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T-2 MYCOTOXIN SLOWS DOWN THE DEVELOPMENT OF MOUSE BLASTOCYSTS, DECREASES THEIR BLASTOMERE NUMBER AND INCREASES CHROMATIN DAMAGE

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The mycotoxin T-2 has many harmful effects on mammalian cells and reproductive functions. In the present study, the *in vitro* effect of T-2 toxin on mouse blastocysts was examined. Embryos were cultured in media supplemented with 0.5, 0.75 and 1 ng/ml T-2. Different exposure times were applied [96 h (treatment I) or 24 h following 72 h in toxin-free media (treatment II)]. Blastomere number, nuclear chromatin status and blastocoel formation were investigated in blastocysts. Our data show that the effect of T-2 toxin may vary depending on the stage of the embryo at the start of exposure. At 96 h of exposure, the blastocysts had blastomeres with normal chromatin quality but their developmental potential was decreased. After 24 h of exposure applied following a 72-h culture, blastomeres had a higher level of chromatin damage, although their developmental potential was the same as in the control embryos. In both cases, decreased mitotic rate was found, which resulted in decreased blastomere number even at low toxin concentration.

Key words: T-2 toxin, embryotoxicity, nuclear chromatin, mouse embryos, blastocoel, *in vitro* culture

T-2 toxin (T-2) is a type A trichothecene mycotoxin produced by phytopathogenic fungi of the *Fusarium* genus, mainly by *F. sporotrichoides* and *F. poae,* which can infect crop plants such as wheat, barley, oat and rice in temperate climates (Glenn, 2007). In Europe, especially in the Nordic countries, the contamination of cereals with T-2 and HT-2 toxins is also a serious problem (Beyer et al., 2009). According to the latest mycotoxin survey report from 2015 (Biomin Holding GmbH, 2015), 57% of feed samples were contaminated with T-2 in Central Europe. T-2 toxin, which has the highest toxicity among trichothecenes, may cause gastrointestinal, dermatological, immunological and neurologic symptoms in experimental and farm animals (Bennett and Klich, 2003). Recently, it

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has been reported that T-2 toxin enters the brain via the blood–brain barrier (Weidner et al., 2013).

Besides the well-documented harmful effects of T-2, many studies in animals have revealed that T-2 can cause reproductive disorders in both males and females. These abnormalities include retarded ovulation and decreased progesterone production (Huszenicza et al., 2000), disrupted corpus luteum maturation (Ványi et al., 1995), inhibited granulosa cell proliferation (Caloni et al., 2009), as well as reduced sperm motility and testosterone production (Kovács et al., 2011).

Although wide-scale studies about T-2 are available, effects of the toxin on early embryo development have not been revealed to date. A recent study conducted by our group showed that T-2 can cause developmental arrest in mouse embryos and can delay their development by 24 h, resulting in late formation of the blastocoel (Somoskői et al., 2014). The aim of this study was to assess the effect of T-2 on preimplantation mouse embryos being in the morula and blastocyst stage (following 72 and 96 h of culture in a medium supplemented with the toxin at different concentrations) and having normal morphology.

Materials and methods

Animal housing and mating

Procedures with animals were performed following good veterinary practice for animal welfare according to the Hungarian national laws in force. The protocol of the animal experiment was approved by the Food Chain Safety and Animal Health Directorate of Pest County's Government Office (11/1/2015). Six weeks old BDF1 (National Institute of Oncology, Budapest, Hungary) mice were kept under a 12 hours light/12 hours dark schedule at a temperature of 21 °C. Feed and drinking water were available *ad libitum*. Superovulated [Day 1: 7.5 IU eCG ip.; Day 3: 7.5 IU hCG ip. (Alvetra und Werfft, Austria)] female mice were placed together overnight with mature males after hCG treatment.

Embryo culture and treatment

One-cell zygotes were obtained from females sacrificed by cervical dislocation 20 h after hCG treatment, pooled and transferred randomly to culture medium (Cleavage Medium, Cook Medical, Roskilde, Denmark). Embryos were exposed to T-2 toxin (Sigma, St. Louis, Missouri, USA) in the following structure:

Treatment I: embryos were cultured *in vitro* for 96 h in culture media supplemented with T-2 at different concentrations (0.5 ng/ml, 0.75 ng/ml and 1.0 ng/ml).

Treatment II: to investigate the stress tolerance against the toxin in compacted stages, embryos were cultured *in vitro* in a medium with no toxin for

72 h, and then morphologically normal embryos were transferred into culture media contaminated with 0.5 ng/ml, 0.75 ng/ml and 1.0 ng/ml toxin (group names: Tr05, Tr075 and Tr1, respectively).

Toxin concentrations were based on our previous studies (Somoskői et al., 2012, 2014). Embryos cultured in a medium with no toxin were regarded as the control group. All embryos were cultured at 37.5 °C with 6.5% CO_2 and maximal humidity in air. Average embryo number was 20.6/group/repeat. The experiment was performed in three repeats, with 5 animals/repeat (n = 15).

Embryos were stained with SYBR14 (Life Technologies, USA) and propidium iodide (PI) (Life Technologies, USA) at 72 and 96 h of culture to assess the cell number of embryos, dead (necrotic) cells and the proportion of blastomeres with damaged nuclear chromatin (micronuclei). Staining and comparison were performed only in the case of morphologically normal embryos in each group.

Blastocysts were investigated using an Olympus CKX41 invert microscope and Olympus E-330 digital camera system. Pictures were taken at the 96th h of culture and embryos were classified based on the expansion of blastocoel [early and mid-, expanded and hatched blastocysts (Saiz and Plusa, 2013)].

Statistical analysis

Data were analysed with R v3.0.0 software. ANOVA with *post-hoc* Tukey's test was used for comparing the mean cell number between groups. Differences in blastocoel expansion of blastocysts were measured with chi-squared test. Differences at a probability value (P) of < 0.05 were considered significant.

Results

Cell number of embryos following 72 h of culture

Cell numbers of embryos in treatment I (0.5 ng/ml: 20 ± 7.22; 0.75 ng/ml: 18.77 ± 5.52; 1 ng/ml: 13.11 ± 7.35) were significantly lower ($P < 0.001$) than in the control embryos (27.74 ± 7.35) (Fig. 1). Significant differences between the highest toxin contamination (1 ng/ml) and lower toxin concentrations were found ($P = 0.046$ to 0.75 ng/ml and $P = 0.01$ to 0.5 ng/ml). There was no difference in the cell numbers of embryos between the groups treated with 0.5 ng/ml and 0.75 ng/ml T-2 toxin. The proportion of PI-positive cells was under 1% in the treated and control groups (data not shown).

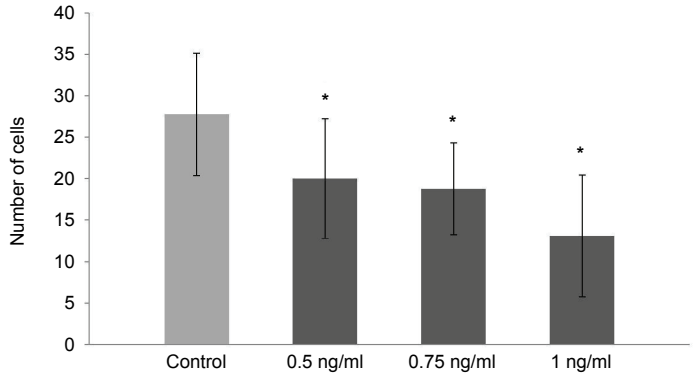

Fig. 1. Number of cells (mean ± SD) in mouse embryos treated with 0.5 ng/ml, 0.75 ng/ml and 1 ng/ml T-2 after 72 h. Control embryos were cultured in a medium without toxin.
*P < 0.001, Tukey's test

Cell number of embryos following 96 h of culture

The mean cell number of control embryos was 91.52 (± 26.09) after 96 h of *in vitro* culture. Every treated group showed a significant difference compared to the control group (0.5 ng/ml: 46.33 ± 19.1; 0.75 ng/ml: 57.93 ± 24.7; 1 ng/ml: 27.77 ± 12.07) (Fig. 2A) in respect of cell number. A difference was found in cell number between the groups treated with 1 ng/ml and 0.75 ng/ml toxin ($P < 0.001$), but no difference was observed between groups treated with 0.5 ng/ml and 0.75 ng/ml toxin concentrations.

The cell number of embryos in treatment II was significantly lower than that of the control embryos (54.86 ± 22.51 in Tr05, 67.66 ± 26.76 in Tr075 and 54.73 ± 38.66 in Tr1). However, no differences were found between the Tr05, Tr075 and Tr1 groups (Fig. 2B). A significant difference between treatments I and II was observed only in the case of 1 ng/ml T-2 concentration ($P = 0.048$) (Fig. 2C).

The mean proportion of PI-positive cells was under 1.5% in all treated and control embryos (the highest rate was 8.82% in the 1 ng/ml group, but the upper quartile was under 2% in all of the treated, transferred and control embryos; data not shown).

A representative image of cell staining is shown in Fig. 3.

Chromatin damage (micronuclei)

The proportion of blastomeres with chromatin damage was 6.15% (± 2.91) in the control group, and 7.68% (± 4.67) in the 0.5 ng/ml, 7.46% (± 5.57) in the 0.75 ng/ml, 7.69% (± 4.21) in the 1 ng/ml, 19.37% (± 7.92) in the Tr05, 12.86% (± 6.09) in the Tr075 and 8.96% (± 5.68) in the Tr1 groups. Embryos in the Tr05 and Tr075 groups contained micronuclei in significantly higher ($P < 0.001$ and

P < 0.01) proportion compared to the control. Transfer of embryos into toxin-contaminated media resulted in a significantly higher rate of micronuclei in the 0.5 ng/ml (P < 0.001) and 0.75 ng/ml (P < 0.05) treatment groups (Fig. 4). No effect was found after culturing in a medium with 1 ng/ml toxin for 96 or 24 h.

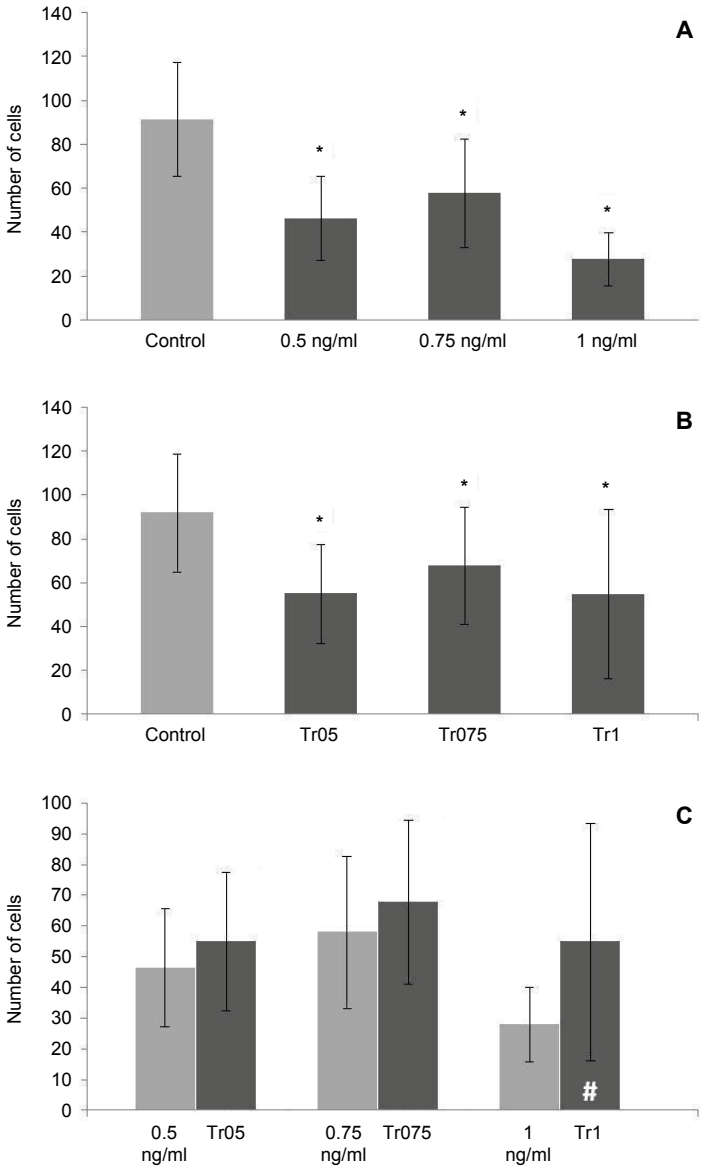

Fig. 2. Comparison of cell number (mean ± SD) in embryos of control and treatment I (A), control and treatment II (B) groups and in toxin-treated embryos (C) at the 96th h of culture.
*Significant difference from control embryos; #P < 0.05 within toxin treatments

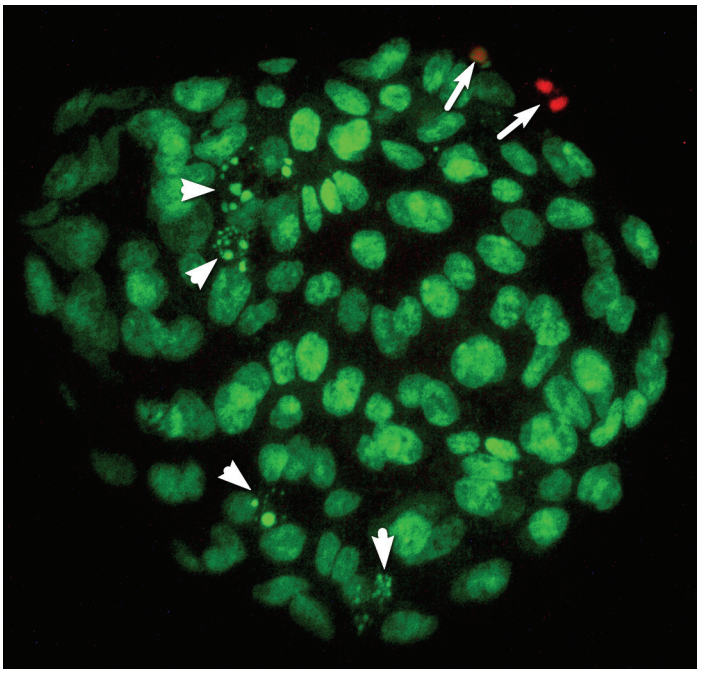

Fig. 3. Representative image of a blastocyst (treated with 0.75 ng/ml T-2) stained at the 96th hour of *in vitro* culture with SYBR14 and propidium iodide. Arrows show PI-positive nuclei and arrowheads show micronuclei (chromatin damage)

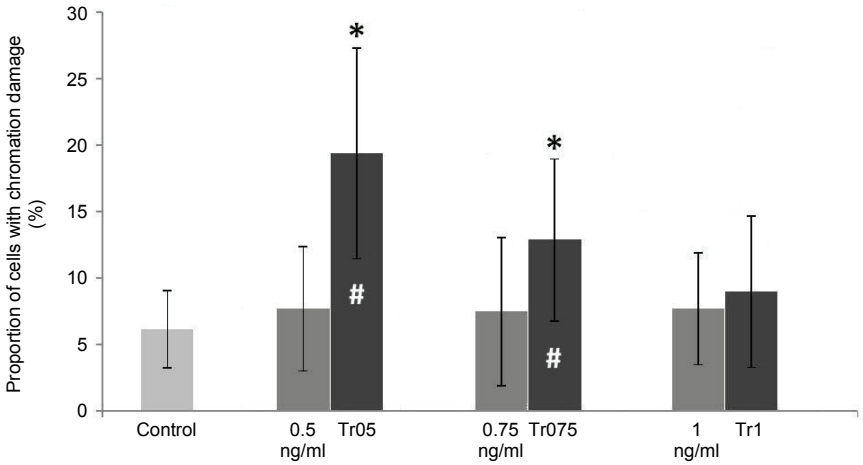

Fig. 4. Proportion of cells (mean ± SD) with chromatin damage in embryos of the control, treatment I and treatment II groups. *Significant difference from control embryos; #significant difference within treatments

Blastocoel expansion

The proportion of different blastocyst types (early and mid-, expanded and hatched; Fig. 5) following 96 h of culture in each group is shown in Fig. 6. A dose-dependent, significantly decreased rate of expanded/hatched blastocysts was found in the groups of treatment I (67.9, 58.6 and 55%) compared to the control (87.7%). No difference was found in blastocoel expansion between the control and the toxin-contaminated embryos in treatment II.

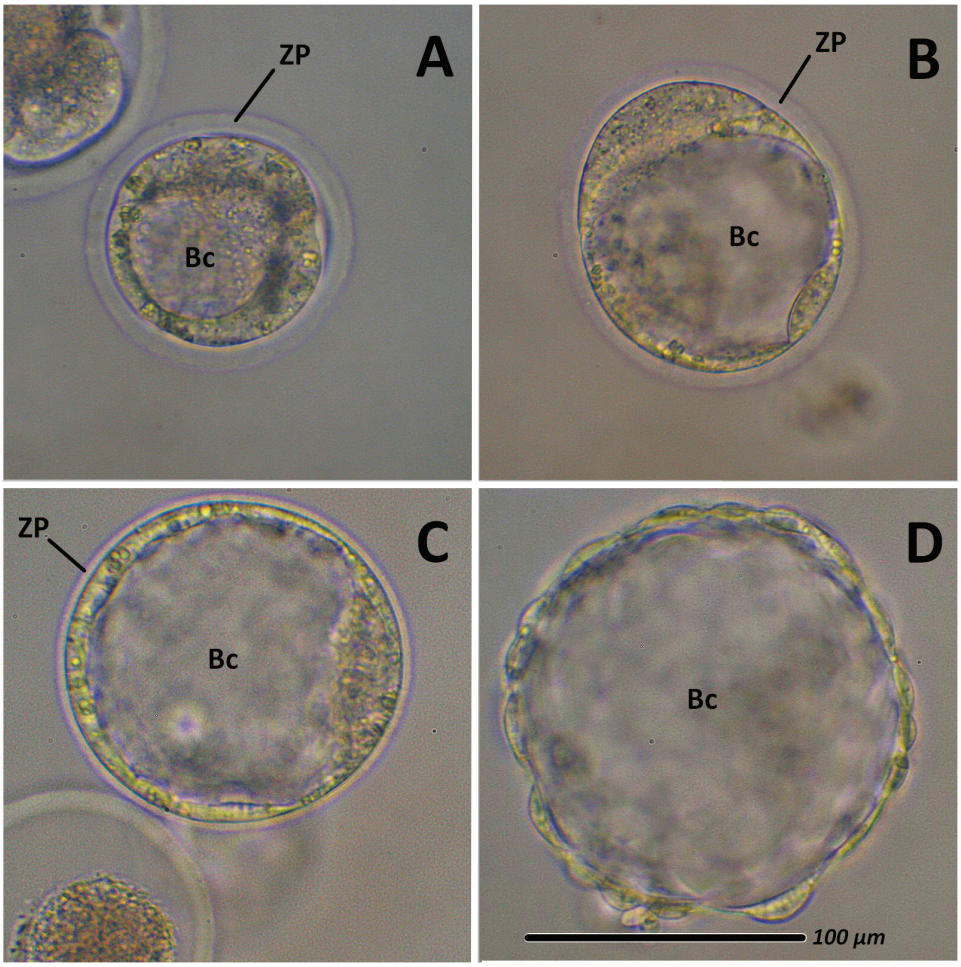

Fig. 5. Representative images of blastocysts showing diverse degrees of blastocoel expansion. A = early blastocyst (cultured in 1 ng/ml T-2); B = mid-blastocyst (cultured in 0.5 ng/ml T-2); C = expanded blastocyst (control) and D = hatched blastocyst (control). Scale bar represents 100 µm. Bc = blastocoel; ZP = zona pellucida. Magnification: ×400

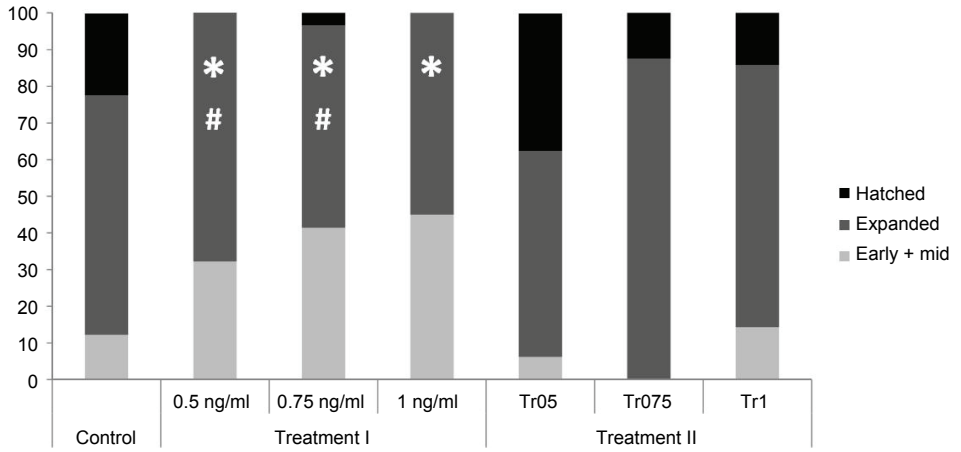

Fig. 6. Proportion of blastocysts being in different stages (early and mid-, expanded and hatched blastocyst stage) following 96 h *in vitro* culture. $^{*}P < 0.05$ between rreatments and control; $^{\#}P < 0.05$ between treatments I and II

Comparing the proportion of expanded/hatched blastocysts at equivalent toxin concentrations in treatments I and II, we found significant differences in the case of 0.5 ng/ml and 0.75 ng/ml toxin concentrations (67.9% vs. 93.8% and 58.6% vs. 100%, respectively). Although the rate of late blastocysts in the 1 ng/ml and Tr1 groups did not differ significantly ($P = 0.059$), a clear tendency was found (55% vs. 85.7%, respectively).

Discussion

The T-2 toxin concentrations used in this experiment were 0.5, 0.75 and 1 ng/ml. According to our previous studies (Somoskői et al., 2012, 2014), 0.75 and 1 ng/ml T-2 concentrations decreased the proportion of blastocysts, caused the formation of micronuclei and lobulated nuclei in the blastomeres; however, already a T-2 concentration of 0.5 ng/ml delayed blastocoel formation.

Our data show that T-2 mycotoxin affects preimplantation embryo development even at low concentrations. Although morulae and blastocysts exhibited normal morphology in a toxin-contaminated environment (treatment I: 0.5 ng/ml, 0.75 ng/ml and 1 ng/ml T-2), these embryos had significantly fewer blastomeres (lower cell number) following both 72 and 96 h of culture than did the control embryos. Since the cell number of embryos indicates developmental capacity, it is a suitable marker of viability. Decreased blastomere number may result in reduced developmental ability (Zakhartchenko et al., 1995; Shapiro et al., 2000). Yuan et al. (2014) investigated the effect of T-2 on zebrafish embryos via 144 h of exposure. They found dose-dependent tail malformation and increased reac-

tive oxygen species (ROS) production. However, the lowest effect level was found to be 93.3 ng/ml. Our results indicate that the mouse embryo model is an effective and sensitive tool to evaluate the harmful effects of T-2 contamination *in vitro*.

The transfer of control embryos into contaminated media (treatment II) was found to have a remarkable effect. The cell number of these blastocysts (Tr05, Tr075 and Tr1) was significantly lower compared to the control ones. Although blastomere number was slightly higher in treatment II than in treatment I, the differences between these groups were not significant, except at 1 ng/ml concentration, however, with a weak significance ($P = 0.048$) and a high SD.

Micronuclei are small fragments of chromatin separated from the main cell nucleus, which are evidence of chromosome breaking or mitotic spindle dysfunction and are frequently produced by genotoxic agents or other stress factors (e.g. cryostress) (Heddle et al., 1991; AbdelHafez, 2011). Jackson et al. (1998) found poor implantation potential associated with a high proportion of fragmented chromatin. Furthermore, micronuclei have been associated with developmental arrest (Moriwaki et al., 2004), defective S phase (Ye et al., 2003), disturbance in mitotic apparatus, as well as impaired topoisomerease II functioning (Tian and Yamanuchi, 2003) and apoptosis (Hnida et al., 2004). Our data show that the proportion of micronuclei in embryos cultured in toxin-containing media for 96 h remains at the same level as in the control embryos. This fact is in accordance with the observation that preimplantation embryos exhibit an amazing plasticity and tolerance when they come to adapting to the environment in which they are cultured (Lonergan et al., 2006). Experiences in assisted reproduction (particularly in cryopreservation) suggest that the stress tolerance of embryos increases with developmental stage (Martino et al., 2013). Although embryos in the Tr1 group did not show significantly higher micronucleus level (but a tendency was apparent), elevated micronucleus proportion was found in embryos transferred into media contaminated with 0.5 and 0.75 ng/ml toxin. These findings show that mouse embryos are sensitive to T-2 toxin even at advanced developmental stages.

Expansion of the blastocoel represents a developmental stage of the blastocyst (Dardik and Shultz, 1991) and the opportunity of implantation presents itself only in the receptive phase of the endometrium (implantation window) (Song et al., 2007). In mice, that implantation window is relatively narrow, about 24 h, on day 4 *(in vivo)*. Consequently, if the blastocyst is not in the late phase on day 4, the implantation will fail. Our results show that embryos cultured in a toxin-contaminated environment from the zygote stage (treatment I) reached the late blastocyst stage at a significantly lower rate than control ones and embryos in treatment II. Furthermore, no hatched blastocyst was found in a 0.5 and 1 ng/ml toxin-contaminated environment.

Propidium iodide staining is a suitable method for detecting necrotic cells or those being in the late apoptotic phase. Although several studies show that T-2 can cause necrosis at extremely high concentrations (475 ng/ml) (Nasri et al., 2006), our data show that the concentrations we used induced necrosis at a negligible rate (mostly in less than 2% of blastomeres).

In conclusion, our data show that T-2 mycotoxin affects the developmental capacity and quality of preimplantation embryos. The effect may vary depending on the stage of the embryo when starting the exposure. At 96 h of exposure (from the zygote stage), the blastocysts have blastomeres with normal chromatin quality (same as the control ones) but their developmental potential is decreased. After 24 h of exposure applied following 72-h culture, blastomeres had a higher level of chromatin damage, although their developmental potential was the same as that of the control embryos. In both cases, we found a decreased mitotic rate, which resulted in a decreased blastomere number even at low T-2 toxin concentrations.

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