Bovine sperm protamination gene expression profile

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Introduction

Protamines (PRM) are the major DNA-binding proteins in the sperm nucleus and can pack the DNA into less than 5% of the volume of a somatic cell nucleus (1). It is known that the bull only have the PRM1 protein on mature spermatozoa, while most mammals also have the PRM2 (2), but there are limited published data about the role of protamine on bull fertility. Transition nuclear proteins (Tnps) and PRM are fundamental for DNA integrity. The inactivation of Tnp2 in mice leads to failure in the PRM2 transduction and a subfertile phenotype, showing less condensed sperm nuclei, and elevated level of breaks in the DNA (3). It was already reported the influence of PRM on chromatin structures, generating low fertility (4). However, molecular mechanisms underlying these are not known. Thus this study aimed to determine the expression of PRM1, PRM2, PRM3, Tnp1 and Tnp2 in bovine testis and verify if there is a correlation between the expressions of these proteins, with the objective of determining specific molecular markers and mechanisms regulating bull fertility.

Material and Methods

Testis from post-pubertal bulls (n = 10) were obtained at a slaughterhouse. The RNA extraction and cDNA synthesis were performed using commercial kits. The expressions of the genes (PRM1, PRM2, PRM3, Tnp1 and Tnp2) were determined by real time RT-PCR, using bovine specific primers and β-actin as endogenous control. The data was analyzed by one-way ANOVA using the SAS 9.1.3. A relative expression software tool (REST) was used to compare all samples of each group (5).

Results and Discussion

Quantification of mRNA relative expression showed a higher expression of PRM1, compared to the other genes (Fig. 1). The relative expression of PRM2 was 5 fold lower than the relative expression of PRM1 ($p < 0.05$). There was no difference between the relative expression of Tnp1 and Tnp2 ($p > 0.05$); and the PRM3 mRNA had the lowest relative expression. The role of PRM3 on bull spermatozoa is still not elucidated and this study is the first that show the expression of this gene on bovine testis. Unexpectedly, the expression of Tnp2 was equal ($p > 0.05$) to Tnp1, which suggests that, different from mice, bulls may have different pathways to DNA compaction. In a spermatozoa transcriptome study, it was demonstrated that bulls of low fertility *in vivo* have a higher expression of PRM2 mRNA, compared to bulls of high fertility (6). Since bulls do not have the PRM2 protein on mature spermatozoa (2), these PRM may play an important role on sire fertility; it is clear that there still are gaps on the knowledge that must be elucidated.

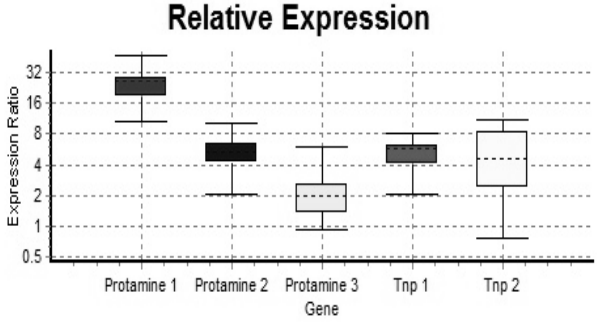

Figure 1. Ratio of mRNA relative expression of PRM1, PRM2, PRM3, Tnp1 and Tnp2 in bull testis.

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Effects of FSH on expression of mRNA encoding fibroblast growth factors receptors (FGFRs) in bovine cumulus cells submitted to *in vitro* maturation

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Introduction

The oocyte actively participates in the regulatory mechanisms of cumulus-oocyte complex (COC) maturation via secretion of paracrine factors. Gonadotrophins are known to modulate the effects of intraovarian factors. Recently, we detected the expression of fibroblast growth factors (FGF) -8 and-10 in immature oocytes and during *in vitro* oocyte maturation. Receptors for FGF10 (FGFR-1b and -2b) and of FGF-8 (FGFR-2c and FGFR-3c) are expressed in cumulus cells (CC) and mRNA abundance varies during *in vitro* maturation, suggesting that oocyte-derived FGFs may regulate CC differentiation. This study tested the hypothesis that FSH stimulates in a dose depent manner FGFR mRNA expression in CC from bovine COC submitted to *in vitro* maturation.

Material and Methods

Immature COCs (grades 1 and 2) were obtained from 2-8 mm follicles from abattoir ovaries (predominantly *Bos indicus*). Pools containing 20 COCs were matured for 12 hours with grading doses of FSH (0, 0.1, 1, 10 and 100 ng/ml; n = 4 per dose). After culture, CC were mechanically separated and stored at -80°C. Total RNA was extracted using RNeasy® (Qiagen), and 100ng of RNA was reverse-transcribed. Relative expression of FGFR-1b, -2b, -2c and -3c was assessed by real time PCR. Expression of Cyclophilin (CYC-A) was used as internal control. Quantification of mRNA expression was determined by Pfaffl equation. The effect of FSH dose was tested by ANOVA and groups were compared by Tukey-Kramer HSD test. Non parametric analysis was used when data were not normally distributed. Level of significance considered was $P < 0.05$.

Results and Discussion

Expression of FGFR-1b, -2c and 3c mRNA was upregulated by FSH at 12 hours of maturation. FGFR-1b and -2c mRNA expression was stimulated by 10ng/ml FSH while FGFR-3c mRNA was increased at the 0.1 ng/ml dose. FGFR-2b mRNA abundance was not affected by FSH treatment. In conclusion, expression of FGF receptors was upregulated by FSH in CC submitted to *in vitro* maturation, suggesting that FSH enhances oocyte-derived FGF actions in the control of CC differentiation.

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Estimate of the population of preantral follicles in the ovaries of *Bos taurus indicus* and *Bos taurus taurus* females

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Introduction

The number of oocytes recovered from *Bos taurus indicus* females submitted to ovum pick-up is on average two to four times greater compared to *Bos taurus taurus* females. This aspect has strongly contributed to the success and expansion of the embryo industry in Brazil. However, little information is available to explain the difference in oocyte production between *B.indicus* and *B.taurus* cows. Therefore, our objective was to test the hypothesis that the difference in oocyte yield is due to a higher number of preantral follicles in the ovaries of *B. indicus* females.

Materials and Methods

Ovaries (n = 64) collected at slaughterhouses from 180 to 240-days-old *B. indicus* (Nelore, n=10) and *B.taurus* (Aberdeen Angus, n = 10) fetuses, 20 to 24-months-old heifers (Nelore, n = 12, and Aberdeen Angus, n = 12) and 25 to 48- months-old cows (Nelore, n = 10, and Aberdeen Angus, n = 10) were cut longitudinally into two halves, fixed in Bouin's solution, and processed for histological evaluation. The number of preantral follicles was estimated through the counting of follicles using a correction factor (1). Only one ovary per female was analyzed. Preantral follicles were classified as primordial, primary, or secondary. The number of preantral follicles was compared using the Mann-Whitney test.

Results and Discussion

There was no difference (P > 0.05) between the average number of preantral follicles in the ovaries of *B.indicus* and *B.taurus* females (Fig. 1).

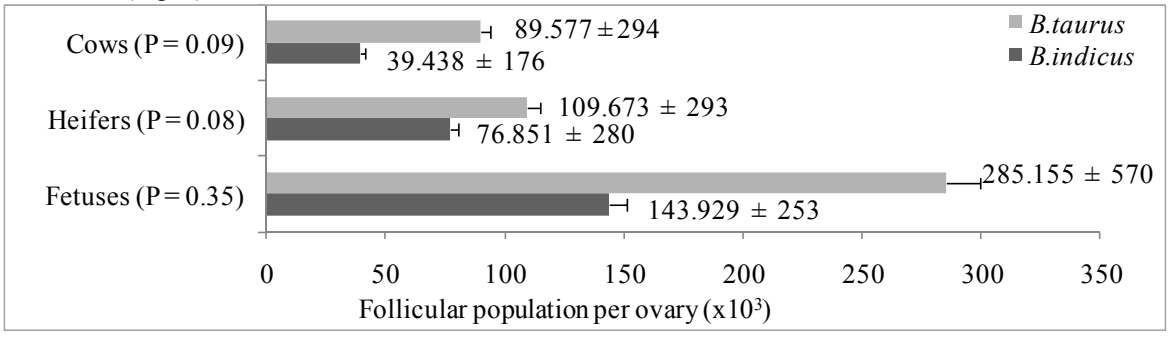

Figure 1. Average number of preantral follicles in the ovaries of *B.indicus* and *B.taurus* females (mean ±SEM).

A large variation in numbers of preantral follicles was observed among individuals within the same category and between breeds. Some ovaries assessed presented multinucleate follicles and cell cords. Our findings suggest that the higher oocyte yield from *B. indicus* females is not due to a greater ovarian reserve of preantral follicles. Therefore, mechanisms controlling follicle development after the preantral stage are likely to explain the differences between *B. indicus* and *B. Taurus* females.

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FGF10 inhibits follicular growth and decreases aromatase mRNA expression and estradiol secretion in bovine granulosa cells

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Introduction

FGF10 expression in theca cells is negatively correlated with estradiol levels in the follicular fluid (1). Also, bovine subordinate follicles express more FGF10 than dominants before deviation (2). FGF10 receptor (FGFR2IIIb) is expressed by mural and cumulus granulosa cells (1, 3). When added to cultured granulosa cells, FGF10 decreases estradiol secretion (1). We have previously shown that dominant follicle growth is interrupted by a single intrafollicular (IF) injection of FGF10. To gain more insight into the mechanisms through which FGF10 prevents follicular growth, we sought to identify genes regulated by FGF10 around deviation.

Materials and Methods

A new follicular wave was induced with a hormonal synchronization protocol and cows were monitored daily by ultrasound. When the largest follicle reached 7-8 mm in diameter, an IF injection of human recombinant FGF10 (1μg/ml; n = 4) or PBS (control group; n = 4) was performed. Twenty four hours after IF injection, follicle diameters were evaluated by ultrasound and cows were ovariectomized via colpotomy. Granulosa and theca cells were obtained by dissection of the follicle wall and submitted to total RNA extraction. mRNA levels were measured by real time RT-PCR and normalized to the expression of GAPDH. Follicular fluid estradiol levels were determined by ELISA. The effect of FGF10 injection on follicular growth, gene expression and estradiol levels was tested by ANOVA.

Results and Discussion

Growth of follicles treated with FGF10 was significantly lower than that of the control group (-0.2 ± 0.07 *vs*. 1.1 ± 0.38mm; Fig. 1A). In theca cells, there was no difference between control and FGF10 treated follicles in mRNA encoding steroidogenic enzymes (17α-hydroxilase, P450scc, StAR, 3βHSD), LH receptor (LHr) and IGFBP-2 and -3 (data not shown). Aromatase mRNA expression in granulosa cells was downregulated by FGF10 treatment (Fig. 1B), which was accompanied by a decrease in estradiol production (Fig. 1D). Expression of 3βHSD, FSHr, LHr, IGFBP-2 and -5, and X-linked Inhibitor of Apoptosis Protein (XIAP) was not affected by FGF10, but FGF10 decreased cyclinD2 mRNA abundance in granulosa cells (Fig. 1C). As cyclinD2 regulates cell proliferation by controlling G1 to S transition and is regulated in part by estradiol, suppression of follicle growth by FGF10 may be a result of its effect on aromatase and estradiol production. These results reinforce the hypothesis that FGF10 acts as an important regulator of follicular growth in cattle. We propose that FGF10 expression needs to be suppressed in order to allow the continuation of follicle growth after deviation

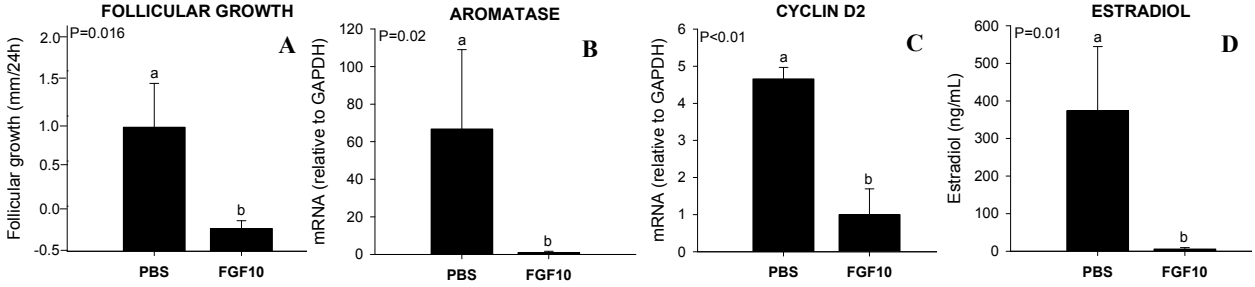

Figure 1. Follicular growth, mRNA expression and estradiol levels in injected follicles. Different letters indicate statistical significance.

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Investigation of potential specific markers for spermatogonial stem cells in the Nile Tilapia (*Oreochromis niloticus*)

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Introduction

Successful spermatogonial stem cells (SSCs) transplantation in adult Nile tilapia has recently been reported (1). The application of this technique in fish offers huge potential for studies involving biotechnology and genetic engineering in animal production and aquaculture, allowing also the preservation of genetic stocks of valuable or endangered fish species. The identification and isolation of SSCs, a subset of type A spermatogonia, are critical for the development of efficient transplantation methodologies and also for better understanding SSCs biology and spermatogenesis in fish. However, specific molecular markers for identifying these cells are not yet available for non-mammalian vertebrate species, including fish. Thus, the present study aimed to investigate specific markers that could potentially be used to characterize and select SSCs, or other particular spermatogonial types in the Nile tilapia.

Material and Methods

Adult tilapia (*Oreochromis niloticus*) testes were evaluated by immunohistochemistry (IHC) for the presence of Notch-1 and GFRα-1 receptors, which are potential or well established surface markers of undifferentiated spermatogonia in mammals (2). Testes from five tilapias were fixed overnight in 10% neutral-buffered formalin. Tissues were embedded in paraplast and sectioned at 5 μm. IHC reactions were performed according to standard protocols using the immunoperoxidase technique. The primary antibodies used were a polyclonal rabbit anti-human Notch Homolog 1 (1:100; Lifespan Bioscience) and a polyclonal goat anti-rat GFRα-1 (1:500; Santa Cruz Biotechnology). Peroxidase-labeled polymer conjugated with goat anti-rabbit IgG (DakoCytomation EnVision) and a biotinylated rabbit anti-goat IgG (1:100; Abcam) were used as secondary antibodies. The peroxidase reactions were developed with DAB-H_2O_2, followed by Mayer hematoxylin counterstaining.

Results and Discussion

In all animals investigated, immunoreactivity for both anti-Notch-1 and anti-GFRα-1 was observed in the seminiferous epithelium, exclusively in single type A spermatogonia. Sertoli cells, differentiating/differentiated germ cells or interstitial cells did not show any evident labeling. The positive spermatogonial cells were found preferentially at the blind ending of the seminiferous tubules, near to the tunica albuginea, where a high density of type A undifferentiated spermatogonia, characterized by morphological criteria, were previously reported to be located (3). This specific pattern of Notch-1 and GFRα-1 labeling in the tilapia testis indicates that these proteins are potentially useful markers for SSCs isolation and characterization in this teleost species. Moreover, these findings suggest a conserved role for Notch-1 and GFRα-1 signaling in SSCs at the onset of vertebrate spermatogenesis. We are currently developing studies, using germ cells transplantation in adult tilapias, aiming to evaluate the potential stemness of Notch-1 and GFRα-1 positive spermatogonial cells.

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Kininogen and bradykinin receptor type 2 expression and kallikrein-like activity in bovine follicles during the ovulatory process

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Introduction

The kinin-kallikrein system (KKS) has been described as an important mediator of ovulatory process (1, 2, 3). Bradykinin, generated from kininogen (KNG) by kallikrein, acts mainly through bradykinin receptor type 2 (B_2) and is the principal active peptide of the KKS (4). Despite the increasing evidence about the role of KKS in the ovaries of mammals, little is know about the presence and regulation of this system in bovine ovaries. The objective of this study was to characterize the mRNA expression pattern of B_2 receptors and KNG in theca and granulosa cells and the kallikrein-like activity in follicular fluid of bovine peri-ovulatory follicles.

Material and Methods

In order to obtain a peri-ovulatory follicle (≥12 mm), twenty-seven cows were submitted to estrus synchronization protocol and ovariectomized by colpotomy at 0, 3, 6, 12 or 24 hours after a GnRH-analog injection (gonadorelin; 100 µg, IM). Follicular fluid was aspirated for enzymatic activity assay and granulosa and theca cells were harvested for mRNA analysis. The expression of B_2 receptor and KNG mRNA in follicular cells were evaluated by real-time RT-PCR and data represented as relative to housekeeping gene cyclophilin. Kallikrein-like activity was measured in follicular fluid by absorbance using a plate reader.

Results and Discussion

The B_2 receptor mRNA expression in theca cells (Fig. 1A) and kallikrein-like activity in follicular fluid (Fig. 1B) showed different profiles during peri-ovulatory period ($P < 0.05$). However, differences were not observed in granulosa cells for B_2 receptor mRNA expression ($P > 0.05$). KNG mRNA expression was similar for both follicular cell types ($P > 0.05$). These results suggest that regulation of gene expression differ in each follicular cell type, indicating that, similar to other species, there is regulation of B_2 receptor mRNA in cattle (2, 3, 4). The results showed that KNG mRNA expression and kallikrein-like activity have a similar regulation during ovulatory process as previously described in rats (2). We concluded that KKS is present and regulated during bovine ovulation cascade.

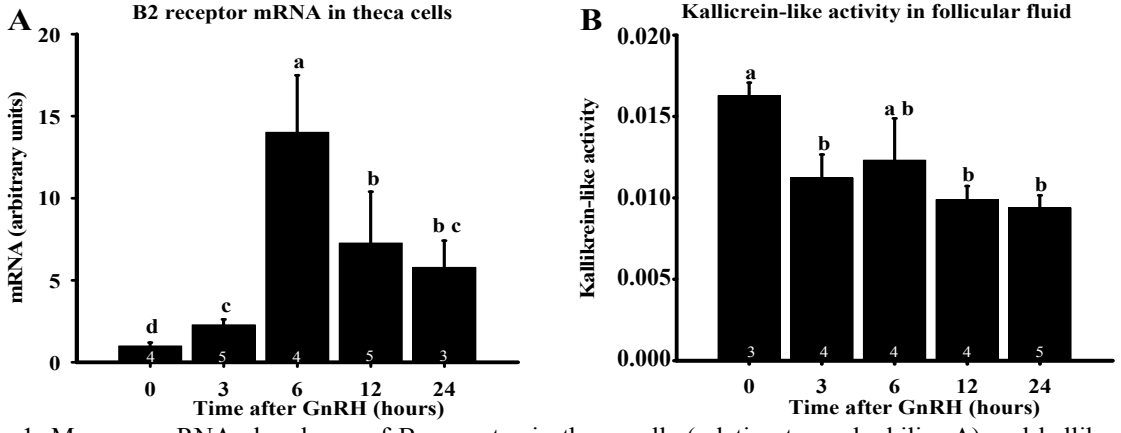

Figure 1. Messenger RNA abundance of B_2 receptor in theca cells (relative to cyclophilin; A) and kallikrein-like activity in follicular fluid (nm/mg protein/min; B) at different time after GnRH injection. Values inside the bars represent the number of follicles per group. Different letters represent statistical difference ($P < 0.05$).

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Leydig and Sertoli cell numbers are significantly increased in Inducible Nitric Oxide Synthase (iNOS) adult mutant-mice

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Introduction

Nitric oxide (NO) has been shown to potently reduce testosterone (T) production in vivo (1) and to directly suppress Leydig cells in vitro. In biological systems, NO is produced via the oxidation of L-arginine by enzymes known as nitric oxide synthases (NOSs). Three traditional NOS, specified as endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) are found in the testis. Particularly, several reports have provided evidences that iNOS is expressed constitutively in Leydig cells (LC) and within Sertoli cells (SC) and spermatogenic cells at specific phases of development in the normal rat (2) and mouse. In the present investigation we sought to provide further insights into the effects of the deficiency of iNOS on the LC and SC proliferative activity.

Materials and Methods

Twenty seven fully sexually mature male mice were used in the present work [12 wild-type controls and 15 iNOS–deficient mice (B6.129P2- $Nos2^{tm1Lau}$/J, stock number 002609, purchased from The Jackson Laboratory). After being perfused-fixed trough the left ventricle with 4% glutaraldehyde these animals had their testis dissected and weighed. Testis tissue fragments were embedded in plastic (glycol methacrylate) and routinely prepared and stained with blue toluidine for histological and morphometric analyses.

Results and Discussion

Both Leydig cell nuclear volume and Leydig cell individual size were significantly decreased in iNOS-deficient mice. In contrast, the total number of Leydig cells per testis in these mice was increased ($P < 0.05$) by approximately 16%. However, compared with wild-type the Leydig cell number per gram of testis was ~18% lower ($P < 0.05$) in iNOS-deficient mice.The possible functional role of iNOS on spermatogenesis was assessed by enumeration of primary pachytene spermatocytes and round spermatids per seminiferous tubules cross-sections at the stage VII of the cycle. The numbers of Sertoli cell nucleoli as well as the Sertoli cell efficiency, measured as the number of spermatids supported by individual Sertoli cell, were also investigated in this stage. Compared to the wild-type, the numbers obtained for both germ cells evaluated and for the Sertoli cells were significantly increased in iNOS-deficient mice. However, no significant changes were observed for the Sertoli cell efficiency and the meiotic index (number of spermatids per pachytene). The number of apoptotic germ cells per seminiferous tubules cross-sections was also evaluated. Except for stages V-VI and VII-VIII, the iNOS-deficient mice presented much less germ cell loss that was approximately 3.5-fold lower in these mice. Both number of Sertoli cells per gram of testis and the total number of Sertoli cells per testis were increased in iNOS-deficient mice by 9 and 76%, respectively. The testis weight, the daily sperm production per gram of testis (spermatogenic efficiency) and per testis followed the same tendency observed for Sertoli cells in the iNOS-deficient mice. We hypothesize that these increases may be related to higher levels of androgens which promoted increased Sertoli cells mitotic activity during testis development, and less germ cells loss in adult mice probably due to the Rhox5 androgen-induced gene (3) expressed in Sertoli cells.

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Loss of methylation at H19 DMR is associated with low implantation rates of somatic cell nuclear transfer bovine blastocysts

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Introduction

Somatic cell nuclear transfer (SCNT) has been achieved in a wide range of species so far. It can be applied as a biological tool to multiply desired alleles, and to generate transgenic animals for pharmaceutical and medical applications. However, the technique is still limited by the low efficiency, pregnancy failures and placental structural and functional disorders observed in SCNT gestations (1). In most of cases, abnormalities have been attributed to failures in epigenetic reprogramming and abnormal DNA methylation patterns (2). In this study we analyzed the parental expression of the imprinted gene H19, as well as the parental methylation status of the bovine H19 differentially methylated region (DMR) in pre and post implantation embryos.

Material and Methods

By using a bovine interspecies model [*Bos indicus* (paternal genome) × *Bos taurus* (maternal genome)], we analyzed the parental expression of the imprinted gene H19 by real time PCR, and the H19 DMR parental methylation patterns by bisulfite reaction in day 17 and day 40 embryos.

Results and Discussion

We demonstrate in this study that H19 is biallelic expressed in clone embryos at day 17 and for the first time we associate this abnormal expression with a severe paternal demethylation pattern at the H19 DMR (Fig. 1 and 2a). Furthermore we demonstrate that day 40 SCNT fetuses showed paternally hypermethylated CTCF binding site for the CTCF transcription factor at the paternal H19 DMR, suggesting that abnormal reprogramming of the bovine H19 gene might be associated with pre-implantation losses (Fig. 2b).

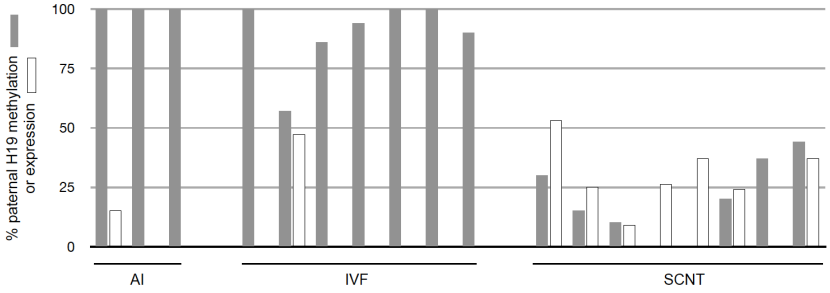

Figure 1. Paternal methylation of H19 DMR and paternal expression of H19 gene transcript.

Figure 2. Paternal CTCF methylation levels of H19 DMR in D17 embryos (a) and fetus (b) produced by AI, IVF and NT. $P < 0.05$.

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Pre-and postimplantational development of bovine cloned embryos derived after treatment with Trichostatin A

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Introduction

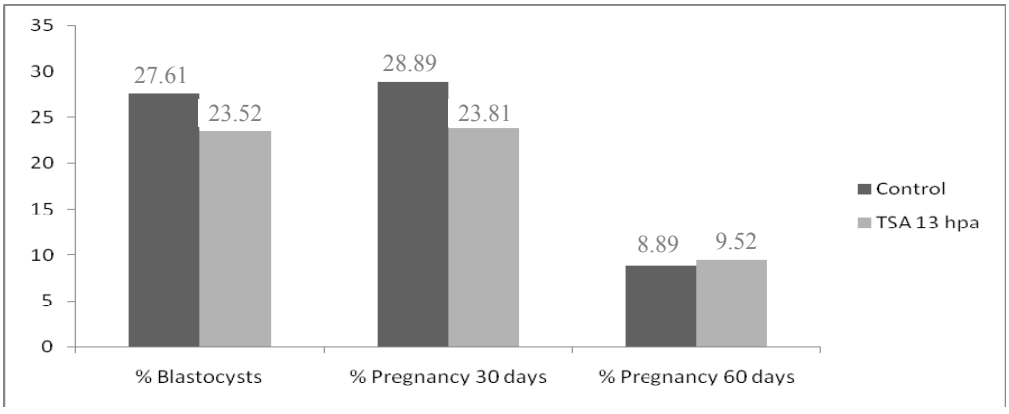
Somatic cell nuclear transfer (SCNT) to derive cloned embryos is a promising technology with potential applications in agriculture (1). However, SCNT is still inefficient, less than 10% of embryos transferred into uterus produce a viable offspring (2). Faulty epigenetic reprogramming of somatic nuclei is likely to be a major cause of low success observed in all mammals produced through this technique (1). Therefore, the use of chromatin modifying agents has been proposed to facilitate nuclear reprogramming and ameliorate the epigenetic abnormalities seen in clones (3). Here, we tested the use of Trichostatin A (TSA), a histone deacetilase inhibitor in bovine cloned embryos after activation, and evaluated the effects on pre- and post-implantation development.

Materials and Methods

To investigate the effect of TSA in bovine cloned embryos development nine replicates were performed. Cumulus-oocyte complexes obtained from the slaughterhouse were *in vitro* matured, denuded, enucleated and fused with synchronized bovine adult fibroblasts cells as described previously (4). Fused couplets were activated with ionomycin (5µM, 5 minutes) and then randomly divided in two groups. Control group was incubated in culture medium CR2aa supplemented with 2 mM dimethylaminopurine (DMAP) for 4 hours. Treated group was cultured with the medium describe above supplemented with 50 nM TSA for 4 hours. Incubation continued for another 9 hours in CR2aa + 50 nM TSA, totalizing 13 hours of incubation with TSA for treated group. After TSA treatment embryos were cultured *in vitro* until D7. Blastocysts were transferred to surrogate cows synchronized previously to assess pregnancy rate at 30 and 60 days of development.

Results and Discussion

Blastocyst rates were similar between groups ($p > 0.05$). In the control group 79 embryos were produced, resulting in 27.61% ± 1.99 blastocyst rate. The treated group resulted in 68 embryos with 23.52% ± 3.37 blastocyst rate. To evaluate the effect of TSA on postimplantation development, 45 blastocysts from the control group and 42 from the treated group were transferred to surrogate cows resulting in similar pregnancy rates ($p>0,05$). The data are summarized on figure 1.



Altogether these results are indicative that the deacetilase inhibition, induced by TSA treatment has no beneficial effect on pregnancies rates until D60. Further evaluation is necessary to ascertain whether this treatment reflects on calving efficiency.

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Supplementation of histone deacetylase inhibitor trichostatin A on *in vitro* c ulture of bovine embryos

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Introduction

Trichostatin A (TSA) is a histone deacetylase inhibitor that induces histone hyperacetylation and leads to increases in gene expression levels. The present study aimed to use TSA on *in vitro* culture of bovine embryos in order to increase blastocyst rate by improving chromatin remodeling on embryonic genome activation and increasing expression of crucial genes for early development.

Material and Methods

For testing this hypothesis, four TSA concentrations (5nM, 15nM, 25nM and 50nM) were applied to IVC medium (SOF supplemented with 2.5% FCS and 5 mg/ml BSA) during different exposure times (12 h, 24 h, 48 h and 144 h) on 8-cell embryos (70 h.p.i.) to determine suitable protocols. On a second experiment, three experimental groups (5nM 48 h, 5nM 144 h and 15nM 48 h) were selected for assessment of embryonic quality using following parameters: apoptosis (In Situ Cell Death Detection Kit, Fluorescein, Roche Applied Science, Mannheim, Germany), total cell number and hatching rate. The blastocyst rate, apoptosis and total cell number were evaluated 7 days after IVF, and blastocyst hatching was evaluated 9 days after IVF. Differences in blastocyst rate, apoptosis and hatching between groups were analyzed by the chi-square (χ^2) test using the MINITAB software, Release 14.1 (Minitab Inc., State College, PA). The total cell number was analyzed by one-way ANOVA and Tukey post test using the GraphPad Prism 4.0 software (GraphPad Prism, Inc., San Diego, CA). A 0.05 level of significance was set for the study.

Results and Discussion

On the first experiment, TSA promoted embryonic arrest and degeneration in concentrations 15nM, 25nM and 50nM. Three replicates corresponding to 1136 oocytes were analyzed. No difference was detected in the blastocyst rate between 8-cell embryos exposed to 5nM TSA for 12, 24, 48 or 144 h and control group (control:37.83, 12 h:27.94, 24 h:33.78, 48 h:24.09, 144 h:30.55). For embryos treated with 15nM, blastocyst development was compromised for exposure times higher than 12 h (control: 37.83^{a}, 12 h:23.07^{a}, 24 h:10.00^{b}, 48h:10.25^{b}, 144 h:8.82^{b}). Treatment with 25nM and 50nM reduced the blastocyst rate in comparison to control group at all exposure times (control:37.83^{a}, 25nM - 12h:16.66^{b}, 24 h:13.79^{b}, 48h:0.00^{c}, 144 h:0.00^{c}; 50nM – 12 h:10.52^{b}, 24 h:0.00^{c}, 48 h:0.00^{c}, 144 h:0.00^{c}). In a second experiment, we carefully investigated the effect of adequate TSA treatment on embryonic development. For that, cleaved embryos (n = 1732) obtained in nine replicates were exposed to 5nM for 48 h (group A), 15nM for 48 h (group B) and 5nM for 144 h (group C). All of the TSA concentrations tested promoted a decrease in blastocyst rate (Control: 44.57^{a}, A:36.38^{b}, B:21.44^{c}, C:24.66^{c}). Blastocyst hatching was decreased in embryos exposed to 5nM for 144 h and 15nM for 48 h (Control: 60.37^{a}, A:55.00^{a}, B:38.46^{b}, C:22.05^{c}). However, the TUNEL assay revealed similar apoptosis rates and total cell numbers in all evaluated groups (apoptosis rate/ total cell number. Control: 5.33 ± 0.83/ 90.18 ± 5.81, A:4.63 ± 0.75/ 81.3 ± 5.47, B:7.72 ± 0.97/ 75.31 ± 4.29, C:7.93 ± 1.22/ 88.18 ± 4.73). The results of the present study suggest that TSA treatment has no beneficial effects on the *in vitro* production of bovine embryos during preimplantation development considering the evaluated parameters. However, histone acetylation is increased in TSA-treated embryos (1), suggesting that gene expression levels may be altered in these embryos. In this respect, further experiments accessing gene expression profiles and embryonic post implantation development are required to better understand the roles of histone deacetylase inhibition over IVC of bovine embryos.

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The association between pre- and postnatal protein undernourishment and testicular temperature after weaning over somatic and germ cell numbers in adult rats

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Introduction

It has been shown that at earlier stages of fetal development, the normal ontogeny of gonadal development and function can be disrupted by undernutrition (1). Moreover, malnutrition can result in low birth weight newborns, which increases the risk of undescended testicles (2). Considering that, the aim of this study was to investigate the effects of malnutrition in adult rat testes submitted to prenatal protein deficiency, associated to the increase of the testicular temperature through experimental cryptorchidism.

Material and Methods

Female rats received either a control (20% crude protein, group C) or a hipoproteic (8% crude protein, group T) diet from five weeks before mating until weaning their pups. After weaning, the male pups were submitted to surgical procedures to maintain the right testis inside the abdominal cavity. After that, they were divided into four groups and fed, as follows: **a**) *Group CC*: pups from group C mothers that received the control diet; **b**) *Group CT*: pups from group C mothers that received the hipoproteic diet; **c**) *Group TT*: pups from group T mothers that received the hipoproteic diet; and **d**) *Group TC*: pups from group T mothers that received the control diet. These male rats were killed at 70 days of age and biometrical data were obtained. The testes were fixed by vascular perfusion with 5% glutaraldehyde and embedded in plastic resin. From histological sections, stained with toluidine blue-borate, the numbers of spermatogonia, spermatocyte, Sertoli cell and Leydig cell were evaluated comparatively among the cryptorchidic testes of the four experimental groups (CC, CT, TT and TC).

Results and Discussion

Undernutrition reduced body weight as well as the criptorchidic testicular weight of the CT and TT groups when compared with the cryptorchidic testes of the control group (Table 1). However, the gonadossomatic index was significantly greater in the criptorchidic testicle of the TT group when compared to the criptorchidic testes of the other groups showing that prenatal malnutrition, associated to the elevation of testicular temperature after weaning, affected body growth more severely than the testicular development. The more advanced germ cells in the criptorchidic testes of all groups evaluated, including the control, were the pachytene spermatocytes and the number of spermatogonia, spermatocytes (exception for TC) and Leydig cells were similar in all experimental groups. These results showed that the increase of the testicular temperature had a more detrimental effect for spermatogenesis than protein malnutrition. Unexpectedly, the raise in the testicular temperature increased only the number of Sertoli cells in the cryptorchidic testes of the undernourished animals since the fetal period (TT; Table 1 and Fig. 1). The testicular development in protein deficient animals was retarded and, in consequence, presented a delay in the onset of puberty (3) that could affect the production of the thyroidian hormones (4). Probably, this condition could prolong the proliferation of Sertoli cells increasing their total number in the testis. Further studies are necessary to confirm this hypothesis.

Table 1. Body weight , testicular weight and cell numbers (mean ± SE) from the cryptorchidic testes after different protein undernutrition conditions . [a,b,c,d] Means within rows with different superscripts differ (P< 0,05).

Groups	CC	CT	TT	TC
Body weight (g)	284.5 ± 4.9[a]	111.3 ± 12[b]	57.6 ± 13.4[c]	195 ± 14.4[d]
Testis weight (g)	0.23 ± 0.02[a]	0.14 ± 0.01[b]	0.14 ± 0.02[b]	0.22 ± 0.01[a]
IGS (%)	0.09 ± 0.01[a]	0.13 ± 0.02[a]	0.25 ± 0.04[b]	0.12 ± 0.01[a]
Spermatogonia (10^6)	7.20 ± 1.0[a]	5.9 ± 0.8[a]	6.91 ± 1.0[a]	6.12 ± 1.5[a]
Spermatocytes (10^6)	18.8 ± 2.2[a]	18 ± 2.0[a]	19.6 ± 3.5[a]	10.8 ± 0.9[b]
Sertoli cells (10^6)	34.4 ± 3.4[a]	32.9 ± 3.8[a]	50.9 ± 3.6[b]	33.9 ± 3.9[a]
Leydig cells (10^6)	10.3 ± 1.5[a]	6.2 ± 1.3[a]	5.5 ± 1.7[a]	11.7 ± 2.0[a]

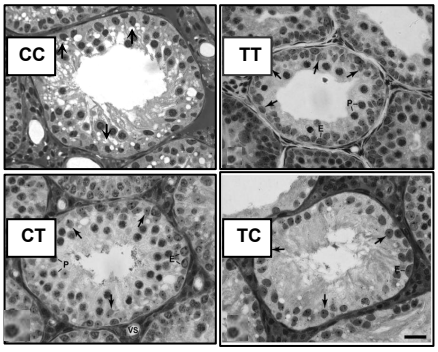

Figure 1. Photomicrographies of cross-sectioned seminiferous tubules from cryptorchidic rat testes after different protein undernutrition conditions. Bar: 32 μm.

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The nuclear receptor Nr5a2 regulates connexin 43 expression in mouse ovarian granulosa cells *in vivo*

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Introduction

The nuclear receptor Nr5a2 (liver receptor homolog-1) is a critical regulator of multiple mechanisms essential for female fertility. Mice lacking *Nr5a2* in granulosa cells fail to undergo cumulus expansion and ovulation (1). A gap-junctional protein, known as connexin 43 (Cx43), participates in the network of cell-to-cell communication established in the cumulus/granulosa cells, and the process of cumulus expansion is related to modifications of gap junctions (2). Our preliminary electron microscopy result revealed that the mural granulosa cells of preovulatory follicles in granulosa-specific *Nr5a2* knockout ($Nr5a2^{gc-/-}$) mice display an absence of normal cell-cell contacts in comparison with wild type (WT) animals. These data led us to hypothesize that *Nr5a2* regulates cell-cell communication and intercellular connections through Cx43 in mammalian ovarian follicles.

Materials and Methods

$Nr5a2^{gc-/-}$ mice were generated crossing animals expressing Cre-recombinase from the anti-Müllerian hormone receptor-2 locus ($Amhr2^{Cre/+}$) with $Nr5a2^{flox/flox}$ mice. Non-transgenic females littermates were used as control groups ($Nr5a2^{gc+/+}$). Immature females were hormonally superstimulated with equine chorionic gonadotropin (eCG; 5IU) followed by human chorionic gonadotropin (hCG; 5IU) 48 h later. Ovaries were collected 2 h, 4 h or 8 h after the hCG injection (n = 3 animals/ time point/ group). From each animal, one ovary was embedded in paraffin for Cx43 protein immunolocalization. The remaining ovary was stored at -80°C; mural granulosa and cumulus cells were excised from all apparently nonatretic antral follicles by laser microdissection of frozen sections of ovaries for RNA extraction and Real-time PCR analysis.

Results and Discussion

The abundance of mRNA for Cx43 in the $Nr5a2^{gc-/-}$ mural granulosa and cumulus cells was downregulated compared to $Nr5a2^{gc+/+}$ ($P < 0.01$). We found that Cx43 expression progressively decreased in both $Nr5a2^{gc-/-}$ and $Nr5a2^{gc+/+}$ groups from 2h to 4h and 8h post-hCG injection ($P < 0.01$). There was no difference between $Nr5a2^{gc-/-}$ and $Nr5a2^{gc+/+}$ at 4 h and 8 h post-hCG ($P > 0.05$), but at the time 2h the expression in the knockout group was downregulated compared to the control group ($P < 0.01$). These results were confirmed by the immunohistochemistry. It is known that the junctions between granulosa cells and the oocyte permit the transfer of metabolites and play an important role in the maintenance of meiotic arrest of the oocyte (3). Cx43 is suggested to be involved in follicle development and oocyte growth, and the amount of Cx43 in the large antral follicles is relatively elevated in a stage of high serum concentrations of FSH (4). It is possible that $Nr5a2^{gc-/-}$ oocytes failed to receive important signals for the acquisition of developmental competence due to decreased gap junctional communication prior to and at the time of hCG injection. In WT mice, the preovulatory surge of LH is followed by a decline in levels of Cx43, blocking the conduction of meiosis inhibitory signals and initiating meiotic resumption (5). Cx43 downregulation occurred in both knockout and control groups during the time post-hCG from 2h to 8h. However, at 12h post-hCG, Cx43 was showed to be upregulated in $Nr5a2^{gc-/-}$ granulosa cells compared to $Nr5a2^{gc+/+}$ according to our previous microarray analysis. The Cx43 immunostaining showed an increase in this protein expression comparing $Nr5a2^{gc-/-}$ granulosa cells at 2 h and at 12 h post-hCG. These high levels of Cx43 by the time of ovulation may be one explanation for the lack of cumulus expansion, preventing the cells to detach and maintaining the inhibitory signals for meiotic arrest of the oocyte. We conclude that Cx43 is one of the downstream genes under *Nr5a2* control and its dysregulation can be one reason for the defect in cumulus expansion in $Nr5a2^{gc-/-}$ females.

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