



Bovine embryos cultured *in vitro* in the presence of antioxidants: implications for blastocysts development, quality and cryotolerance

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Introduction

The increase in intracellular reactive oxygen species (ROS) due to the high oxygen tension during *in vitro* culture (IVC) induces oxidative stress, leading to apoptosis and embryonic developmental failure (1). Addition of antioxidants during IVC appears to increase the resistance of bovine embryos to the oxidative stress and consequently improves cryotolerance (2). Thus, the aim of this study was to evaluate the effects of intracellular (cysteine and β -mercaptoethanol) and extracellular antioxidants (catalase) during IVC on the embryo development and cryoresistance, as well as the amounts of intracellular ROS and the percentage of blastomeres undergoing apoptosis.

Materials and Methods

Oocytes (n=733) were matured and fertilized *in vitro* for 24 h. Presumptive zygotes were IVC during the first 72 h (up to day 3) in SOF with 0.6 mM cysteine (CIST), 100 μ M β -mercaptoethanol (β ME), 100 UI catalase (CAT) or without antioxidants (Contr). From day 3 to the end (day 7), all embryos were cultured in SOF medium. All cultures were conducted at 38.5°C in 5% CO₂ in air. The cleavage and blastocysts rates were evaluated, respectively, at 72 and 168 hours post-insemination, when the blastocysts were vitrified (n=151; Ingámed®, Maringá-PR, Brasil), stained (n=45) with 5 μ M of the fluorescent probe H₂DCFDA or stained (n=71) for TUNEL according to the technique (3). The embryos vitrified were thawed and cultured for 24 h to evaluate the re-expansion rates. Embryos stained with H₂DCFDA and TUNEL were evaluated under an epifluorescence inverted microscope (excitation 495/510-550nm and emission 520/590 nm, respectively) and the levels of intracellular ROS (arbitrary fluorescence units) were measured by Q-Capture Pro image software. The cleavage, embryo development, levels of ROS and percentage of apoptosis were analyzed by ANOVA followed by Tukey's test, and re-expansion rates by Chi-square test (P<0.05). Data are presented as Mean \pm SEM.

Results and Discussion

Results are summarized in Table 1. The fluorescence intensity were lower in CIST and CAT compared to the Control (P<0.05). The percentage of apoptotic cells was reduced in CIST compared to the Control (P<0.05). Then, data showed that the reduction in the levels of intracellular ROS resulted in a decrease in the incidence of apoptosis in IVP bovine embryos. In conclusion, addition of cysteine or catalase during IVC improves the quality of bovine embryos measured by the intracellular levels of ROS and rates of apoptosis, however did not affect the embryo survival after vitrification.

Table 1. Cleavage, development to blastocyst, embryo survival after vitrification, measurement of intracellular ROS (Fluorescent intensity) and the incidence of apoptotic cells in bovine embryos cultured in medium supplemented with antioxidants

Groups	Cleavage *	Blastocysts *	Re-expansion 24h † (%)	Arbitrary Fluorescence Units *	Number of blastomeres *	Apoptotic cells *
Control	82.9 \pm 3.2	48.7 \pm 3.4	76.0	1.0 \pm 0.07 ^a	85.7 \pm 4.6	4.32 \pm 1.2 ^a
CIST	80.5 \pm 3.1	37.1 \pm 3.2	71.0	0.6 \pm 0.06 ^c	81.9 \pm 3.3	1.96 \pm 0.4 ^b
β ME	79.6 \pm 3.0	33.4 \pm 9.4	73.3	0.9 \pm 0.05 ^{ab}	80.5 \pm 4.8	2.17 \pm 0.6 ^{ab}
CAT	83.6 \pm 3.7	53.0 \pm 7.1	83.6	0.7 \pm 0.06 ^{bc}	90.9 \pm 4.1	2.05 \pm 0.5 ^{ab}

*Means followed by different letters in the same collum differ (P < 0.05) by Tukey's test. † Means followed by different letters in the same collum differ (P < 0.05) by Chi-square.

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Acknowledgements: Alta Genetics Brazil, Deoxi Biotecnologia, Ingámed and Brasfrigo.

Financial support: CNPq.

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Heat shock protein 70 messenger rna is present in ejaculated sperm from hairy rams

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Introduction

Spermatozoa are highly differentiated and specialized cells, designed to deliver the paternal genome to an oocyte (1). Although it is well-known that these cells are transcriptionally inactive, reports show that they contain RNAs that are delivered to the oocyte during fertilization (2). Many of these RNAs are not present in the unfertilized oocyte, and affect embryo development, such as the case for clusterin (1). We recently reported that clusterin is one of the major proteins of cauda epididymal fluid and seminal plasma in ruminants (3). Although studies have been conducted with humans, no information is available on the composition of sheep sperm RNAs. Thus, our goal was to determine if ovine sperm presents transcripts for *CLU* and heat shock protein 70 (*HSP70.1*), two chaperones we previously identified in the ram seminal plasma.

Material and Methods

Fresh semen was collected from 3 mature, reproductively sound rams and evaluated for motility and concentration. High quality samples were snap-frozen in liquid nitrogen and stored at -80°C until used. Total sperm RNA was isolated using the RNeasy kit, with adaptations. Briefly, 40-100µL of semen were incubated in lysis buffer for 5 minutes, and extensively homogenized using a 26G needle. RNase block and DTT were added to the RNA-rich sample. Purity and concentration of the resulting RNA were determined by measuring 260/280 OD ratios. RNA samples were treated with DNase I to prevent contamination with genomic DNA. Protamine 2 was used as internal control. Two primers for HSP70 and two for clusterin were designed using expressed sequence tags (ESTs) obtained from the NCBI database, and manually annotated by us. All primers were designed using Primer3Plus and Oligo Analyzer online tool. Total RNA was reversely transcribed to cDNA using ~670-800ng of RNA, which was incubated for 5' at 65°C with oligo(dT) and 10mM dNTP mix. The first strand synthesis reaction was performed by incubating these samples at 50°C during 60 min. with a mixture containing 0.1M dithiothreitol (DTT), RNase Block, 5X First-Strand Buffer, and SuperScript III RT. Two microliters of each product were used to run PCR reactions, in duplicate. Water and testis cDNA (2ng/RXN) served as negative and positive quantitative PCR controls, respectively. PCR was performed as follows: 15min. at 95°C, and then 40 cycles of 30sec. at 94°C denaturation, 30sec. annealing at the primer specific temperature, and 30sec. at 72°C extension. Melting curve analysis was initiated at 60°C, with 1°C increments for 10sec. to a final temperature of 95°C.

Results

To the best of our knowledge, this is the first study focused on identification of transcripts present on ram spermatozoa. Only HSP70.1 primers amplified the expected products. Our results showed, for the first time, that HSP70 transcripts are present in ram spermatozoa. *HSP70.1* transcripts were detected in ovine sperm using both primers, with similar Ct values (between 30 and 34). In the case of *CLU*, no transcripts could be identified in ram spermatozoa, using either primer set. The fact that we could not detect *CLU* transcripts is intriguing. In human, *CLU* RNA is present in the sperm and affects embryo development (1). Considering that genomic information for the *CLU* gene is lacking for sheep, we used ESTs available in the NCBI database. Thus, we were limited in our choices for primer designing. Facing an unexpected result, we designed two additional primers from a different location in the genome. However, none of them amplified testis cDNA, suggesting that they are not present on testis. Considering that this sequence does not have protein-coding ability in the testis, it is possible that this second sequence is a pseudogene. In humans, *CLU* gene displays a high number of splicing variants (4) and those available in EST databases might not reflect those present in the ram sperm. Our results showed the presence *HSP70.1* RNAs in ovine sperm cells. These results suggest that HSP70.1 transcripts might play a role in post-fertilization events, such as oocyte activation and embryo development. However, this hypothesis requires further validation.

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Effect of nitric system on the *in vitro* culture of bovine embryos

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Introduction

Nitric Oxide (NO⁻) is a cell signaling molecule involved in several cell pathways (1). We have now investigated the role of NO⁻ production on culture of bovine embryos produced *in vitro*, using L-arginine (ARG), a precursor for NO, in different periods of culture (embryo genome activation and compaction).

Material and methods

NO⁻ effects were assessed by developmental rates, kinetics of development and embryo quality. Oocytes obtained from abattoir derived ovaries were *in vitro* matured and *in vitro* fertilized according to previously described (2). Embryos were cultivated in medium SOF for 8 days, and NO⁻ production was measured by Griess method (3). In the experiment 1, in order to investigate the relationship between NO⁻ production and the period of embryo genome activation, the embryos were exposed to 10mM N-Nitro-L-Arginine Methyl-Ester (L-NAME) during the entire culture period (from 1st to 8th day [group N1-8]), also during the first (1st to 4th day [N1-4]) and the last half (4th to 8th day [N4-8]) of culture period. In the experiment 2, the embryos were cultivated with different concentrations of ARG. In the experiment 3, the best concentration of ARG was added to the period of compaction (from the 5th to 8th day of culture).

Results and discussion

In the experiment 1, NO⁻ inhibition was detrimental to embryo development starting from the day 4th of culture (N4-8 and N1-8 groups), decreasing the blastocyst hatching (17.3%±13.44 and 13.7%±14.51, respectively, p<0.05). However the most negative effect occurred from the 1st to 8th day of culture which the blastocyst rate was significantly decreased compared to control (29.4%±3.72 vs 47.8%±11.34, respectively, p<0.05). Due to, in experiment 2, ARG (1, 10 and 50mM) was added since the 1st day of culture. The blastocyst rates using ARG at 1 and 10mM were similar to control (48%±13.03 e 34.2%±3.92 vs 49.4%±4.82, respectively, p>0.05), but 50mM was found to impair embryo development (10.7%±7.24, p<0.001). In experiment 3, ARG at 1mM was added from the 5th to 8th day of culture did not affect blastocyst yield in comparison to Control (49.4%±6.5 vs 49.4%±4.8, respectively, p>0.05) but resulted in improved blastocyst hatching (54.8%±6.9 vs 41.4%±11.47, respectively, p<0.05) and embryo quality (84.8%±2.63 vs 52%±8.62, respectively, p<0.05). NO⁻ production was positively correlated with blastocyst hatching (R²=96.4%, p<0.001) and embryo quality (R²=75.5%, p<0.05). These data provide evidence that NO⁻ contributes to hatching and embryo quality improvement, especially at the period between morula and blastocyst stages. NO⁻ production is required to preimplantational development of bovine embryos cultured *in vitro* and can be mediated by supplementation of medium culture with ARG.

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Financial support: UNOPAR and CNPq.

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Evaluation of the establishment of full spermatogenesis from testis tissue and testis cell suspensions from the Collared peccary (*Tayassu tajacu*) ectopically xenografted in immunodeficient mice

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Introduction

Studies from our laboratory demonstrated that the collared peccary (*Tayassu tajacu*) presents a unique Leydig cells (LCs) cytoarchitecture in the testis (1,2), making this species an excellent model for investigating spermatogonial stem cell (SSC) biology and niche. Therefore, we found that peccaries' LCs may play a substantial role on SSCs differentiation (2). Testis xenograft is a fascinating approach in which testis tissue or isolated testis cells are able to develop after being ectopically grafted under the back skin of an immunodeficient mouse. Hence, this technique is a powerful approach for investigating the mechanisms and regulation of testis cells interactions, testis development, and SSCs physiology and niche. Moreover, among other possibilities, such as toxicological studies, testis xenograft from both testis tissue fragments (TTX) and testis cell suspensions (TCSX) have a very high potential for producing male gametes and for the preservation of male germplasm. In this context, our main objectives were to investigate the feasibility of this technique using prepubertal peccaries as donors.

Material and Methods

Both TTX and TCSX were obtained from prepubertal peccaries (n=4; 3 months old). The size of each TTX was 3x3x3mm and the TCSX were obtained by enzymatic digestion (collagenase, hyaluronidase and trypsin). Particularly for TCSX, after enzymatic digestion a cell population with 18.5%±0.1 of gonocytes (VASA positive) and 35%±5.1 of Sertoli cells (GATA4 positive) was obtained with 88.7%±4.5 of cell viability (Annexin/PI negative). The TTX and the TCSX were xenografted in SCID mice (total n=32) and the grafts were collected in different time periods (one to six months) after xenograft.

Results and Discussion

One month after TCSX the cells interacted and *de novo* testis morphogenesis, with large clusters of LC and primitive testis cords, showing a similar arrangement to that observed *in situ*, was found. Three and five months after TCSX, seminiferous cords/tubules containing spermatogonia and pachytene spermatocytes were respectively observed. Based on these results, knowing that TCSX is delayed in comparison to TTX, we expect to find full spermatogenesis in about 7 months after grafting. Noticeably, full progression of spermatogenesis (presence of spermatozoa in the tubular lumen) was observed six months after TTX. In conclusion, our findings indicate that both TTX and TCSX are useful approaches to investigate reproductive biology in peccaries. Also, to our knowledge this is the first study showing the formation of spermatozoa using TTX in wild mammals. We are now planning to perform new experiments aiming to evaluate if these sperm are fertile and able to form viable peccaries embryos. (CETEA/UFMG/protocol#070/2005).

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Financial Support: CAPES, CNPq and FAPEMIG.

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The periovulatory endocrine milieu alters the expression of immune response-related genes in bovine endometrium on day 7 post-estrus

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Introduction

The timely combination of progesterone (P4) and estradiol production has been linked to positive regulation of uterine protein expression and secretion, concept development and pregnancy success. It has been demonstrated that the local and peripheral immune cell populations are associated with pregnancy status. Despite the current debate discussing whether pregnancy is compatible or not with a pro-inflammatory state, the recruitment and local differentiation of specific immune cell types, and switch in chemokine and cytokine profile has been reported, with evidence suggesting a role for P4. Our aim was to determine the gene expression response of the endometrium on day 7 (D7) to distinct periovulatory endocrine milieus. Specifically, genes associated with immune response (DPP4, LCP1, CD34 and BMP4) were investigated.

Material and Methods

Twenty-two cyclic and non-lactating Nelore cows received a P4 device along with estradiol benzoate on D-10. Animals were divided to receive cloprostenol (PGF; high P4 group; HP; N=11) or not (low P4 group; LP; N=11) on D-10. Progesterone devices were withdrawn and PGF injected on D-2.5 for cows of the HP group and on D-1.5 for cows of the LP group. Ovulation was induced with GnRH on D0. Plasma P4 concentrations were measured daily from D0 to D7, when endometrial fragments were collected post-mortem. Total RNA was isolated from endometrium and cDNA synthesized for gene expression assessment by qPCR. Relative expression rates were normalized by the reference gene cyclophilin A and calculated by the $\Delta\Delta C_t$ method. Comparison of mean expressions rates between groups was performed by student's t test.

Results and Discussion

Differential gene expression was observed for genes DPP4 (2.6 ± 0.3 vs. 1.3 ± 0.3 ; mean relative fold difference \pm standard error of the mean), LCP1 (0.8 ± 0.1 vs. 1.0 ± 0.1), CD34 (1.7 ± 0.3 vs. 1.1 ± 0.1) and BMP4 (1.7 ± 0.2 vs. 1.1 ± 0.1) in HP versus LP group, respectively. Furthermore, significant linear regressions were observed between P4 concentrations on D3 and expression values for CD34 and BMP4 genes on D7 (R^2 0.25 and 0.46, respectively). With the exception of CD34, the other 3 genes are acknowledged to act on a variety of tissues, however all 4 identified genes share a commonality: their specific biological functions are of major relevance to the regulation of the immune response, targeting specifically immune cells. Organization of the actin filaments during cell migration in response to chemotaxis (LCP1; Freeley et al. 2012), inhibition of hematopoietic cell differentiation into the T lymphocyte lineage (BMP4; Varas et al. 2003), marker of hematopoietic progenitor cells (CD34; Vacca et al. 2011), and co-stimulation of T lymphocyte activation (DPP4; Ohnuma et al. 2008) are characteristics that suggest an interplay among these genes. Overall, our results suggest the suppression of the T cell differentiation pathway and an increased number of CD34+ hematopoietic progenitor cells, which are potential precursors of uterine natural killer cells. Moreover, it is also suggested that the uterine population of immune cells has an enhanced capacity to respond to stimulus (e.g. increased proliferation and chemokine synthesis and secretion). Reduction in LCP1 expression indicates decreased cell migration, which in the context of this work may indicate that immune cells allocated in the uterine environment have a reduced capacity of migrating out of the uterus. Although at this point our results can only support speculations, it is clear that distinct periovulatory endocrine milieus differentially modulate the endometrial pattern of gene expression, particularly concerning the local immune response.

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Acknowledgments: FAPESP (2010/01284-4 to FSM, 2011/15242-4 to RSR, 2011/03226-4 to MB), CNPq (481087/2010-9 to MB), Ourofino, CCPS-USP and Deoxi Biotecnologia.

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Fecal sex steroids in rats exposed to Di(2-ethylhexyl) phthalate (DEHP): a longitudinal noninvasive approach to monitor hormonal status in experimental studies

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Introduction

Phthalate esters, including DEHP, are known for being able to disrupt steroidogenesis in several species and causing disruption of pre- and post-natal androgen dependent development in males. The aim of this study was to characterize, on a daily basis, the androgen insufficiency induced by a high DEHP dose in pubertal rats by measuring fecal androgen metabolites, as well as to detect possible changes in the daily concentrations of fecal estrogen, progesterin and androgen metabolites throughout gestation in female rats exposed to DEHP.

Materials and Methods

Wistar male rats, aged 22 or 23 days, were divided into two groups (n=15 rats/group) and treated for 30 days by oral gavage with corn oil (control) or 750 mg DEHP/kg/day. Fecal samples were collected from cage bedding on days 1, 5, 10, 15, 20, 25 and 30 of treatment. To minimize stress, animals were kept isolated only from day 19 onwards. Therefore, fecal samples from days 1 to 19 of treatment represent a "pool" of three different rats and from day 20 to 30 are individual samples. On day 30 of treatment, animals were sacrificed and reproductive organs and tissues were collected and weighed. Pregnant Wistar rats were treated by oral gavage with corn oil (control; n=10) or 750 mg DEHP/kg/day (n=7) from gestation day (GD) 13 to 20, which represents the pre-natal period of male androgen dependent development. Dams were kept isolated and feces were collected from each cage on GD6, 13, 14, 16, 18 and 20. Fecal steroids were extracted with ethanol and estrogen, progesterin and androgen metabolites were quantified by enzyme immunoassay (1).

Results and Discussion

In the pubertal study, control animals showed a sharp increase in fecal androgen metabolites, as expected for a normal pubertal development. Such increase was not seen in rats treated with DEHP (Fig.1 A), which also displayed lower weights of reproductive organs and tissues than controls (P<0.01). In pregnant rats, the main result was a significant increase in the concentration of fecal progesterin metabolites from GD14 onwards in the DEHP-group (Fig. 1B). DEHP treatment also induced a transient increase in estrogen and androgen metabolites on GD 14 and GD16, respectively. The consistent increase in progesterin may be related to an effect on maternal and/or fetal adrenal gland axis. Our results indicate that longitudinal measurement of fecal steroid hormones can be successfully used for monitoring hormonal status in toxicological studies.

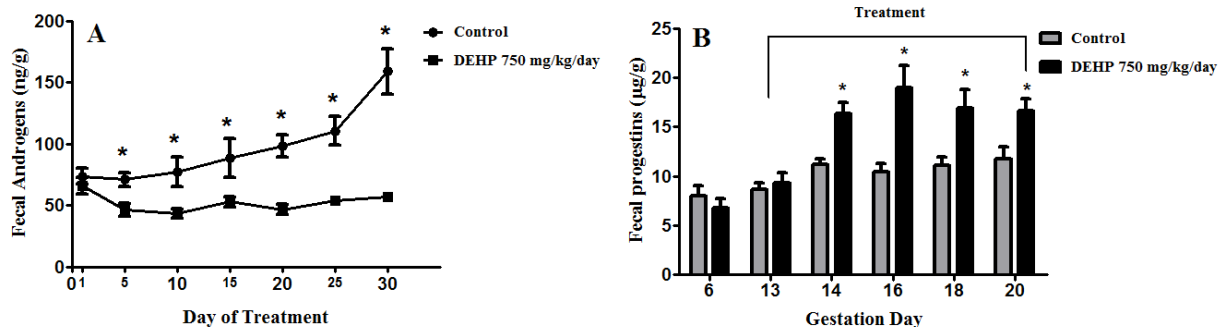


Figure 1. Fecal androgen metabolites (ng/g of feces) in control and DEHP treated pubertal rats (A) and fecal progesterin metabolites ($\mu\text{g/g}$ of feces) in control and in DEHP treated pregnant rats (B). (* $p < 0.01$).

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Regulation of fibroblast growth factor 18 (FGF18) and its effect on bovine granulosa cell viability

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Introduction

Fibroblast growth factors (FGFs) are involved in the paracrine modulation of ovarian function. In a previous study, we measured FGF18 mRNA in bovine granulosa and theca cells in vivo, and abundance of mRNA was higher in atretic compared to healthy follicles [1]. Granulosa cells cultured in serum-free conditions presented negligible amounts of FGF18 mRNA, and addition of FGF18 to granulosa cells culture decreased estradiol synthesis and increased the proportion of apoptotic cells [1]. The objective of the present study was to explore further the role of FGF18 in apoptosis of granulosa cells in a non-luteinizing serum-free culture system.

Material and Methods

Granulosa cells were collected from 2-5 mm diameter follicles and cultured in serum-free conditions. In the first 2 days, cell culture medium was supplemented with 1 ng/ml insulin. On day 2, the medium was changed to one containing insulin and FSH at 1 ng/ml each, and on day 4 the medium was replaced with medium devoid of FSH and insulin (unstimulated control) or containing insulin, IGF1, FGF2 or EGF, all at a final concentration of 10 ng/ml. In the second experiment to test a potential interaction between estradiol and FGF18 on cell viability, cells were cultured with FSH (1ng/ml) and insulin (10ng/ml) for 4 days. At medium change on day 4, cells were treated with FGF18 (10ng/ml) or FGF18+estradiol (10ng/ml). The effect of treatment on mRNA levels was measured by real-time PCR, and flow cytometry was used to determine the proportion of dead cells (sub-G1 peak).

Results and Discussion

In the first experiment, FGF18 mRNA abundance was very low in the presence of FSH and insulin, but increased significantly ($P<0.05$) in cells cultured without insulin and FSH. This was associated with an increase in abundance of mRNA encoding the pro-apoptotic factors GADD45B and FasL, and a decrease in CYP19A1 mRNA levels (encoding aromatase) indicating that FGF18 expression increases in unhealthy granulosa cells. As previously reported (ref) treatment of FSH-stimulated cells with FGF18 increased the proportion of apoptotic cells, and the effect of FGF18 on apoptosis was not a general effect of receptor tyrosine kinase activity as neither FGF2, FGF10 nor EGF increased the proportion of apoptotic cells. Estradiol is anti-apoptotic, therefore we cultured cells without FSH but with estradiol; addition of estradiol failed to prevent the increase in FGF18 mRNA. Culture of cells with FSH and estrogen receptor antagonist likewise failed to alter FGF18 mRNA abundance, suggesting that estradiol does not directly alter FGF18 expression. However, addition of estradiol abrogated the effect of FGF18 on the proportion of apoptotic cells ($p<0.05$). In conclusion, FGF18 is implicated in granulosa cell apoptosis, and the ability of estradiol to increase cell survival is downstream of FGF18 action.

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Acknowledgments: CNPq, Brazil and NSERC, Canada.

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Evaluation of equine spermatogonial stem cell viability after cryopreservation

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Introduction

The establishment of proper conditions for germ cells cryopreservation and storage represents an important biotechnological procedure for studies involving germ cells transplantation and is a very useful tool for the preservation of the genetic stock of valuable animals. In this regard, in the present study we have addressed the effects of different cryopreservation protocols on the viability and survival rates of spermatogonial stem cell in horse.

Material and Methods

Spermatogonial stem cells (SSCs) were enzymatically isolated from testis of 8 adult horses. In order to evaluate the presence of SSCs in the obtained cell suspension after Percoll gradient enrichment, we performed immunolabeling and western blot for GFRA1 receptor, which is considered so far the best marker for undifferentiated spermatogonia. Because most germ cells present in the obtained suspension were GFRA1⁺, we evaluated the feasibility of several SSCs cryopreservation protocols. Three different cryoprotectants media [dimethyl sulfoxide + DMEM + 10%BFS (1); ethylene glycol (2); dimethyl sulfoxide + sucrose (3)], associated with different methods (vitrification, slow-freezing and fast-freezing) were tested. The cell viability was evaluated before and after thawing by trypan blue staining assay using the Neubauer chamber. Annexin V and propidium iodide staining was used to estimate the rate of apoptotic and necrotic cells, after thawing, by flow cytometry (Facsan, BD Pharmingen). The cell metabolic activity (cell viability) and “stemness” potential were also evaluated post-thawing, maintaining these cells in culture for 24 days. The metabolic activity was measured performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, and the stemness potential was investigated through immunohistochemistry using specific SSCs markers (GFRA1 and NANOS2).

Results and Discussion

Based on the rates of viable SSCs found before and after thawing, as well as the number of recovered cells after the cryopreservation, the best results were obtained utilizing the DMSO-based cryoprotectants using fast (Medium 3) and slow freezing method (Media 1 and 3). In addition, when these cells were kept in culture (24 days), the MTT test data have indicated that the cryopreserved cells were as metabolically active as the fresh cells, and also they were expressing typical stem cell proteins (GFRA1 and NANOS2). Thus, the results obtained so far have indicated that equine SSCs could be cryopreserved without impairment of their metabolic activity and “stemness”. Further studies are now being performed, including BrDU immunostaining and germ cell transplantation, in order to evaluate *in vivo* the most efficient SSC cryoprotectant media. (CETEA/UFGM/Protocol#56/2011).

Financial support: FAPEMIG and CNPq.

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