RABBITS IN MYCOTOXIN RESEARCH AT KAPOSVÁR UNIVERSITY

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ABSTRACT – Rabbits in mycotoxin research at Kaposvár University
In toxicity testing a lot of animals have been used as models with rodents being the most frequently used due to their small size and inexpensive purchase. Rabbits are large enough to permit easy handling and at the same time to facilitate easy blood and semen collection as well as other more intrusive practices like embryo transfer. Thus rabbits have been used in many studies on developmental and reproductive toxicity. Mycotoxin research is another field in which rabbits have been the animal of choice. Mycotoxins are the secondary metabolites of fungi which can cause a series of syndromes and health implications to both animals and humans. Rabbits are highly susceptible to aflatoxin B1 (AFB1). Ochratoxin A (OTA) can exert teratogenic effects whereas fumonisins can cause renal or liver toxicity to rabbits. In Kaposvár University both single (FB1, T-2, DON) and combined (FB1+T-2, DON+ZEA and FB1+DON+ZEA) experiments have been conducted. T-2 is the most studied mycotoxin and the No observed Adverse Effect Level (NOAEL) has been determined in rabbit bucks. The effect of FB1 has also been studied confirming the renal toxicity in pregnant rabbit does and the foetuses. ZEA has also been studied in combination with only DON or FB1 and DON. The effect of DON in concentration twice as much as the guidance value set by the European Commision for complementary and complete feedstuffs; rabbits were not severely affected. The two targets were the liver (altered morphology) and the immune system with no negative secondary effect.

Keywords: rabbit, toxicity studies, mycotoxins

ÖSSZEFOGLALÁS – Házinyúllal végzett kísérletek a Kaposvári Egyetem mikotoxin kutatásaiban
A házinyúl elterjedten alkalmazott modell állat a toxikológiai kutatásokban, különösen a reprodukciós toxicitás vizsgálatokban. A nyúl ugyanakkor, mint gazdasági állat is jelentős (nyúlhús előállítás), így a mikotoxinok termeléscsökkentő és állategészségügyi hatásai miatt is tárgya a kutatásoknak. A mikotoxinok a penészgombák másodlagos, toxikus anyagcsere termékei, amelyek számos állat- és humánegészségkárosító hatást fejtenek ki. A nyulak kifejezetten érzékenyek az aflatoxin B1-re (AFB1). Az ochratoxin A teratogén, míg a fumonizinek máj- és vesekárosító hatásúak. A Kaposvári Egyetemen több mikotoxin önálló (FB1, T-2, DON) és együttes (FB1+T-2, DON+ZEA and FB1+DON+ZEA) hatását vizsgálták nyulakban. Kifejlett baknyulakban meghatározásra került a T-2 toxin No observed Adverse Effect Level (NOAEL) értéke. Kimutatták a FB1 vesekárosító hatását vemhes anyanyulakban és az újszülött nyulakban. Ugyanakkor az EU takarmányokra vonatkozó határérték ajánlásában szereplő DON koncentráció kétszeres mennyisége sem okozott jelentős károsító hatást. A FB1, DON és ZEA együttes hatásának vizsgálatakor eltérő jellegű interakciókat mutatták ki. Hasonlóképpen élettani paraméterenként változó jellegű interakció lépett fel a FB1 és a T-2 toxin között.

INTRODUCTION

In order to assess the effects of various toxic agents different animal species have been used as models. Rodents are most frequently used because they are inexpensive to purchase, easy to handle and have a high reproduction rate. Despite their advantages rodents lack some features that larger animals possess.

Rabbit is the most representative example due to its easy handling and high reproduction rate that at the same time is the smallest animal in which a lot of parameters (reproductive or toxicological) can be measured (FOOTE and CARNEY, 2000). Rabbit is relatively cheap in comparison with other animals such as pig (or minipig), dogs and monkeys (KIRHCNER and HENWOOD, 2012). In rabbits (unlike with rodents) repeated measurements can be performed on both males and females (MORTON, 1988; FOOTE and CARNEY, 2000). For example blood collection can be performed from the marginal ear vein which can provide sufficient blood for most assays (MANNING *et al*., 1994). Furthermore their size is adequate to conduct ocular and

dermal irritations (KIRHCNER and HENWOOD, 2012). Another advantage of rabbit is the variety of exposure routes that can be used to administer the tested compound e.g. feed, drinking water, dermal or injections-subcutaneous (s.c.) or intraperitoneal (i.p.) (FOOTE and CARNEY, 2000). For all the aforementioned reasons rabbit has been widely used as a model in reproductive and developmental toxicity studies (FOOTE and CARNEY, 2000).

Interventions in male rabbits

In rodents semen collection cannot be performed non-surgically whereas rabbit bucks can be easily trained to serve the artificial vagina facilitating thus the collection of semen. Semen collection can be performed daily or twice per day for 3 or 4 days per week (BREDDERMAN *et al.*, 1964). As above mentioned blood collection is quite feasible in rabbits (both males and females). Serum hormone screening is important because in male rabbits the quality of the sperm and the libido are related to the hormonal patterns (FOOTE *et al.*, 1986; MORTON, 1988).

Interventions in female rabbits

Due to induced ovulation rabbit does can be injected at any time with gonadotropin and 10 to 12 hours later ovulation takes place; this ensures accurate timing of artificial insemination (AI). The combination of AI with injection of an ovulation-inducing hormone is advantageous over natural mating in which the quality of each ejaculate is unknown and variable unlikely with AI (FOOTE and CARNEY, 2000). Another advantage of rabbit does (like humans) is that their conceptuses have large volume of extraembryonic fluid in respect to the embryo size; by mid-gestation up to at least 1.0 ml of fluid is contained in the blastocyst so fluid can be used in assays when it is needed (FOOTE and CARNEY, 2000).

MYCOTOXIN RESEARCH

Mycotoxins are products of the secondary metabolism of filamentous fungi. Major mycotoxins are aflatoxins, citrinin, ergot alkaloids, fumonisins, ochratoxin, patulin, trichothecenes (deoxynivalenol, T-2 etc.) and zearalenone (BENNET and KLICH, 2003). Mycotoxins can cause a series of syndromes and health implications to both animals and humans. They can be carcinogenic (aflatoxin B_1; IARC, 2002), nephrotoxic (citrinin and ochratoxin A; EFSA, 2012) or hepatotoxic (fumonisin B1; GELDERBLOM *et al.*, 1996). Mycotoxin co-occurrence is another important issue in mycotoxin research that has received special attention recently. The reasons are that some fungi genes can produce more than one mycotoxin concurrently, a commodity can be infected by different fungi at the same time and animal feed usually consists of a variety of commodities.

Rabbits have been extensively used in mycotoxin (single and combined) research because they are quite sensitive to certain mycotoxins (MÉZES, 2008). Rabbits are highly susceptible to aflatoxins with the LD_{50} of aflatoxin B1 (AFB1) being 300 μg/kg of body weight and in concentrations as low as 15 μg/kg of feed high morbidity and mortality can occur (MAKKAR and SINGH, 1991; FAO, 2000). Ochratoxin A (OTA) exerted carcinogenic effects to rabbit does that were exposed to the toxin by gastric intubation from day 6 to day 18 of gestation. The highest dose (0.1 mg/kg of body weight) increased the incidence of gross and skeletal anomalies (WAGNIKAR *et al.*, 2004). In the study of WAGNIKAR *et al.* (2005) combination of AFB1 and OTA resulted in higher percentage of resorbed implants compared to control group. Fumonisins can cause renal and hepatic toxicity to adult rabbits and pregnant rabbit does respectively (GUMPRECHT *et al.*, 1995; KOVÁCS *et al.*, 2003).

RABBIT AND MYCOTOXIN RESEARCH AT KAPOSVÁR UNIVERSITY

Several studies on the effects of mycotoxins in rabbits have been performed in Kaposvár University (*Table 1*). These studies include single (KOVÁCS *et al.*, 2011, 2013; KACHLEK *et al.*, under review) and combined *Fusarium* mycotoxins (SZABÓ *et al.*, 2014; SZABÓ-FODOR *et al.*, 2015; HAFNER *et al.*, under review). A prebiotic, mannan oligosaccharides (MOS) has also been used in combination with T-2 to assess their potential protective effect (HAFNER *et al.*, 2012). MOS are usually obtained from the cell walls of *Saccharomyces cerevisiae.*

*Table 1***.** Mycotoxin studies in rabbits at Kaposvár University

Mycotoxin	Gender, age	Parameters	References
FB1[1]	female (pregnant does)	histopathology, clinical chemistry	Kovács *et al.*, 2003
T-2	male	productive performance, semen quality, testosterone levels	Kovács *et al.*, 2011
T-2 (and MOS[2])	suckling and growing rabbits, mixed genders	genotoxicity	Hafner *et al.*, 2012
T-2	male, adult	semen quality, clinical chemistry, antioxidant status, histopathology, testosterone levels	Kovács *et al.*, 2013
FB1+T-2	growing rabbits, mixed genders	productive performance, hematology, RBC[3] na^+/k^+ atpase activity, RBC membrane FA[4] composition	Szabó *et al.*, 2014
FB1+T-2	growing rabbits, mixed genders	antioxidant parameters, histopathology, genotoxicity	Hafner *et al.*, under review
FB1+DON[5]+ZEA[6]	male, adult	productive performance, hematology, clinical chemistry, antioxidant status, genotoxicity	Szabó-Fodor *et al.*, 2015
DON	male and female (growing rabbits)	productive performance, hematology, clinical chemistry, antioxidant status, genotoxicity	Kachlek *et al.*, under review

[1]fumonisin B1; [2]mannan oligosaccharide; [3]red blood cell; [4]fatty acid; [5]deoxynivalenol; [6]zearalenone

The first study was conducted on pregnant does that were receiving fumonisin B1 (FB1) in the concentration of 5 mg/animal/day for 5 days from the 25th to the 30th day of gestation (KOVÁCS *et al.*, 2003). Toxin was administered as a solution which was extracted from the fungal culture of *Fusarium moniliforme*. The foetuses were examined on the 28th and 30th days of pregnancy. After the dissection of the does pathological changes were found mainly in the liver and kidneys; liver and kidney were the target organs in the foetuses as well. Some of the clinical chemistry parameters (alkaline phosphatase, aspartate transaminase and alanine transaminase activities, creatinine and urea) were significantly higher in both does and foetuses in comparison to control animals.

The quality of semen as well as the hormone levels can be adversely affected by mycotoxin exposure in rabbit bucks. Exposure of rabbit bucks to T-2 toxin (0.78 to 0.99 mg/kg of body weight) by gavage for 3 days resulted in decreased forward motility, increased ratio of spermatozoa with abnormal morphology, decreased citric acid concentration of semen and

decreased of basic testosterone level as compared to control animals, even after 48 days of the 3-days toxin treatment. Feed refusal was observed from the second day of T-2 exposure and the feed intake remained lower than the control group for two weeks even after the withdrawal of the contaminated diet (KOVÁCS *et al.*, 2011).
T-2 has been shown to be genotoxic in human and animal cells (JARADAT, 2005; FRANKIC *et al.*, 2006). Comet assay is commonly used to assess genotoxicity. Evaluation is performed by either visual or electronic scoring. In visual scoring the values vary from 0 (no damage) to 5 (complete damage). The genotoxic effect of T-2 on rabbit lymphocytes was studied along with the potential protective effect of MOS (HAFNER *et al.*, 2012). The rabbit does were divided into two groups control (C) and the MOS (P) group. From the 17th day the suckling rabbits were allowed to consume their mothers feed. At 7 weeks of age both groups (C and P) were subdivided to introduce the toxin (CT and PT). In group C all the cells had a score of 0 (which means zero damage). In CT group only 33% of the cells had a score of 0 (i.e. not affected by the toxin) and some cells were detected with a score of 5 (complete disintegration) whereas in PT group 55% of the cells were intact with score of 0 and no cells with a score of 5 were observed showing a protective effect of MOS against T-2.
Sub-chronic exposure to mycotoxins is very likely to occur in complete feed. Two experiments each lasting 65 days were performed on male rabbits exposed to T-2 toxin by gavage (first experiment) or in diet (second experiment) (KOVÁCS *et al.*, 2013). In the first experiment the concentrations used were 0.05, 0.1 and 0.2 mg/animal/day. Feed intake was affected on the first week on the groups receiving 0.1 and 0.2 mg/animal whereas on the third week only the 0.2 mg/animal/day group had significantly decreased feed intake. A temporary decrease of albumin and urea concentrations were observed on day 30 but no other clinical parameters were altered. The toxin did not alter significantly sperm quality parameters except from the increased ratio of spermatozoa with cytoplasmic droplets in the highest dose group. Based on the results from the first experiment a second trial was performed by dietary exposure. The concentrations used were 0.05 and 0.1 mg/animal/day which were the no observed adverse effect level (NOAEL). In diet these concentrations were 0.33 and 0.66 mg/kg of feed respectively. Although there were differences in the sperm quality parameters and testosterone concentrations between the control and the toxin groups, these differences were not statistically significant.

Trichothecenes are known to show hematotoxic effects. The individual and combined hematotoxic effects of T-2 individually and FB1 were investigated on rabbits in the study of SZABÓ *et al.* (2014). Weaned rabbits were exposed to T-2 (2 mg/kg of feed), FB1 (10 mg/kg of feed) or T-2 and FB1 (2 and 10 mg/kg of feed, respectively) for a period of 4 weeks. In T-2 fed group body weight was significantly lower whereas liver weight singificanlty increased. The activity of red blood cell Na^+/K^+ ATPase was decreased in the T-2 group, increased in the FB1 group and their combination caused an antagonistic effect (same levels as the control group's).
The same combination adminestered to growing rabbits as well, resulted in a significant increase in the concentration of plasma total protein, albumin, fructosamine and creatinine in the group treated with FB_1 compared to the control (HAFNER *et al.*, under review). The liver and the kidney of most animals treated with T-2 toxin, FB_1 and their combination showed pathological changes, the occurrence of which was more frequent in animals exposed to both toxins. T-2 resulted in depletion of spleen lymphocytes. FB_1 and T-2 exerted additive effect on the antioxidant/oxidative parameters after 2 weeks of exposure, manifesting in less glutathione and glutathione peroxidase, while more malondialdehyde production. Both toxins caused DNA damage in the lymphocytes, which was more pronounced in the group fed T-2 toxin and T-2 combined with FB_1, without additive or synergistic effects.

The combined effects of mycotoxins have not been studied extensively in rabbits. In Kaposvár University the first study on the interactive effects of three fusariotoxins that are very likely to co-occur in nature was performed (SZABÓ-FODOR *et al.* 2015). FB1, DON and ZEA were used in binary (DON and ZEA; DZ) or tertiary (FB1, DON and ZEA; FDZ) mixtures in low doses (subchronic exposure) with a special focus on reproductive toxicity in rabbit bucks. Antagonism was observed for several parameters (lipid peroxidation, spleen weight and genotoxicity) whereas synergism was observed in testosterone production. Additive effect was observed in spermatogenesis (histological analysis) and a less than additive effect was observed in the morphology of sperm cells (DZ).

Although DON is the least toxic of the trichothecenes it is the most frequent contaminant of grains. The data on DON and rabbits are scarce (KHERA *et al.*, 1986; HEWITT *et al.*, 2012). High dose of DON (10 mg/kg) had no adverse effect on productive performance, clinical chemistry parameters, genotoxicity and antioxidant status (KACHLEK *et al.*, under review). The morphology of the liver was affected in some of the animals receiving the contaminated diet but this was not translated in degenerative alteration of the hepatocytes. The immune system was the other target in this study which is typical of DON which is known for its immunomodulative effects. Monocytes and eosinophils percentages were significantly higher in the toxin group in comparison to the control whereas neutrophils' percentage was significantly lower but these alterations did not result in negative secondary response (infection).

CONCLUSIONS

In summary, rabbits have a lot of advantages as animal models in toxicity studies. They have also been used extensively in mycotoxin research as well. A lot of studies have been performed using various mycotoxins (single and in combination), different genders and different ages (kits, growing rabbits, does and bucks). In Kaposvár University several studies with rabbits have been conducted with a focus on *Fusarium* mycotoxins single or in combinations (binary and tertiary mixtures). Rabbits seem to be less sensitive to DON in comparison with other farm animals like pig even after esposure to the dietary concetration of DON twice as much as the guidance value set by the European Commision for complementary and complete feedstuffs. On the other hand the NOAEL of T-2 for adult male rabbits is <0.1 mg/animal/day (<0.02 mg/kg b.w./day), which is about one order of magnitude less than identified for other rodents (mice and rats), and less than the least NOAEL, which is 0.03 mg/kg b.w./day as observed in the short term toxicity study on pigs (SCF, 2001).

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