University of Veterinary Medicine

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EFFECTS OF MYCOTOXINS AND CRYOPRESERVATION ON PREIMPLANTATION MOUSE EMBRYOS

Brief version of PhD thesis

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MTA-KE Mycotoxins in the Food Chain

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1. Effects of Fusarium mycotoxins on early embryo development

1.1. General introduction

Fusarium mycotoxins are secondary metabolites produced by several species of Fusarium moulds. These moulds are the most common pathogens in the temperate region in cereals and the species of the Fusarium genus producing fusariotoxins (139 out of 300 toxic fungal metabolites). Fusarium mycotoxins are endowed with both acute and chronic aspects of toxicity and have shown to cause a broad variety of toxic effects in animals and human. These toxins account for millions of dollars annually in losses worldwide in human health, animal health, and condemned agricultural products. It is important to emphasise that it is very common for an array of mycotoxins to occur together at low concentrations. This is on the one hand due to the ability of various fungi to simultaneously produce a variety of mycotoxins and on the other hand due to the fact that any given commodity is likely to be infected with different types of fungi.

Beside the well documented critical effects of T-2, many studies in animals revealed that T-2 can cause reproductive disorders both in males and females. The effect of T-2 on ovarian function was assessed both *in vitro* and *in vivo*. Peroral T-2 intake can significantly delay follicle maturation, postpone the subsequent ovulation, and may also possibly retard the consecutive luteinisation. T-2 was found to have potent direct dose-dependent inhibitory effects on granulosa cell proliferation and steroidogenesis. In a recent study, T-2 exposure resulted in a decrease in sperm motility, an increase in the number of spermatozoa with morphological abnormalities, a drop in the concentration of citric acid in seminal plasma, and a decrease in testosterone levels in rabbit bucks. All of these changes by themselves or in combination may result in subfertility or infertility in domestic animals. Numerous evidences were found about the fetotoxic effect of T-2 mycotoxin. The toxin passes through the placenta and may adversely affect the development of fetus, causes abnormailities in the blood-brain barrier and may induce brain lesions.

Fumonisins (B1 and B2) are cancer-promoting metabolites of Fusarium proliferatum and Fusarium verticillioides that have a long-chain hydrocarbon unit (similar to that of sphingosine and sphinganine) which play a role in their toxicity. Fumonisin B1 (FB_1) is the most toxic and has been shown to promote tumor formation in rats and cause equine leukoencephalomalacia and porcine pulmonary edema. The effect of FB1 exposure on the ovary in domestic animals has not been extensively studied so far. The potential of FB1 to impair porcine granulosa cell function in vitro has been investigated and results suggesting that this mycotoxin may compromise the normal follicle growth and oocyte survival in swine.

These findings demonstrating the ability of these Fusarium mycotoxins to disturb the critical well-balanced endocrine regulation of the developing follicle, which is essential to reach the preovulatory stage. Fumonisin B1 has adverse effects on male reproductive functions, such as sperm mass activities, motility, and live spermatozoa of the rabbits' semen significantly declined with an increase in the dietary FB1.

Despite the above mentioned knowledge of Fusarium toxins on reproduction, there are limited data on the effects of T-2 on preimplantation development either in single doses or in combination with other toxins.

Objectives of this part of the thesis:

1. To investigate embryo development under different T-2 concentrations (Experiment 1)
2. To investigate the effect of T-2 on developmental dynamics and blastocyst quality (Experiment 2)
3. To investigate the effect of different T-2 exposition times on blastocyst quality (Experiment 3)
4. To investigate the effect of mycotoxin co-contamination on preimplantation embryo development (Experiment 4)

1.2. Materials and methods

Embryo production and collection were carried out in all of the experiments with the following method: six weeks old mice were kept under a 12 hours light/12 hours dark schedule at a temperature of 21 °C. Superovulated (Day 1: 7.5 IU eCG ip.; Day 3: 7.5 IU hCG ip.) female mice were placed together overnight with mature males after hCG treatment. One-cell zygotes were obtained 20 hours after hCG treatment and transferred to culture medium supplemented with mycotoxins in different concentrations. The embryos obtained were pooled and randomly divided into treated (medium with different concentrations of toxin) and control (medium without toxin) groups. Toxin concentrations were based on our previous studies. Embryos were cultured for 96 hours at 37.5°C with 6.5% CO2 and maximal humidity in air.

In Experiment 1, the culture media were contaminated with the following T-2 concentrations: 0.1; 0.5; 1.0; 1.25; 1.5; 1.75; 2.0; 2.5 and 3 ng/ml. Developmental potency, mitochondrial activity and distribution and ROS production were assessed.

In Experiment 2, we investigated the effect of 0.5, 0.75 and 1 ng/ml T-2 concentrations on preimplantational development and the quality of blastocysts based on the chromatin damage rate.

In Experiment 3, different exposition times (96 hours [Treatment I] and 24 hours after compaction [Treatment II]) were applied during the in vitro embryo culture. Embryos were analysed morphologically (to distinguish different blastocyst stages) and were stained with SYBR Green and propidium-iodide to assess the cell number of embryos, necrotic cells and proportion of blastomers with damaged nucler chromatin (micronuclei). Staining and comparison were performed only in case of morphologically normal embryos in each group.

In Experiment 4, three types of toxin treatment were applied. In this study, simple T-2 (0.5 ng/ml), simple FB1 (1, 2 and 10 ng/ml) and co-contamination (1.5 ng/ml T-2 and 1, 2 or 10 ng/ml FB1) treatments were investigated. The assessed parameters were the same as in the Experiment 3.

1.3. Results and discussion

Our data show that majoritiy of embryos cultured under low toxin contaminated environment reached the blastocyst stage and were morphologically normal. 0.5 ng/ml T-2 concentration did not cause difference either in the mitochondrial activity or in the ROS production. During normal embryo development blastomeres show pericortical and perinuclear (heterogenous) pattern. Embryos cultured in 2.5 ng/ml T-2 concentration – and stopped in very early stage – show diffuse (homogenous) mitochondrial distribution inside the cytoplasm.

Our data show that whereas the normal cleavage rate is equal to control in the low toxin-treated group, one and a half fold concentration caused reduction to less than 40%. However, we found delayed cleavage already on the lowest (0.5 ng/ml) toxin concentration since embryos in this group reached blastocyst stage 24 h later than in the control group.

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 Our results show that blastocysts denoted as morphologically normal can contain damaged chromatin in trichotecene-contaminated environment. It may cause disturbed implantation following high toxin impact.

Although morulae and blastocysts denoted normal morphology in 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 hours of culture than control ones. Since cell number of embryos indicates developmental capacity, therefore it is a suitable marker of viability. Decreased blastomere number may result in reduced developmental ability. Remarkable effect of the transfer of control embryos into contaminated media (Treatment II) was found. Cell number of these blastocysts (Tr05, Tr075 and Tr1) was significantly lower compared to control ones. Although blastomere number was slightly higher in Treatment II than in Treatment I, differences between these groups were not significant (except in 1 ng/ml concentration, however, with weak significancy ($p=0.048$) with high SD).

Expansion of the blastocoel represents the developmental stage of the blastocyst and the opportunity of implantation presents only in the receptive phase of endometrium (implantation window). In mice, the implantation window is relatively tight, about 24 hours, on Day4 (in vivo). Consequently, if the blastocyst is not in the late phase on Day4, the implantation will be failed. Our results show that embryos cultured in toxin-contaminated environment from zygote stage (Treatment I) reached the late blastocyst stage in significantly

lower rate than control ones and embryos in the Treatment II. Furthermore, no hatched blastocyst was found in 0.5 and 1 ng/ml toxin-contaminated environment.

In conclusion, our data show that T-2 mycotoxin affects 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 exposure (from zygote stage), the blastocysts have blastomeres with normal chromatin quality (same as the control ones) but their developmental potential is decreased. After 24 h exposure applied following 72 hour culture, blastomeres have higher level of chromatin damage, although, the developmental potential was the same as in the control embryos. In both cases, we found decreased mitotic rate, resulted in decreased blastomere number even in small concentration.

In Experiment 4, our goal was to assess the toxicity of low concentration of T-2 and different concentrations of FB_1 alone and in combination on early embryo development before implantation. Based on our knowledge, there are no data reported about the effect of FB1 on preimplantation embryo development in mammals. In our study, neither T-2 nor FB_1 contamination affected the blastocyst rate negatively, although, co-contamination of the two toxins radically decreased the number of embryos being in blastocyst stage. Our data show type I synergistic interaction between T-2 and FB_1 related to the late blastocyst rate.

All the toxin treatments caused decreased blastomer number in the blastocysts. However, T-2 contamination decreased the cell number much stronger, in a higher ratio compared to FB_1. Since co-contamination decreased cell number in a lower level than T-2 used alone but higher level than FB_1 treatments, our data suggest additive effect for the combined treatments on this indicator.

In conclusion, our study show that the studied concentrations of the T-2 and FB_1 toxins do not decrease the developmental rate of mouse embryos, but co-contamination resulted in synergistic negative effects on blastocyst development, blastocoel expansion and rate of expanded blastocyst. All of the toxin treatments decreased the cell number of blastocysts, with a higher decreasing rate after treatment with a combination of different mycotoxins.

2. Effects of cryopreservation on embryos being in compacted stages

2.1. General introduction

Over the last few decades, cryopreservation techniques have progressed rapidly. This progress has made a significant impact in many fields, with reproductive medicine possibly the most significant. From initial success in cryopreservation of sperm, the ability to cryopreserve mammalian embryos has become an integral part of assisted reproductive technologies (ART) in both human and veterinary medicine. Cryopreservation (CP) is a process by which biological cells or tissues are preserved at subzero temperatures resulting in a radical decrease in the rate of metabolic processes and the ability to store samples for extended periods. Despite differences in the size and physiological characteristics of embryos from various species, most embryos have been frozen by either one of two procedures: the traditional slow (equilibrium) cooling and freezing method of cryopreservation (CP), namely slow freezing (SF), and the rapid procedure (non-equilibrium cooling) referred to as vitrification (VF). Embryo CP has decreased the number of fresh embryo transfers and maximized the effectiveness of the IVF cycle. Similarly, embryo CP is a crucial tool in cases of cancelled embryo transfer (ET) due to ovarian hyperstimulation risk, endometrial bleeding, elevated serum progesterone levels on the day of triggering, or any other unplanned events. There is still a large debate on the best stage, protocol/procedure, and cryoprotective additives (CPA) to use. However, it is unquestionable that successful CP of zygotes/embryos has greatly enhanced the clinical benefits and cumulative conception rates possible for couples following a single cycle of ovarian stimulation and IVF.

Objectives of this part of the thesis:

1. To investigate the effect of vitrification on chromatin integrity, mt distribution, energy status and intracellular levels of ROS in mouse embryos being in different stages (early to blastocyst). (Experiment 5)

2. To investigate and compare the effects of slow freezing and vitrification on chromatin integrity, mt distribution, energy status and intracellular levels of ROS in mouse embryos being in morula and blastocyst stages. (Experiment 6)

2.2. Materials and methods

Embryo recovery and embryo culture were carried out in the same way as in the Chapter 1.

Vitrification (VF) was performed with the VitroLoop vitrification procedure, using 2-step loading of the CPA solution, ethylene glycol (EG) and propylene glycol (PG), before being placed on a thin filmy layer formed from the vitrification solution in a small nylon loop, then they were rapidly submerged in liquid nitrogen (LN_2). All manipulations of the embryos during their preparation for vitrification were carried out at 37^{o}C (on a heated stage). Embryos were warmed and rehydrated by a 3-step dilution of the CPA performed at 37°C. At warming, the embryos were moved through a series of G-MOPS^{TM} solutions containing the sucrose in decreasing concentrations.

Slow programmable freezing (SF): After equilibration in DPBS containing 10% glycerol, for 10-15 min, the embryos were sucked up into straws (5 embryo per straw). The straws containing the embryos were then transferred into a Planer freezing machine pre-cooled to minus 7°C. . After a 10 min waiting period, the samples were allowed to cool down to minus 7°C. Once reached this temperature, artificial induction of the ice formation with a pre-cooled forceps was performed (seeding). After 10 min waiting, cooling down the embryos to minus 33°C was performed with cooling speed 0.3 °C/minute. Finally, the embryos were transferred into liquid nitrogen (LN_2) and stored for one week. Thawing was performed by keeping the straw in air for 20 sec (air thaw), followed by 30 sec in warm water (25°C). Cryoprotectant (CPA) was removed from the embryos in 4 steps with decreasing concentrations of glycerol and sucrose.

Mitochodria and intracellular ROS were stained with MitoTracker Orange CMTM Ros and 2',7'-dichlorodihydrofluorescein diacetate, respectively. To evaluate nuclear chromatin, embryos were stained with 2.5 µg/ml Hoechst 33258 in 3:1 (v/v) glycerol/PBS.

Mitochondrial distribution pattern, intracellular ROS localization, Mitotracker Orange CMTM Ros and DCF fluorescence intensity and Mitochondria/ROS colocalization assessment were performed with Nikon C1/TE2000-U laser scanning confocal microscope and analysis was carried out with EZ-C1 Gold Version 3.70 software

2.3. Results and discussion

Vitrification slightly affected embryo morphology in Experiment 5 by increasing the percentage of embryos showing low grade (<20%) blastomere cytofragmentation. This technique increased low level chromatin damage, although, only intermediate stages of development were affected. Both in embryos at the morula and blastocyst stage, significant increase of intracellular ROS production was found after vitification.

In the comparison of slow freezing and vitrification (Experiment 6), we found that both SF and VF significantly reduced the percentages of grade A embryos (<20% of blastocysts contained micronuclei), thus impairing the nuclear chromatin integrity. The extent of chromatin damage was higher after SF than VF, as only 32% of slow frozen embryos , but 60% of vitrified embryos had grad A ($P<0.0001$). Moreover, chromatin damage induced by SF was much more evident in embryos at the morula than the blastocyst stage. In the group of morulae only 16% of the embryos, however in the blastocyst group 61% of the embryos had grade A ($P<0.001$). We found that both CP procedures, SF and VF, significantly reduced the rates of embryos showing the pericortical/perinuclear mitochondrial pattern which indicative of cytoplasmic activity and maturity. Furthermore, the effects of CP on mt pattern varied according to embryo grade with more serious effects on grade C embryos, thereby emphasizing the synergy between chromatin and mitochondrial patter. Quantification analysis revealed a statistically significant reduction of MitoTracker fluorescence intensity in VF and SF morulae compared with their fresh counterparts, indicating significant reduction of mitochondrial activity at this stage of development after both CP procedures. Cryopreservation altered the quantitative bioenergy/redox parameters at a greater extent in morulae than in blastocysts.

Summary of main scientific results

- We confirmed the applicability of preimplantation mouse embryos in *in vitro* toxicological studies which provides more detailed informations and denies the all-or-nothing theory in connection with early embryonal stages.

- We defined the lowest inhibitory level (0.75 ng/ml) of trichotecene T-2 fusariotoxin on the viability and development of preimplantation mouse embryos (on the 0.5-0.75-1 ng/ml concentration range). Delayed tendency of development under low T-2 fusariotoxin concentration (0.5 ng/ml) was demonstrated instead of the appropriate mouse blastocyst rate after 96 hours.

- We found that the mouse embryos showed different tendency in development under different exposure time to T-2 fusariotoxin (on the 0.5-0.75-1 ng/ml concentration range). The chromatin quality and the developmental potential depend on the stage in the time of exposure.

- We investigated the effect of trichotecene T-2 and Fumonisin B1 fusariotoxins and we also analysed the coincidence of these two mycotoxins on early mouse embryo development for the first time. We found synergistic effect when co-occurence existed.

- We detected that vitrification and slow freezing preserve the developmental potential of mouse embryos. However, both methods induce higher chromatin damage, intracellular reactive oxygene species (ROS) level and alter the mithochondrial pattern, nevertheless the vitrification affects less adversely. Furthermore, we confirmed that heterogenous (perinuclear/pericortical) mitochondrial pattern indicates higher energy status in morulae and blastocysts.

- We studied for the first time the chromatin damage and mithochondrial energy potential jointly in cryopreservation of mouse embryos. The comparative assessment allows the detailed and objective comparison of the effects of vitrification and slow freezing (the two most commoly used cryopreservation techniqes) on morula and blastocyst stage embryos.

Publications in peer-reviewed journals related to the thesis

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