University of Veterinary Medicine

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EFFECTS OF MYCOTOXINS AND CRYOPRESERVATION ON PREIMPLANTATION MOUSE EMBRYOS

PhD thesis

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MTA-KE Mycotoxins in the Food Chain

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List of abbreviations

ANOVA   Analysis of variance

AR   Assisted reproduction

ART   Assisted reproductive techniqes

ATP   Adenosine triphosphate

BSA   Bovine serum albumin

CP   Cryopreservation

CPA   Cryoprotective agent

DAPI   4',6-diamidino-2-phenylindole

DCDHFDA 2',7'-dichlorodihydrofluorescein-diacetate

DCF   2',7'-dichlorofluorescein

DON   Deoxynivalenol mycotoxin

DPBS   Dulbecco's phosphate buffered saline

EG   Ethylene glycol

FB1   Fumonisin B1 mycotoxin

FDA   Food and Drug Administration

FSH   Follicle-stimulating hormone

H2O2   Hydrogen peroxide

hCG   Human chorionic gonadotropin

HSA   Human serum albumin

HT-2   Deacetylated form of T-2

IETS   International Embryo Technology Society

IGF-1   Insulin-like growth factor 1

MEA   Mouse embryo assay

MGV	Mean gray value
mt	Mitochondrium
OS	Oxidative stress
PG	Propylene glycol
PMSG	Pregnant mare serum gonatropin
ROI	Region of interest
ROS	Reactive oxigene species
SF	Slow freezing
T-2	Trichotecene-2 mycotoxin
VF	Vitrification
ZEA	Zearalenone mycotoxin

Summary

During my PhD studies, I focused on embryo development and quality from two different aspects. These two are mycotoxin contamination and cryopreservation.

In the toxicological investigation the effect of different T-2 doses were studied. The objectives of these experiments were to investigate the developmental potential, dynamics and blastocyst quality of mouse embryos in toxin contaminated environment. Our data on the effect on preimplantation mouse embryos showed that the *in vitro* lowest inhibitory dose of T-2 is 0.75ng/ml (on the 0.5-0.75-1 ng/ml range). However, lower T-2 contamination even cause developmental delay. Instead of the normal morphology and blastocyst rate, we found detention in the blastocoel formation at 0.5 ng/ml. When different exposition times (96 and 24 hours) were applied, we found different effects in each treatment. Long term exposition caused lower cell number and higher early blastocyst rate than that in the control group. Short term exposition did not affect the late (expanded and hatched) blastocyst proportion, although, induced significantly higher chromatin damage rate.

Beside the T-2, the effects of different FB1 concentrations were investigated as well as the co-contamination. Presence of both fusariotoxins in the culture media caused significantly lower rate of blastocysts, and furthermore, late blastocysts compared to control and single-dose T-2 and FB1 groups. The combined and single T-2 treatment negatively affected the blastomer number, instead of the single doses of FB1, which did not induced lower cell number.

The other part of my thesis is the assessment of embryo quality after applying two cryopreservation methods: vitrification and slow freezing. Furthermore, we compared the effect of cryopreservation techniques on compacted embryo stages (morulae and blastocysts). Chromatin damage rate, mitochondrial distribution, energy status and intracellular ROS levels were examined in different developmental stages (2/16-cell, morula and blastocyst). Our data show that chromatin damage induced by slow freezing was more relevant than that induced by vitrification. Vitrification and slow freezing similarly affected mitochondrial distribution pattern. Greater damage extent was observed at the morula stage. Cryopreservation altered the quantitative bioenergy parameters at a greater extent in morulae than in blastocysts.

Chapter 1. Effects of Fusarium mycotoxins on early embryo development

1.1. General introduction

1.1.1. Fusarium mycotoxins

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) (Placinta et al., 1999). 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. Spontaneous outbreaks of Fusarium mycotoxicoses have been reported in Europe, Asia, Africa, New Zealand, and South America. Moreover, chronic intake of these mycotoxins is reported on a regular and more widespread basis due to their global occurrence (D'Mello et al., 1999). The main classes of Fusarium mycotoxins with respect to animal health and production are trichothecenes such as deoxynivalenol (DON) and T-2 toxin (T-2), fumonisins, and zearalenone (ZEA) (Glenn 2007). Mycotoxin producing Fusarium (and some other) species are listed in Table 1.

Genera	Species	Mycotoxins produced	Invaded foods
Aspergillus	flavus	Aflatoxins, sterigmatocystin	Groundnuts, Brazil nuts, pistachios
	parasiticus	Aflatoxins	
	versicolor	Sterigmatocystin	
	niger	Ochratoxins	Coffee, cocoa, raisins
	ochraceus	Ochratoxins	Diverse
Penicillium	expansum	Patulin	Apples
	verrucosum	Ochratoxin A	Diverse
	roqueforti	Roquefortins, PR toxin	Milk products
Fusarium	graminearum	Deoxynivalenol and further type B trichothecenes, zearalenone	Cereals
	sporotrichoides	T2- and HT2-toxins and other type A trichothecenes	
	culmorum	Deoxynivalenol and other type B trichothecenes	
	tricinctum	Beauvericin and enniatins	
	verticilloides	Fumonisins, deoxynivalenol and other type B trichothecenes, moniliformin	
	proliferatum	Fumonisins	
Alternaria	alternata	Alternariol, alternariol methyl ether, altenuene	Diverse
	tenuis	Tenuazonic acid	
Claviceps	purpurea	Ergot alkaloids	Cereals

Table 1. Mycotoxin producing fungi species and their prevalence. (Rychlik, 2012)

These toxins account for millions of dollars annually in losses worldwide in human health, animal health, and condemned agricultural products (Shane, 1994; Vasanthi and Bhat,1998). Factors contributing to the presence or production of mycotoxins in foods or feeds include storage, environmental, and ecological conditions. Often times most factors are beyond human control.Under field conditions, mycotoxins usually occur in concentrations leading to reduced animal performance and/or immune suppression without causing any obvious clinical symptoms (Bryden, 2004; Marquardt, 1996). 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 (Bottalico,1998; Atalla, et al. 2003) and on the other hand due to the fact that any given commodity is likely to be infected with different types of fungi. Moreover, compound feed is made up of a number of different commodities contributing to the final mycotoxin profile.

1.1.2. Reproductive toxicology of Fusariotoxins

Fusariotoxins have well known adverse effects in general and on different phases of reproduction both in males and females (Cristina Cortinovis et al., 2013).The detailed overview of these effects of different toxins is beyond this thesis, therefore, this section focuses on the T-2 and FB1.

T-2 toxin (T-2) is a type A trichothecene which can infect crop plants such as wheat, barley and rice in the field and/or during storage (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.

Many studies have shown the adverse effect of T-2 mycotoxin on physiological processes of different types of cells. Trichotecenes are lipophilic compounds that can pass into the cell and after binding to the peptydil-transferase, the protein-synthesis and indirectly the DNA- and RNA-synthesis are inhibited (Thomson & Wannemacher, 1990). Oxidative stress is also involved in the toxicities of trichothecene mycotoxins including T-2 toxin. The T-2 toxin-induced oxidative stress may cause DNA damage and apoptosis in the cell (Chaudhari et al., 2009; Wu et al., 2011). T-2 treatment can cause decreased RNA content, nuclear globules and cytoplasmic vacuoles in pig kidney cell culture (Bodon and Zöldág, 1974). Immunosuppressive effect of the toxin was also found both in vivo and in vitro (Gutleb et al., 2002). T-2 toxin with the highest toxicity among trichothecenes may cause gastrointestinal, dermatological, immunological and neurologic symptoms in experimental and farm animals

(Bennett & Klich, 2003; Dohnal et al., 2008). Recently, it has been reported that T-2 toxin enters the brain via the blood–brain barrier (Weidner et al., 2013).

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*. The effect of oral exposure to low doses of T-2 on the ovarian function was evaluated in ewes and heifers (Huszenicza et al. 2000). The study showed that the 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. Specifically, T-2 strongly inhibited FSH plus IGF-I induced progesterone and to a greater extent estradiol production as well as cell numbers. It was concluded that these direct ovarian effects could be one mechanism whereby the presence of T-2 in feedstuffs could impact reproductive performance in swine (Caloni et al., 2009). In 1998 at a Finnish artificial insemination bull station, impairment of semen quality, as detected by low progressive motility and poor morphology, was connected with mouldy hay. Analysis of the hay revealed the occurrence of T-2 and HT-2 that may have been responsible for the impaired semen quality (Alm et al., 2000). Recently, the effects of high doses of T-2 on spermatozoa, seminal plasma, and testosterone production have been investigated in rabbits. 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 (Kovács et al., 2011). 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 (Lafarge-Frayssinet et al., 1990). Thymus atrophy was observed after T-2 toxin exposure in pregnant mice (Holladay et al., 1993). The apoptosis-inducing effect of the toxin was shown in developing fetal central nervous system (Ishigami et al., 1999). Rousseaux and Schiefer (1987) reported bone malformation, absence of bones, wavy and fused bones in mouse offspring following T-2 mycotoxin administration. Sehata et al. (2004) found abnormalities in the blood-brain barrier and fetal brain lesions in rats.

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. FB1 is the most abundant of the numerous fumonisin analogues and was classified by the IARC (International Agency for Research on Cancer) as a Group 2B carcinogen (possibly carcinogenic in humans) (IARC, 2002). The effect of FB1 exposure on the ovary in domestic animals has not been extensively studied so far. Gbore et al. found that the toxin may reduce serum gonadotropin levels (Gbore et al., 2012). 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 (Cortinovis et al, 2014). 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 (Petro et al., 2012). The embryotoxic potential of FB1 in New Zealand white rabbits was investigated by LaBorde et al. (1997). It was indicated that FB1 did not cross the placenta. However, subsequent studies showed that FB1 crossed the placenta of LM/Bc mice that were given FB_1 i.v. on GD10.5 (Gelineau-van Waes et al., 2005) or fed diets containing 150 ppm FB_1 for more than 5 weeks (Voss et al., 2006). Fumonisin B1 has adverse effects on male reproductive functions. In a study carried out by Ewuola and Egbunike (Ewuola and Egbunike, 2010), sperm mass activities, motility, and live spermatozoa of the rabbits' semen significantly declined with an increase in the dietary FB1. Moreover, FB1 has been found to affect some functional parameters of equine spermatozoa in vitro, such as sperm chromatin stability and motility, with a potential to determine subfertility in stallions (Minervini et al., 2010). In a recent study by our research team, FB1-contaminated forage was fed with rabbit males, in single doses and in combination with DON and ZEA. Data show that after 65 days of diet spermatogenesis decreased by 43% which was reflected by lack of differentiated spermatozoa, thinning of germinal epithelium, the appearance of multinuclear giant cells, indicative of the disturbance of meiosis and mitosis of the germinal epithelial cells and in some cases the lack of spermatozoa (Szabó-Fodor et al., 2015).

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.

1.1.3. Preimplantation embryos in toxicology

Mice preimplantation embryos are simultaneously cytological and embryological test models being both few-cells structures or self-determined system and integrated (whole) organisms. Based on the model, it is possible to estimate both cytotoxic and embryotoxic effects after pathogenic influence has occurred. Even though the preimplantation period is considered to

be fairly refractory (Wilson, 1973), there is some evidence for high vulnerability of embryos in this period: a large proportion of early embryos perish before implantation and up to 60% during implantation and in the early postimplantation period (Norwitz et al., 2001). The threat of embryo damage in the preimplantation period (in humans, 1–7 days of pregnancy) is probably associated with the fact that women are most commonly not aware of the initiated pregnancy and do not protect the embryo from extreme exposures. Consequently, a human fetus requires protection from exposure to pathogenic factors in the preimplantation period, and to include this period in the practice of experimental research on the hazards of environmental factors is a prerequisite for objective assessment of their impact on the course and outcome of pregnancy. Thus, experimental identification of hazards from one or other factors for the earliest stages of embryogenesis can serve prognostic and preventive purposes.

1.2. Objectives

Our reseach group (Andrology/Assisted Reproduction Research Group) joined in the MTA-KE Mycotoxin in the Food Chain Research Team in 2012. The aim of this cooperation is to investigate the effect of several mycotoxins (mainly T-2 and Fumonisin B1) on the reproductive processes, especially on the early embryo development. Within the framework of this project, present part of my PhD research focuses on the preimplantation development of mouse embryos. Aims of this study are:

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)

1.3. Experiment 1

1.3.1. Methods

Animal housing and mating

6 weeks old C57Bl/6 mice were kept under 21 °C, 12h/12h light program in the experimental animal house of the Department of Obstetrics and Reproduction. Female ones were superovulated (7.5 IU PMSG i.p. + 7.5 IU hCG i.p. 48 h later [Alvetra und Werfft AG, Austria]) and mated with males (one male with each female). 20 hours after hCG injection zygotes were obtained from donor females and transferred into toxin-contaminated Cleavage Medium (Cleavage Stage Medium, Cook Medical, Roskilde, Denmark).

1st trial

10 treatment groups were made based on the T-2 concentrations: 0,1; 0,5; 1,0; 1,25; 1,5; 1,75; 2,0; 2,5 and 3 ng/ml. Embryos cultured in medium with no toxin are referred to control. Embryos were cultured for 96 h at 37.5 °C with 6.5% carbon dioxide and maximal humidity in air. Morphology was checked following the culture period.

2nd trial

In the second experiment the mitochondrial activity and ROS production of blastocysts were investigated. In this part we examined 4 groups:

1. embryos cultured in highly (2,5 ng/ml) contaminated medium and stopped in 2-cell stage

2. 4-cell stage embryos cultured in toxin-free medium

3. embryos cultured in lowly (0,5 ng/ml) contaminated medium and reached the blastocyst stage

4. blastocysts from the control group

Mitochondrial activity was measured with MitoTracker CMTM Ros (Molecular Probes, USA). Reactive oxygen species (ROS) were stained with 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, USA). Results of staining were detected with confocal laser scanning

microscope (C1/TE2000-U Nikon) in Veterinary Clinics and Animal Productions Unit, Department of Emergency and Organ Trasplantation (DETO), University of Bari Aldo Moro Valenzano, Bari, Italy. Fluorescence intensity was measured with Image J software. The region of interest (ROI) was drawn around the embryos and mitochondrial/ROS quantitative fluorescence intensities were reported as mean grey value (MGV) (Figure 1).. This factor was generated by the software and used for statistical analysis.
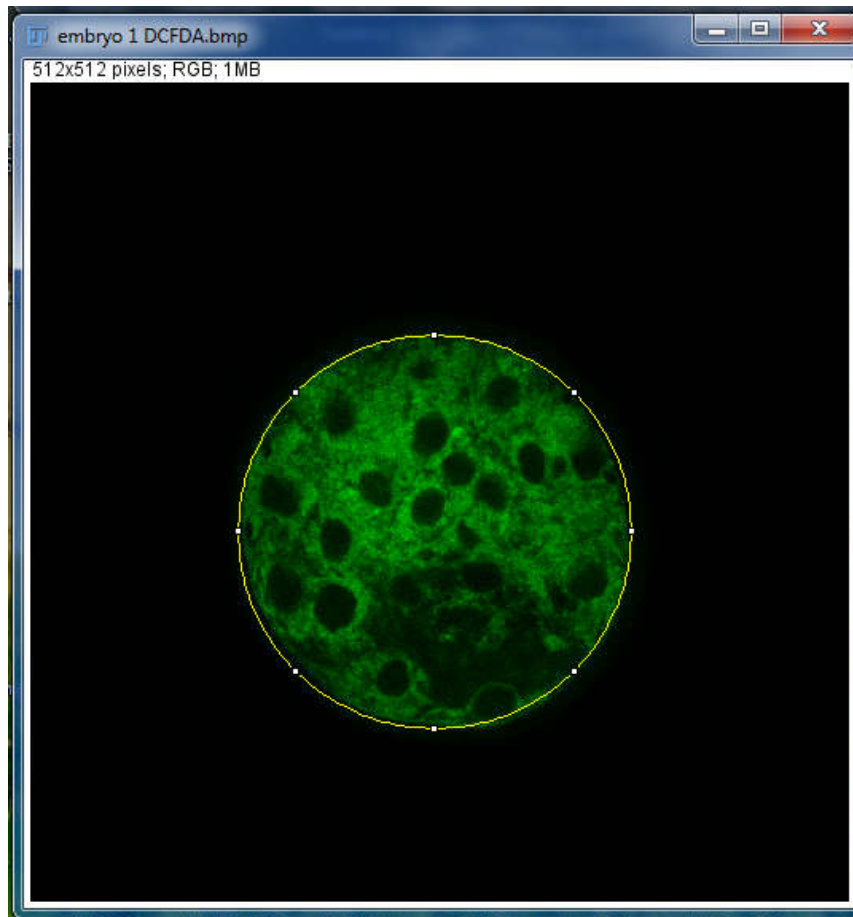
Fig 1. Representative image of the ROI (yellow circle) measurement of a control blastocyst.

Statistical analysis

R 2.12.0 was used for analysis. Blastocyst rate of each treatments were compared with Chi-squared test for independence and pairwise comparison. Mitochondrial activity and ROS-production data were analysed with two-sided Welch-test. Differences with $p < 0.05$ were considered as statistically significant.

1.3.2. Results

1st trial

In the first part of our study, we compared the blastocyst rate of control group to treated ones. Results are shown in Table 2.

| Treratment (ng/ml T-2)	No. of zygotes	No. of blastocysts	Surv. rate[1] (%)	p-value[2]
0	66	55	83,33	-
0,1	36	28	77,78	0,6726
0,5	41	32	78,05	0,6696
1	73	39	53,42	**0,00034**
1,25	33	13	39,39	**2,51E-05**
1,5	21	11	52,38	**0,00948**
1,75	16	5	31,25	**9,46E-05**
2	26	3	11,54	**6,25E-10**
2,5	50	0	0	**2,20E-16**
3	19	0	0	**1,32E-10**

Table 2. Blastocyst rate in each treatments after 96 hours of *in vitro* culture. [1]rate of blastocsts[4]bold numbers indicate significant difference

Significant difference from control was not detected in the two lowest T-2 contaminated groups (0.1 and 0.5 ng/ml). However, above 1 ng/ml toxin concentration the embryos reached the blastocyst stage in significantly lower rate. Embryos cultured in medium with 2.5 or 3 ng/ml T-2 stopped in the 2-cel stageand did not show any further development.

2nd trial

In the second part of the study we investigated the effect of T-2 toxin on the activity and distribution of mitochondria and the production and distribution of ROS in blastomeres.

Embryos stopped in 2-cell stage in the 2.5 ng/ml treatment showed altered mitochondrial distribution, compared to control ones. Instead of the normal perinuclear and pericortical pattern in control balstomeres, these embryos show diffuse mitochondrial distribution.(Figure 2.).
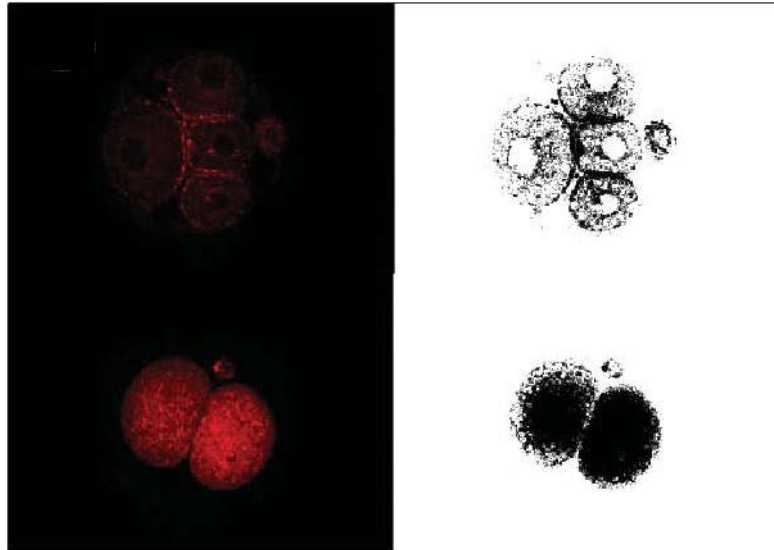
Figure 2. Mitochondrial pattern of control (upper row) and treated (2.5 ng/ml T-2) 2-4 cell stage embryos.

In both control and high toxin-treated embryos, ROS distribution appeared diffuse In the cytoplasm of all blastomeres (Figure 3.).
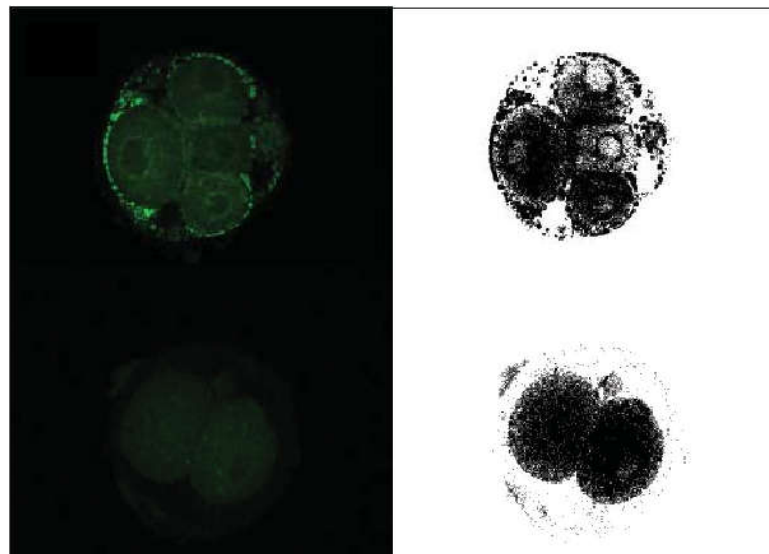
**Figure 3.** Distribution of reactive oxygen species in control (upper row) and high toxin-treated embryos being in 2-4 cell stage.

We investigated the mitochondrial activity and ROS production in low (0.5 ng/ml) toxin treated and control blastocysts. We did not found any significant differences neither in the mitochondrial activity (9.67 ± 3.73 vs. 7.18 ± 2.53 in control and treated embryos,

[16]

respectively), nor in the ROS production (12.02 ± 3.5 vs. 14.99 ± 3.65 in control and treated embryos, respectively) (Figure 4.).

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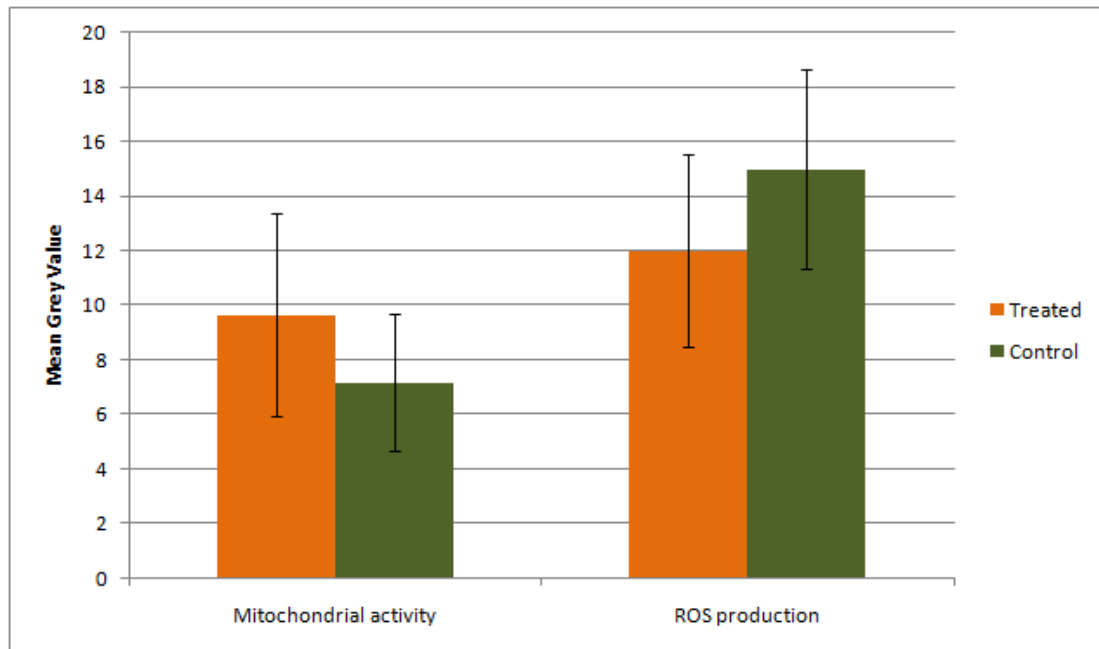
Figure 4. Differences in mitochondiral activity and ROS production between control and treated (0.5 ng/ml T-2) blastocysts.

1.3.3. Discussion

Localization of different organelles and the biochemical composition of the cytoplasm, and changes in the genetic material play important role both in oocyte maturation and embryo development (Sun et al., 2004). While numerous authors investigated the effect of T-2 on pregnancy and fetus (Nagai et al., 2006; Nelson et al., 1994; Sehata et al., 2004; Van Soom et al., 2003), based on our knowledge, it was the first study on the preimplantation mouse embryo.

Our study focused on the effect of T-2 on early embryo development (from zygote to blastocyst stage) in an in vitro system. The toxin concentration that we used in this study for in vitro culture of embryos can be found in human peripheral blood (0.2–1800 ng/ml) (Berek et al., 2001). Based on our data we can suspect that T-2 has a strong negative effect on early embryo development which can cause early embryonic death and absorbtion (even before the implantation). The routinely used quality assessment of embryos during *in vitro* fertilization, cryopreservation and embryo transfer in human healthcare and veterinary practice based on morphological characteristic. The main disadvantage of this method is that

the embryo can be classified sometimes as acceptable for transfer (no morphological abnormality) instead of the physiological abnormalities. In that case, the embryologist can not realise that and the embryo will be transferred (Nagai et al., 2006). Main causes of physiological abnormalities are low activity and/or inappropriate distribution of mitochondria which prevent the production and localization of sufficient amount of energy (Harvey et al., 2011). 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 (Bavister et al., 2000). Embryos cultured in 2.5 ng/ml T-2 concentration – and stopped in very early stage – show diffuse (homogenous) mitochondrial distribution inside the cytoplasm. These results confirm the findings by Sun et al. (2006). In that study mitochondrial pattern of porcine embryos were examined after adding nocodazol, a microtubule assembly inhibitor, into the culture medium. Homogenous mitochondrial pattern was found in nocodazol-treated group. Schoevers et al. (2010) investigated the effect of deoxynivalenol (an other trichotecene) on the cell division. They found that the mycotoxin inhibited the assembly of mitotic spindle (Schoevers et al., 2010). In conclusion, our data suggest that T-2 can disturb the function of microtubules, through the inproper mitochondrial pattern it can cause developmental arrest in the early embryos.

1.4. Experiment 2

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### 1.4.1. Methods

Embryo recovery and in vitro culture

Procedures with animals were performed following good veterinary practice for animal welfare according to Hungarian national laws in force. The protocol of the animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Science Szent Istvan University of Budapest (40/2013.II.14.). Six weeks old BDF1 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. (Alvetra und Werfft, Austria)) 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 (Cleavage Medium, Cook Medical, Roskilde, Denmark) supplemented with T-2 mycotoxin (Sigma Aldrich, USA) in different concentrations (0.5 ng/ml, 0.75 ng/ml and 1 ng/ml). 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 (Somoskői et al., 2012). Embryos were cultured for 96 hours at 37.5°C with 6.5% CO_2 and maximal humidity in air. Average embryo number was 19.6/group/repeat.

Embryo development was examined on 72nd and 96th hours of culture to assess developmental dynamics. Embryos were classified as un-compacted and/or damaged, morula and blastocyst (early, mid and late/expanded stage).

Detection of the nuclear chromatin status

After 96 hours the nuclear chromatin status of blastocysts was examined. To evaluate nuclear chromatin status, embryos were stained with 2.5 µg/ml DAPI in DPBS (Sigma Aldrich, USA), kept in 4% paraformaldehyde at 4°C in the dark until observation and mounted on microscope slides for microscopy. Nuclear chromatin was observed under a Nikon A1 confocal microscope equipped with DAPI filter set on 400X magnification. Image analysis was performed with Image J software (NIH, USA). Embryos were classified as normal (grade A) when the presence of a regular-shaped nucleus inside each blastomere was observed. The formation of micronuclei and lobulated nuclei was considered as signs of

chromatin damage. Embryos showing affected blastomeres less than 20% were classified as grade B and embryos with more than 20% affected blastomeres were classified as grade C (Martino et al., 2013) (Figure 5.).
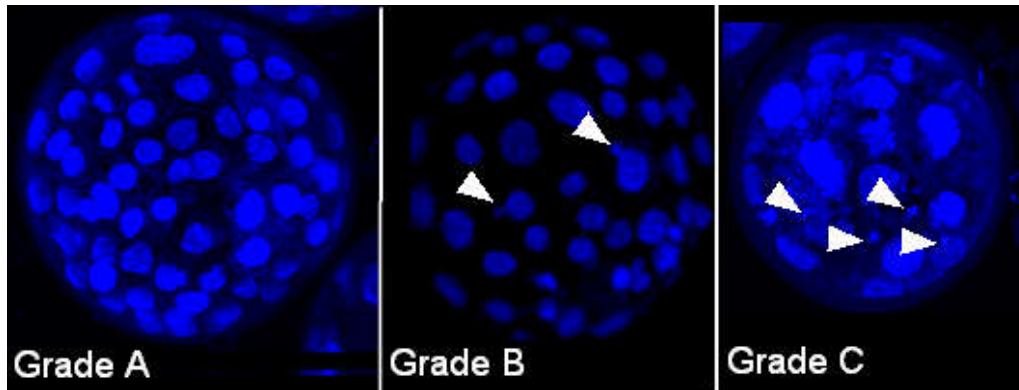
Figure 5. Representative pictures of grades A, B, and C blastocysts. Arrowheads show micronuclei.

Statistical analysis

Data were analyzed with R v3.0.0 software. The rates of blastocysts on the 72nd and 96th hours and chromatin status compared between control and treated groups by Chi-square test. ANOVA with Tukey-test was used for compare blastocyst proportion after all repeats. Differences at a probability value (P) < 0.05 were considered significant.

1.4.2. Results

After 72 h 61.5% of control embryos reached the blastocyst stage whereas no blastocyst was found in any of the treated groups. 24 h later blastocysts appeared in all groups however with significantly lower proportion in the high toxin-contaminated groups (p<0.001) (Figure 6.).

	72 h	96 h	72 h	96 h	72 h	96 h	72 h	96 h
	control		0.5 ng/ml		0.75 ng/ml		1.0 ng/ml	
Blastocyst (%)	61,5	84,78	0	73,17	0	46,48	0	27,27
Morula (%)	23,1	11,96	93,3	8,94	66,7	21,13	75	20,45
Uncompacted/crashed (%)	15,4	3,27	6,7	17,9	33,3	32,4	25	52,27
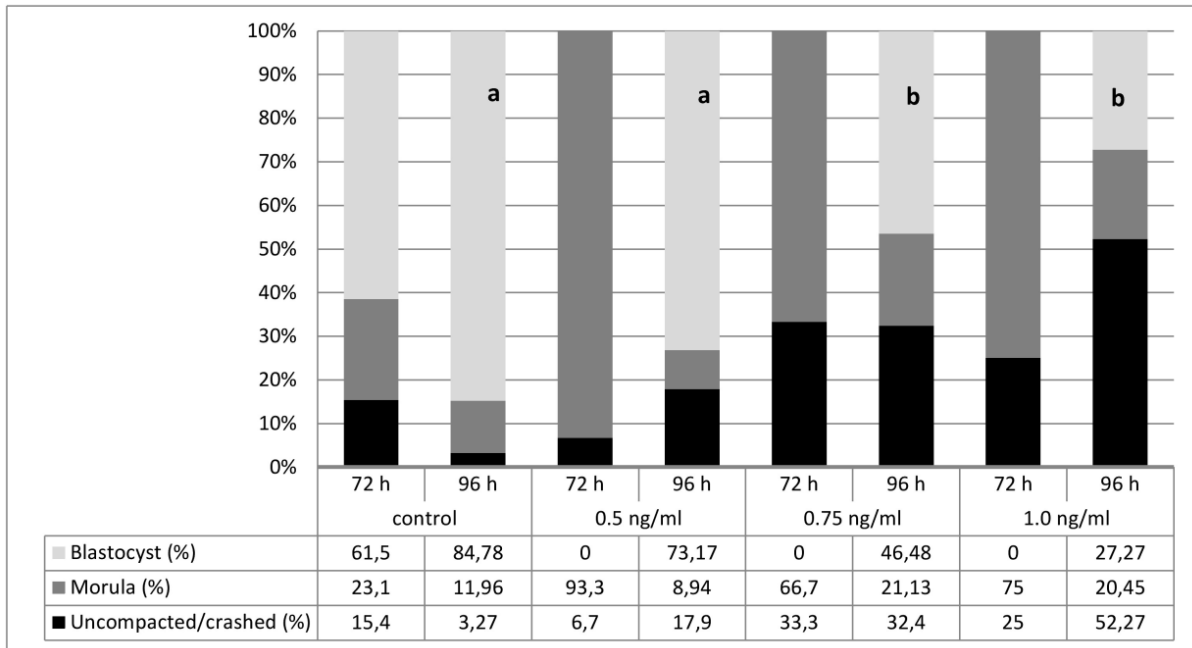
Figure 6. Development dynamics in control and treated groups. χ^2 test: (a,b) p < 0.001.

All the treated blastocysts showed stage-specific morphology (Fujimori et al., 2010) and considered normal as the control ones.

After 96 hours of culture the developmental stage of embryos was assessed. Table 3. shows the number and proportion of different embryo stages in the toxin-treated and control groups.

Treatment (ng/ml T-2)	No. of repeats	1-cell	2-cell	4-cell	6-cell	morula	blastocyst
0.5 **(n=138)**	6	2 (1.4%)	15 (10.9%)	5 (3.6%)	0 (0.0%)	12 (8.7%)	104 (75.4%)
0.75 **(n=142)**	6	7 (4.9%)	23 (16.2%)	14 (9.9%)	2 (1.4%)	30 (21.1%)	66 (46.5%)
1 **(n=132)**	7	23 (17.4%)	25 (18.9%)	21 (15.9%)	0 (0.0%)	27 (20.5%)	36 (27.3%)
control **(n=104)**	6	2 (1.9%)	1 (1.0%)	1 (1.0%)	0 (0.0%)	11 (10.6%)	89 (85.6%)

Table 3. Numbers and ratios of embryos in different developmental stages after 96 hours of culture in medium with and without T-2 toxin.

Data show that in the group cultured in medium supplemented with 0.5 ng/ml T-2 toxin the blastocyst rate was 10% lower compared to the control group. In the groups in which the embryos were cultured in mediums enriched with 0.75 ng/ml or 1 ng/ml T-2 toxin decreased blastocyst formation rates were found (by >38 % and >57 % compared to control).

In each group, the proportions of blastocysts were assessed after each repeat. Data show that the blastocyst rate in the low toxin group is similar to the control group. However, in the high toxin-treated groups significant decrease was detected in it (Figure 7.).
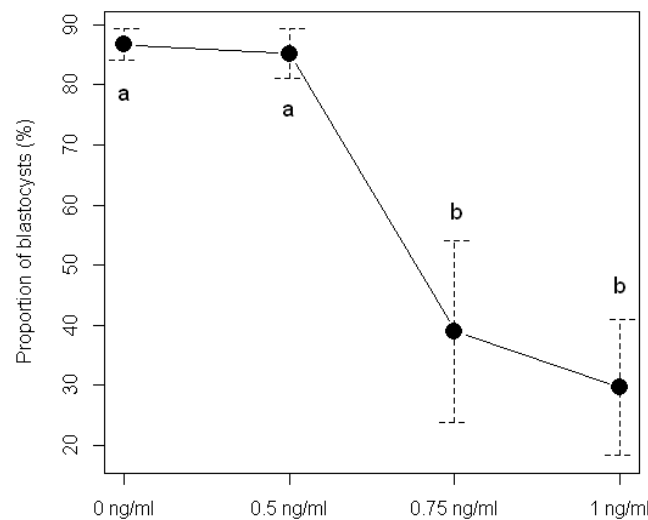

Figure 7. Proportion of blastocysts following 96 h in vitro culture after all replicates (six or seven replicates,19.6 average embryo/group/repeat). Tukey's test: (a,b)p < 0.05.

Statistical analysis of the obtained data shows significant negative effect of the T-2 toxin concentration on blastocyst formation (p<0.001). In the pair wise comparison we found significant differences in the blastocyst rates between the control and treated groups with high (0.75 and 1 ng/ml) toxin concentration. There was also significant difference in the blastocyst proportions between the high and low (0.5 ng/ml) toxin-supplemented groups. It was found that 1 ng/ml T-2 toxin contamination did not cause further significant decrease compared to 0.75 ng/ml.

Nuclear chromatin status of treated and untreated control blastocysts embryos was also evaluated (Figure 8.). All the examined blastocysts either in control or treated groups were morphologically normal. Only grade A and grade B embryos were found in the control and low toxin contaminated groups. Ten percent of the blastocysts in the 0.75 ng/ml T-2 toxin treated group was qualified as grade C. Significant increase (33.3%) of grade C embryos was found in the group of embryos treated with the highest (1 ng/ml) toxin concentration

compared to control group. Figure 8. shows representative pictures of blastocysts with different grades.
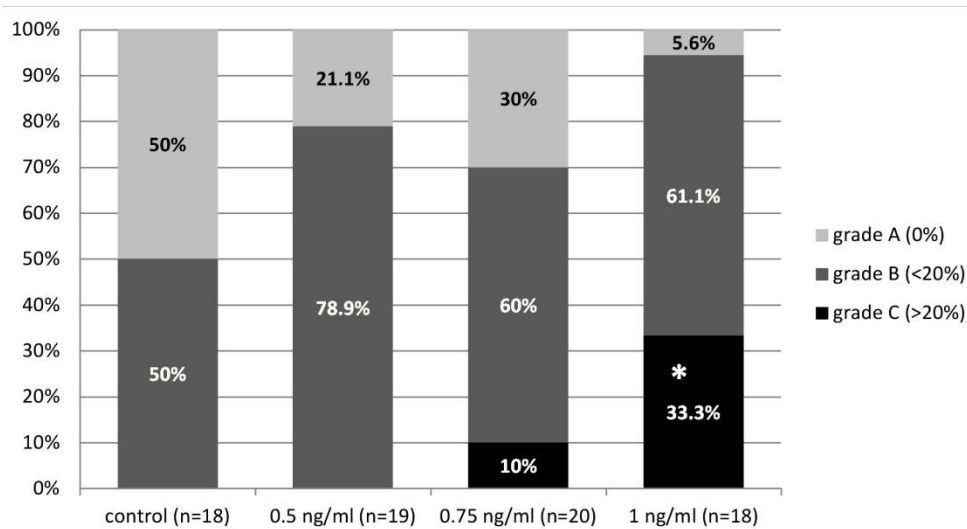
Figure 8. Proportion of grades A, B, and C blastocysts in the control and treated groups. Asterisk (*) means $p<0.05$ (χ^2 test) compared with the control.

1.4.3. Discussion

Although fetotoxic effect of T-2 toxin is well documented in the advanced stage of gestation, to the best of our knowledge no data is available on that how T-2 mycotoxin affects early preimplantation stage embryo development. *Fang et al.* (2012) found that T-2 induces significant increase of DNA fragmentation in 0.5 ng/ml concentration and results in significantly higher ROS production above 1 ng/ml exposure in differentiated murine ES cells after 24 h incubation. Our findings show that the lowest inhibitory dose of T-2 toxin on mouse preimplantation embryos is 0.75 ng/ml. Embryo model is considered to be a more relevant system for assessing toxicological effects on reproduction compared to cell culture. Embryos are simultaneously cytological and embryological test models being both few-cells structures or self-determined systems and whole organisms. Based on the model, it is possible to estimate both cytotoxic and embryotoxic effects after pathogenic influence has occurred (Popov & Protasova, 2011)*.*

The toxin concentration that we used in this study for in vitro culture of embryos can be found in human peripheral blood (0.2-1,800 ng/ml) (Berek et al., 2001) and can occur if TDI dose (100 ng/bw/day) is ingested by a human (70 kg body weight) calculated with 60 % absorption rate (EFSA, 2011). Our data show that in a concentration dependent way the toxin has harmful effect on early embryo development as well as the blastulation dynamics. 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 (Heddle et al., 1991). High proportion of micronuclei present poor implantation potential (Jackson et al., 1998) and has been associated with developmental arrest (Moriwaki et al., 2004) and apoptosis (Hnida et al., 2004). 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.

Our data contribute to reveal the mode of action of T-2 mycotoxin which is the most toxic trichotecene, has an established tolerable daily intake (TDI) of 100 ng/kg b.w. for the sum of T-2 and HT-2 toxins (EFSA, 2011) and still has only recommendation values on the presence in cereals and cereal products within the European Union (EC, 2013).

1.5. Experiment 3

Authors: Bence Somoskői, Melinda Kovács, Sándor Cseh

1.5.1. Methods

Animal housing and mating

Procedures with animals were performed following good veterinary practice for animal welfare according to 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 cervically dislocated females 20 hours 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 hours in culture media supplemented with T-2 in 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 medium with no toxin for 72 hours, after that morphologically normal ones were transferred into culture medium with 0.5 ng/ml, 0.75 ng/ml and 1.0 ng/ml toxin contaminated media (Group names: Tr05, Tr075 and Tr1, respectively).

Toxin concentrations were based on our previous studies (Somoskői et al., 2012 and 2014). Embryos cultured in medium with no toxin considered as 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) on 72nd and 96th hours of culture to assess the cell number of embryos, dead (necrotic) cells and proportion of blastomeres with damaged nuclear chromatin (micronuclei). Staining and comparison were performed only in case of morphologically normal embryos in each group.

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

Statistical analysis

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

1.5.2. Results

Cell number of embryos following 72 h 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 control ones (27.74±7.35) (Fig. 9.). Significant differences between the highest toxin contamination (1 ng/ml) and lower ones were found (p=0.046 to 0.75 ng/ml and p=0.01 to 0.5 ng/ml). There was no difference in cell numbers of the embryos between 0.5 ng/ml and 0.75 ng/ml treated groups. Proportion of PI-positive cells was under 1% in the treated and control groups (data not shown).
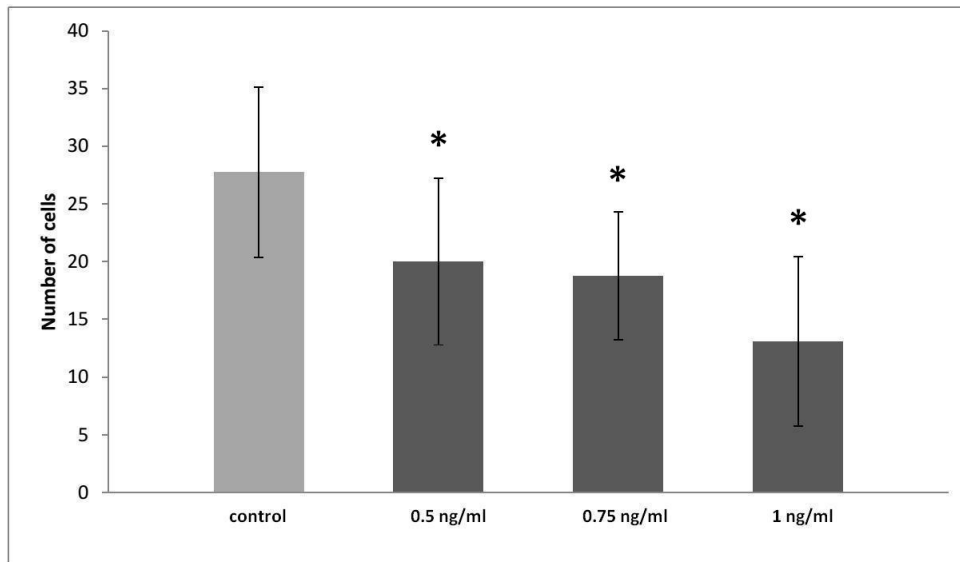
Figure 9. Number of cells (mean±SD) in embryos treated with 0.5 ng/ml, 0.75 ng/ml and 1 ng/ml T-2 after 72 hours. Control embryos were cultured in medium without toxin. (*) p<0.001, Tukey-test.

Cell number of embryos following 96 h culture

Mean cell number of control embryos was 91.52 (±26.09) after 96 h in vitro culture. Every treated group showed significant difference compared to the control one (0.5 ng/ml: 46.33±19.1; 0.75 ng/ml: 57.93±24.7; 1 ng/ml: 27.77±12.07) (Fig.10A) in respect of cell number. Difference was found in cell number between 1 ng/ml and 0.75 ng/ml toxin treated groups (p<0.001) but no difference was observed between groups treated with 0.5 ng/ml and 0.75 ng/ml toxin concentration.

Cell number of the embryos in Treatment II were significantly lower than that of control embryos (54.86±22.51 in Tr05; 67.66±26.76 in Tr075 and 54.73±38.66 in Tr1). Differences between Tr05, Tr075 and Tr1 groups were not found (Fig.10B).

Significant difference between Treatment I and II was only observed in the case of 1 ng/ml T-2 concentration (p=0.048) (Fig.10C).

Mean proportion of PI-positive cells was under 1.5% in all treated and control embryos (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).
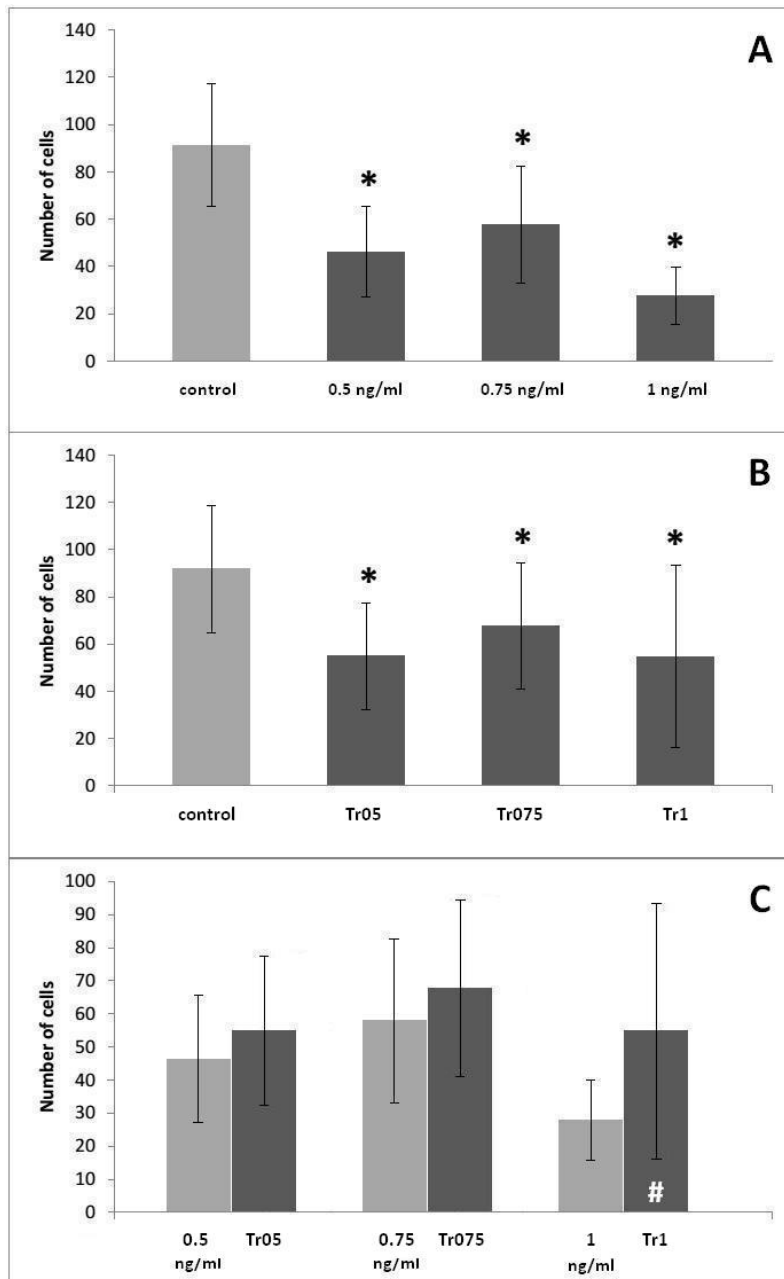
Figure 10. Comparison of cell number (mean±SD) in embryos of control and Treatment I (A), control and Treatment II (B) and in toxin-treated embryos (C) on the 96th hour of culture. (*) means significant difference from control embryos. (#) means $p < 0.05$ within toxin treatments.

Representative image of cell staining is shown on Figure 11.
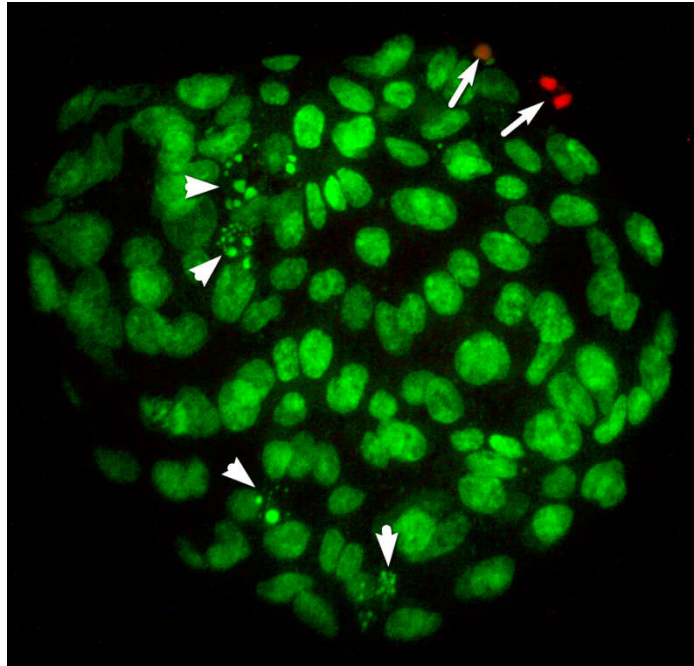
Figure 11. Representative image of a blastocyst (treated with 0.75 ng/ml T-2) stained at 96th hour of in vitro culture with SYBR14 and propidium-iodide. Arrows show PI-positive nuclei and arrowheads show micronuclei (chromatin damage).

Chromatin damage (micronuclei)

Proportion of blastomeres with chromatin damage was 6.15% (±2.91) in the control group, 7.68% (±4.67) in 0.5 ng/ml, 7.46% (±5.57) in 0.75 ng/ml, 7.69% (±4.21) in 1 ng/ml, 19.37% (±7.92) in Tr05, 12.86% (±6.09) in Tr07 5 and 8.96% (±5.68) in Tr1 groups. Embryos in Tr05 and Tr075 groups contained micronuclei at significantly higher ($p<0.001$ and $p<0.01$) proportion compared to control ones. Transfer of embryos into toxin-contaminated media resulted in significantly higher rate of micronuclei in 0.5 ng/ml ($p<0.001$) and 0.75 ng/ml ($p<0.05$) treatment groups (Fig.12.). No effect was found after culturing in medium with 1 ng/ml toxin for 96 or 24 hours.
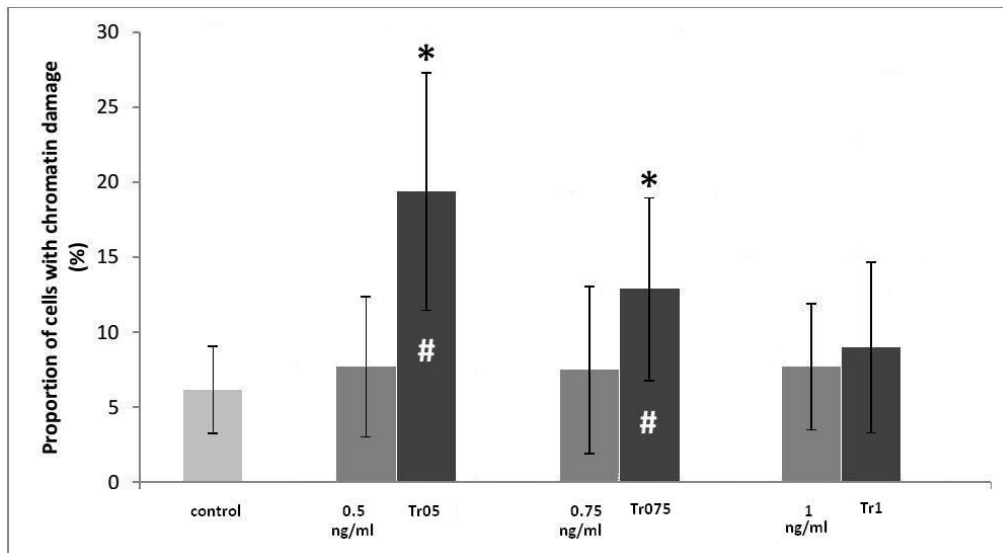
Figure 12. Proportion of cells (mean±SD) with chromatin damage in embryos of control. Treatment I and Treatment II. Groups show (*) significant difference from control embryos. Symbol of (#) indicates significant difference within treatment.

Blastocoel expansion

Proportion of different blastocyst types (early & mid-, expanded and hatched; Figure 13.) following 96 h culture in each group is shown in Figure 14. A dose dependent significantly decreased rate of expanded/hatched blastocysts was found in groups of Treatment I (67.9, 58.6 and 55%) compared to control (87.7%). Difference between blastocoel expansion of control and contaminated embryos in Treatment II was not found.

Comparing the proportion of expanded/hatched blastocysts in equivalent concentrations of Treatment I and II, we found significant differences in case of 0.5 ng/ml and 0.75 ng/ml (67.9% vs 93.8% and 58.6% vs 100%, respectively). Although, the rate of late blastocysts in 1 ng/ml and Tr1 groups did not differ significantly (p=0.059), a sharp tendency was found (55% vs 85.7%, respectively).
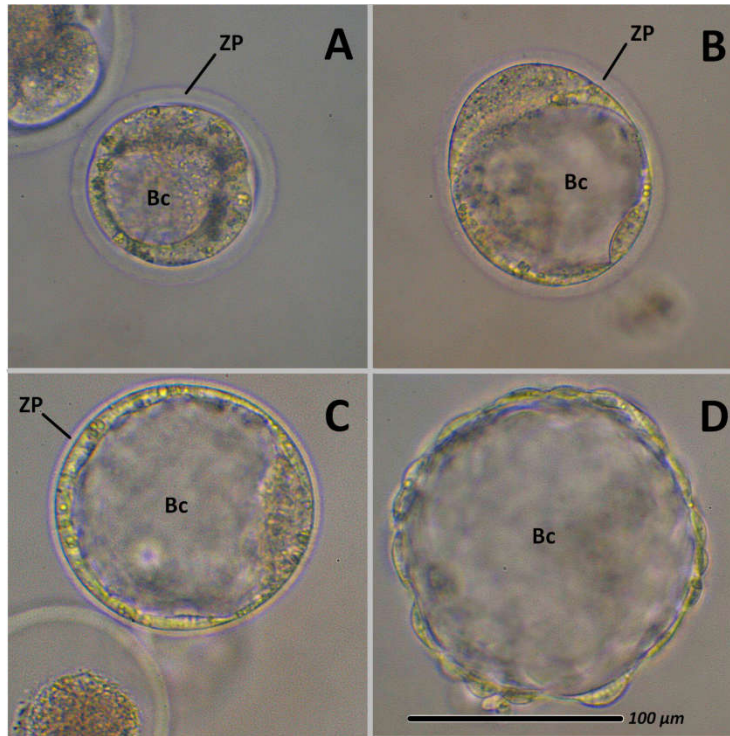
Figure 13. Representative images of blastocysts showing diverse degree of blastocoel expansion. A=early blastocyst (cultured in 1 ng/ml T-2); B=mid-blastocyst (cultured in 0.5 ng/ml T-2); C=expanded blastocyst (control) and D=hatched blastocyst (control). Scale bar represents 100 µm. Bc=blastocoel; ZP=zona pellucida. Magnification=400X
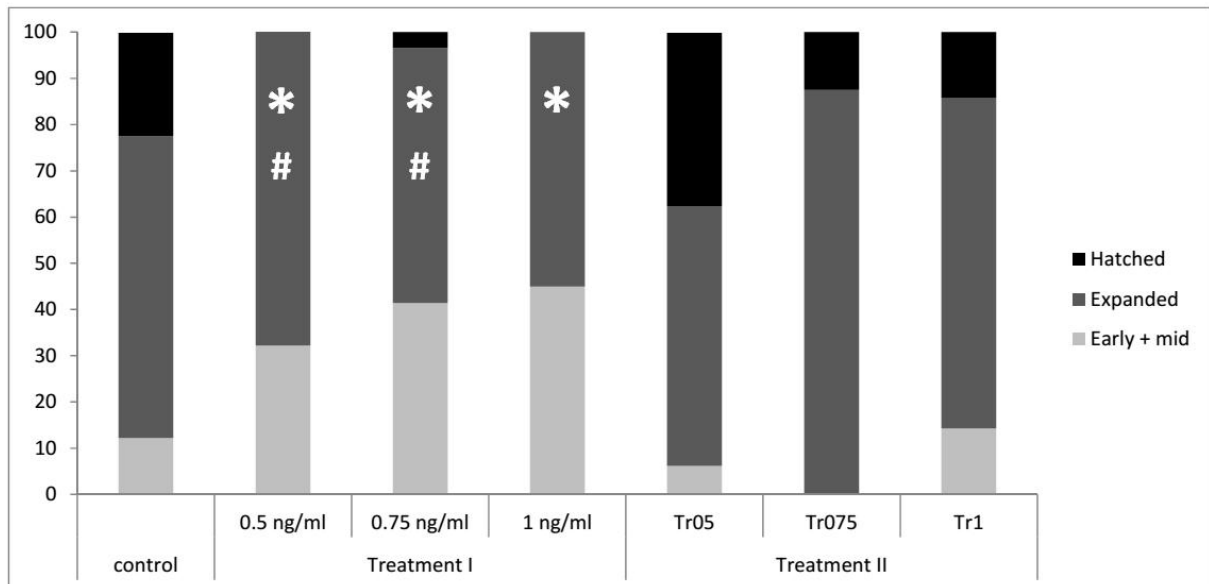
Figure 14. Proportion of blastocysts being in different stages (early & mid-, expanded and hatched) following 96 h *in vitro* culture. (*) means P<0.05 between Treatments and control; (#) means P<0.05 between Treatment I and II.

1.5.3. Discussion

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

Our data show that T-2 mycotoxin affects preimplantation embryo development even in small concentration. 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 (Zakhartchenko et al., 1995; Shapiro et al., 2000). Yuan et al. (2014) investigated the effect of T-2 on zebrafish embryos via 144 hours of exposure. They found dose-dependent tail malformation and increased ROS production. However the lowest effect level was found to be 93.3 ng/ml. Our results indicate that mouse embryo model is an effective and sensitive tool to evaluate the harmful effect of T-2 contamination *in vitro*.

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).

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 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 & Yamanuchi, 2003) and apoptosis (Hnida et al., 2004). Our data show that proportion of micronuclei in embryos cultured in media with toxin for 96 hours remains in 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 the assisted

reproduction (particularly in cryopreservation) suggest that stress tolerance of the embryos increases with developmental stage (Martino et al., 2013). Although embryos in Tr1 group did not show significantly higher micronucleus level (but tendency was apparent), elevated micronuclei proportion was found in embryos transferred into 0.5 and 0.75 ng/ml toxin contaminated media. These findings show that mouse embryos are sensitive to the toxin even in elder developmental stages.

Expansion of the blastocoel represents the developmental stage of the blastocyst (Dardik & Shultz, 1991) and the opportunity of implantation presents only in the receptive phase of endometrium (implantation window) (Song et al., 2007). 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.

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

1.5.4. Conclusions

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.

1.6. Experiment 4

Authors: Bence Somoskői, Melinda Kovács, Sándor Cseh

1.6.1. Methods

Animal housing and mating

Procedures with animals were performed following good veterinary practice for animal welfare according to 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 with 30% relative humidity in air. Feed and drinking water were available *ad libitum*. Superovulated (Day 1: 7.5 IU eCGip.; Day 3: 7.5 IU hCGip. (Alvetra und Werfft, Austria)) female mice were placed together overnight with mature males after hCG treatment.

Embryo culture and treatment

Zygotes were obtained from the oviduct 20 hours after hCG treatment following cervical dislocation of the donor females. The obtained zygotes were pooled and randomly transferred into culture media (Cleavage Medium, Cook Medical, Roskilde, Denmark) contaminated with T-2 and FB1 in different concentrations (Table 4.).

Group	No. of embryos	Toxin concentration (ng/ml)	
		T-2	FB1
T-2	108	0.5	-
A	99	-	1
B	107	-	2
C	90	-	10
TA	128	0.5	1
TB	137	0.5	2
TC	202	0.5	10
Control	140	-	-

Table 4. Toxin concentrations and embryo numbers of each group.

T-2 concentration was based on our previous studies (Somoskői et al., 2016), since it was the highest no effect level on blastocyst rate. Embryos cultured in medium with no toxin were considered as control group. All embryos were cultured for 96 hours at 37.5°C with 6.5% CO_2 and maximal humidity in air.

Embryo quality assessment

Following 96 hours of culture, the proportion of blastocysts (developmental rate) was checked in each treated groups. Further on, only morphologically normal blastrocysts were examined. Blastocysts with poor quality and earlier embryo stages (e.g. morula, 8-cell) were excluded from the analysis.

Blastocysts were investigated using Olympus CKX41 invert microscope and Olympus E-330 digital camera system and were classified based on the expansion of blastocoel (Early [early and mid blastocysts] and Late [expanded and hatched blastocysts]) (Saiz and Plusa, 2013).

After the morphological assessment, blastocysts were stained with SYBR14 (Life Technologies, USA) to check the cell number and proportion of blastomers with damaged nuclear chromatin (micronuclei).

Statistical analysis

Data were analyzed with R v3.0.0 software. Differences in blastocyst rate and blastocoel expansion were measured with Chi-squared test. ANOVA with post-hoc Tukey-test was used for comparison of mean cell number and micronuclei-containing blastomer number between groups. Differences at a probability value (P) < 0.05 were considered significant.

1.6.2. Results

Developmental rate and blastocoel expansion

Following 96 hours of culture, the proportion of blastocysts in group T-2 (83.33%), A (78.79%), B (85.98%) and C (86.67%) were not different from the control (86.43%). Opposite to this tendency, the combined treatment with two toxins decreased the blastocyst rate significantly in group TA, TB and TC (14.5, 33.6 and 22.8%, respectively) compared to control, T-2 and FB_1-contaminated treatment groups. Embryos cultured in T-2 contaminated medium did not show different blastocyst rate from A, B and C (Figure 15.).
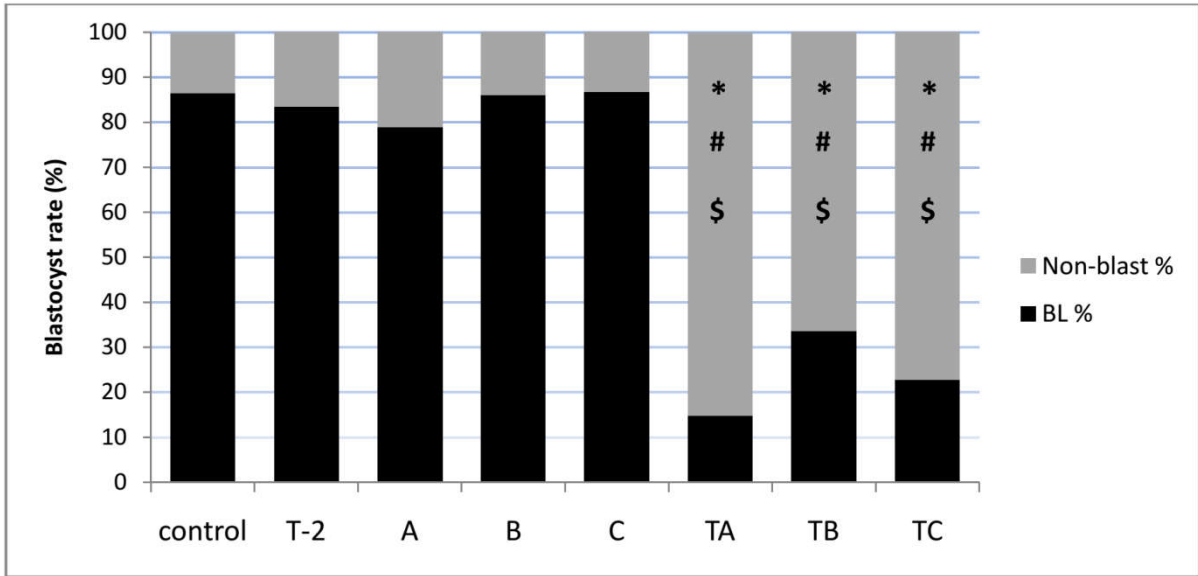
Figure 15. Blastocyst rate in control and toxin-treated groups after 96 hours of culture.(*, #, $)P<0.01, compared to control, T-2 and correspondent FB$_1$ treatment (e.g. A vs. TA), respectively.
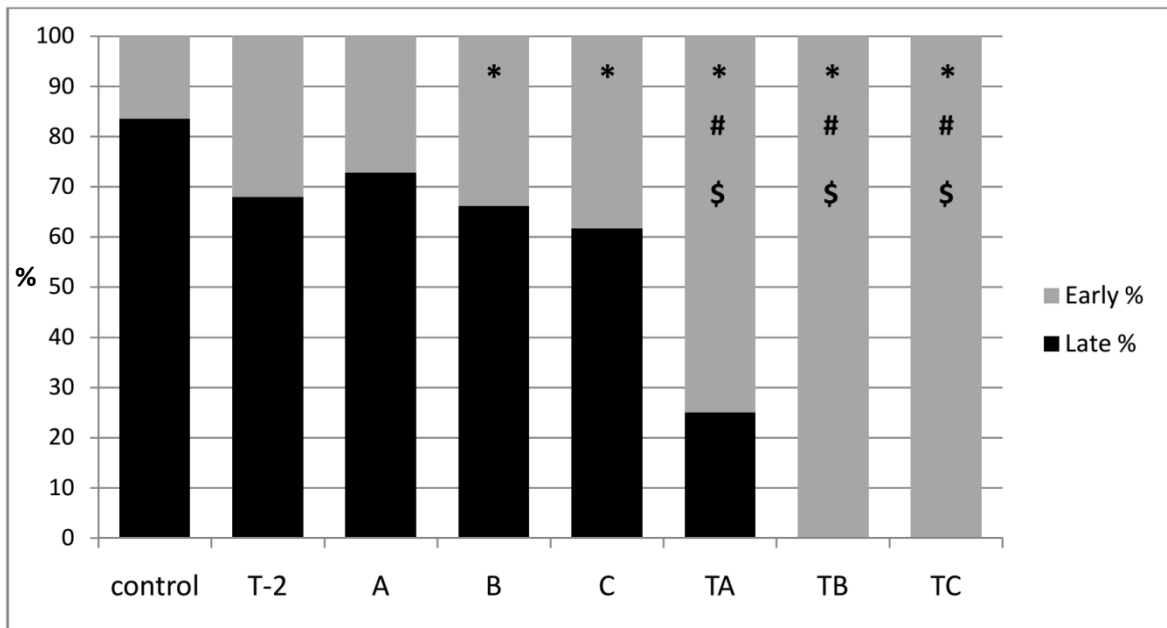
Figure 16. The proportions of early and late blastocysts in control and toxin-treated groups after 96 hours of culture. Early = early blastocyst stages (early- and mid); Late = late blastocyst stages (expanded and hatched). (*, #, $)P<0.01 compared to control, T-2 and correspondent FB$_1$ treatment (e.g. A vs. TA), respectively.

Compared to the control group, decreased numbers of expanded and hatched blastocysts were observed in each toxin treated groups. However, the differences were not significant in groups of T-2 and A (83.6 % vs. 67.9 and 72.8%, respectively). Higher concentrations of FB1

(group B and C) caused slight, but significant decrease in the rate of later developmental stages (66.2 and 61.7%, respectively). In cases of the combined toxin expositions, remarkable effects were found. In the TA group, the proportion of late blastocysts went under 30 %, while in the groups of TB and TC only early blastocysts were found (Figure 16.).

Beside the blastocyst morphology, the rate of morulae was also evaluated. In the control group, 5.26% of the embryos stopped the development at the morula stage (< 16 cells). The rate of morula was 33.3% in the T-2 group and 23.8% in the A, 13.3% in B and 33.33% in the C group. Combined toxin treatment resulted in less than 20% rate of morulae in TA (13.8%), TB (15.4%) and TC (11.5%) groups.

Cell number and micronucleus rate

Compared to control blastocysts, the toxin treatment decreased the cell/blastomer number in each treated group (Figure 17.). However, significant decrease was found only between the groups of control vs. T-2 (85.92 ± 3.28 vs. 46.33 ± 3.33; mean$\pm$SE), control vs. group C (85.92 ± 3.28 vs. 61.27 ± 5.09) and control vs. co-contamination treatments (85.92 ± 3.28 vs.49.28 ± 7.47, 44.63 ± 3.97 and 44.62 ± 4.91 for TA, TB and TC, respectively). FB1-treated blastocysts showed higher blastomer numbers compared to embryos cultured in medium containing 0.5 ng/ml T-2 alone, although, significant difference was found only between T-2 and B groups (46.33 ± 3.33 vs. 69.48 ± 5.61). Among the groups containing FB1, significant difference was found only between B and TB groups (69.48 ± 5.61 and 44.63 ± 3.97; $p < 0.01$). However, a tendency was detected in the decrease of blastomer number in the embryos between the combined treatment groups and corresponding FB_1-exposition (e.g. A vs. TA). Our data show, that the same concentration of mycotoxin did not have any harmful effect alone, however, in case of combined treatment it did.

Investigating the rate of blastomers with micronuclei, no significant difference was found between control and toxin-treated blastocysts (Figure 18.).
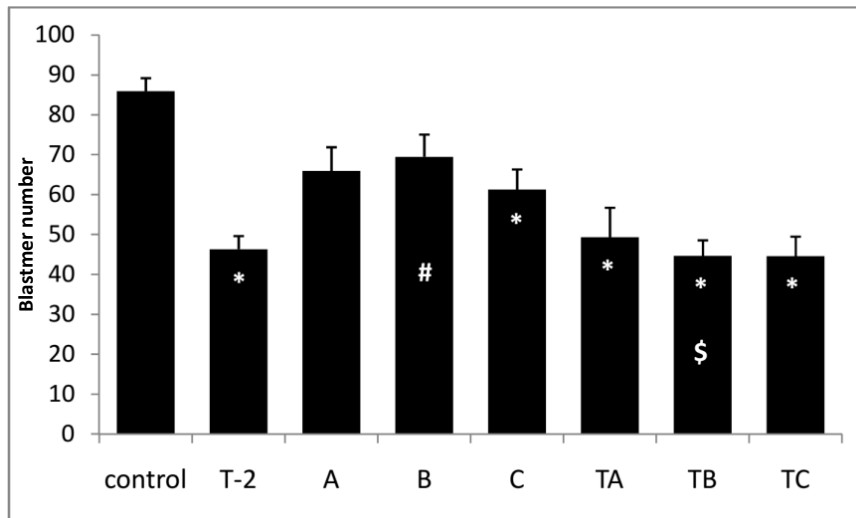
Figure 17. Number of blastomers (mean ± SE) in the blastocysts in the control and toxin-treated groups after 96 hours of culture.(*, #, $)P<0.01, compared to control, T-2 and correspondent FB_1 treatment (e.g. A vs. TA), respectively.
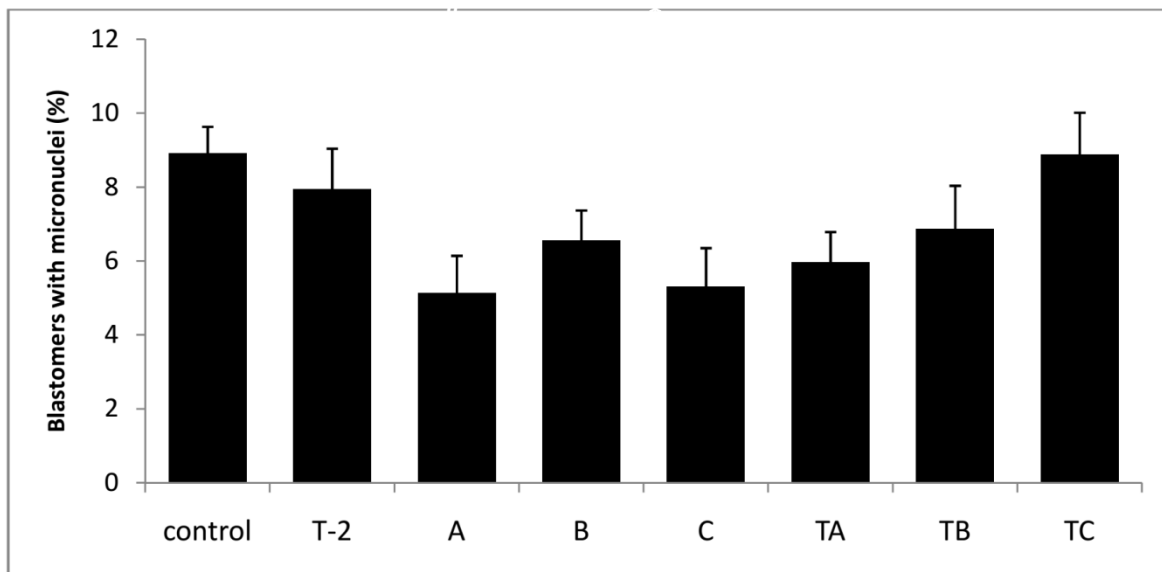
Fig.18. Rate of blastomers with micronuclei (mean ± SE) in blastocysts of control and toxin treated groups after 96 hours of culture.

1.6.3. Discussion

Both T-2 and FB1 are fusariotoxins, produced by members of *Fusarium* genus which has spacious common area in the temperate region (Schatzmayr and Streit, 2013).The occurrence of T-2 and FB_1 was measured in wheat grain samples in the studies of Stankovic et al. (2012) and Stepanic et al. (2011). It was found that the frequency of positive samples was high and ranged between 60-90.2% and 82-92%, respectively. The probability of co-occurrence was also high and ranged between 52 and 75.6%. These data show that co-occurrence of different mycotoxins (two or more mycotoxins are detected in the sample) may occur frequently and therefore it is of serious concern (more than half of the samples). Although individual effects of mycotoxins are mostly well-known, co-contamination (combined effect) studies are less current, especially in case of fusariotoxins, therefore this area requires more study (Speijers and Speijers, 2004). The combined effect (toxicity) of more mycotoxins cannot always be predicted based upon their individual toxicities. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive, or synergistic (Greiner and Oswald, 2011).

Reproductive efficiency is a very important economic factor in economical animal production. However, exposure to several *Fusarium* mycotoxins have been linked to reproductive disorders (Cortinovis et al., 2013). 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. Hlywka et al. (1997) using chicken embryo screening test found that 25 µg/egg FB1 decreased the survival rate below 60% (LD_{50} was 52 µg/egg). Sheu et al. (1996) examined the effect of 0.01 to 1000 µg/ml FB1 on BALB/3T3 A31-1-1 mouse embryo-derived cells. They found no reduction in cell number after 48 hr of treatment and only a moderate reduction (less than 50%) was observed after 6 days of treatment at concentrations of l0 µg/ml or higher. In an *in vivo* study by Collins et al. (1998), no difference was found between control and FB_1-treated (1.875-15 mg/kg) female rats in the implantation sites (15.3±0.5 vs. 14.5±0.6 to 15.6±0.4) and implantation efficiency (90.8%±2.3 vs. 89.3%±3.0 to 93.6%±2.5). In case of T-2, beside our experiments (Somoskői et al. 2012 and 2016) there are no data on the effect of the toxin on early mammalian embryo development. Yuan et al. (2014) investigated the effect of T-2 on zebrafish embryos via 144 hours of exposure. They found dose-dependent tail malformation and increased ROS production. The lowest effect was observed at 93.3 ng/ml. Fang et al. (2012) found that T-2 induces significant increase of DNA fragmentation at 0.5 ng/ml

concentration and results in significantly higher production of ROS over 1 ng/ml, after 24 h of exposure. Although, they used differentiated murine embryonic stem cells.

We examined the proportion of blastocysts after 96 hour of culture in order to measure the survival rate of embryos under different expositions. 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. This method, called MEA (Mouse Embryo Assay) is thought to be an appropriate indicator for potential toxicity of different chemicals (Punt–van der Zalm et al., 2009) and therefore it is approved by the FDA (FDA, 1998). There is a debate on the usage of mammalian preimplantation (cleavage stage) embryos in the frame of in vitro model based on the „all-or-nothing answer" (A/N): embryo can develop further or dies (Popov and Protasova, 2011). However, investigating the development in detail, with deeper insight into blastocyst-morula-un-compacted scale and toxin effect mechanism, it is possible to collect more information about the developmental capacity. In our study, the control embryos showed typical A/N: approximately 95% of non-blastocyst embryos remained uncompacted, i.e., embryos reaching the compacted stage can develop further to blastocysts. Conversely, in the single-dose groups only 13.3-33.3% of the embryos reached the compacted/morula stage and did not develop to blastocyst within the 96 hour of culture. In the co-contaminated groups, low ratios of compaction were found (10-15%). Developing from the morula to the blastocyst stage (forming the fluid filled blastocoel and inner cell mass, etc.) is a complex, very well-organized process, including the first differentiation event (Saiz and Plusa, 2013). Our data show that this developmental process can fail/can be disturbed (stopped) by the presence of mycotoxins on a dose dependent form. With other words, not only the presence of the mycotixin/mycotoxins, but the types of combinations and concentrations are both important in the seriousness of the generated effect. This fact indicates the critical review of A/N theory.

Our data show type I synergistic interaction (based on Greiner and Oswald, 2011) between T-2 and FB_1 related to the late blastocyst rate. In this study, expanded and hatched blastocysts were classified together as "late" stages, since mouse embryo hatching frequently unavailing in *in vitro* environment. (Lin et al., 2001). Expansion of the blastocoel indicates the developmental stage of blastocysts (Dardik and Shultz, 1991). The formation and expansion, furthermore the hatching provide the opportunity for the implantation of the embryo. There is a receptive phase of the endometrium (implantation window) which is a relatively short interval, 24 hours, on the 4th day of the cycle of the mouse (Song et al., 2007). Consequently, if the blastocyst is not in the late phase on Day4, the implantation will be failed, since without hatching, the blastocyst is not able to implant (Cheng et al., 2004). Our data indicate that T-2 and FB_1 co-occurrence may delay the development to

expanded/hatched blastocyst with 18-20 hours which may cause implantation failure, because the embryo misses the implantation window.

Cell number of embryos indicates developmental capacity (developmental strength), therefore it is a suitable marker/predictor of viability evaluation (Mori et al., 2002). Decreased blastomer number may result in reduced developmental ability/capacity (Zakhartchenko et al., 1995; Shapiro et al., 2000). In our study, 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.

Assessing micronuclei in the bastomers of the embryo is a useful tool to measure the rate of chromatin damage. Recently the analysis of micronuclei in preimplantation embryos has been used to assess the cytogenetic effects of maternal or paternal treatment with chemicals such as chlorambucil, cyclophosphamide, cis-platinum, adriamycin, acrylamide, triethylenemelamine, and X-irradiation (Ishikawa et al., 2000). This technique is also used in the assessment of chromatin damage in embryos being in different stages (Martino et al., 2013). In our study, difference between treatments was not found. Standard deviations were relatively high in all groups indicating individual discrepancy in the response of the embryos to the toxins. Further investigations are needed to see clearer picture connected with the role of mycotoxins in the chromatin damage, and the role of chromatin damage in the develeopmental capacity of early mammalian embryo development.

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.

Chapter 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 (*Polge et al., 1949*), the ability to cryopreserve mammalian embryos has become an integral part of assisted reproductive technologies (ART) in both human and veterinary medicine. Cryopreservation 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 (Armitage, 1987)). 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) (Leibo and Songsasen, 2002; Fuller et al., 2004). The same fundamental cryobiological principles operate to determine the survival of embryos cryopreserved by both methods (Rall, 1987; Shaw et al., 2000; Kasai and Mukuida, 2004). In order to avoid the so-called freezing damage during CP of embryos, the freezing solution is supplemented with cryoprotective additives (CPAs). There are differences in the permeability to permeating CPAs and sensitivity to cooling among the embryos of different species. Furthermore, the earlier the stages of development, the less permeable are the embryos and the survival rates of cryopreserved embryos increases as the developmental stage proceeded (Leibo and Songsasen, 2002;Massip, 2001; Shaw and Jones, 2003). This higher cryotolerance of late stage embryos is the outcome of higher nucleus-cytoplasm ratio (Leibo, 1993). At the traditional SF, the progressive dehydration of the embryo is based on 1) the equilibration of embryos prior to cooling in freezing solution supplemented with low concentration of CPA (1-2 M) and 2) slow cooling to minus 30-40°C, before plunging into liquid nitrogen for long term storage at minus 196°C. Vitrification is an alternative approach for cryopreservation, which avoids the formation of ice crystals in the intracellular and extracellular space (Rall and Fahy,1985; Vajta, 2000; Fuller et al., 2004). This process is achieved by a combination of a very high concentration of CPAs (4-8 M, a solution with very high viscosity) and an extremely high cooling rate which result in the solidification of the solution without ice crystal formation (the solution vitrifies; Figure19.) (Rall and Fahy,1985; Liebermann et al., 2002).
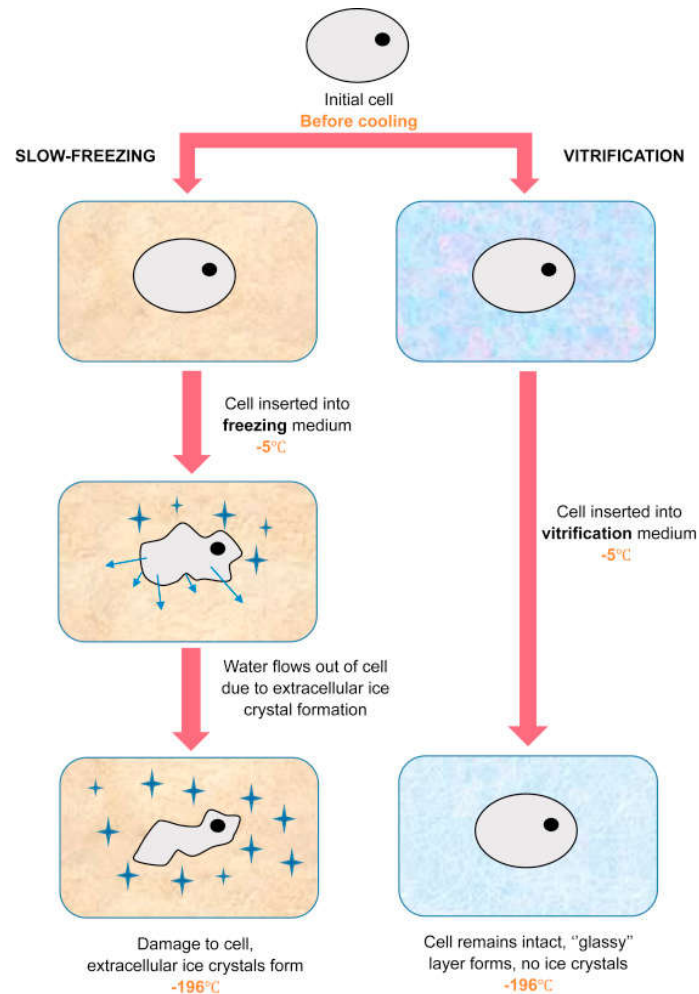
Figure 19. Differences between slow freezing and vitrification, with special regard to ice cristal formation (*Mandawala et al., 2016*).

Appropriate mitochondrial (mt) distribution and membrane potential in embryos are very important for their developmental potential and for a variety of cellular activities, including ATP synthesis and specific cell functions (Van Blerkom et al., 2000; Van Blerkom, 2011). However, there is little information available about the effects of CP (traditional SF and VF) on mt dynamics/distribution and reactive oxygen species (ROS) production in embryos. In an early study, Noto et al. (1993) found that rapid freezing did not affect subcellular structures. The well organized and specific mt distribution appeared still to be present after frozen storage and subcellular structures seemed to be rather resistant targets for cryoinjury (Noto et al., 1993). Zhao et al. (2009) found that the mt ring rate decreased in mouse 2-PN (fertilized egg) embryos after VF, an event which may affect the subsequent developmental viability of the embryos (Zhao et al., 2009). Similar results were found by Shi et al. (2007) indicating that VF alters mt distribution in porcine metaphase II (MII, matured) oocytes (Shi et al., 2007). Tanim et al. (2014) analyzed the impact of different factors on the outcome of

human ART. Their data indicate that among other things, gamete/embryo CP may be associated with mitochondria, genetic and epigenetic alterations to gametes/embryos. A recent study shows remarkable decrease in mt activity in slow frozen and vitrified sheep embryos compared with fresh ones (Dalcin et al., 2013).

Informations about the effects of CP on chromatin integrity are incomplete and contradictory. Kader et al (2010) investigated the chromatin integrity index in fresh, vitrified and slow-frozen blastocysts and found significant decrease in vitrified embryos. However, Li et al (2011) found higher DNA integrity in vitrified human and mouse blastocysts than in the slow-frozen ones and similar to fresh blastocysts. Isachenko et al. (2008) investigated the effect of integrity rate of pronuclei after the CP of pronuclear-zygotes on subsequent embryo development and pregnancy. Their observation indicates that it is a predictor of future embryo development and implantation. The high integrity rate resulted in high pregnancy rate, while zygotes with low integrity of pronuclei after CP had low developmental potential.

It has been reported that oxidative stress (OS) may be an important mechanism underlying the toxic effects of CP procedures which then may trigger the apoptotic cascade leading to a decrease in the survival rate and developmental rate of gametes/embryos after thawing (Bilodeau et al., 2000; Ahn et al., 2002; Somfai et al., 2007; Tatone et al., 2010). Oxidative stress occurs if disequilibrium takes place between ROS production and antioxidative capacity of the cell (Finkel et al., 2000) and it has also been implicated in the etiology of some forms of female infertility (Agarwai et al., 2004). Mitochondria represent the major source of ROS, in which they are produced in a stepwise process (Cadenas and Davies, 2000). Under physiological conditions, ROS are neutralized by an elaborate defense system consisting of enzymes (e.g. catalase, superoxide dismutase, glutathione peroxidase or reductase) and non enzymatic antioxidants (e.g. vitamin C, E, A, pyruvate, glutathione, ubiquinone, taurine, hypotaurine) (Winyard et al., 2005). Thus, any perturbation in mt activity or in the activity of scavenger systems can lead to profound implications in ROS production, OS induction, and mt cytochrome c release, which is an important step for apoptosis (Brookes et al., 2004).

Embryos, as other aerobic cells, produce ATP and ROS by means of mt oxidative phosphorylation. Blastocyst freezing was abandoned for years, since only 25% of the zygotes were able to reach the blastocyst stage in vitro in usual culture media and low pregnancy rates were reported. Recently the situation has been changed, because the improved embryo culture systems, the newly developed sequential media, and furthermore the improved CP procedures have been increased the outcome of in vitro blastocyst

production, the survival of cryopreserved blastocysts and the pregnancy rates of frozen-thawed IVF cycles (Konc et al., 2005).

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. The average potential of a frozen stored embryo to become a living child lies in the order of 4%, and babies born from cryopreserved embryos do not represent more than 8−10% of the total number of babies born from AR (de Jong et al., 2002). 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.

According to the International Embryo Transfer Society (IETS) the total number of transferred embryos, produced in vitro or in vivo, for cattle and small ruminant species were approximately 800.000 and 2.000, respectively (Amiridis and Cseh, 2012). The best stages for CP and transfer of frozen cattle embryos are compact morulae/early blastocysts at Day 6 (D6, when IVF is on D0) and expanded blastocysts (D7-8). Most of the assessment for either cryopreservation or fresh transfer is at D7-8. To our knowledge, no comparative research regarding the effect of SF and VF on mitochondrial distribution, chromatin integrity, energy status and ROS production in different developmental stages (mouse morulae and blastocysts) of embryos has been documented.

2.2. Objectives

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)

2.3.Experiment 5

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2.3.1. Methods

Embryo recovery and in vitro culture

Procedures with animals were performed following good veterinary practice for animal welfare according to Hungarian national laws in force. The protocol of the animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Science Szent István University, Budapest. Embryos were produced as reported by Klambauer et al., 2009. Briefly, eight weeks old BDF1 female mice (Institute of Oncology, Animal Care Facility, Budapest, Hungary) were superovulated by 10 IU eCG i.p., followed by 10 IU hCG i.p. 48 h later, in order to induce the final maturation of the oocytes and ovulation. After the hCG injection the females were paired with males (1 female/male), then 20 to 24 h later the embryos were collected (Day 1). Until vitrification or analysis (fresh control embryos), embryos were cultured in G1 medium (Vitrolife, Goteborg, Sweden) at 37.5 °C with 6.5% CO_2 and maximal humidity in air for further 20 to 96h. Only morphologically normal embryos, being in cleavage stage (4- to 16-cell) or at the morula or blastocyst stage, were randomly destined to either vitrification or fresh control groups.

Vitrification

Embryo vitrification was performed with the VitroLoop vitrification procedure as previously described (Klambauer et al., 2009) and unless otherwise specified, all materials were provided by Vitrolife. Briefly, embryos were exposed to a 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). Vitrification was carried out in RapidVit Cleave vitrification solutions (Vitrolife; solution 1: holding medium, solution 2: equilibration medium, and solution 3: vitrification medium) and embryos were manipulated in 4-well culture dish (Nunc Intermed, Roskilde, Denmark) held on a warming plate at 37°C. The holding or basic solution is based on G-MOPS™ and was supplemented with gentamycin and human serum albumin (HSA, 5 μg/ml). Both the equilibration and the vitrification media are based on the holding/basic solution, but the equilibration medium was supplemented with 8% EG + 8% PG and the vitrification solution was enriched with 16% EG, 16% PG, F (Ficoll, F-400, 10 mg/ml) and S

(Sucrose, 0.65 mol/l). All manipulations of the embryos during their preparation for vitrification were carried out at 37°C (on a heated stage). Embryos were suspended from solution 1 into the equilibration medium (solution 2) for 2 min. Thereafter, they were transferred and washed quickly in small drops of vitrification medium (solution 3). The cryoloop was dipped into the vitrification medium to create a thin filmy layer of the solution on the nylon loop where embryos (max 3 embryos) were quickly transferred from the vitrification medium. Within 30 sec of suspension in the vitrification medium, the loop with the embryos was plunged into LN$_2$. 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 S in decreasing concentrations (warming solution 1: 0.65 mol/l; 30 sec, warming solution 2: 0.25 mol/l; 1 min, warming solution 3: 0.125 mol/l; 2 min and warming solution 4: 0.0 mol/l; 5 min) (RapidWarm Cleave; Vitrolife, Goteborg, Sweden).

Mitochondria and ROS staining

Fresh and vitrified-warmed embryos underwent mitochondria (mt) and ROS staining following the procedure by Ambruosi et al., 2011 and Martino et al., 2012. Embryos were washed three times in PBS with 3% bovine serum albumin (BSA) and incubated for 30 min in the same medium containing 280 nM MitoTracker Orange CMTM Ros (Molecular Probes M-7510, Oregon, USA) at 38.5°C under 5% CO$_2$ in air. The cell-permeant probe contains a thiol-reactive chloromethyl moiety. Once the MitoTracker probe accumulates in the mitochondria, it can react with accessible thiol groups on peptides and proteins to form an aldehyde-fixable conjugate. This cell-permeant probe is readily sequestered only by actively respiring organelles depending on their oxidative activity (Poot et al., 1996; Torner et al., 2004). After incubation with MitoTracker Orange CMTM Ros, embryos were washed three times in PBS with 0.3% BSA and incubated for 15 min in the same media containing 10 mM 2',7'-dichlorodihydrofluorescein diacetate (DCDHF DA). The non-ionized DCDHF DA is membrane permeant and therefore is able to diffuse readily into cells. Once within the cell, the acetate groups are hydrolysed by intracellular esterase activity forming 2',7'-dichlorodihydrofluorescein (DCDHF) which is polar and thus trapped within the cell. DCHF fluoresces when it is oxidized by H2O2 or lipid peroxides to yield 2',7'-dichlorofluorescein (DCF). The level of DCF produced within the cells is linearly related to that of peroxides present and thus its fluorescent emission provides a measure of the peroxide levels (Yang et al., 1998). After incubation, embryos were washed three times in prewarmed PBS without BSA and fixed with 3.7% paraformaldehyde solution in PBS. All procedures after thawing/warming were performed within 1 hour. Embryos were kept in fixative at 4°C for no longer than two to three days. The organelle-specificity of the mt probe was assessed, as

reported by Valentini et al., 2010, in control samples which were imaged after incubation in MitoTracker Orange and further incubation for 5 min in the presence of 5 mM of the mt membrane potential (Delta Psi)-collapsing uncoupler carbonyl cyanide 3-chloro phenylhydrazone (CCCP; Molecular Probes), which inhibits mt respiratory activity thus reducing fluorescence intensity. Particular attention was paid to avoid sample exposure to the light during staining and fixing procedures in order to reduce photobleaching.

Embryo morphology assessment

Embryos at the different examined developmental stages were recovered from vials and their morphological appearance was assessed by evaluating blastomere cytofragmentation following the criteria described in previous studies in the mouse as well as in other species. Embryos were examined at 400x magnification phase contrast microscopy and scored as either unfragmented (Grade 0), or with fragmentation graded into three categories as reported by Van Soom et al., 2003 and Han et al., 2005. Grade 1 fragmentation was defined as the presence of one or more cytofragments smaller than the size of a polar body and often clustered at one or both poles or in the crevice between blastomeres. Grade 2 fragmentation was defined as the presence of many more fragments, with total volume of fragments comprising an equivalent of less than one-half the volume of a blastomere. Grade 3 fragmentation was assigned to embryos with many large fragments, with the total volume of fragments being approximately one-half of one blastomere or greater.

Detection of the nuclear chromatin status

To evaluate nuclear chromatin, embryos were stained with 2.5 µg/ml Hoechst 33258 in 3:1 (v/v) glycerol/PBS, mounted on microscope slides, covered with cover-up micro slides, sealed with nail polish and kept at 4°C in the dark until observation (Ambrousi et al., 2011). Nuclear chromatin status was observed under a Nikon Eclipse 600 fluorescent microscope equipped with B2A (346 nm excitation/ 460 nm emission) filter. Embryos were classified as normal (grade A) when the presence of a regular-shaped nucleus inside each blastomere was observed. The formation of micronuclei and lobulated nuclei was considered as signs of chromatin damage (Liu et al., 2010). Embryos showing 0 to 20% affected blastomeres were classified as grade B and embryos with more than 20% affected blastomeres were classified as grade C).

Mitochondrial distribution pattern and intracellular ROS localization

For mt distribution pattern evaluation, embryos were observed at 600 x magnification in oil immersion with a Nikon C1/TE2000-U laser scanning confocal microscope. A helium/neon laser ray at 543 nm and the G-2 A filter (551 nm exposure/576 nm emission) were used to observe the MitoTracker Orange CMTM Ros. An argon ions laser ray at 488 nm and the B-2 A filter (495 nm exposure/519 nm emission) was used to observe the DCF. Scanning was conducted with 25 optical series from the top to the bottom of the embryo with a step size of 0.45 µm to allow three-dimensional distribution analysis. General criteria for mt pattern definition were adopted on the basis of previous studies in mouse and human oocytes and embryos (Van Blerkom, 2011; Zhao et al., 2009; Van Blerkom et al., 2002; Van Blerkom, 2006), as well as in oocytes of other species (Ambrousi et al., 2009). Thus, an homogeneous/even distribution of small mt aggregates throughout the cytoplasm was considered as an indication of low energy cytoplasmic condition. Heterogeneous/uneven distribution of small and/or large mt aggregates indicated metabolically active cytoplasm. In particular, the accumulation of active mitochondria in the peripheral cytoplasm (pericortical mt pattern) and/or around the nucleus (perinuclear and perinuclear/pericortical mt pattern, P/P) was considered as characteristic of healthy cytoplasmic condition. Embryos showing irregular distribution of large mt clusters unrelated to the specific cell compartments were classified as abnormal. To our knowledge, few studies have reported to date on intracellular ROS localization and levels in mouse embryos (Zhang et al., 2010; Kawamura et al., 2010) and no studies on cryopreserved mouse embryos have been reported.

Quantification of Mitotracker Orange CMTM Ros and DCF fluorescence intensity

Measurements of fluorescence intensities were performed in embryos having either heterogeneous (perinuclear/pericortical) or homogeneous (small aggregates) mt distribution pattern. Embryos showing abnormal mt distribution pattern were excluded from this analysis. In each individual embryo, the fluorescence intensity was measured at the equatorial plane, with the aid of the EZ-C1 Gold Version 3.70 software platform for Nikon C1 confocal microscope. A circle of an area (arbitrary value = 100 in diameter) was drawn in order to measure only the cytoplasmic area. Fluorescence intensity encountered within the programmed scan area was recorded and plotted against the conventional pixel unit scale (0–255). Quantification analysis was performed only on embryos at the morula or blastocyst stage. In fact, due to their round shape, late stage embryos allow the software set-up for quantification analysis as reported for oocytes (Martino et al., 2012). Parameters related to fluorescence intensity were maintained at constant values for all evaluations. In detail,

images were taken under fixed scanning conditions with respect to laser energy, signal detection (gain) and pinhole size.

Mitochondria/ROS colocalization analysis

Colocalization analysis of mitochondria and ROS was performed by using the EZ-C1 Gold Version 3.70 software. For each channel, the same threshold, set to the zero value, was used for the data set. Degree of colocalization was reported as a Pearson's correlation coefficient quantifying the overlap degree between MitoTracker and DCDHF DA fluorescence signals.

Developmental ability of vitrified embryos

In order to demonstrate whether vitrified embryos retain the developmental ability, a group of vitrified embryos were warmed and cultured in vitro as described above. Their developmental ability was compared with that of non vitrified control embryos. Moreover, the possible toxic effect of the cryoprotectant mixture was assessed, by comparing the developmental rate of non vitrified embryos exposed to cryoprotectants (non-vitrified/exposed) with that of non vitrified/non exposed embryos.

Statistical analysis

For each examined embryo developmental stage, embryo morphology (the rates of embryos showing cytoplasmic fragmentation and chromatin damage) and the rates of embryos showing the different mt distribution patterns and ROS intracellular localization were compared between the fresh control and vitrified-warmed embryos by Chi square-analysis with the Yates correction for continuity. Fisher's exact test was used when a value of less than 5 was expected in any cell. Mean values (mean±SD) of mt and ROS fluorescence intensities, expressed as arbitrary density units (ADU) and mt/ROS colocalization, expressed as Pearson's correlation coefficient, were compared by the Student's t-test. Differences with $P<0.05$ were considered statistically significant.

2.3.2. Results

Two hundred and sixty-seven mouse embryos, 99 of which at the early stages of development (including 4/8-cell, n=42 and 8/16-cell, n=57), 122 at the morula stage (including 16/32-cell, n=76 and >32 cell, n=46) and 46 at the blastocyst stage, were randomly divided into vitrified (n=118 embryos) and control (n=149 embryos) groups.

*Vitrification increases low grade embryo blastomere cytofragmentation*

The overall proportion of fragmented embryos (grade 1 + grade 2) was increased by vitrification (26% vs 5%, for vitrified-warmed vs control embryos, respectively; P<0.05). However, most of fragmented embryos were found to be of low grade, i.e. of grade 1 (24% and 5% grade 1 fragmented embryos in vitrified and control groups, respectively; P<0.05) and very few embryos were found to be of intermediate grade, i.e. of grade 2 (2% and 0% grade 2 fragmented embryos in vitrified and control groups, respectively; NS). No high grade fragmentation (grade 3) was found in either group.

Vitrification increases low level embryo chromatin damage

Vitrification induced low level nuclear chromatin damage (26% vs 5% in vitrified and control embryos, respectively; P<0.05; Figure 20, panel A). Chromatin damage increased with embryo development and became statistically significant in embryos at the morula stage (P<0.01). On the contrary, chromatin integrity was preserved in embryos at the blastocyst stage. Figure 20 (panel A) shows the overall (grade B + grade C) proportion of embryos with damaged chromatin observed after vitrification (black bars), grouped according to their developmental stage, and compared with controls (white bars). In Figure 21, the proportions of embryos showing grade B (0 to 20%; panel A) or grade C (>20%; panel B) chromatin damage are detailed. As depicted in panel A, the proportions of grade B affected embryos were significantly higher after vitrification (24%) compared with controls (5%, P<0.05) while vitrification had no effect on the rates of embryos showing grade C chromatin damage (3/118; 2.5%).In Figure 20 panel A and in the Figure 21, values plotted on the y axis are the proportion of embryos with chromatin damage, thus complementary percentages are referred to normal (grade A) embryos.
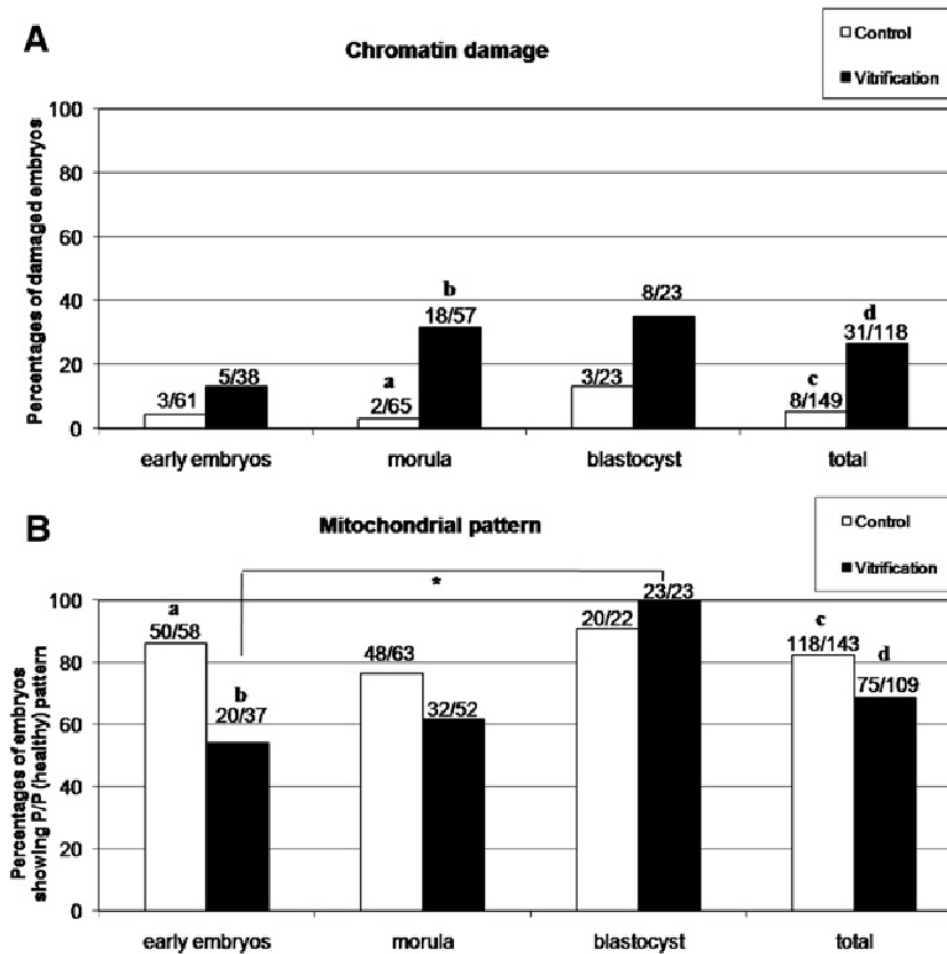
Figure 20. Effects of vitrification on chromatin integrity and mitochondrial distribution pattern of mouse 4/16-cell, morula and blastocyst stage embryos. Panel A: overall percentages of embryos (grade B + grade C) with damaged chromatin observed after vitrification (black bars), grouped according to their developmental stage, and compared with controls (white bars). In detail, in embryos at the morula stage, vitrification increased the rate of embryos showing chromatin damage whereas it had no effect in early embryos and in blastocysts. Chi square test: within each stage: a,b P<0.001; c,d P<0.05. Panel B: Percentages of embryos showing the P/P mt pattern observed after vitrification (black bars), grouped according to examined developmental stages, and compared with controls (white bars). In detail, in embryos at the 4/16-cell stage, vitrification reduced the rate of embryos showing P/P mt pattern whereas it had no effect in embryos at the morula and blastocyst stages. Chi square test: a,b P<0.001; c,d P<0.05; * P<0.001. Numbers of analyzed oocytes per group are indicated on the top of each histogram.
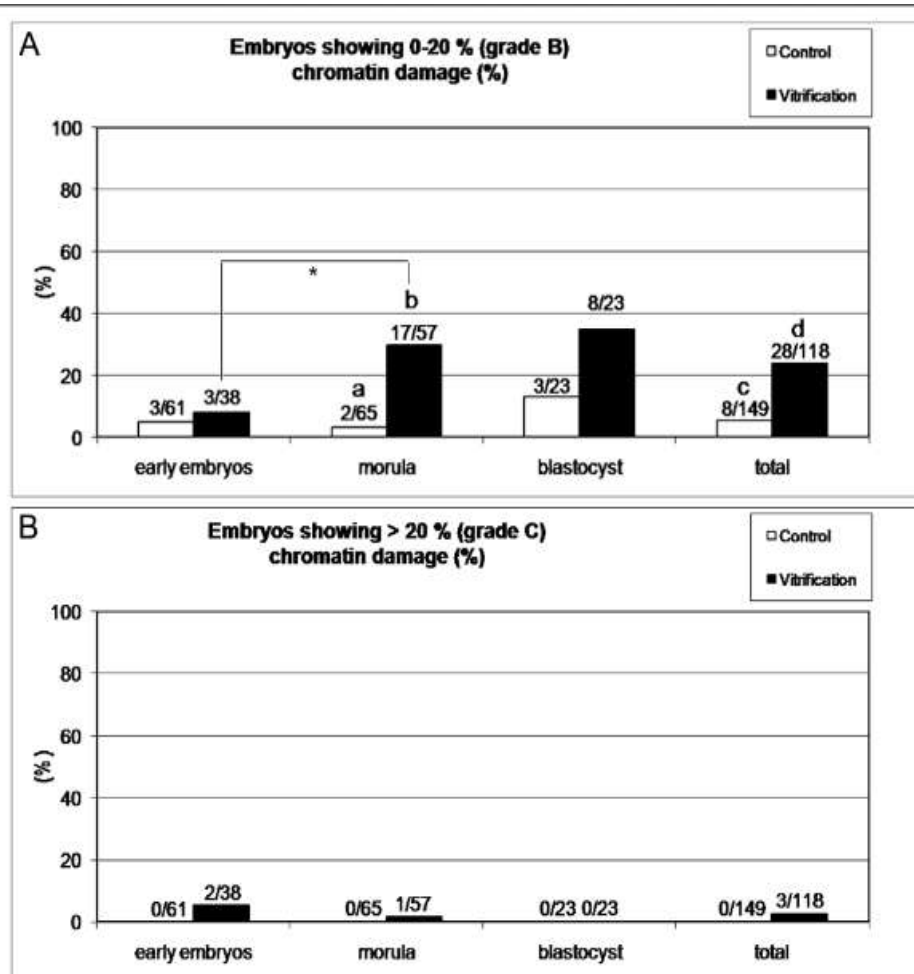
Figure 21. Percentages of embryos showing either 0 to 20% (Panel A: grade B) or >20% (Panel B: grade C) chromatin damage. Numbers of analyzed embryos per group are indicated on the top of each histogram. Chi square test: within each stage: a,b P < 0.001; c,d P < 0.05; between stages: (*) P < 0.05.

Vitrification preserves mt distribution pattern and ROS localizationin mouse embryos

In Figure 20 (panel B), the percentages of embryos showing the P/P (healthy) mt pattern observed after vitrification (black bars), grouped according to their developmental stage, and compared with controls (white bars), are reported. Apparently, vitrification reduced the ratio of embryos showing P/P mt pattern compared to controls (overall data: P<0.05). However, the developmentally-related data showed that vitrification significantly reduced the ratio of embryos showing the P/P mt pattern only at early stages (P<0.001).

In Figure 22, representative photomicrographs of embryo blastomere cytoplasmic shape and texture, as observed after staining and fixing procedures (lane 1) and nuclear chromatin (lane 2) of vitrified-warmed embryos at different developmental stages (rows B, D and F) and

their non-vitrified control counterparts (rows A, C and E), are shown. In addition, Figure 3shows representative photomicrographs of heterogeneous and homogeneous mt distribution pattern (lane 3) with corresponding intracellular ROS localization (lane 4) and mt/ROS merge (lane 5) in vitrified-warmed and control embryos at the 4/16-cell, morula and blastocyst stages. In control fresh embryos (Figure 22, rows A, C, E), MitoTracker signals were detected in all blastomeres in the form of continuous rings around the nuclei and clusters of mitochondria at the cortex (heterogeneous, perinuclear/pericortical, P/P mt pattern), which has been reported in previous studies as an indication of healthy embryos (Zhao et al., 2009 for 2 pronuclear mouse embryos;[8]). Intracellular ROS appeared diffused throughout the cytoplasm in embryonic blastomeres at any stage of development and in both groups (vitrification and controls) apart areas/sites of mt/ROS overlapping (Figure 22, lane 4). In Figure 22, in embryos at the blastocyst stage, a higher number of red fluorescent spots was found on the trophoectoderm compared with the inner cell mass (ICM; E3 and F3), indicating differences in mt number or activation status per cell, between these two embryo lineages. Possibly, an higher mt number and/or aggregate formation of active mitochondria is found in the trophoectoderm compared with ICM. This feature was observed in all groups (23% and 22% for control and vitrified embryos: NS) and thus was not influenced by vitrification procedure.
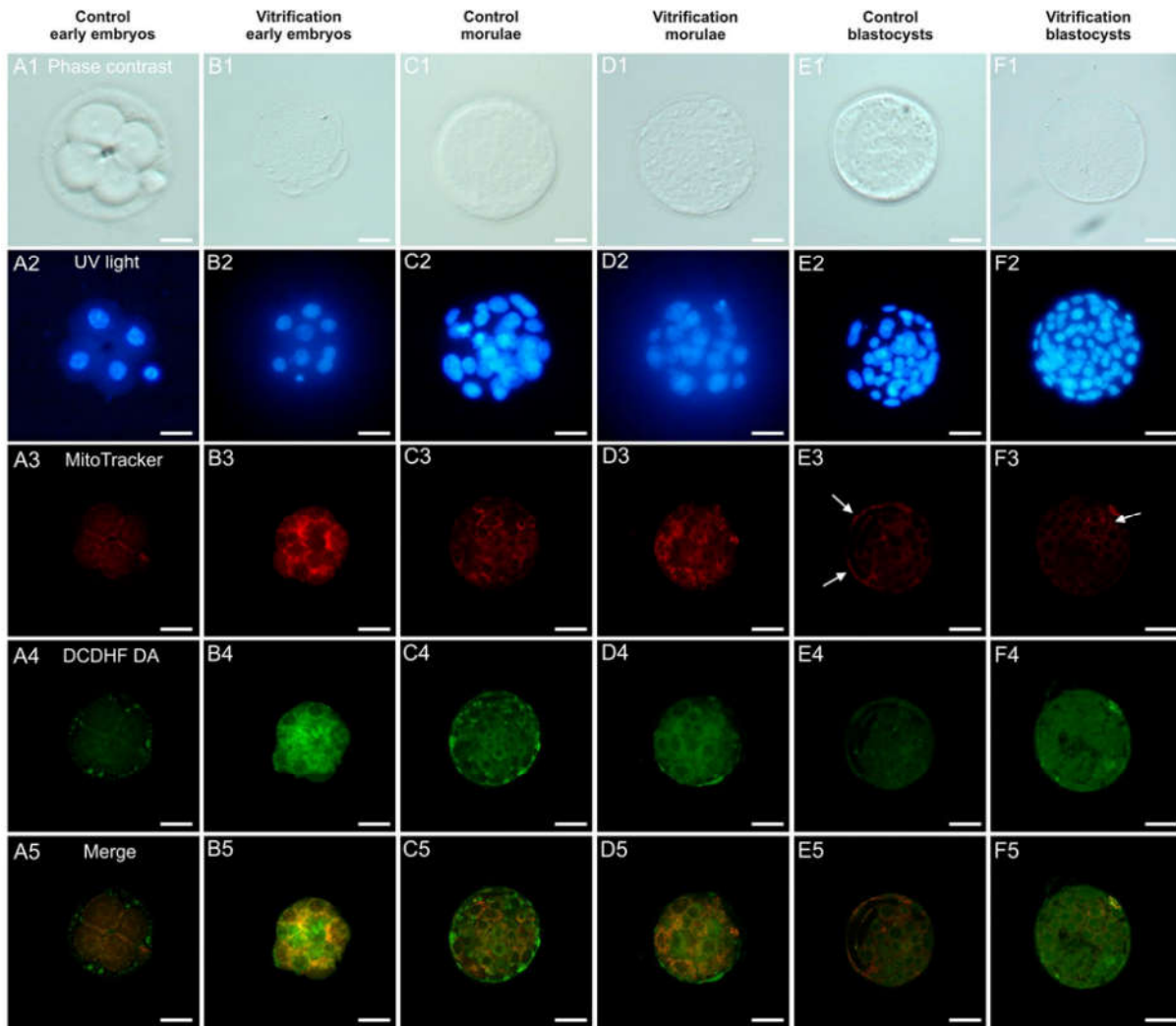
Figure 22. Photomicrographs of fresh and vitrified/warmed mouse embryos at early (4/16-cell), morula and blastocyst stages of development as assessed for their nuclear chromatin and bioenergy/oxidative potential. For each embryo, corresponding bright-field (phase contrast; lane 1), UV light (lane 2) and confocal images showing mt distribution pattern (lane 3), ROS localization (lane 4) and mt/ROS merge (lane 5), are shown. Representative photomicrographs of control non-vitrified and vitrified/warmed embryos at the 4- (row A) and 8-cell (row B), morula (rows C and D) and blastocyst stages (rows E and F), are shown. Nuclear chromatin was stained with Hoechst 33258. MitoTracker Orange, and DCDH FDA were used to label mitochondria and ROS, respectively. In control fresh early embryos and in morulae, in all blastomeres, there were detectable MitoTracker signals in the form of continuous rings around the nuclei and clusters of mitochondria at the cortex, namely perinuclear/pericortical mt pattern (heterogeneous, healthy P/P mt pattern) (A3, C3). Vitrified early embryos (B3) showed a uniform/diffused (homogeneous) mt distribution pattern throughout the blastomere cytoplasm. In embryos at the morula and blastocyst stage, the mt pattern was apparently not affected by vitrification (D3 vs C3 and F3 vs E3). A higher number of red fluorescent spots is evident on the trophoectoderma (white arrows) compared with the inner cell mass, indicating differences in mt number/cell between these two embryo lineages and higher mt/number and

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aggregate formation in the trophoectoderma compared with ICM. This feature can be observed in embryos of both groups, thus it was not influenced by vitrification. ROS intracellular localization (lane 4) corresponded to the distribution pattern of mitochondria. In fresh control embryos, apart areas/sites of mt/ROS overlapping (merge, lane 5), intracellular ROS appeared diffused throughout the cytoplasm (A4, C4, E4). In vitrified (B4, D4 and F4) embryos, diffused MitoTracker and DCDH FDA labelling were evident throughout the cytoplasm. Scale bar represents 20 μm.

Vitrification alters mt activity and ROS levels but preserves mt/ROS colocalization in mouse preimplantation embryos

Mitochondrial activity and intracellular ROS levels were evaluated at the equatorial plane of embryos which were vitrified at the morula or blastocyst stage, having a round shape and thus allowing the confocal quantification software set-up in areas describing continuous surfaces as for oocyte analysis [21]. Mitochondrial activity did not change upon vitrification in embryos at the morula stage, whereas it was reduced in embryos at the blastocyst stage (Figure 22, Panel a; $P<0.05$). Intracellular ROS levels significantly increased in both morulae and blastocyst stage embryos (Figure 23, Panel b; $P<0.001$). Mitochondria/ROS colocalization significantly increased ($P<0.05$) in vitrified versus control embryos in embryos at the morula stage whereas it did not change in embryos at the blastocyst stage (Figure 23, Panel c). In Figure 23, representative samples of mt/ROS colocalization scatterplots for a fresh (panel d) and a vitrified (panel e) blastocyst, are also shown.
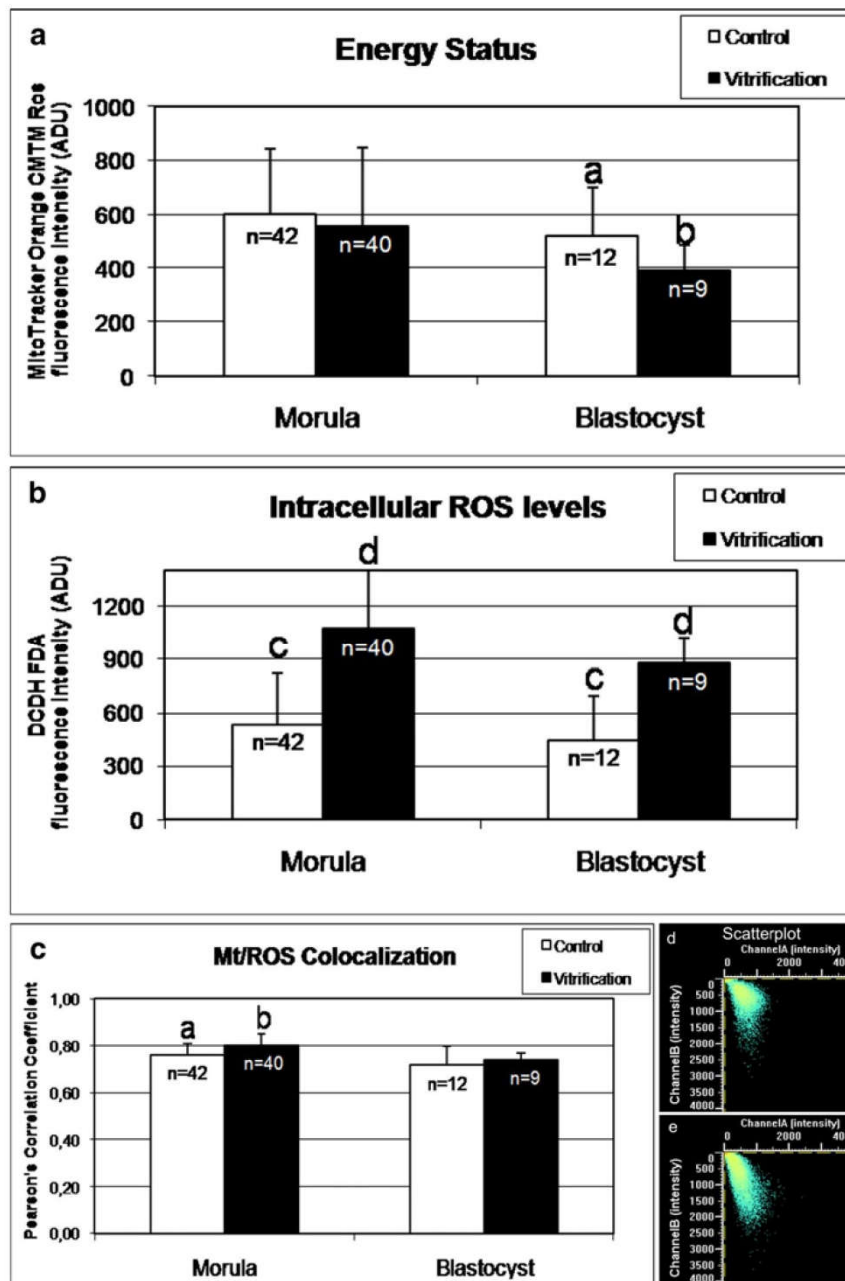
Figure 23. Effects of vitrification on mitochondrial activity, intracellular ROS levels and mt/ROS colocalization in single mouse early (4/16 cell stage) embryos, morulae and blastocysts. In each group, energy status and ROS intracellular levels are expressed as mean±SD of Mitotracker Orange CMTM Ros (a) and DCF (b) fluorescence intensity of individual embryos in arbitrary densitometric units (ADU). In each group, mt/ROS colocalization is expressed as mean±SD of Pearson's correlation coefficient of individual embryos (c). Representative mt/ROS colocalization scatterplots graph of a fresh (panel d) and a vitrified (panel e) blastocyst are shown. In embryos at the morula stage, mt activity did not change after vitrification whereas in embryos at the blastocyst stage, mt activity was significantly reduced (P<0.05). ROS levels significantly increased in vitrified embryos at morula and blastocyst stages (P<0.001). Mt/ROS colocalization significantly increased in vitrified morulae. Numbers of analyzed embryos per group are indicated on the top of each histogram. Student's t-Test: a,b P<0.05; c,d P<0.001.

Vitrification does not affect mouse embryo developmental ability

The results of in vitro embryo culture obtained in the different treatment groups (vitrified, non-vitrified but exposed and non-vitrified/non-exposed to cryoprotectants control embryos) are presented in Table 1. A total of 229 cleavage stage embryos were vitrified/warmed, and out of them 11 were lost (11/229; 4.8%). From the remaining 218 embryos, 202 survived vitrification (202/218; 92.7%) and 180 developed further to expanded blastocysts during in vitro culture (180/202; 89.1%). In the group of embryos non-vitrified but exposed, 86.6% of the embryos developed to expanded blastocysts (65/75). In the group of control embryos (non-vitrified/non-exposed), 91.4% of the embryos developed to expanded blastocysts (75/82).

Groups	N° (%) of cleavage stage embryos	N° (%) of embryos developed in vitro to expanded blastocysts
Vitrified embryos	202	180 (81.9)
Non-vitrified embryos exposed to cryoprotectants	75	65 (86.6)
Non-vitrified/non-exposed embryos	82	75 (91.4)

Table 5. In vitro developmental ability of vitrified mouse embryos.Chi-square test: NS

2.3.3. Discussion

Vitrification slightly affected embryo morphology by increasing the percentage of embryos showing low grade (less than 20%) blastomere cytofragmentation. Embryo fragmentation is clinically considered as an indicator of reduced embryo viability and developmental ability, reduced number of high quality embryos available for establishing pregnancies, thus as a relevant problem for assisted reproduction outcome. In fresh embryos, blastomere fragmentation has been reported to be associated to apoptosis (Yang et al., 1998; Jurisicova et al., 1996; Han et al., 2010) or to apoptosis and necrosis (Chi et al., 2011). A recent study (Han et al., 2010) revealed novel early transcription mechanisms by which maternal genotype affects cytofragmentation by alterating regular cytoskeletal functions. In the present study, in which embryos were examined no more than two hours after warming (including staining procedures), these mechanisms could be only hypothesized. Thus, it can be concluded that vitrification increases only mild cytofragmentation, thus allowing the

preservation of embryo blastomeres integrity, and not reducing the number of embryos available for transfer.

Vitrification increased low level chromatin damage P<0.05). Moreover, only intermediate stages of development were affected. Although not many studies have been published so far, our observations are in line with results of previous studies demonstrating, with different methods, lower damaging effects of vitrification compared with slow freezing on embryo chromatin integrity and function. Vutyavanich et al., 2009 reported significantly higher average number of nuclei in blastocysts derived from embryos vitrified at the 2-cell stage, and cultured in vitro, compared with those obtained after slow freezing. Other studies examined the effects of cryopreservation methods on DNA integrity and stability, as assessed by TUNEL test. Tsang and Chow, 2010 reported significant reduction of DNA integrity after both procedures. Abdel Hafez et al., 2011, reported higher DNA damage extent in vitrified blastocyst versus in early cleavage stage vitrified embryos, may be attributable to differences in blastocoel shrinkage after exposure to vitrification solutions. To our knowledge, this is the first study including all developmental stages and comparing stage-specific effects of vitrification on chromatin integrity of mouse embryos.

Bioenergy/oxidative stress analysis can be performed with several molecular and biochemical methods (Piccoli et al., 2008). Global assessment strategies, such as OMICS technologies, such as Transcriptomics, Proteomics, Metabolomics, are becoming increasingly valuable in this area of investigation (Dell'Aquila et al., 2012). For the specific purpose of assisted reproduction, particularly for research on oocytes and embryos, confocal imaging allows global qualitative and quantitative evaluation of bioenergy/redox parameters in individual samples, also enabling the localization and quantification of functional aberration. As for parameters of cytoplasmic maturity, reduced percentages of embryos showing mt P/P distribution pattern were observed only at early stages of development (4/16-cell stage, P<0.001) compared with fresh control embryos. Moreover, at any stage of development the rate of vitrified embryos showing P/P pattern never dropped below 50% and in total samples, more than 70% of embryos retained this mt pattern. The qualitative analysis of fluorescence, indicative of mt activity, conducted on fresh 4/16-cell stage embryos demonstrated that: 1) mt localization was perinuclear and pericortical, 2) there were blastomeres with intense mt activity while others with almost no activity, and 3) spots of intense mt activity were evident at the level of blastomere cell junctions. This observation is in agreement with those reported by Van Blerkom, 2009, who showed that mt activity can influence or can be influenced by intercellular contacts. Our observations are in agreement with a previous study by Zhao et al., 2009 who reported that in fresh 2PN mouse embryos stained with JC-1, red colored mitochondria (high $\Delta\psi$) were distributed primarily around

pronuclei and along the cell membrane whereas in vitrified-warmed 2 PN embryos, red mitochondria were greatly diminished with green mitochondria (low $\Delta\psi$) evenly distributed throughout the cytoplasm. At the same time, these authors found that the proportion of fresh 2PN embryos with normal aggregation of high $\Delta\psi$ mitochondria (84%) was significantly higher than that of vitrified 2 PN embryos (27%). Observed altered mt distribution could be due to modifications of cytoskeletal elements which have been reported to be involved in cellular movement on a rapid timescale of these organelles (Brookes et al., 2004), other cytoplasmic components, such as endoplasmic reticulum, or to modifications of specific proteins involved in mt anchoring to cytoskeletal microfilaments or microtubules (Perkins et al., 2010). Previous studies in the mouse reported no significant differences after vitrification in microfilament distribution in zygotes, 2 cell embryos, morulae and blastocysts (Tsang and Chow, 2010) and in microtubule formation in 2PN embryos (Zhao et al., 2009).

In embryos at the morula and blastocyst stage, the qualitative analysis showed that mt compartmentalization which at these stages is indicative of developmental stage-dependent acquisition of blastomeres cytoplasmic maturity, was not affected by vitrification. This observation could be related to major cryotolerance but also to a greater difficulty in visualizing mt distribution modification in embryos at these stages due to reduced blastomere cytoplasmic size. Qualitative analysis also showed that mt activity of trophoectodermal cells in blastocyst stage embryos is more intense than that observed in the cells of the inner cell mass and that blastomeres showing strong mt activity were located nearby the blastocoelic cavity. This observation is in agreement with those reported by Van Blerkom, 2011, who showed that the maintenance of the blastocoele and its rapid recovery after collapse and hatching phase, are morphodynamics activities that require huge production of ATP from the cells of the trophoectoderma. Instead, cells in the inner cell mass, which are not involved in these activities, appear to be metabolically quiescent in these developmental phases. Quantitative analysis of ATP production in mouse blastocysts showed that approximately 80% of ATP produced by the embryo is from the trophoectoderma and that the number of mitochondria observed by confocal laser microscopy are located well below in the inner cell mass.

Quantification analysis in the present study was performed in embryos at the morula and blastocyst stage. A statistically significant reduction of MitoTracker fluorescence intensity was found in vitrified blastocysts compared with their fresh counterparts, indicating significant reduction of mt activity at this stage of development. In embryos at the morula stage, vitrification had no effects on mt activity. More interestingly, both in embryos at the morula and blastocyst stage, significant increase of DCF fluorescence intensity, indicative of an excess production of ROS, was found after vitrification. This finding could be interpreted

considering that the conditions used for the vitrification method could have resulted in the onset of oxidative stress condition. This observation could be consistent with the reported up-regulation of genes involved in the mechanisms of oxidative stress (Hsp70, MnSOD, CuSOD) in mouse vitrified embryos (Tsang and Chow, 2010). Increased oxidative stress in vitrified/warmed embryos in the present study, kept in vitro no more than two hours after warming, could be an initial effect, as suggested by Tsang and Chow, (2010) who reported that stress-related gene expression dropped down to normal levels within 7 hours after warming. Colocalization of intracellular free radicals (ROS) and actively respiring mitochondria has been reported as indicative of higher ATP turnover resulting from a more intense mt activity and thus indicative of healthy cell conditions in ovine in vivo matured ovulated metaphase II oocytes (Martion et al., 2012) and in hepatocytes. By this analysis, it came out that mt/ROS colocalization was not affected by vitrification in blastocyst stage embryos and it was increased in embryos at the morula stage, may be due to increased ROS generation. To our knowledge, this is the first study reporting mt/ROS colocalization, objectively expressed as Pearson's correlation coefficient, for the comparison between fresh and vitrified mouse embryos.

Taken together our data allow to confirm that morula and blastocyst are good stages from the standpoint of embryo viability after vitrification. In vitrified morulae, only low level chromatin damage was found and bioenergy/redox parameters were positively affected. In fact, mt pattern was not affected and increased oxidative activity and consequent increased mt/ROS colocalization were found. In vitrified blastocysts, neither nuclear chromatin nor mt pattern were affected; a significant reduction of mt activity was found but ADU absolute values remained at consistent levels (395.1±89.3 versus 522.4±176.8 for vitrified vs fresh, respectively), indicating that vitrified embryos retained/kept a good/substantial part of mt activity. As well, in vitrified blastocysts increased respiratory/oxidative activity was found, as observed by increased ROS generation.

2.3.4. Conclusions

The vitrification technology only slightly affects embryo morphology, chromatin integrity and energy/oxidative status in a developmentally-related manner. This embryo cryopreservation method is of great scientific and clinical interest and application in both human and animal assisted reproduction. Global assessment strategies for embryo quality evaluation, such as confocal 3D imaging, can significantly contribute to the improvement of routine use crypreservation protocols, as well as to identify appropriate conditions to preserve nuclear and cytoplasmic integrity and competence to ensure proper embryonic development in the uterine environment and that the pregnancy could come to term. This study shows for the

first time the joint assessment of mitochondrial activity and levels of ROS in mouse embryos in relation to their developmental stage and the application of a vitrification procedure.

2.4. Experiment 6

2.4.1. Methods

Embryo recovery and in vitro culture

Embryo recovery and in vitro culture of embryos were carried out in the same way as in Experiment 5.

Slow programmable freezing

After equilibration in a medium containing 10% glycerol, Dulbecco phosphate bufferd saline (DPBS + 10% FCS + 10% glycerol) for 10-15 min, the embryos were sucked up into straws (5 embryo per straw) (34-37). The straws containing the embryos were then transferred into a Planer freezing machine (PLANER R 205, Planer, Sunbury-on-Thames, Middlesex UK) 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: 5 min in medium containing 6% glycerol + 0.3 M sucrose; 5 min in medium containing 3% glycerol + 0.3 M sucrose; 5 min in medium containing 0.3 M sucrose and, finally, 5 min in PBS + 20% FCS.

Vitrification

Vitrification of embryos was carried out in the same way as in the Experiment 5.

Mitochondria and ROS staining

Mitochondria and ROS staining were carried out in the same way as in the Experiment 5.

Detection of the nuclear chromatin status

Detection of nuclear chromatin status was carried out in the same way as in the Experiment 5.

Mitochondrial distribution pattern and intracellular ROS localization

Investigation of mt distribution pattern and intracellular ROS localization were carried out in the same way as in the Experiment 5.

Quantification of Mitotracker Orange CMTM Ros and DCF fluorescence intensity

The same method as in the Experiment 5 was applied.

Mitochondria/ROS colocalization analysis

The same method as in the Experiment 5 was applied.

Statistical analysis

The rates of embryos showing different degrees of chromatin damage were compared between treated (SF or VF) and control groups and between treatments (FS vs VF) by the Chi-square test with the Yates correction for continuity. The rates of embryos showing different mt distribution pattern were compared between treated (SF or VF) and control groups and between treatments (FS vs VF), as a whole or as separated data according to chromatin damage level, whether of grade A, B or C, by the Chi-square test with the Yates correction for continuity. The Fisher's exact test was used when a value of less than 5 was expected in any cell. For confocal quantification analysis of mt activity and intracellular ROS levels, the least-square means of the dependent variable (Mitotracker CMTM Ros and DCF fluorescence intensity) were calculated in examined samples and the statistical significance of the least-square means between treated and control groups was calculated by one-way ANOVA followed by Multiple Comparison Dunn's or Dunnett's methods (SigmaPlot software). For mt/ROS colocalization, mean values of Pearson's correlation coefficient were compared between treated and control groups by one-way ANOVA followed by Multiple Comparison Dunn's or Dunnett's methods (SigmaPlot software). Differences with p<0.05 were considered as statistically significant.

2.4.2. Results

Three hundred and six mouse embryos, 69% of which were at the morula (M, n=210) and 31% at the blastocyst (Bl, n=96) stage, were randomly allocated as non-frozen controls (n= 88 embryos, 65 morulae and 23 blastocysts) or slowly frozen/controlled rate freezing (SF, n=75 embryos, 49 morulae and 26 blastocysts) or vitrified/ultrarapid freezing (VF, n= 143 embryos, 96 morulae and 47 blastocysts) groups. Survival rates after freezing-thawing were at least 80% in both procedures (not significant, data not shown).

Cryopreservation affects chromatin integrity at a greater extent at the morula than the blastocyst stage

After both CP procedures, either SF or VF, chromatin damage was observed as formation of micronuclei or lobulated nuclei. In Figure 24 (Panel A), a percentage bar graph is reported in which embryos were graded as A, B and C, according to the described criteria (see Materials and Methods): grade A, embryos having all blastomeres with intact chromatin (Figure 24, Panel A, white segments), grade B, embryos having less than 20% blastomeres with damaged chromatin (gray segments) and grade C, embryos having more than 20% blastomeres with chromatin damage (black segments). In Figure 24 (Panel B), representative photomicrographs of embryos at the morula and blastocyst stage, and classified as grade A, B or C, are shown.
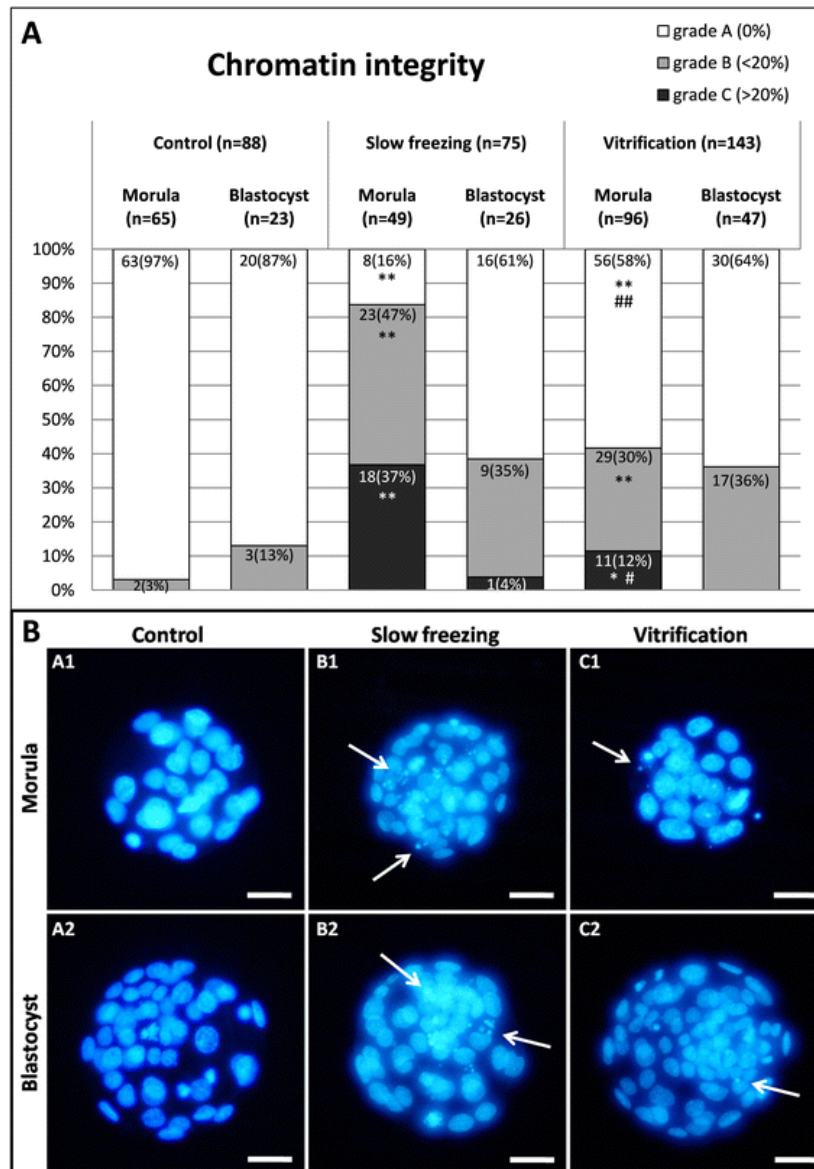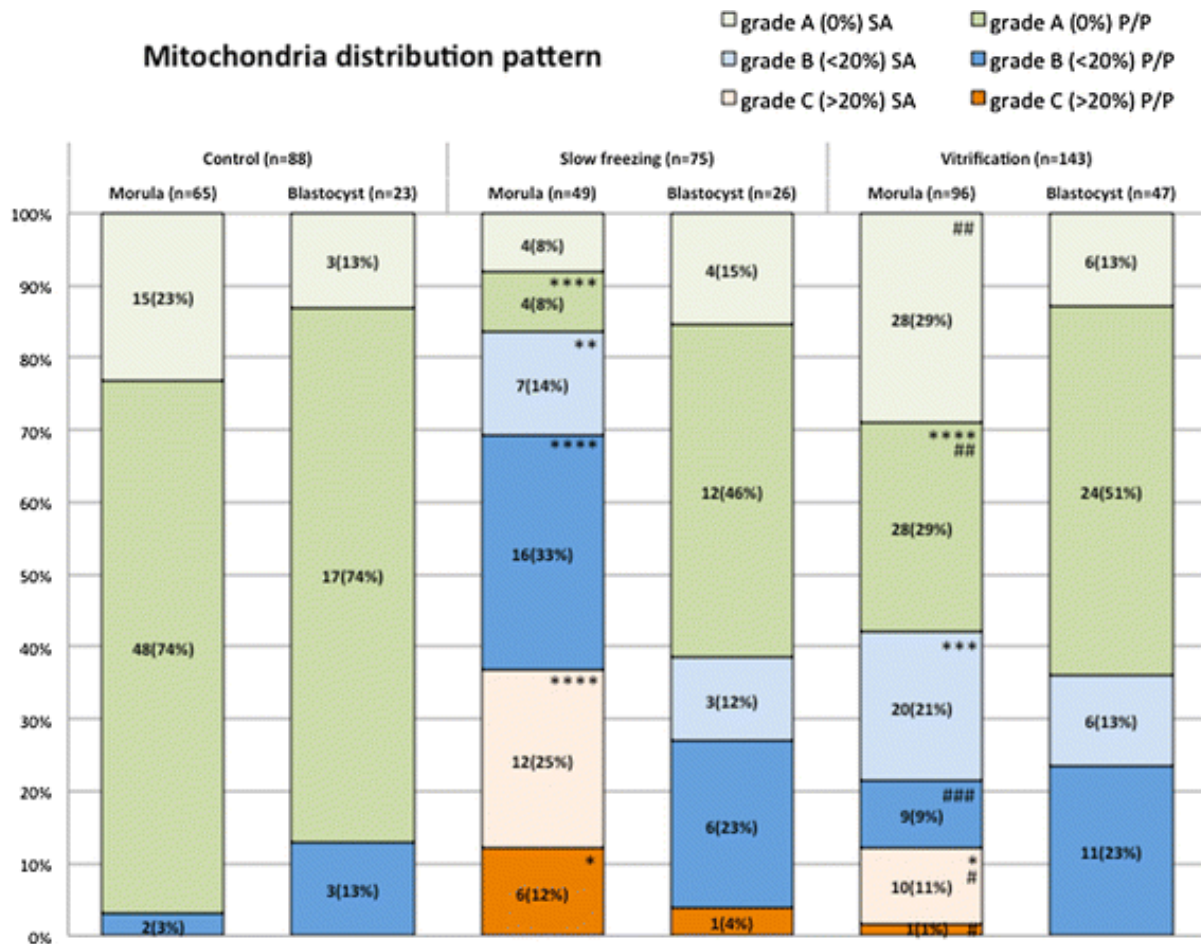
Figure 24. Effects of slow freezing and vitrification on chromatin integrity of mouse embryos at the morula and blastocyst stage. Panel A: percentages of embryos graded according to chromatin damage (for details and criteria see M&M) as grade A (no damage, white segments), grade B (slight damage, gray segments) or grade C (severe damage, black segments). Embryos were grouped according to their developmental stage, observed after slow freezing/thawing or vitrification/warming and compared with controls. Numbers of analyzed embryos per group are indicated on each histogram and segment. Chi square test with the Yates correction: comparisons slow freezing vs control and vitrification vs control: *P<0.05; **P<0.0001; comparisons slow freezing vs vitrification: # P<0.001; ## P<0.0001. Panel B: Representative photomicrographs of control grade A morula (A1) and control grade A blastocyst (A2), slow frozen grade C morula (B1) and slow frozen grade C blastocyst (B2), vitrified grade B morula (C1) and vitrified grade B blastocyst (C2) are shown. The nuclei of embryos were stained with Hoechst 33258. For each embryo, UV light images are shown. Arrows indicate signs of chromatin damage: white thin arrows indicate micronuclei and white thick arrows indicate lobulated nuclei. Scale bar represents 20 μm.

In the control group, the majority of embryos were of grade A (for M+Bl, 94%, 83/88), few embryos were of grade B (for M+Bl, 6%, 5/88) and no grade C embryos were found. In the SF group, the rates of grade A embryos were consistently reduced. In fact, significantly higher rates of grade B and C embryos were found (for M+Bl: 32/75, 43% vs 5/88, 6%, $P<0.0001$ and 19/75, 25% vs 0/88, 0%, $P<0.0001$ for B and C, respectively) compared with controls. In this group, 14 embryos (out of 19 grade C embryos) had less than 50% cells with chromatin damage and 5 embryos had more than 50% blastomeres with chromatin damage. In the VF group, the rates of grade B and grade C embryos also increased but at a lesser extent than in the SF group (for M+Bl: 46/143, 32% vs 5/88, 6%, $P<0.0001$ and 11/143, 8% vs 0/88, 0%, $P<0.05$ for B and C, respectively). In this group, all C embryos showed less than 50% blastomeres with chromatin damage. Furthermore, SF induced significantly higher chromatin damage than VF. In fact, even if the rates of grade B embryos did not change between the two methods (32/75, 43% vs 46/143, 32%, for SF and VF respectively, not significant) the rate of grade C embryos was significantly higher in the SF group than in the VF one (19/75, 25% vs 11/143, 8%, $P<0.001$).

As for developmental stage-related effects, both CP procedures affected chromatin integrity of mouse pre-implantation embryos, with greater extent in morulae compared with blastocysts (Figure 24, Panel A). The rates of grade B and grade C morulae significantly increased after SF (23/49, 47% vs 2/65, 3%, $P<0.0001$ and 18/49, 37% vs 0/65, 0%, $P<0.0001$ for B and C morulae, respectively) and VF (29/96, 30% vs 2/65, 3%, $P<0.0001$ and 11/96, 12% vs 0/65, 0%, $P<0.05$ for B and C morulae, respectively) compared with controls. As well, even if the rates of grade B morulae did not change between the two methods (23/49, 47% vs 29/96, 30%, for SF and VF respectively, not significant), the rates of grade C morulae was significantly higher after SF than VF (18/49, 37% vs 11/96, 11%, $P<0.001$). Conversely, chromatin integrity of embryos at the blastocyst stage was not affected by both CP methods, as no statistical differences were found among groups and comparable rates of grade A and grade B blastocysts were found, irrespectively of the CP treatment.

Cryopreservation affects mitochondrial aggregation/distribution pattern at a greater extent at the morula than the blastocyst stage

In both CP techniques, mt aggregation/distribution pattern in mouse pre-implantation embryos was found as either heterogeneous (pericortical/perinuclear distribution of mt clusters, P/P; Figure 25, grainy segments) or homogeneous (small mt aggregates, SA; smooth segments).

Figure 25. Effects of slow freezing and vitrification on mitochondrial distribution pattern of mouse embryos at the morula and blastocyst stage. Percentages of embryos graded according to chromatin damage as grade A, grade B or grade C, as in Figure 1, and further divided as having perinuclear/pericortical (P/P; apple-green, blue and brick-red, respectively) or homogeneous mt distribution pattern in small aggregates (SA; pale colours). Embryos were grouped according to their developmental stage, observed after slow freezing or vitrification and compared with controls. Numbers of analyzed embryos per group are indicated on each histogram and segment. Chi square test with the Yates correction: comparisons control vs slow freezing and control vs vitrification: *P<0.05; **P<0.01;***P<0.001; ****P<0.0001; comparisons slow freezing vs vitrification: #P<0.05; ##P<0.01; ###P<0.001.

In the control group, the majority of embryos had P/P mt pattern. In fact, 80% (70/88) P/P and 20% SA embryos (18/88) were found. In both CP groups, the rates of embryos showing P/P pattern tended to decrease and corresponding increased rates of embryos showing the SA mt pattern were observed. In fact, significantly lower rate of embryos with P/P mt pattern was found in the SF (for M+Bl, grade A+B+C: 45/75, 60% vs 70/88, 79%, for SF vs controls, respectively; P<0.05) and VF group (for M+Bl, grade A+B+C: 73/143, 51% vs 70/88, 79%, for VF vs controls, respectively; P<0.0001) compared with controls. Furthermore, no differences

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were revealed between the two methods, as the comparisons between the rates of embryos with P/P pattern was not significantly different between SF and VF embryos (for M+Bl, grade A+B+C: 73/143, 51% vs 45/75, 60%, for VF vs SF, respectively; NS).

Cryopreservation methods differently affected mt pattern of mouse embryos according to their developmental stage, with greater extent in morulae compared with blastocysts. The rates of morulae with P/P pattern was significantly reduced after SF (26/49, 53% vs 50/65, 77%, for SF and controls respectively; $P<0.05$) and VF (38/96, 40% vs 50/65, 77%, for VF and controls respectively; $P<0.0001$) compared with controls. However, the comparison between the rates of heterogeneous morulae issuing from the two methods was not statistically significant (26/49, 53% vs 38/96, 40%, for SF and VF respectively, NS). In embryos at the blastocyst stage, mt aggregation/distribution pattern was not affected by both CP procedures, as no significant differences were found among groups. Comparable rates of blastocysts showing P/P mt pattern were found, irrespectively of the CP treatment (19/26, 73% vs 20/23, 87% for SF and controls, respectively, NS; 35/47, 74% vs 20/23, 87% for VF and controls, respectively, NS; 19/26, 73% vs 35/47, 74% for SF and VF, respectively; NS).

Further, the effects of CP on mt pattern varied according to embryo grade with heavier effects observed in grade C embryos. As overall data (including morulae and blastocysts), no differences were found between grade A and grade B embryos (133/193, 69% vs 47/83, 57% for grade A and B, respectively; NS) whereas significantly lower rates of embryos showing P/P mt pattern were found in grade C embryos compared with grade A (8/30, 36% vs 133/193, 69% for grade C and A, respectively; $P<0.0001$) and grade B embryos (8/30, 36% vs 47/83, 57%, for grade C and B, respectively, $P<0.01$). In grade A morulae, the P/P pattern was found at comparable rates (white grainy segments) in SF (4/8, 50%) and control groups (48/63, 76%; NS) whereas it was significantly reduced in the VF group (28/56, 50%) compared with controls (48/63, 76%; $P<0.01$). In this group (grade A morulae), no statistical differences were found between methods (4/8 50%vs 28/56, 50% for SF vs VF grade A morulae, respectively; NS). In grade B morulae (gray grainy segments), no differences were found in the rates of embryos showing the P/P pattern between each CP method and controls (16/23, 69%, for FS 9/29, 31% for VF and 2/2, 100% for controls; NS). However, significantly lower rates were found in the VF group compared with the SF one (9/29, 31% vs 16/23 69% for VF vs SF, respectively; $P<0.05$). In grade C morulae (black grainy segments), statistical comparisons for SF vs controls and VF vs controls were not feasible, and the comparison SF vs VF was not statistically significant. For any embryo category, no statistical differences were found between treatments groups in embryos at the blastocyst stage, as all comparisons were not statistically significant.
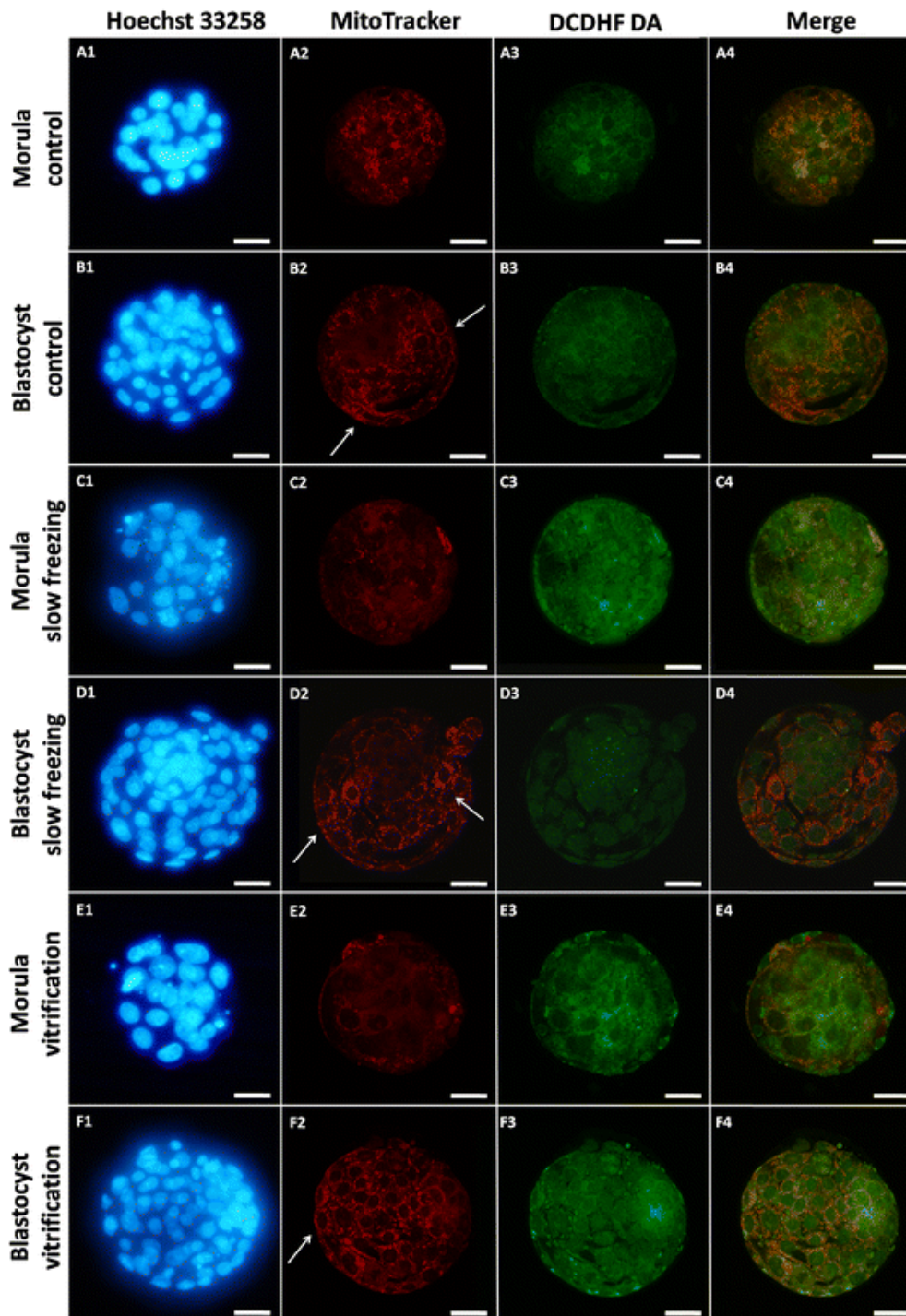
Figure 26. Photomicrographs of frozen/thawed and vitrified/warmed mouse embryos at the morula and blastocyst stage of development as assessed for their nuclear chromatin and bioenergy/oxidative potential. MitoTracker Orange, and DCDH FDA were used to label mitochondria and ROS, respectively. Nuclear chromatin was stained with Hoechst 33258. Representative photomicrographs showing mt distribution pattern and ROS intracellular localization in a control morula (row A) and a control blastocyst (row B) with P/P mt pattern, a SF morula with SA pattern (row C), a SF blastocyst with P/P pattern (row D), a VF morula with SA pattern (row E) and a VF blastocyst with P/P pattern (row F). In embryos at the blastocyst stage, a higher number of red fluorescent spots is

evident on the trophoectoderma (white arrows) compared with the inner cell mass, indicating differences in mt number/cell between these two embryo lineages and higher mt/number and aggregate formation in the trophoectoderma compared with ICM. This feature can be observed in embryos of both groups, thus it was not influenced by cryopreservation. For each embryo, the corresponding epifluorescence images showing nuclear chromatin (line 1) and confocal images showing mt distribution pattern (line 2), ROS localization (line 3), mt/ROS merge (line 4) are shown. Scale bar represents 20 µm.

In Figure 26, representative photomicrographs of mouse morulae and blastocysts of control (rows A, B), slow freezing (rows C, D) and vitrification (rows E, F) groups and showing nuclear chromatin configuration (lane 1), and corresponding P/P or SA mt pattern (lane 2), intracellular ROS localization (lane 3) and mt/ROS merge (lane 4) are reported. In morulae and blastocysts with heterogeneous P/P mt pattern, in all blastomeres, there were detectable highly fluorescent signals of the mt-specific probe in the form of continuous rings around the nuclei and clusters of mitochondria at the cortex (Figure 3, A2, B2, D2, F2) which was reported in previous studies as indication of healthy embryos (51-52]. Due to reduced blastomere cytoplasmic size in embryos at the morula or blastocyst developmental stages, P/P mt clustering were almost overlapping. In morulae and blastocysts with homogeneous SA pattern, small mt aggregates diffused throughout the blastomere cytoplasm were observed (Figure 26, C2, E2). In addition, in embryos at the blastocyst stage, a higher number of red fluorescent spots was found on the trophoectoderm compared with the inner cell mass, indicating differences in mt number/cell between these two embryo lineages and higher mt/number and aggregate formation in the trophoectoderm compared with ICM (Figure 3, B2, D2, F2). This feature was observed in all groups (22% (5/23], 23% (6/26] and 32% (15/47] for control, SF and VF embryos: not significant) thus it was not influenced by CP procedures. Intracellular ROS localization did not vary upon CP and intracellular ROS appeared diffused throughout the cytoplasm of embryonic blastomeres at any stage of development (Figure 26, lane 3) apart areas/sites of mitochondria/ROS overlapping (Figure 26, lane 4).

Cryopreservation alters quantitative bioenergy/redox parameters at a greater extent at the morula than the blastocyst stage

Mitochondrial activity, intracellular ROS levels and mitochondria/ROS colocalization were evaluated at the equatorial plane of embryos which were cryopreserved at the morula or blastocyst stage, having a round shape and thus allowing the confocal quantification software set-up in areas describing continuous surfaces (39, 52]. Energy status, expressing embryonic mt activity, was significantly reduced upon application of both cryopreservation

procedures in embryos at the morula stage, whereas it did not change in embryos at the blastocyst stage (Figure 27, Panel a; P<0.05). Intracellular ROS levels were significantly increased in VF embryos at the morula stage (Figure 27, Panel b; P<0.05) compared with controls whereas they did not change in blastocyst stage embryos. Moreover, ROS levels were significantly increased in VF compared with SF embryos. Mitochondria/ROS colocalization significantly increased in VF embryos compared with controls in both developmental stages (Figure 27, Panel c; P<0.05).
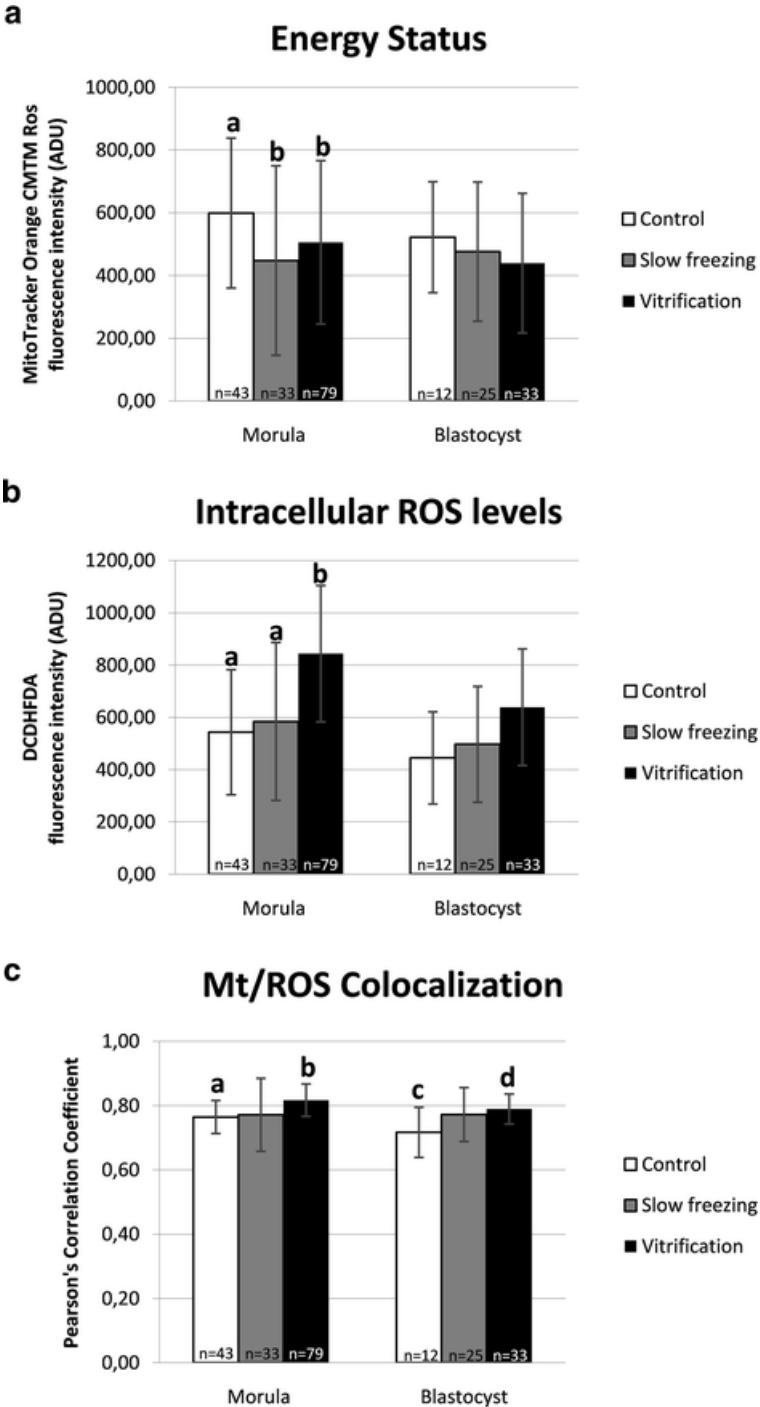
Figure 27. Effects of slow freezing and vitrification on mitochondrial activity, intracellular ROS levels and mt/ROS colocalization in single mouse morulae and blastocysts. In each group, energy status and ROS intracellular levels are expressed as mean±SD of Mitotracker Orange CMTM Ros (Panel a) and DCF (Panel b) fluorescence intensity of individual embryos in arbitrary densitometric units (ADU) and mt/ROS colocalization is expressed as mean±SD of Pearson's correlation coefficient of individual embryos (Panel c). In embryos at the morula stage, energy status, expressing embryonic mt activity, was reduced by cryopreservation (P<0.05) whereas in embryos at the blastocyst stage, it did not change. In embryos at the morula stage, ROS levels increased after vitrification (P<0.05) whereas no changes were observed in embryos at the blastocyst stage. Vitrified morulae and blastocysts showed significantly higher Pearson's correlation coefficient than controls (P<0.05). Numbers of analyzed embryos per group are indicated on the bottom of each histogram. One-way ANOVA followed by Multiple Comparison Dunn's method: comparisons among morula stage embryos: a,b P<0.05; comparisons among blastocyst stage embryos: c,d P<0.05.

In Figure 28, quantification bioenergy/redox data were separated according to embryo grade. Due to the absence of grade C embryos in control morulae and blastocysts and in vitrified blastocysts, the column representing grade C embryos is lacking in these groups. In addition, only one grade B morula, one grade B blastocyst and one grade C blastocyst were examined, thus data of these samples are represented as single values and were not statistically analyzed. Statistical analysis revealed that energy status of SF grade C morulae was significantly lower than that of control grade A morulae (Figure 5, Panel a; P<0.05). No differences were found for this parameter between treatments (SF vs controls, VF vs controls and SF vs VF) among grade A and among grade B embryos. No differences were found for intracellular ROS levels and mt/ROS colocalization between treatment and for any embryo grade.
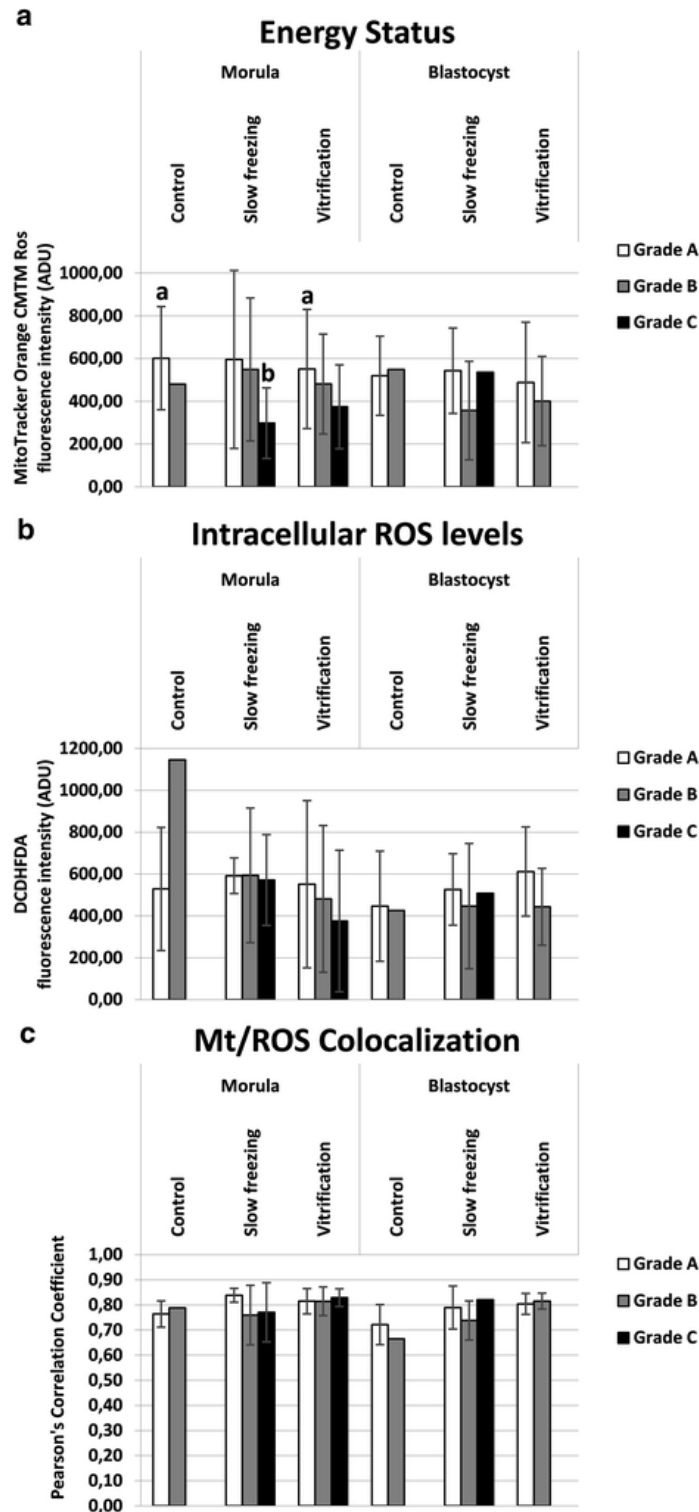
Figure 28. Effects of slow freezing and vitrification on mitochondrial activity, intracellular ROS levels and mt/ROS colocalization in single mouse morulae and blastocysts, as related to embryo grade. In each group, energy status, intracellular ROS levels and mt/ROS colocalization are expressed as in Figure 4. In grade C SF morulae, energy status was significantly lower than in control grade A and in vitrified grade A morulae ($P<0.05$). Numbers of analyzed embryos per group are indicated on the bottom of each histogram. One-way ANOVA followed by Multiple Comparison Dunn's method: a,b $P<0.05$.

Figure 29 shows quantification bioenergy/redox data separated according to mt pattern. Due to the absence of control blastocyst with SA pattern with regular round shape, the corresponding column is lacking. Statistical analysis revealed that, for energy status, no significant differences between SF and controls for embryos having either P/P or SA pattern were observed, indicating that both embryo types contributed equally to energy status reduction observed after SF. Instead, energy status was significantly higher in VF embryos with P/P mt pattern compared with those having SA mt pattern, both at the morula and the blastocyst stage, indicating that, mt activity reduction in vitrified embryos is associated with the appearance of the SA pattern (Figure 29, Panel a; P<0.05); 2) for intracellular ROS levels, significantly higher values were observed in VF embryos with P/P pattern compared with fresh ones with P/P pattern, both in morulae and blastocysts (Figure 29, Panel b; P<0.05); 3) the same significances were revealed for mt/ROS colocalization (Figure 29, Panel c; P<0.05). In embryos showing SA mt pattern, no differences were observed for any bioenergy/redox parameter between both treatments and controls.
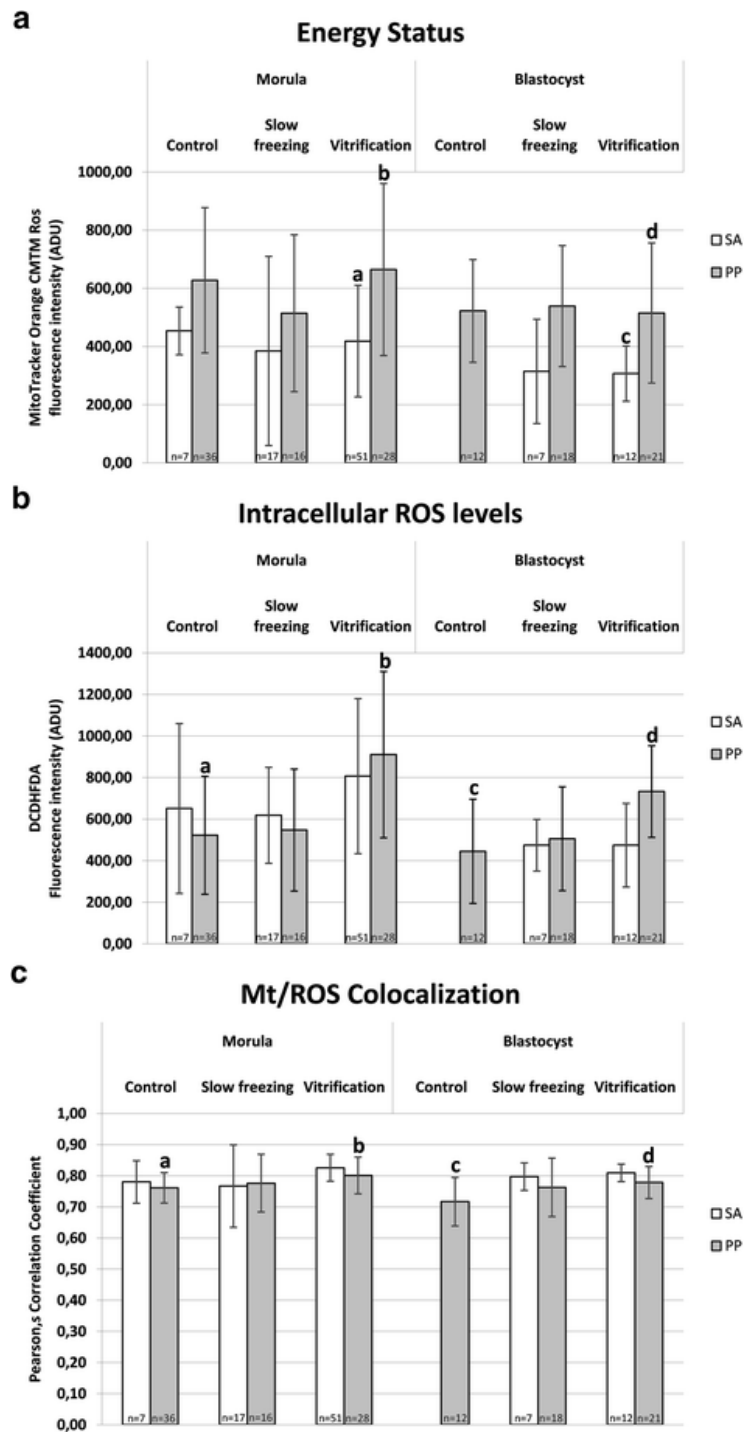
Figure 29. Effects of slow freezing and vitrification on mitochondrial activity, intracellular ROS levels and mt/ROS colocalization in single mouse morulae and blastocysts, as related to mt pattern. In each group, energy status, intracellular ROS levels and mt/ROS colocalization are expressed as in Figure 4 and Figure 5. Vitrified embryos with P/P pattern had significantly higher energy status than their SA counterparts and significantly higher ROS levels and mt/ROS colocalization than P/P controls (P<0.05). Numbers of analyzed embryos per group are indicated on the bottom of each histogram. One-way ANOVA followed by Multiple Comparison Dunn's or Dunnett's met hods: comparisons among morula stage embryos: a,b P<0.05; comparisons among blastocyst stage embryos: c,d P<0.05.

2.4.3. Discussion

While the impact of CP on the integrity of the oocyte/embryo plasma membrane, organelles, and spindle cytoskeleton have been the focus of most studies to date, the short- and long-term consequences of CP and cryoprotectants on the nuclear chromatin integrity has received much less attention (Albertini and Olsen, 2013). In our study, both SF and VF significantly reduced the percentages of grade A embryos, thus impairing 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 grade 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 ($P<0.001$). Conversely, VF-induced chromatin damage was lower compared to SF (as assessed by prevailing appearance of grade B but no grade C embryos) and was equivalent in the morula and the blastocyst stage, as 58% of vitrified morulae and 64% of vitrified blastocysts had grade A (not significant).

Although not many studies have been published so far, our observations are in agreement with the results of previous studies. Even with different detection methods, damaging effects of CP procedures (SF and VF) on embryo chromatin integrity and function have been reported (Vutyavanich et al., 2009; Jurisicova et al., 1996; Han et al., 2010). Isachenko et al (2008) found that the nuclear chromatin integrity rate after CP of pronuclear zygotes was a predictor of future embryo development and implantation. Tsang and Chow (2010) reported significant reduction of DNA integrity in embryos after both SF and VF. Vutyavanich et al., (2009) obtained significantly higher average number of nuclei in blastocysts derived from embryos vitrified at the 2-cell stage, and cultured in vitro, compared with those obtained after SF. Although the fact of morula being more cryosusceptible is well known (Massip, 2001), to our knowledge, our study is the first comparing developmental stage-specific effects of SF and VF on chromatin integrity of mouse morulae and blastocyst embryos.

Cryopreservation may have an effect on the structures of the embryo, but very mismatching data have been published up to now. Zhao et al (2009) observed that VF affected mitochondrial distribution, microtubule distribution and reduced the mitochondrial membrane potential in mouse 2-PN embryos, events which may have an influence on subsequent developmental viability of such embryos. However, Noto et al (1993) found that freezing did not affect subcellular structures and the mitochondrial distribution appeared untouched after freezing – thawing. Their conclusion was that subcellular structures are rather resistant targets for cryoinjury. Whole embryo confocal imaging allows qualitative and quantitative evaluation of several aspects of mt activity in single embryos, thus allowing to localize and

quantify functional aberrations. Taking into consideration the mt aggregation and distribution pattern as a qualitative parameter of mt activity and cytoplasmic maturity, we found that both CP procedures, SF and VF, significantly reduced the rates of embryos showing the P/P mt pattern which indicative of cytoplasmic activity and maturity. In the group of grade B morulae, higher rates of SA patterns were found after VF than SF. It also came out that SF and VF affected mt pattern differently in relation to embryo developmental stage, with more relevant damage at the morula than the blastocyst stage. 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 pattern, which could correlate each other with loop mode. Experiences obtained with fresh morulae and blastocyst embryos indicated that: 1) mt localization was P/P in blastomeres of embryos at both the morula and blastocyst stage, 2) there were blastomeres with intense mt activity while others with lower activity, and 3) in embryos at the blastocyst stage, mt activity of trofoectodermal cells was more intense compared to cells located in the inner cell mass and blastomeres with strong mt activity were located nearby the blastocoelic cavity. Similarly, Van Blerkom (2011) found that the maintenance of the blastocoel and its rapid recovery after the collapse and the hatching phase, are morphodynamics activities that require huge production of ATP from the trofoectodermal cells than the inner cell mass. Data obtained in mouse blastocysts show that 80% of ATP produced by the trofoectodermal cells and the number of mitochondria detected by confocal laser microscopy is very low in the inner cell mass. 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 mt activity at this stage of development after both CP procedures. In blastocysts, no significant effects were observed, even though data tended toward reduction after both procedures (Figure 27), as it was observed in a previous study from our unit (Martino et al., 2013). Interestingly, energy status reduction observed in slow frozen morula-stage embryos cannot be associated with a specific level of chromatin damage, as slow frozen morulae did not differ for energy status among grade groups. Nevertheless, slow frozen grade C morulae showed significantly lower mt activity than grade A control and vitrified morulae (P<0.05, Figure 28). Thus indicates that SF could induce a joint chromatin and energy status damage, however further studies are needed to confirm this hypothesis. In the VF group, energy status was also associated with P/P mt pattern, as morulae and blastocysts with P/P mt pattern showed higher mt activity than those with SA mt pattern (P<0.05 for VF embryos, Figure 29). In embryos at the morula stage, a statistically significant increase of DCF fluorescence intensity, indicative of an excess production of ROS, was found after VF whereas ROS generation did not change after SF. ROS level variations were neither associated with embryo chromatin grade (Figure 28, NS) nor with mt pattern. Nevertheless,

VF morulae and blastocysts with P/P mt pattern showed higher ROS staining than their corresponding controls (P<0.05, Figure 29). This finding confirms previous observations from our unit (Martino et al., 2013) and allow us to lean more towards the hypothesis that the conditions used for the VF method may have stimulated oxidative activity and therefore cell viability rather than resulting in the onset of oxidative stress conditions. On the other hand, SF could have induced loss of embryo viability, as observed by reduced energy status and non increased ROS levels.

In the present study, mt/ROS colocalization was significantly increased by VF, both at the morula and blastocyst stage, but it was not increased by SF. These variations were neither associated with embryo chromatin grade (Figure 28, NS) nor with mt pattern (Figure 29, NS). To our knowledge, this is the first study reporting mt/ROS colocalization, objectively expressed as Pearson's correlation coefficient, for the comparison between SF and VF in mouse preimplantation stage embryos.

2.4.4. Conclusions

Cryopreservation affected chromatin integrity in mouse pre-implantation embryos at a greater extent at the morula than the blastocyst stage. Chromatin damage induced by SF was more relevant than that induced by VF (percentages of affected embryos and damage extent). Slow freezing and VF similarly affected mitochondrial aggregation/distribution pattern. Greater damage extent was observed at the morula stage and was associated with embryo grade, as lower rates of embryos showing P/P mt pattern were found in grade C compared with grade A and B embryos. Cryopreservation altered the quantitative bioenergy/redox parameters at a greater extent in morulae than in blastocysts. Effects induced by SF were not related to embryo grade or mt pattern, as affected embryos were of all grades and both mt patterns. Instead, effects induced by VF were related to mt pattern, as only embryos with SA mt pattern had reduced energy status. This study shows for the first time the joint assessment of chromatin damage and mitochondrial energy/redox potential in mouse embryos at the morula and blastocyst stage allowing the comparison of the effects of the two most commonly used cryopreservation procedures.

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.

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