

PRELIMINARY INVESTIGATIONS INTO THE EFFECT OF FEEDING MANNAN OLIGOSACCHARIDE (MOS) ON THE GENOTOXIC EFFECT OF T-2 TOXIN IN RABBITS MEASURED BY COMET ASSAY

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ABSTRACT

T-2 toxin is a secondary fungal metabolite produced by *Fusarium* species. Several *in vitro* and *in vivo* studies described genotoxic potential of T-2 toxin, which is generally accepted to be caused by oxidative stress. There are some data showing that colonic probiotic bacteria can remove mycotoxins via physical binding. Mannan oligosaccharides (MOS) are widely used animal feed to improve gastrointestinal health. Because of their interaction with microbes, the aim of our study was to determine the possible protective effect of MOS in T-2 toxicosis. Sucking rabbits were randomly assigned into two experimental groups, the control (C, n = 20) and the prebiotic (P, n = 20) group. In group P the feed of the does was completed with MOS. The young rabbits were allowed to consume the feed of the does from about the 17th days of age. The rabbits were weaned on the 35th day. At 7 weeks of age both groups (C and P) were divided into two (n = 10 in each), and half of the C and P rabbits were fed with the same diet as before, but contaminated with 2 mg/kg feed T-2 toxin (groups CT and PT). The animals were fed the toxin containing diet for the duration of 21 days. At the end of the 10th week blood samples were collected from 6 animals from group C, CT and PT. Mononuclear cells obtained from the rabbits were tested in comet assay to detect the genotoxic effect of T-2. All control samples (C) were negative in the test, i.e. all cells were scored as '0'. T-2 toxin in 2 mg/kg feed concentration had genotoxic effect on the rabbits' lymphocytes, as could be concluded from the comet values. MOS supplementation in the feed had significant protective effect against T-2 as seen by the lower comet score frequencies compared to T-2 treated animals. These results demonstrate that MOS may reduce risk associated with the uptake of mycotoxins probably by their binding capacity or antioxidative properties.

Key words: rabbits / animal nutrition / T-2 toxin / mannan oligosaccharide / genotoxicity / comet assay / rabbit lymphocytes

1 INTRODUCTION

T-2 toxin is a secondary fungal metabolite produced by *Fusarium* species and is the most toxic member of the type A trichothecenes. The ability of trichothecenes to inhibit protein, RNA and DNA synthesis, moreover apoptotic effects was investigated using T-2 toxin (Battilani *et al.*, 2009; Rocha *et al.*, 2005). Many publications reported apoptotic effects of T-2 toxin *in vitro* and *in vivo* as well and it can induce apoptosis, especially in high proliferating cell types, e.g., in haematopoietic, lymphoid, gas-

trointestinal cells and foetal tissue (SCF, 2001; Doi *et al.*, 2006; Jaradat, 2005). Several *in vitro* and *in vivo* studies described the genotoxic potential of T-2 toxin (Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001). The SCF (2001) and WHO (2001) reported inhibition of DNA and RNA synthesis by T-2 toxin in *ex vivo* cell cultures and *in vitro*, furthermore, total inhibition of DNA synthesis in phytohaemagglutinin-stimulated human peripheral blood lymphocytes. In another experiment T-2 toxin induced formation of free radicals and DNA damage *in vitro* and *in vivo* in mice liver cells (Jaradat, 2005). DNA

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fragmentation was found in chicken spleen leukocytes in a feeding experiment with T-2 toxin (Frankic *et al.*, 2006). The same authors observed increased DNA fragmentation in blood lymphocytes of pigs, after feeding diet with T-2 toxin (3 mg/kg) for 14 days (Frankic *et al.*, 2008). In another feeding experiment, DNA fragmentation was observed in spleen leukocytes of male broiler chicks when the diet contained T-2 toxin (13.5 mg/kg) for 17 days (Rezar *et al.*, 2007).

Food contaminants are directly exposed to the action of gut microbiota. Several studies showed that colonic probiotic bacteria can remove mycotoxins via physical binding as a mechanism for mutagen removal (El-Nezami *et al.*, 2000; Gratz *et al.*, 2006).

Mannan oligosaccharides (MOS) are widely used in animal feed to improve gastrointestinal health, energy levels and performance. They are normally obtained from the yeast cell walls of *Saccharomyces cerevisiae*. MOS are able to bind the mannose receptors on the type 1 fimbriae of some pathogen bacteria (such as *Escherichia coli* and *Salmonella enteritidis*) in order to prevent their attachment to intestinal mucosa (Firon *et al.*, 1983; Spring *et al.*, 2000). Because of their interaction with microbes, MOS could have an effect on microbial components of intestinal microbiota and/or on their activity.

The aim of the study was to determine the possible protective effect of MOS in T-2 toxicosis of rabbits.

2 MATERIALS AND METHODS

2.1 CHEMICALS

All chemical standards used in this study were obtained from Sigma/Hungary. Specialised chemicals used were: T-2 mycotoxin standard, Histopaque-1077, RPMI-1640 medium, and 4',6-diamine-2-phenylindol dihydrochloride (DAPI) for staining.

2.2 ANIMAL TREATMENT SCHEDULE

Suckling rabbits were randomly assigned into two experimental groups, the control (C, n = 20) and the prebiotic (P, n = 20) group. In group P the feed of the does was completed with MOS (Bio-Mos, Alltech Hungary, Budapest). The young rabbits were allowed to consume the feed of the does from about the 17th days of age. The rabbits were weaned on the 35th day. At 7 weeks of age both groups (C and P) were divided into two (n = 10 in each), and half of the C and P rabbits were fed with the same diet as before, but contaminated with 2 mg/kg feed T-2 toxin (groups CT and PT). The animals were fed the

toxin containing diet for during 21 days. At the end of the 10th week blood samples were collected from 6 animals from group C, CT and PT.

The does and their kits were housed in flat-deck cages (85x55 cm) while after weaning the growing rabbits were housed in two-level wire mesh cages (2 kits/cage) in a closed building. Average temperature ranged from 21 to 29 °C, the lighting was on between 05:00 a.m. and 09:00 p.m., and the farm had overpressure ventilation.

The diet contained 17% crude protein, 10% starch, 5% soluble fibre and 22% acid detergent fibre (ADF).

T-2 toxin was produced experimentally on corn grits by *Fusarium sporotrichioides* strain NRRL 3299, as described by Fodor *et al.* (2006).

The experimental protocol was authorised by the Food Chain Safety and Animal Health Directorate of the Somogy County Agricultural Office, under permission number 23.1/02322/007/2008.

2.3 ISOLATION AND PURIFICATION OF MONONUCLEAR CELLS

Venous blood from the marginal ear vein of the rabbits was put into EDTA tubes using a 2 ml sterile syringe with immediate transference. The collected blood was then mixed with an equal volume of RPMI-1640 tissue culture medium. The mixture was overlaid on Histopaque 1077 and centrifuged at 3000 rpm for 30 minutes and the interface layer consisting of mononuclear cells was carefully removed with a sterile pipette. The mononuclear cells (lymphocytes) were washed 3 times with 15 ml RPMI-1640 and each time centrifuged at 3000 rpm for 10 minutes. Finally, the pelleted cells were re-suspended in 10 ml of complete culture media (90% foetal calf serum / 10% DMSO), transferred to Eppendorf tube and were cultured at 37°C in 5% CO₂ humidified incubator for 24 hours.

2.4 COMET ASSAY

One percent NMP (normal melting point agarose), was prepared by dissolving 0.5 g in 50 ml autoclaved distilled water in a beaker and heating in microwave until boiling with occasional mixing until it completely dissolved. New slides were coated with warm 1% NMP Agarose gel prepared by dipping the slide vertically into gel in a beaker, and then they were allowed to dry overnight. About 140 µl of 1% warm low melting point agarose (LMP) Agarose gel (37 °C) in autoclaved distilled water was prepared as above and was added to incubated cells. The suspension was mixed gently and then quickly

70 µl was transferred to two spots on the previously coated slides. The suspensions were covered with cover slips (20x20 mm) and allowed to set for at least 10 minutes at 4 °C. The cover slips were removed and mononuclear cellular membranes were lysed with lysing buffer solution (pH 10) for 1 hour at 4 °C. The resulting nucleoids were unwound in alkaline electrophoresis buffer (pH13) in an electrophoresis tank (Cleaver Scientific Ltd. Warwickshire, UK) for 40 minutes at 4 °C and followed by electrophoresis at 25 V (300 mA) for 30 minutes at 4 °C. The slides were placed in neutralizing buffer (pH 7.5) with 3 washes of 5 minutes each and finally washed in bi-distilled water for 5 minutes. The gels were allowed to dry overnight, stained with 30 µl of working solution of DAPI, covered with cover slips. The confocal fluorescence images were generated using an Olympus Fluoview FV-1000 laser scanning confocal imaging system (Japan).

2.5 COMET ASSAY (DNA DAMAGE) SCORING

Manual scoring was done by following methods of Singh *et al.* (1988) and Collins *et al.* (1997) in which comets are classified into '0', '1', '2', '3' and '4' according to the relative intensity of DNA fluorescence in the tail. Fully disintegrated cells obtained the score of '5'. A minimum of 100 cells per samples were scored according to the length or shape of the comet that was formed (Hartmann *et al.*, 2003).

2.6 STATISTICAL ANALYSIS

Statistics were calculated with SPSS 19. software (SPSS Inc., Chicago, IL, USA). The occurrence frequency of the comet assay scores was different in the two

groups, as determined by Chi-Square test and two way contingency table analysis ($P < 0.0001$). This refers to the condition that the distribution of the individual comet scores (from 0 as undamaged to 5 as strongly damaged) falls apart from the theoretic 50–50% distribution by all 6 score values.

3 RESULTS AND DISCUSSION

Mononuclear cells obtained from control (C), T-2 exposed (CT) and T-2 exposed plus MOS supplemented rabbits (PT) were tested in comet assay to detect the genotoxic effect of T-2. All control samples (C) were negative in the test, i.e., all these cells were scored as '0'. T-2 toxin in 2 mg/kg feed concentration had genotoxic effect on the rabbits' lymphocytes, as concluded from the comet values (Fig. 1). Only 33% of the cells had 0 value (i.e., not affected by the toxin) and some cells with a '5' score could be detected, which indicates their complete disintegration.

MOS supplementation in the feed had significant protective effect against T-2 as seen by the different comet score frequencies of cells compared to those in T-2 treated animals. In PT animals 55% of the cells were intact, scored as '0'. Significantly lower frequencies were found in case of scores '1', '2', and '4'. Disintegration of the cells (score 5) was not observed.

In vivo genotoxic effect of T-2 toxin on mononuclear cells (lymphocytes) has been barely studied by comet assay. In male broiler chickens fed T-2 toxin of 10 mg/kg feed for 17 days DNA damage in spleen leukocytes was detected by comet assay (Frankic *et al.*, 2006). When T-2 toxin was added to the diet of the chickens for a 17-d period, the rate of DNA damage in spleen leukocytes

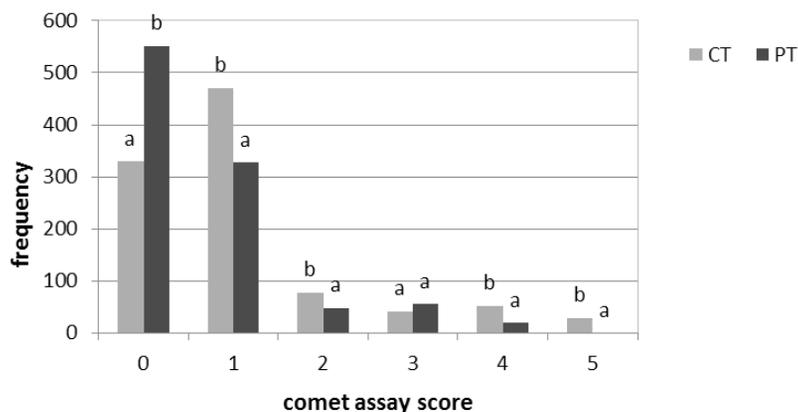


Figure 1: Frequency of comet scores of mononuclear peripheral blood cells obtained from T-2 exposed (CT) and T-2 exposed plus MOS consuming (PT) rabbits. ^{ab} indices mean significant ($P < 0.05$) difference between treatments.

increased significantly in the group fed 13.5 mg of T-2 toxin/kg of feed compared to the control group (Rezar *et al.*, 2007). Sokolovic *et al.* (2007) administered a single dose of T- toxin (0.5 mg/kg of body weight) to male 5 days old chickens which was genotoxic to their blood lymphocytes. Male crossbred pigs were fed 3 mg/kg T-2 toxin for 14 days, which increased the amount of DNA damage in peripheral blood lymphocytes by 27%, (Frankic *et al.*, 2008). In our study, lower concentrations of T-2 toxin than those used in the studies above were administered to rabbits still caused significant DNA damage in peripheral lymphocytes. This is in accordance with the observations of Wannemacher and Wiener (1997) who considered rabbits as rather sensitive to T-2 toxin. Caecotrophy, by which substantial toxin quantities get back into the digestive tract so resulting in prolonged excretion times, may play a role in rabbits' high sensitivity to toxic substances (Ványi and Glavits, 1988).

MOS are widely used as prebiotic substances in animal nutrition, which act by competitive exclusion of enteric pathogens. There are several papers referring the positive effect of MOS on performance and health of rabbits (cited in Maertens *et al.*, 2006) but few data relating mycotoxin effects to dietary fibres, like glucomannans. However, it was shown that the supplementation of the diet with glucomannan protected against the immunotoxicity of aflatoxin B1 and T-2 toxin (Meissonnier *et al.*, 2009). Rabassa *et al.* (2010) reported reduced liver damage in ewes caused by AFB1 when adding dietary fibre glucomannan. In the study of Meca *et al.* (2012) soluble alimentary dietary fibres decreased the duodenal bio-accessibility of the *Fusarium* micotoxin beauvericin, while bio-accessibility ranged from 1.3 to 38-fold higher after colonic bacterial fermentation of the fibre. This indicates that fibre has the property to bind mycotoxins, which is supported by the fibre fermentation in the hindgut increasing mycotoxin concentration and bio-accessibility in the colon. These results demonstrate that some prebiotic substances, such as MOS, may reduce risk associated with the uptake of mycotoxins by their binding capacity.

Another possible way how MOS exerts protective effect against the genotoxic effect of T-2 may be by its antioxidative properties. It is generally accepted (Schuhmacher-Wolz *et al.*, 2010), that DNA damage caused by T-2 is caused by oxidative stress (marked by depletion of glutathione levels and increased lipid peroxidation). Such antioxidative and antimutagenic effect of yeast cell wall mannans were described by Križková *et al.* (2001, 2006).

According to our results and those found in the literature, MOS appear to be promising, not only as natural growth promoters but also as protective agents against mycotoxins in feeds.

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