higher concentration than the T-2 toxin.
5. I found that employing T-2 and HT-2 mycotoxin collectively in case of pig lymphocytes results in constantly lower cell survival compared to individual effects. However, since the measured cytotoxicity, as the combined effect of the two toxins, was significantly lower than the expected (calculated) cytotoxicity, the effects of the two toxins are not additive.

6. RECOMMENDATIONS

Further *in vivo* examinations are needed to determine how different concentrations of T-2 toxin exposure influence the fertilizing ability of male rabbits. There are several mechanisms of action yet to be explored regarding the T-2's effect on male reproductive function too (spermatogenesis, sperm maturation, seminal plasma production, testosterone production, etc.). Feedstuff limitation, namely the effects of decreased nutrient intake is recommended to investigate further too, as results are still not clear (increase in morphological abnormality, however, has no effect on motility and plasma composition; causes mild weight loss).

Further *in vivo* examinations are needed to determine the IC_{50} value of T-2 and HT-2 in pig lymphocytes, as well as to study the two toxins' interaction in case of lower concentration. It is needed to identify the possible T-2/HT-2 metabolizing capability of pig lymphocytes, similarly to the proven mechanisms in case of the other cells (Königs et al., 2009).

7. SCIENTIFIC PUBLICATIONS WRITTEN ON THE TOPIC OF THE THESIS; EDUCATIONAL PUBLICATIONS, PRESENTATIONS

Scientific publications in foreign languages:

- Mwanza, M., Kametler, L., Bonai A., Rajli, V., Kovacs, M., Dutton, M.F.: The cytotoxic effects of fumonisin B1 and ochratoxin A on human and pig lymphocytes using Methyl thiazol tetrazolium (MTT) assay. *Mycotoxin Research*, 2009. 25. 4. 233-238. Q3
- Kovács, M., Tornyos, G., Matics, Zs., Kametler, L., Rajli, V., Bodnár, Zs., Kulcsár, M., Huszenicza, Gy., Keresztes, Zs., Cseh, S.: Subsequent effect of subacute T-2 toxicosis on spermatozoa, seminal plasma and testosterone production in rabbits. *Animal*, 2011.10.1563-1569. Q1, IF: 1,744
- Tornyos, G., Cseh, S., Matics, Zs., Kametler, L., Rajli, V., Bodnár, Zs., Rusvai, M., Mándoki, M., Kovács, M.: Preliminary results on the effect of chronic T-2 toxin exposure in rabbit bucks. *Agriculturae Conspectus Scientificus*, 2011. 76. 369-373. Q3
- Rajli, V., Bodnár, Zs., Horvatovich, K., Kametler, L., Dutton, M. F., Kovács, M.: Preliminary investigations on the single and combined cytotoxic effect of T-2 and HT-2 toxin measured by methyl thiazol tetrazolium (MTT) cytotoxicity test using pig lymphocytes. *Acta Agriculturae Slovenica*, 2012., Supplement 3, 253-257. Q3
- Kovács, M., Tornyos, G., Matics, Zs., Mézes, M., Balogh, K., Rajli, V., Bloch-Bodnár Zs., Rusvai, M., Mándoki, M., Cseh, S.: Effect of chronic T-2 toxin exposure in rabbit bucks, determination of the No Observed Adverse Effect Level (NOAEL). *Animal Reproduction Science*, 2013. 137. 3-4. 245-252. (2013) Q1, IF: 0,60

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- Rajli V., Tornyos G., Cseh S., Matics Zs., Kametler L., Bodnár Zs., Rusvai M., Mándoki M. and Kovács M.: Effect of T-2 toxin in low doses on sperm quality in rabbit as a model animal. Budapest, 2. June 2012. CEELA-II- 2012 Conference Budapest. 124-125.
- Rajli, V., Bodnár, Zs., Horvatovich, K., Kametler, L., Dutton, M. F., Kovács, M.: Preliminary investigations on the single and combined cytotoxic effect of T-2 and HT-2 toxin measured by methyl thiazol tetrazolium (MTT) cytotoxicity test using pig lymphocytes. 20th Int. Symp. „Animal Science Days”, Krajnska gora, Slovenia, Sept. 19-21, 2012. *Acta agriculturae Slovenica*, Supplement 3, Ljubljana 2012, p. 253-257.

Publications in proceedings in Hungarian:

Rajli V., Cseh S., Tornyos G., Matics Zs., Huszenicza Gy., Kulcsár M., Rusvai M., Mándoki M., Kovács M.: A T-2 toxin reprodukciós toxicitása baknyulakban. 24. Nyúltenyésztési Tudományos Nap, Kaposvár, 2012. 99-104.

Abstract in foreign languages:

Mwanza, M., Kovacs, M., Rajli, V., Dutton, M.F.: The cytotoxic effects of fumonisin B1 and ochratoxin A on human and pig lymphocytes using Methyl thiazol tetrazolium (MTT) assay. ESTIV 2010 16th International Congress on In Vitro Toxicology, Linz, 2-4 September, 2010., ALTEX, 27. Suppl. p. 94.

Tornyos, G., Cseh, S., Matics, Zs., Kametler, L., Rajli, V., Bodnár, Zs., Rusvai, M., Mándoki, M., Kovács, M.: Subsequent effect of subacute T-2 toxicosis and effect of chronic T-2 exposure in rabbit bucks. Conference on 'Power of fungi and mycotoxins in health and disease', 19-22 Oct 2011, Primosten, Croatia, p. 35.

Rajli, V., Cseh, S., Tornyos, G., Keresztes, Zs., Matics, Zs., Huszenicza, Gy., Kulcsár, M., Kovács, M.: Response of male rabbits to T-2 exposure. 10th WRSA World Rabbit Congress, 3-6. September 2012, Sharm El Sheikh, Egypt, p. 139.

Abstract in Hungarian:

Rajli V., Cseh S., Tornyos G., Matics Zs., Huszenicza Gy., Kulcsár M., Rusvai M., Mándoki M., Kovács M.: A szubakut és krónikus T-2 expozíció hatásai baknyulaknál. 38. Akadémiai Beszámoló, Budapest, 2012. jan. 16-19. 9. http://www.vmri.hu/Nagyb/allathigi_2011.pdf

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Rajli V., Cseh S., Tornyos G., Matics Zs., Huszenicza Gy., Kulcsár M., Rusvai M., Mándoki M., Kovács M.: A T-2 toxin reprodukciós toxicitása baknyulakban. 24. Nyúltenyésztési Tudományos Nap, Kaposvár, 2012.

Rajli V., Cseh S., Tornyos G., Matics Zs., Huszenicza Gy., Kulcsár M., Rusvai M., Mándoki M., Kovács M.: A szubakut és krónikus T-2 expozíció hatásai baknyulaknál. 38. Akadémiai Beszámoló, Budapest, 2012. jan. 16-19.