Immunolocalization of Na^+/K^+-ATPase and proliferative activity of enterocytes after administration of glucan in chickens fed T-2 toxin

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Abstract

The protective effect of polysaccharide glucan in chickens fed low doses of T-2 toxin was assessed. The binder effect of ß-D-glucan on jejunal mucosa in relation to the expression of Na^+/K^+-ATPase, proliferative activity of enterocytes and number of goblet cells was investigated. A total of 40 one-day-old chickens were allocated to four groups: control (C), ß-D-glucan (G), T-2 toxin (T) and combined ß-D-glucan+T-2 toxin (GT). The chickens were individually administrated *per os* 1.0 mg/bird/day of ß-D-glucan derived from *Candida albicans* on days 11, 12, and 21 of the experiment (totally 3 mg per bird). T-2 toxin at a concentration of 1.45 $\mu g \cdot kg^{-1}$ was added to the feed from day 14 to day 28 of the experiment. The α subunit-specific anti-Na^+/K^+-ATPase antibody was used to identify the protein by immunofluorescence in the cell membrane of jejunal enterocytes. Higher expression of Na^+/K^+-ATPase was found in the jejunal epithelial cells and lamina propria in the chickens fed T-2 toxin and administered glucan ($P < 0.05$) compared to control. The number of proliferated enterocytes was higher in group T compared to group G and control ($P < 0.001$), as well group GT ($P < 0.01$). Goblet cell density did not present significant differences between groups of chickens, but group G showed the highest values. These data suggest that administration of pure T-2 toxin at low doses affects primarily the protein synthesis of actively dividing cells. Higher distribution of Na^+/K^+-ATPase in enterocytes of chickens in GT group suggests positive influence of glucan and mycotoxin on the ion pump. A binding effect of this immunomodulator on the digestive tract mucosa in the applied setup was not observed.

Poultry, intestine, yeast, trichotecene, ion pump, immunofluorescence

T-2 toxin, one of the members of the trichothecene group, is a naturally occurring mycotoxin produced by several species of fungi in the genus *Fusarium.* T-2 toxin at small doses can damage the mucosa of the digestive tract and impair the resorption of nutrients (Sokolović et al. 2008; Kanora and Maes 2009). The absorption of mycotoxins and their fate within the gastrointestinal tract suggest that the epithelium is repeatedly exposed to these toxins, and at higher concentration than other tissues (Grenier and Applegate 2013). *Fusarium* mycotoxins alter the different intestinal defence mechanisms including epithelial integrity, cell proliferation, mucus layer and immunoglobulins (Bouhet and Oswald 2005). In a study of low and moderate T-2 toxin doses, Antonissen et al. (2014) reported a decrease of the mentioned indices.

The proliferative activity of enterocytes is a sign of healthy tissue turnover and maintenance (Garcia et al. 2007). Unlike in mammals, chicken enterocyte proliferation

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is not localized only in the crypt region, and the site of enterocyte differentiation is not precisely localized (Uni et al. 1988).

NA^+/K^+-ATPase is a key transport element required for the establishment of electrochemical gradients driving the cellular transport and substrate flow across epithelia. In addition, the enzyme is involved in basic processes such as maintenance and proliferation (Zouzoulas et al. 2005). A large proportion of the energy demands of the gastrointestinal tract is associated with the activity of Na^+/K^+-ATPase, therefore, the energy costs involved in the absorption of glucose and other nutrients are considerable (Wang et al. 2009).

Many reports have been published in recent years about mycotoxin inactivation agents (mycotoxin binders) in order to reduce the toxic effects of mycotoxins in animals. The application of microorganisms capable of biotransforming certain mycotoxins into less toxic metabolites has been proposed (Jouany et al. 2005; Shetty and Jespersen 2006). The microorganisms act in the animal intestinal tract prior to the absorption of mycotoxins. Many species of bacteria and fungi have been shown to enzymatically degrade mycotoxins (Avantaggiato et al. 2004; Jouany et al. 2005). Yeast or yeast cell walls show the potential as mycotoxin binders. The cell walls harbouring polysaccharides (glucan, mannan), proteins and lipids exhibit numerous different and easy accessible adsorption centres including adsorption mechanisms (Huwig et al. 2001).

The effect of the interaction of small doses of T-2 toxin and polysaccharides on the functional activity of intestinal mucosa is not known. The aim of this study was, therefore, to investigate the activity of Na^+/K^+-ATPase, proliferative activity of enterocytes, and goblet cell density in intestinal mucosa after 14 days administration of T-2 toxin in feed and oral application of ß-D glucan.

Materials and Methods

Animals

A total of 40 one-day-old chickens of the Lohmann Brown hybrid were placed in wire cages with solid floors. They were allocated to four groups: control (C), ß-D-glucan (G), T-2 toxin (T), ß-D-glucan + T-2 toxin (GT); each group containing 10 chickens that were fed a diet prepared at the Institute of Pathological Anatomy of University of Veterinary Medicine and Pharmacy in Košice, corresponding to the commercial diet for chickens (HYD 02/a) but free of antimycotics. The chicks were kept in the menagerie of the Institute of Pathological Anatomy, UVMP, Košice, Slovakia (SK P 52004), in accordance with the rules and with the approval of the Ethics Committee; the experiment was authorized by the State Veterinary and Food Administration of the Slovak Republic (No. Ro-270710-221).

Preparing of β-D-glucan and T-2 toxin

Beta-D-glucan in powder form was obtained from *Candida albicans* (donated by RNDr. Ema Pavlovičová, CSc., Chemical Institute of the Slovak Academy of Science, Bratislava, Slovak Republic). One hundred mg of lyophilised ß-D-glucan were dissolved in *aqua pro injectione* with the final concentration of 3 $mg \cdot ml^{-1}$. The chickens were individually administered 1.0 mg/bird/day of dissolved polysaccharide *per os*.

T-2 toxin in powder form (OEKANAL®, Sigma-Aldrich, Germany, mol. w. 466.52 $g \cdot mol^{-1}$) was dissolved in 50 ml of 96% ethanol; 2 ml were added to 1 kg of feed and then mixed thoroughly with a hand-mixer. The concentration of 1.45 $\mu g \cdot kg^{-1}$ of food was confirmed by the HPLC method at the State Veterinary and Food Institute (Bratislava, SNAS Reg. No. 050/S-127, Slovak Republic) together with other mycotoxins.

Experimental design

The experiment lasted from day 1 to day 28 of the chickens' life. Group C served as a negative control and was fed a diet without ß-D-glucan or T-2 toxin. Chicks in groups G and GT were individually administered ß-D-glucan (1 mg) *per os* on days 11, 12, and 21 of the experiment (3 mg per bird in total). Chicks of groups T and GT were fed *ad libitum* a diet prepared daily with the addition of T-2 toxin (1.45 $\mu g \cdot kg^{-1}$) from day 14 to day 28 of the experiment. Jejunum samples were collected from six chickens of different groups slaughtered on day 28 of the experiment, after having consumed the feed with T-2 toxin for two weeks.

Detection of NA^+/K^+-ATPase by immunoflourescence

The small intestine (mid jejunum) was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight, washed in PBS, frozen in liquid nitrogen, and cryosectioned on a Leica CM3050 S cryostat (Leica,

Bensheim, Germany). Sections (6 µm) were washed three times with PBS, permeabilized by incubation for 10 min in PBS with 0.1% Triton X-100 (PBS-T), and blocked with 10% goat serum in PBS-T for 15 min (all at room temperature). A step of staining with 0.1% Sudan black B in 70% ethanol was included to reduce disturbance to autofluorescence. Subsequently, the sections were incubated for 1 h at room temperature in a moist chamber with primary antibody Na+/K+-ATPase (mouse monoclonal antibody specific for the α subunit Na+/K+-ATPase; M7-PB-E9; Affinity Bioreagents, Golden, CO, USA) diluted 1:100 with 2% normal goat serum in PBS-T. After being washed three times in PBS-T, the sections were incubated for 45 min at room temperature in the dark with secondary antibody (Alexa Fluor 488 conjugated goat anti-mouse IgG1; Invitrogen, Darmstadt, Germany) diluted 1:500 in PBS-T. The nuclei were counterstained with 2 µg/ml propidium iodide in PBS. Negative control samples were incubated in PBS-T containing 2% normal goat serum (NGS) instead of the primary antibody. No unspecific binding of the secondary antibody was observed.

Digital pictures of histological slides were collected using the Nikon fluorescence microscope (Nikon Instruments Europe B.V., Netherlands) and an image analysis system equipped with the CELL^F image analysis software as well as CC-12 high resolution colour camera (OSIS, Münster, Germany). Fluorescence filters in the Nikon microscope were as follows: blue excitation filter set for Alexa Fluor 488: excitation 420-490 nm, dichroic mirror 510 nm, barrier filter longpass 520 nm; green excitation filter set for propidium iodide (PI): excitation 510–560 nm, dichroic mirror 580 nm, barrier filter longpass 590 nm. Additional bandpass barriers filter was used to exclude red fluorescence of PI 535/40 nm. Excitation time for green fluorescence was always 2 s.

The intensity of immunofluorescence in the recorded pictures was measured by NIS-Elements 3.0 software (Nikon, Japan).

Quantification of proliferative cells in the jejunum

Samples for proliferating cell nuclear antigen (PCNA) assay were taken from the mid jejunum on day 28 of the experiment, fixed in 10% neutral buffered formalin and embedded in paraffin. The PCNA was carried out on 5 µm thick tissue sections with the commercial Animal Research Kit (ARK) according to the included protocol (DAKO, Denmark). The kit contained monoclonal mouse anti-PCNA antibody (Clone PC 10, DAKO, Denmark) and all components (peroxidase block, streptavidin HRP, blocking reagent, biotinylation reagent and DAB tablets) needed for examination. Negative controls were obtained by omitting the primary antibody.

The cells in the jejunum were counted in the villus epithelium and lamina propria from the villus basal lamina (which coincide with the upper crypt end) toward the villus apex. Twenty appropriate areas were chosen at random from each of these sites in the gut. Measurements were taken using a light microscope at × 400 magnification. The positive stained cells within each randomly-selected area were counted using a calibrated ocular graticule with 0.25 mm Id × Grd (Electronmicroscopy, UK). The appearance of positive enterocytes is expressed in numbers per mm^2.

Staining and counting of goblet cells

A commercial kit was used for the staining of goblet cells on paraffin sections (Alcian blue pH 2.5 P.A.S., 04-163802, Bio-Optica, Italy). Mid jejunum samples were taken on day 28 of the experiment from five chickens in each group. The density of goblet cells was calculated as the number of goblet cells per unit of surface area (mm^2). All measurements were performed from photos using the NIS-Elements 3.0 software (Nikon, Japan).

Statistical analysis

All data were statistically tested using one-way ANOVA and Tukey test in Minitab 16 (SC&C Partner, Brno, Czech Republic). The results are given as means ± SD (standard deviation). Differences between mean values for the groups of chickens were considered significant at $P < 0.05$.

Results

The α subunit-specific anti-Na^+/K^+-ATPase antibody identified the protein in the cell membrane of jejunal enterocytes showing a bright green fluorescence signal. This green fluorescence was demonstrated not only in the epithelial cells but also in the lamina propria mucosae (Plate XI, Fig. 1 A-D). Higher distribution of Na^+/K^+-ATPase was found in the jejunal epithelial cells and lamina propria mucosae of chickens that were fed T-2 toxin as well as ß-D-glucan (GT group, $P < 0.05$) compared to control chickens (Fig. 2). The intensity of signal distribution of Na^+/K^+-ATPase in the epithelial cells gradually increased in the basal direction toward the cells of the lamina propria mucosae. This characterizes the highly metabolic activity of cells in middle jejunum villi (Fig. 1D). Groups G (Fig. 1B) and T (Fig. 1C) alone showed no significant changes in the distribution of Na^+/K^+-ATPase,

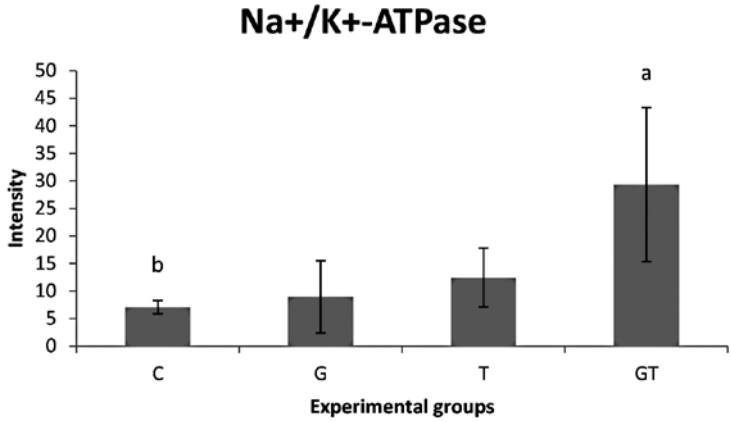

Fig. 2. Intensity of Na^+/K^+-ATPase distribution in jejunal mucosae of control and experimental groups on 28 d, after 2 week of T-2 toxin feed contamination (n = 6; mean ± SD, $^{ab}P < 0.05$; C - control group; G - ß-D-glucan; T - T-2 toxin; GT - ß-D-glucan + T-2 toxin)

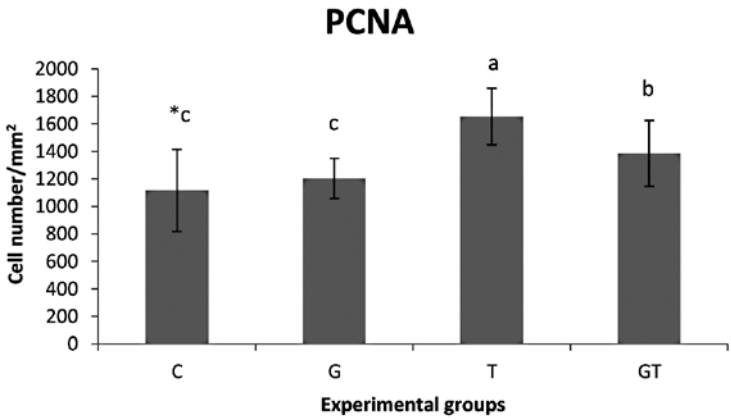

Fig. 3. Number of PCNA (proliferating cell nuclear antigen) positive cells in the jejunum (positive enterocytes are expressed in numbers per mm²) of chickens in control and experimental groups on d 28, after 2 weeks of T-2 toxin feed contamination (n = 6; mean ± SD; C - control group; G - ß-D-glucan; T - T-2 toxin; GT - ß-D-glucan + T-2 toxin). Different index in column is significant: $^{ab}P < 0.01$ (T vs GT), $^{b*}P < 0.01$ (GT vs C), $^{ac}P < 0.001$ (T vs C and G)

although there was a slight increase in the activity in the basal direction. Epithelial cells in the jejunum of control chickens demonstrated a gradual decrease in Na^+/K^+-ATPase activity toward the cells of the lamina propria mucosae (Fig. 1A).

The number of PCNA positive enterocytes was the highest in chickens of group T ($P < 0.001$) comparing to G and C groups, as well as group GT ($P < 0.01$). Moreover, PCNA positive cells in chickens of the combined group GT were higher in number ($P < 0.01$) than PCNA positive enterocytes in group C (Fig. 3).

The density of goblet cells did not show significant differences between the groups of chickens (Fig. 4).

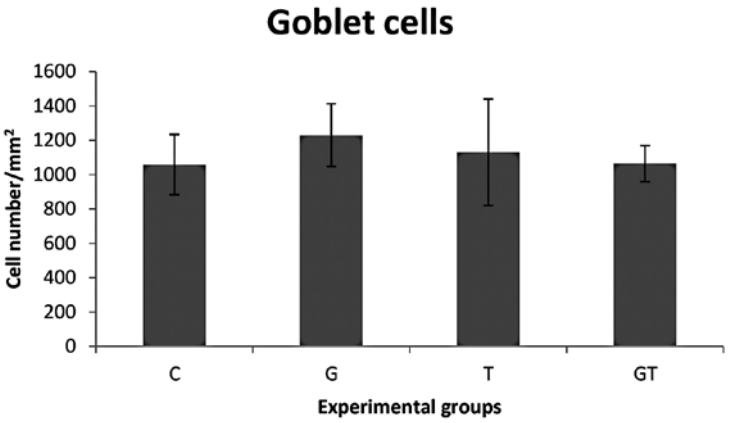

Fig. 4. Number of goblet cells in the jejunum (per mm²) of chickens after administration of ß-D-glucan and T-2 toxin on d 28, after 2 weeks of T-2 toxin feed contamination (n = 6; mean ± SD; C - control group; G - ß-D-glucan; T - T-2 toxin; GT - ß-D-glucan + T-2 toxin)

Discussion

Recent research results have shown that in many cases membrane active properties of various mycotoxins determine their toxicity (Zain 2011). Previously, we have reported that administration of feed contaminated with low doses of T-2 toxin to chickens increased the expression of *MUC-2* and *IgA* genes in the jejunum. On the other hand, application of β-D-glucan as binder for mycotoxins and T-2 toxin demonstrated suppressed density of IgA+ lymphocytes and increased *pIgR* expression (Levkut et al. 2015).

In order to identify the changes in energy metabolism in the intestine (Wang et al. 2009), ion transport and cell proliferation including goblet cells were evaluated. In the current paper we focused on changes in the jejunum caused by absorption of ingested mycotoxin in the proximal part of the gastrointestinal tract. Immunolocalization of the Na^+/K^+-ATPase α subunit demonstrated its distribution in the basolateral membrane of the intestinal epithelial cells which is consistent with previous findings in ruminants (Kuzinski et al. 2011).

In the current trial, higher distribution of Na^+/K^+-ATPase in enterocytes of chickens in the combined group (ß-D-glucan+T-2 toxin) suggests a positive influence of glucan and the administered mycotoxin on the ion pump in these cells. It is known that β-glucans reinforce the host immune defence by activating the complement system, and enhancing macrophages and the natural killer cell function (Kolodzieyski et al. 1995; Akremiené et al. 2007). Similarly, macrophages are the main target cells for T-2 toxin (Seeboth et al. 2012). On the other hand, proinflammatory interleukins increase nutrient absorption in the intestine and stimulate the activity of Na^+/K^+-ATPase (Hardin et al. 2000). If we take into account previous results (Rezar et al. 2007; Levkut et al. 2015) showing that low doses of T-2 toxin stimulate the immune system, subsequently the synergic effect of both modulators, i.e. T-2 toxin and ß-glucans could increase Na^+/K^+-ATPase activity. On the other hand, the results obtained in the current trial indicate that administration of β-glucans did not decrease the effect of T-2 toxin on Na^+/K^+-ATPase.

The current trial demonstrates the stimulatory effect of 1.45 $\mu g \cdot kg^{-1}$ T-2 toxin on proliferating cell nuclear antigen in enterocytes. This is assumed to occur through a direct interaction of T-2 toxin with deoxyribonucleic acid (DNA). This study shows that

the effects of T-2 toxin are dose-dependent, because higher doses of the toxin (4.5 mg of T-2 toxin·kg^{-1} of feed) cause DNA fragmentation (Rezar et al. 2007). The indication of intestinal changes in group T is supported by our previous results (Levkut et al. 2015) which demonstrated increased mRNA of IgA and pIgR after administration of low doses of T-2 toxin. Finally, promotion of IgA production confirms the exposure of gastrointestinal lymphoid tissue to more feed antigens. However, the administration of β-D-glucan did not confirm the tested polysaccharide as a mycotoxin binder.

Goblet cells are present throughout the gastrointestinal tract and are the main source of mucin there (Specian and Oliver 1991). Mucins are likely to be the first molecules which invading pathogens interact with at the cell surface and can thus limit their binding to other glycoproteins and neutralize the pathogens (Kim and Khan 2013). The data obtained in our current study do not show hyperplasia of goblet cells in the intestine of chickens after administration of T-2 toxin and β-D-glucan. On the other hand, our previous report (Levkut et al. 2015) showed a higher expression of *MUC-2* gene in the jejunum of the T-2 toxin group. It seems that a low dose of T-2 toxin stimulates only the mucin gene and does not affect, at least during the two-week experimental period, the goblet cell dynamic.

In conclusion, these data suggest that administration of pure T-2 toxin at low doses primarily affects the protein synthesis of actively dividing cells which was demonstrated by increased numbers of positive PCNA cells in groups T and GT. The higher distribution of Na^+/K^+-ATPase α subunit found in group GT indicates a synergistic effect with the administered immunomodulator as β-D glucan. A binding effect of the immunomodulator on mucosa of the digestive tract was not observed. However, we proved that administration of β-D-glucan to chickens had a beneficial effect as it did not affect the nutritional properties of enterocytes yet improved the goblet cell production which could result into enhancement of the natural and acquired immunity to mycotoxins.

Acknowledgements

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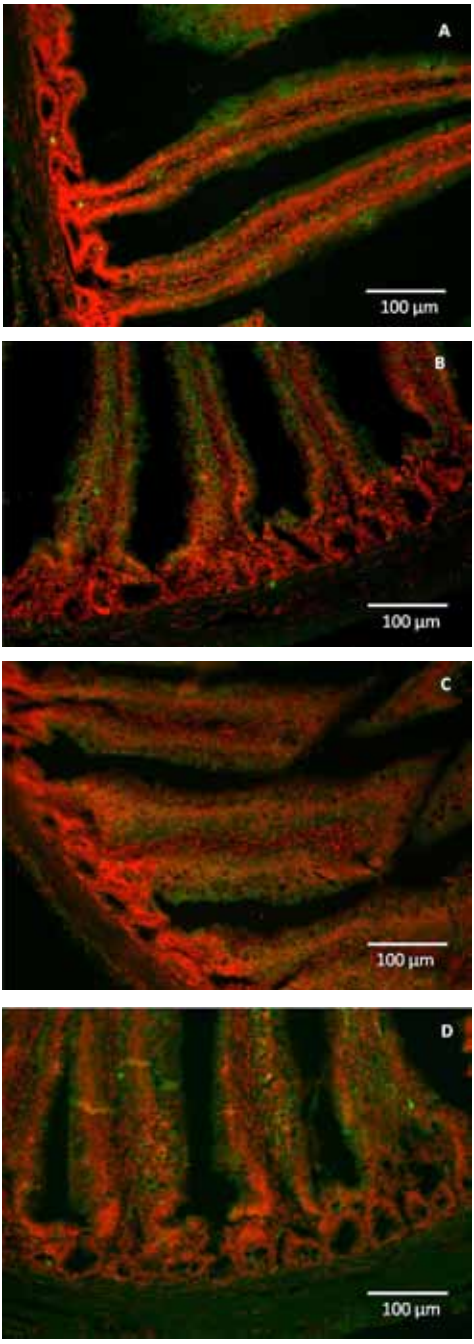

Fig. 1. Representative images of α subunit NA^+/K^+-ATPase detection in jejunum of chickens. Fluorescence signal (green colour) in epithelial cells and lamina propria of jejunum in control (A), ß-D-glucan (B), T-2 toxin (C) and ß-D-glucan + T-2 toxin (D) groups. Cells were counterstained with propidium iodide (red colour) to label nuclei (n = 6); scale bar 100 µm.