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Expression of molecular markers for bovine spermatogonial stem cells in prepubertal and adults Nelore

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Keywords: molecular marker, bovine, Spermatogonial stem cell.

The little knowledge about expression of molecular markers for bovine's Spermatogonial Stem Cells (SSCs) and the negative effect of ageing over these cells (Kokkinaki et al., 2010, Reproduction, 139, 1011-20) reduce the sorting efficiency by flow cytometry and immunocytochemistry. The goals of this study were to evaluate and to compare the expression of SSCs molecular markers by flow cytometry (SSEA4) and by immunocytochemistry (SSEA4, PGP 9.5 and alpha-6-Integrin (A6Int)) in cells from prepubertal (aged of 5 months, n = 10) or adults Nellore males (aged 3-4 years age, n = 10) before and after differential plating. In order to perform the experiment, biopsies from testicular parenchyma were minced and digested with collagenase (1mg/ml, 30min at 37ºC followed by trypsin (2.5mg/ml, 5 min at 37ºC). Viable cells were plated on cell culture dish (100mm) previously covered with bovine serum albumin (0.5 mg/ml) and cultured overnight in high humidity atmosphere with 5% of CO_2 at 37ºC. Viable cells from the supernatant were fixed with cold ethanol 70% and incubated with antibody anti A6Int labeled with Alexa Fluor 488 (BioLegends®, San Diego, CA, USA), antibody anti PGP 9.5, anti SSEA4, anti Vimentin or anti Cytokeratin (all antibodies form Abcam®, Cambridge,MA, USA). Samples previously incubated with antibody anti PGP 9.5 and SSEA4 were also incubated with a second antibody labeled with FITC (Abcam®, Cambridge, MA, USA) whereas samples previously incubated with antibody anti Vimentin and Cytokeratin were incubated with secondary antibody but labeled with Texas Red Sulfonyl Chloride (Abcam®, Cambridge,MA, USA). Percentage of positive cells for SSEA4 was determined by flow cytometry (Atunne, Applied Biosystems, Foster City, CA, USA) and the immunocytochemistry evaluation were performed by fluorescence microscope (Olympus IX-81, Olympus, Tokyo, Japan). Flow cytometry data was analyzed by FlowJow (Tree Star, Ashland, OR, USA) and Wilcox statistical test was performed (STATA, College Station, Texas, USA). Immunocytochemistry results strongly suggest that SSEA4 is a molecular marker for bovine SSCs. Positive staining also was observed for PGP 9.5, A6Int, Vimentin and Cytokeratin. SSC purification by differential plating was not as efficient as expected because positive staining for vimentin was observed after the treatment. This result suggests that there is a subpopulation of Sertoli cells after the purification. Furthermore, no effects of differential plating and ageing were observed on percentage of positives SSCs for SSEA4 by flow cytometry. Thus, the expected high number of SSEA4 positive cells after the cell purification was not confirmed. In conclusion, SSEA4 is a potential molecular marker for bovine SSC and the purification of SSCs by overnight differential platting with bovine serum albumin treatment was not as efficient as expected.

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Cryopreservation of mesenchymal stem cells derived from bovine adipose tissue with propylene glycol or DMSO

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Keywords: DMSO, Mesenchymal stem cells, propylene glycol.

Cryopreservation is a strategy used for the conservation of cells for extended periods, however, it is important to maintain their characteristics during this process. The use of DMSO as cryoprotectant for stem cells is controversial because it can potentially interfere in the undifferentiated condition of these ones. The objective of this study was to evaluate the use of Propylene Glycol (PG) as an alternative to Dimethylsulfoxide (DMSO) in the cryopreservation of mesenchymal stem cells (MSCs) obtained from bovine adipose tissue. For this, a sample of adipose tissue was collected from the tail base of a female bovine and submitted to enzymatic digestion with collagenase (0.075%). The isolated cells were grown in DMEM supplemented with 10% FCS, at 37°C and 5% of CO2, and after reaching 70% confluence part of the cells were maintained in culture as a control, and also used for induction of osteogenic differentiation by the MSC Osteogenic Differentiation Medium kit (Lonza Pharma), while another part was used for cryopreservation. For this, the cells were trypsinized, centrifuged and resuspended in medium containing 10% FBS and 10% cryoprotectant (DMSO or PG) at a concentration of $1x10^6$ cells/mL. Freezing was done in cryotubes at -1ºC/min with the Mr. Frosty® apparatus (Nalgene Nunc Cooler, USA) at -80°C and then kept at -196°C. For thawing, cryotubes were placed in the water bath at 37°C, and then the content was transferred to a tube containing 2 ml of DMEM with 10% FCS, centrifuged and resuspended in 500 µL of culture medium. One aliquot was used to determine the immediate survival by trypan blue staining. Of the remainder, 10000 cells were cultured in 1.9 cm^2 wells, in triplicate, and counted after 24, 48 and 72 hours to evaluate the growth curve. Other aliquots were used to perform the Comet assay (Collins, 2004, Mol. Biotechnology, 26, 249-261) and to the induction of differentiation. Immediately after thawing, the survival rate was 85.0% and 68.8% for cells frozen with DMSO and PG, respectively. The cells submitted to growth curve showed no statistical difference between treatments (DMSO x PG) relative to the number of cells considered viable in 24 h (25,667 x 18,667) and 48 h (40,500 x 42,833), however, with 72 h the number of cells was significantly higher (79,333) with DMSO treatment than with PG (67,167). The cells cryopreserved with PG showed higher DNA integrity (69.5%), assessed by the Comet assay, while only 46.5% of cells cryopreserved with DMSO didn't show any damage (p=0,05). The induction of differentiation into osteogenic tissue was positive in all groups, showing their multipotency. Therefore, despite the PG shows lower recovery of viable cells, it promotes low damage in the genetic material of cells and can be used as an alternative to DMSO.

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Pregnancies and births of bovine cloned embryos from amniotic fluid and adipose tissue cells collected in vivo

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Keywords: bovine, embryos, nuclear transfer.

Cells from amniotic fluid (CAF) and adipose tissue (CAT) were characterized as mesenchymal stem cells in some animal species (Mauro et al., 2010, Vet Res Commun 34, S25-S28; De Mattos Carvalho et al., 2009, Vet Immunol Immunopathol, 132, 303-06), and despite its potential for use as nucleus donors, it has not been used in nuclear transfer (NT) in bovine. The objective of this study was to test the efficiency of use of CAF and CAT in NT. The recovery of CAFs were performed by ultrasound-guided intra vaginal aspiration, without damaging the 64-days fetus. The amniotic fluid was centrifuged and the cells cultured in Amniomax Complete II medium (Gibco, Rockville, USA). CATs were collected by perineal biopsy of the same calf with seven months old and explants were cultured in DMEM (Invitrogen Life Science, USA). The morphological characterization was performed by Scanning Electron Microscopy. The isolated cells were used in the NT procedure according Kuroiwa et al. (2002, Nature, 20, 889-894), with modifications. The statistical analysis was performed by ANOVA and Tukey test ($p<0.05$) (SAS 9.1.2). The blastocyst rate based on number of cleaved structures was 45.46±13.03 and 46.47±7.92% for CAF and CAT, respectively, with no significant difference. The pregnancy rate at 35 days was 12.5% (1/8) to CAF and 25% (1/4) to CAT. Hydropsy in pregnant recipient with CAF was observed at 245 days. The birth was induced at 277 days and cesarean section was performed after 36h to remove the calf alive. The animal weighed 58.5 kg and on the external examination was observed teeth not completely developed, thick umbilical cord and bilateral flexion of the metatarsophalangeal joints. Hydrothorax serous, fluid throughout the trachea, fluid in the lung parenchyma, enlarged liver with yellowish and fat deposition in the surface were observed in the necropsy. The cloned calf from the CAT was born at 291 days with 35 kg without birth complications. No clinical alteration was observed in this calf, which is still healthy. The pregnancy rates obtained in this study were higher than those reported in the literature, however, the calf derived from CAF presented the Large Offspring Syndrome (LOS), frequently associated with cloned animals, whereas LOS was not observed in calf from CAT, suggesting that this new cell type can be used in bovine NT but further studies should be carried out.

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Generation of bovine induced pluripotent stem cells (biPS) and production of cloned embryos derived from biPS cells after nuclear transfer

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Keywords: induced pluripotency (iPS), nuclear transfer; stem cells.

Bovine embryo production trough nuclear transfer (NT) is still hampered by low developmental rates, due to the incomplete reprogramming of the nuclei donor. Strategies used to promote a better nuclei reprogramming, for example, the use of more undifferentiated cells as nuclei donors have already showed interesting perspectives. It is known that *in vitro* maintenance of pluripotency in bovine embryonic stem cells-like is difficult, therefore, the present study aimed to generate bovine induced pluripotent stem cells (biPS) and use them as nuclei donors in NT, enhancing therefore the efficiency of nuclei reprogramming. Bovine iPS cells were produced trough lentiviral transduction of murine transcriptional factors related to pluripotency (Oct4, Sox2, c-Myc and Klf4 - OSKM) in bovine fibroblasts. Cells were characterized regarding morphology, gene expression, immunofluorescence of pluripotency factors, alkaline phosphatase detection, embryoid body formation, *in vitro* differentiation, *in vivo* teratomas formation and subsequently used as nuclei donor in NT. Briefly, bovine oocytes obtained from slaughterhouse ovaries were *in vitro* matured for 18h, enucleated and reconstructed with biPS (n=203) or bovine fetal fibroblasts (bFF, n=153), in five repetitions. After reconstruction, zygotes were activated with ionomycin and 6-DMAP and *in vitro* cultured until blastocyst stage. Fusion, cleavage (48h after activation) and blastocyst (192h after activation) rates were evaluated and results were submitted to Chi-square test at 5% de significance. No differences were observed between groups regarding cleavage (81.53 vs 88.96%) or embryo production (22.68 vs 29.63%, respectively), however embryos reconstructed with biPS cells presented a reduced fusion rate (47.78 vs 70.59%). In conclusion, biPS cells were produced and derived cloned embryos after NT. A better understanding of nuclei reprogramming mechanisms and production of animals derived from reprogrammed cells should lead to enhance the efficiency of reproductive biotechnologies.

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Co-culture of bovine embryos with adult stem cells derived from adipose tissue (preliminary results)

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Keywords: co-culture, embryo, stem cells.

Somatic cells used in co-culture of embryos have the ability to remove components harmful to embryonic development, such as reactive oxygen species (ROS), (Mouatassim S. et al., 2000, Eur J Obstet Gynecol Play Biol, 89, 1-6). Stem cells from adipose tissue (SCAT) have the potential for adipogenic, osteogenic, chondrogenic and myogenic differentiation (Zuk et al., 2002, Mol Biol Cell, 13, 4279-95). Nowadays, these cells can be considered as one of the best sources of stem cells for various types of clinical studies, because it is easily isolated and exhibit multipotency (Zhu et al., 2008, Cell Biochem Funct, 26, 664-75). Thus, the objective of this study was to evaluate the use of SCAT in co-culture of bovine embryos, aiming to improve the protocol for in vitro embryo production. The SCAT were obtained from fat of cattle from slaughterhouse, isolated with collagenase type I (0.001 g/ml) for 180 minutes and cultured in IMDM (Iscove's Modified Dulbecco's Medium, Gibco®) supplemented with 10% fetal calf serum and gentamicin (0.01 g/ml). To assure the multipotency of SCAT, cells were differentiated on passage P7 in osteoblasts (confirmed by staining with 2% of alizarin red S), adipocytes (staining with 25% Oil Red) and chondrocytes (staining with Alcian Blue 1 %) with STEMPRO Differentation Kit Gibco®. Cattle`s cumulus-oocytes complex (Cocs) were matured in vitro in TCM-199 medium supplemented with 10% FCS, FSH and LH, for 20 hours, in 38.5°C and 5% CO_2. The Cocs were fertilized in Talp-hepes FERT medium supplemented with heparin, penicillamine, hipotaurine, epinephrine and BSA, and grown under the same conditions cited for the IVM. After 24 hours of fertilization, the zygotes were distributed to droplets of SOF medium supplemented with BSA (6 mg/ml) and 10% FCS, in the following experimental groups: SOF without monolayer (SOF), SOF with granulosa cells (SOF-G), SOF with one thousand SCAT (SOF-ONE THOUSAND) and SOF with ten thousand SCAT (SOF-10 THOUSAND). The cleavage rate was evaluated on the 2nd day of culture and the blastocyst formation on day 7, and the results were analyzed by ANOVA using Bonferroni post-test, adopting the significance level of 5 %. Regarding to cleavage rate, there was no difference ($p>0.05$) between the groups analyzed, however, the group SOF-10 THOUSAND (n= 61) increased significantly ($p< 0.05$) the production of blastocysts in comparison to groups SOF-G (n= 59) and SOF (n= 63) at the end of the 7th day of culture (47.20±1.46 *vs.* 31.43±1.49 *vs.* 19.15±1.29, respectively), though it did not differ ($p>0.05$) from SOF-ONE THOUSAND group (32.73±1.16; (n= 61). These preliminary results show that the use of SCAT significantly increased the rate of bovine embryonic development to the blastocyst stage, which may be due to its effect against ROS in comparison to traditional co-culture with granulosa cells.

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Ovarian follicle-like structures differentiated in vitro from pig skin derived fibroblasts treated with 5-azacitidine

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Keywords: 5-azacitidine, differentiation, fibroblasts.

This study aimed to evaluate the effects of 5-azacitidine (5-Aza) on the expression of mRNA for Oct-4, Nanog, Rex-1 and Sox-2 in pig fibroblasts, and to investigate the influence of follicular fluid (FF) associated or not with bone morphogenetic protein-4 (BMP-4) on differentiation of treated fibroblasts into ovarian follicle-like structures after 21 days of culture. To this end, fibroblasts isolated from skin biopsies were cultured in DMEM supplemented with 1μM 5-Aza, 20% FCS, penicillin and streptomycin in an incubator with 5% CO_2 at 37°C for 18 h. Then, the fibroblasts were cultured in DMEM supplemented with 5% FCS, 0.23mM sodium pyruvate, 0.1mM nonessential amino acids, 2mM L-glutamine, 0.1mM β-mercaptoethanol, 100 IU/mL penicillin and 100 mg/ml streptomycin (control medium). For the treatments, the cells were cultured in control medium supplemented with 5% porcine FF or both 5% FF and 50 ng/mL BMP-4 (Sigma). As a control, fibroblasts that were not treated with 5-Aza were cultured in control medium. Besides evaluating cell morphology after each period of culture (4, 7, 14 and 21 days), the expression of markers for pluripotent cells (Oct-4, Nanog, Rex-1 and Sox-2), primordial germ cells (Dazl and Vasa) and oocytes (GDF-9B) was evaluated by qualitative PCR. After 5 to 7 days of culture, several colonies of round cells in the three different media tested were observed, and some of these cells had a diameter of approximately 25μm. The morphology of these cells resembled that of oocytes before primordial follicles formation. After 14 to 21 days in culture, primordial and primary follicle-like structures, with a diameter of 40-50μm were observed. These structures had a large centrally localized cell surrounded by small cells, being the morphology similar to that of oocyte and granulosa cells. The PCR showed that 5-Aza induced the expression of mRNAs for Oct-4, Nanog, Rex-1 and Sox-2 and the cells continued to express Nanog and Sox-2 for up 21 days of culture in the three tested media. Expression of Oct-4 and Rex-1 persisted for up 7 days after cultured in all media, but after 7 and 14 days, the expression was kept only in medium with both FF and BMP-4. Regarding to primordial germ cell and oocyte markers, transcripts for VASA and GDF-9B were detected from 4 to 21 days of culture in all tested media. In addition, the expression of DAZL was observed in cells cultured either in control medium for 7 days or in medium with FF and BMP-4 for 14 and 21 days. In conclusion, 5-Aza stimulated the expression of Oct-4, Nanog, Rex-1 and Sox-2 in pig fibroblast and these cells were able to differentiate into ovarian follicle-like structures after 21 days of culture.

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Equine induced pluripotent stem cells (iPS) production through exogenous expression of human factors related to pluripotency

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Keywords: equine, iPS, STEMCCA.

Induced reprogramming has been used as a tool to in order to reprogram somatic cells to a pluripotent state similar to the embryonic stem cells (ESC). Transcription factors previously described by TAKAHASHI and YAMANAKA (Cell, 2006, 126, 663-76), that, when associated are capable of reprogramming cells into a pluripotent state, turning them into induced pluripotent stem cells (iPS), were used in this study. Aiming equine iPS cells (eiPS) production, human factors related to pluripotency OCT4, KLF4, SOX2 and c-MYC in a polycistronic lentiviral vector STEMCCA (stem cell cassette) were introduced in mesenchymal cells derived from equine adipose tissue and cultured at 38.5ºC with 5% CO_2 atmosphere and maximum humidity. eiPS cells were characterized regarding their morphology, alkaline phosphatase expression, gene expression, immunofluorescence, embryoid body (EB) formation, spontaneous differentiation *in vitro* and teratoma formation *in vivo*. A non-transduced mesenchymal cell line was used as control group. Equine mesenchymal cells presented colony formation three days after exogenous gene insertion in their genome. These cells were individually cultured after approximately 15 days after transduction and three eiPS cells lines were studied. All three cell lines were tested and presented alkaline phosphatase expression. OCT4 and SOX2 expression was proven by quantitative gene expression and presence of OCT4 protein was confirmed by immunofluorescence for all cells studied. Two cells lines were tested regarding *in vitro* EB formation and both showed efficient generation of EBs. *In vitro* spontaneous differentiation analysis was tested in one cell line, however, it was still possible to observe a high ratio between nucleus/cytoplasm, even after 20 days in culture in fibroblast culture medium, indicating the possibility that full reprogramming and consequent transgene silencing did not occur resulting in continuous exogenous expression of pluripotent factors in these cells. Also, no teratoma formation was observed in a 35 days period after inoculation. Thus, it was possible to demonstrate equine iPS colony formation induced by the introduction of human transcription factors during this study. Studies regarding inactivation of introduced genes and reactivation of endogenous genes, characterizing full reprogramming of these cell lines are still needed and will provide important information about the nuclear reprogramming process for the equine species.

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Epigenetic marks and imprinted genes expression in bovine cells

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Keywords: epigenetics, imprinting, reprogramming.

The obtainment of appropriated cellular reprogramming is a hurdle in animal cloning. Many fetal abnormalities found in cloned animals have been correlated to abnormal epigenetic reprograming of the donor cells. In this context, the disrupted expression of imprinted genes has been highlighted as one of major factor that lead to this increased occurrence of fetal abnormalities such as the large offspring syndrome. Chromatin modifying agents, such as trichostatin-a (TSA), which is an inhibitor of histone deacetylases, have been employed to alter the epigenetic status of donor cells and evaluate the epigenetic mechanisms controlling imprinted genes and its impacts after nuclear transfer. To understand how the TSA can modulate IGF2R expression, we treated non-confluent bovine fibroblast with different concentration of TSA (0.05; 0.25; 1.25 and 6.25 μM for 8, 12 and 24-20 hours of culture). Three bovine fibroblast cell lines were treated and evaluated for cell viability by MTT analysis; global expression of IGF2R by qPCR and global di-methylation levels of lysine 9 of histone protein 3 (H3K9me2) by western blotting. The treatment of 6.25 μM of TSA decreased cell proliferation and there was a significant decrease of cells proliferation with the time. The TSA did not change global expression of IGF2R in any tested concentration and/or time; however there was a decrease of H3K9me2 levels when cells were treated with 6.25 μM TSA for 8 h. Moreover, there was depletion of H3K9me2 levels in the cells treated with 0.25; 1.25 and 6.25 μM TSA but not 0.05 μM after 24 h of TSA treatment. The qualitative western blotting analysis show an increase of H3K9me2 levels in cells treated for 24 h when compared to those treated for 8 h. Despite alterations of H3K9me2 levels and increased levels of 5-hidroxymethylcytosine by TSA have also been previously reported by others (Perecin et al., 2012, Reprod Domest Anim, 47, 503) IGF2R expression remained unaltered, indicating the lack of direct effect of histone methylation levels and 5-hmC levels on IGF2R epigenetic control. The results of this research will contribute to the understanding of the epigenetic mechanisms controlling IGF2R expression.

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Birth of an extremely lighter-than-normal asymmetric cloned Gujarat calf at term: similarity to the pathophysiology of intrauterine growth restriction (IUGR) syndromes in humans

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Keywords: abnormal offspring syndrome, IUGR, SCNT.

The *in vitro* production of bovine embryos by *in vitro* fertilization or nuclear transfer procedures is often associated with developmental abnormalities that appear to interfere with the pattern of fetal and placental growth and life *ex utero*, in a set of symptoms collectively called Abnormal Offspring Syndrome (AOS). One of the most common symptoms of the syndrome is the birth of unusually large calves with lower postnatal survival. However, smaller-than-normal calves may also occur on occasions, at a low frequency (Meirelles *et al.,* 2010, *Reprod Fertil Dev,* 22, 88-97). Disturbances in placentation caused by faulty epigenetic reprogramming may at least partly cause changes in the pattern of fetal growth during pregnancy. In human and sheep, nutrient restriction commonly related to reduced placental development or insufficiency is associated with intrauterine growth restriction (IUGR), which may lead to a pattern of asymmetric fetal growth. In such extreme cases, fetal asymmetry is characterized by an increase in brain-to-liver ratio and in abnormalities in internal organs (Cox & Marton, 2005, *Best Practice & Resarch: Obstetrics & Gynaecology,* 6, 751-64). In this report, we describe a case of fetal growth asymmetry in cattle and the birth of an exceedingly light asymmetric calf after the transfer of cloned embryos. Bovine cloned embryos were produced by Handmade Cloning (HMC) procedures, according to Ribeiro *et al.* (2009, Cloning Stem Cells, 11, 377-86), using skin fibroblast cells from three Gujarat females, two from adult and one from neonatal origin. A total of 56 out of 73 (72.6%) structures fused following embryo reconstruction, with 50 cloned embryos *in vitro*-cultured in aggregates (2 x 100%) in the WOW system. Cleavage and blastocyst rates on Days 2 and 7 of development were 92% (23/25) and 32% (8/25), respectively, on a per WOW basis. Eight cloned blastocysts were transferred to six synchronous Holstein-crossed female recipients, resulting in one pregnancy, diagnosed on Day 30 of gestation by ultrasonography. The viable pregnancy, developed from a grade 3 blastocyst stage (stage 6) embryo, was monitored monthly by ultrasonography, with signs of retarded fetal development observed as early as on Day 90 of gestation. Following the pre-induction for parturition on Day 284 of pregnancy, by the administration of 8 mg triamcinolone acetonide (IM), the recipient female calved vaginally on Day 287, delivering a female Gujarat calf weighting 2.2 kg, more than 90% less than the mean birth weight (BW) for the breed (25-30 kg). The calf died soon after birth, and atelectasia was detected after necropsy. The newborn calf was meconium-stained, with a normal coat color and no skin defects. Also, an increased head-to-liver size, bragnatism, lens opacity, apparent artrogryposis, thicker heart walls, and a poor skeletal muscle development were observed, with underdeveloped internal organs, including kidneys with thinner cortex and a smaller liver. Fetal membranes (FM) were lighter (530 g) and had a smaller cotyledonary surface area (CSA, 366.6 cm^2) for the species and breed, with fewer than normal cotyledons (n=27) and an abnormal BW-to-FM weight ratio (24.1%) and BW-to-CSA (16.7%), indicating an altered development of the placental tissue in prenatal development. The FM vasculature appeared to be underdeveloped. Similar to the case study, head sparing, i.e., growth of the head at normal or near-normal rate, is usually detectable in cases of IUGR in humans, which appears to be a protective mechanism to ascertain brain development, as placental is insufficient to provide the necessary nutrients to the fetus at mid- to late gestation. The understanding of mechanisms of prenatal growth in normal and abnormal development may be of significance for prevention or attenuation of abnormalities of common occurrence in cattle, being also a potential model for the study of pathophysiological processes related to IUGR syndromes in humans, sheep and other species.

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Intracytoplasmic sperm injection (ICSI) mediated transgenesis using bovine sex-sorted semen

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Keywords: ICSI, sex-sorting, transgenesis.

During the production of sex-sorted spermatozoa, the gametes are exposed to damages that reduce the number of viable spermatozoa (Boe-Hansen et al., 2005, Theriogenology, 63, 1789-802) but that could be beneficial for transgenesis. Since ICSI technique requires low numbers of sperm, and allows the use of low quality or even death sperm (Goto K et al., 1990, Vet Rec, 127, 517-20), its application could increase the efficiency of the use of sex-sorted semen, which usually has a high cost. The objective of this work was to test sex-sorted sperm for ICSI mediated transgenesis. COCs were collected from slaughtered cow ovaries and in vitro matured for 21 h. The sorted Y, X and non sorted semen from the same bull were thawed and co-incubated with 50 ng/µl of pCX-EGFP plasmid for 5 min and used for ICSI (ICSI-Y, ICSI-X and ICSI-NS groups respectively). Injected oocytes were activated by a 4 min exposure to 5 µM ionomycin, placed on TCM-199 for 3 h and subsequently treated with 1.9 mM DMAP for 3 h. Sham controls were injected with 50 ng/µl pCX-EGFP. Haploid and diploid parthenogenetic controls were also included (Haplo PA and Diplo PA groups respectively). Embryos were cultured in SOF medium. Cleavage and blastocyst rates were evaluated on Days 2 and 7 post ICSI, respectively. *EGFP* expression was assayed at day 4 and at the blastocyst stage. Differences among treatments were determined by Fisher′s exact test ($P \leq 0.05$). Cleavage rates of ICSI-Y (83%, n=106), ICSI-X (83.2%, n=101), Sham (85.3%, n=116) and Haplo PA (76.1%, n=88) were lower than those of the Diplo PA control (95.6%, n=157), and higher than ICSI-NS (50.9%, n=106) ($P \leq 0.05$). Transgene expression levels at day 4 were significantly higher for ICSI-Y (36.4%) and ICSI-X groups (38.1%) than those of ICSI-NS group (29.6%); and all of them differed from Sham control (1%) ($P \leq 0.05$). Although all the groups showed lower blastocyst rates than Diplo PA control (45.9%), only ICSI-NS (9.3%) did not differ from Haplo PA control, which did not produce any blastocyst. ICSI-Y and ICSI-X blastocyst rates (15.9 and 13.1% respectively) were higher than Haplo PA control group, but did not differ from Sham control (9.1%) ($P \leq 0.05$). The percentage of *EGFP* expressing blastocysts did not vary between ICSI groups (36.4; 28.6 and 40% for ICSI-Y, ICSI-X and ICSI-NS respectively), being all of them different from the Sham group (0%) ($P \leq 0.05$). To our knowledge, this is the first report of ICSI mediated transgenesis using sex sorted semen in bovine, and it demonstrates its utility to produce transgene expressing blastocysts with the same efficiency than non sorted semen. It could be a useful strategy to produce transgenic animals of the desired sex, avoiding the problems carried by somatic cell nuclear transfer.

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Evaluation of cell viability and gene expression of stress and apoptosis in bovine fibroblasts exposed to ethanol extract of *Azadirachta indica*

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Keywords: cell stress, nuclear reprogramming, somatic cell nuclear transfer.

Somatic cell nuclear transfer (SCNT) is an important biotechnological tool, but it still faces many challenges. The synchronization of donor cells has been described as one of the main factors required to achieve a correct nuclear reprogramming (Oback and Wells, 2004, Cloning Stem Cells, 4, 169-174). It has already reported that ethanolic extract of the *Azadirachta indica* (Neem) was efficient to inhibit the cell cycle of bovine fibroblasts (Rabelo et al., 2011, Acta Sci Vet, 39, 338) and that its effect is reversible (Rabelo et al., 2012, Animal Reprod, 9, 652). It is required, however, that the cells remain viable after exposure to the extract in order to support the embryonic development after SCNT. The objective of this study was to evaluate cell viability and expression of stress and apoptosis genes in bovine fibroblasts exposed to ethanol extract of Neem. The cells were cultured in DMEM + 10% fetal calf serum (FCS) and exposed to 100 and 200µg/mL of the extract for 24h. Three repetitions were performed in triplicate. Simultaneously, a serum starvation group (DMEM +0.5% FCS for 72h, without the extract) and a control group (DMEM +10% FCS) were prepared. Cell viability was evaluated by Trypan blue (0.1%) staining, which readings were performed by the Cedex XS Analyser automatic cell counter (ROCHE, Switzerland). Data was analyzed by analysis of variance and means compared by Student Newman Keuls test. Relative quantification of transcripts of stress and apoptosis (HSP70.1A, HSBP1, HSP27.P1, BAX, BCL-2 e WNT5A) was performed by Real-Time PCR and results compared by the comparative Ct method, using the β-actin gene as endogenous reference and control group as calibrator. Data was analyzed by the REST® software using the Pair Wise Fixed Reallocation Randomisation *TEST*®. The products obtained were also subjected to electrophoresis on agarose gel. Values are shown as mean ± SEM. There was no difference ($p>0.05$) for cell viability among serum starvation (51.78±3.04%), control (51.04±0.9%) and treatment with 100µg/mL *A. indica* (50.15±1.64%). However, cell viability decreased ($p<0.05$) when cells were exposed to 200µg/mL (45.1±2.76%). There was a decrease ($p<0.05$) on expression of genes associated to stress and apoptosis in cells exposed to *A. indica*, except for HSP27.P1 gene in 100µg/mL and HSP70.1A gene in 200µg/mL extract, which expression were not altered. These results indicates that 100µg/mL ethanol extract of *A. indica* can be a complement for in vitro cell culture, keeping cell viability and with a potential protective effect against stressful conditions of *in vitro* culture. Taking together, the previous results (Rabelo et al, 2011 and 2012) and the present one suggest that ethanol extract of *A. indica* can be an alternative for synchronizing cells donor nuclei.

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A243 Cloning, Transgenesis and Stem Cells

Clinical findings and neonatal care of two transgenic cloned calves containing coagulation factor IX: a case report

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Keywords: bovine, newborn care protocol, SCNT.

Both basic and applied research benefit from the technology of transgenic animal production. The production of transgenic animals through nuclear transfer (NT) presents advantages over other methods, in special, the possibility to select a transgenic donor cell prior cloning and therefore to produce an homogenous herd without mosaic offspring. Despite all efforts, the neonatal cloned calves mortality is still high, approx. 50%. The pulmonary hypertension is a problem that requires major clinical attention, representing the neonate disability on adapting to the new environment conditions. This study aimed to support the extra uterine life of two transgenic cloned calves by using neonatal care protocol focused on pulmonary function improvement. Bovine epithelial mammary cells, transduced with lentivirus harboring coagulation factor IX (FIX) driven by β-casein promoter, were submitted to NT. From 21 embryos transferred into recipient cows, two pregnancies were maintained until 290 days. For induction of parturition, 25mg (IV) dexamethasone (DEX) were used. Once no cervical dilatation was observed, the two calves were obtained by cesarean, 44h after DEX application. Both calves weighted approximately 40kg. At delivery, both newborn had their upper airway aspirated to remove excess fluid, for about 10 minutes. One minute after birth, both animals presented low APGAR scores, which is a manner to measure the newborn vitality by heart rate, respiratory rate, response to stimulation and mucous coloration; animal 1 (FIX1) presented APGAR score 2 and had more suppressed respiratory movement and became more cyanotic after the APGAR first evaluation, and animal 2 (FIX2) presented APGAR score 3. After 5 min post partum, APGAR score of FIX1 and FIX2 were 2 and 6 respectively. Atropine (0.05mg/kg; IV) was administered in both animals in attempt to revert bradycardia. Also, intranasal oxygen (5L/min), aminophylline (6mg/kg, IV), DEX (0.05mg/kg), bromhexine hydrochloride (0.5mg/kg; SC), and sildenafil (25mg, oral) were administered 10 minutes after delivery, in order to improve pulmonary function. Although treatments, FIX1 died about one hour, and FIX2 died about 6 hours after delivery. Clinically, FIX1 umbilical cord was increased in size, presenting approx. 85mm of diameter. FIX1 necropsy findings include hemorrhagic umbilical arteries increased in size and collapsed nonfunctional lung, fat degeneration of the liver, degenerated kidneys, pericardium excess fat, and hematic cyst at the heart valves. FIX2 had an improvement of physiological parameters after administration of medicines; nevertheless, it was feed by nasogastric tube. Although FIX2 had smaller umbilical cord (about 35mm) it was still augmented. FIX2 necropsy findings included internal bleeding in umbilical cord region next to abdominal ventral wall only. We conclude that neonatal care protocol could improve pulmonary function in some newborns presenting certain degree of lung maturation in cloned calves.

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Generation of bovine induced pluripotent stem cells (iPS) from Rho 0 mesenchymal bovine cells using human transcription factors

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Keywords: cells, iPS, Rho 0.

This study aimed to use bovine Rho 0 mesenchymal cells obtained through treatment with Ethidium Bromide (EtBr) to the conditions necessary to produce induced pluripotent stem cells (iPS) and to evaluate them regarding their mitochondrial DNA (mtDNA) content and gene expression of Tfam, Bax and Bcl-2. Rho 0 cells used in this experiment were produced in previous experiments using defined EtBr concentration (100ng/mL) and culture duration established based on King and Attardi (King 1996, Method in Enzymology, 264, 304-313). Cells were distributed in four experimental groups: control Rho 0 (Rho 0c); transduced Rho 0 (Rho 0t) both kept in culture in the presence of EtBr; control MSC (MSCc) and transduced MSC (MSCt), kept in culture in the absence of EtBr. Groups were then submitted to the process of transduction for viral insertion of the human vector STEMCCA (hSTEMCCA) containing transcriptional factors Oct4, Sox2, Klf4 e c-Myc sequences. These cells were cultured for 21 days, in ideal conditions at 38.5ºC, 5% CO_2 atmosphere and maximum humidity. Until the fifth day after transduction cells were cultured in supplemented IMDM medium (10% fetal calf serum; 1% Antibiotics), after the fifth day cells were cultured in supplemented DMEN/F12 KnockOut medium (20% KnockOut Serum Replacer; 1% Antibiotics; 1% Non-essential Aminoacids; 1% Glutamine; 0,007% β-mercaptoethanol; 0,01% bFGF). After a 21-days period, cells were collected and evaluated considering their mtDNA content and relative quantification of genes Tfam, Bax, Bcl-2 and the ratio Bax/Bcl-2. Regarding mtDNA copy number, there was no statistical difference after transduction between MSC and Rho 0 groups (P=0,10 and P=0,57, respectively) and also no interaction among experimental groups was found. It may be observed that there was no effect of the transduction process and treatment with EtBr during the process. Concerning relative quantification, groups didn't present difference for Tfam after transduction (P=0,54 and P=0,19, respectively for groups MSC and Rho 0). Groups also didn't present difference for Bax expression (P=0,87 and p=0,18, respectively for groups MSC and Rho 0). However, after transduction, groups Rho 0c and Rho 0t presented increase of Bcl-2 expression (P=0,02). After transduction, none of the groups presented expression alterations for Bax/Bcl-2 ratio. Despite there is interaction among studied variables, which is capable of influencing Bcl-2 expression for Rho 0c and Rho 0t groups and presenting tendencies, it can be concluded that the hSTEMCCA vector system associated or not to a mtDNA copy number reduction is not capable to induce bovine mesenchymal cells to a pluripotent state, once cells didn't succeed in forming colonies and didn't present morphological modifications.

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Allele-specific expression of the MAO-A gene and the X chromosome inactivation in bovine embryos produced by nuclear transfer

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Keywords: embryoblast, RT-PCR-RFLP, trophoblast.

The inactivation of one of the X chromosomes (XCI) in females equalizes gene expression between the sexes (Lyon 1961, Nature, 190, 372-373). The XCI in cloned embryos can show some alterations, as such as failures in the pattern of imprinting in the placenta (Yang *et al*. 2007, Nat Genet, 39, 295-302). Therefore, it is suggested that epigenetic reprogramming occurs incompletely at most clone embryos, resulting in an aberrant gene expression and abnormal embryonic development (Morgan *et al*. 2005, Hum Mol Genet, 14, 47-58). Thus, the aim of this study was to characterize the process of XCI in trophoblast (TP) and the inner cell mass (ICM) of bovine embryos produced by nuclear transfer (NT) through the characterization of allele-specific expression of the MAO-A gene (located on the X chromosome and subject to inactivation). We used fibroblasts from a skin biopsy of a female adult Nellore, previously genotyped for MAO-A gene and presenting AG genotype. The cumulus oocyte complexes from slaughterhouse ovaries were evaluated for the presence of the first polar body (PB) after 20 hours of maturation. Oocytes showing PB were subjected to the process of nuclear transfer, which were hold individually by holding pipette and a portion of the cytoplasm adjacent to the PB was removed. A somatic cell was then placed in the perivitelline space and the structures were subjected to electrofusion (ECM 200, BTX®) with two pulses of 2.1kVA with 50μs of duration. After 30 minutes, oocytes were activated using ionomicina/6-DMAP for 4-5 hours followed by *in vitro* culture in SOFaaci medium for eight days in an incubator at 39 ° C and 5% CO2 in air. It was produced a total of 25 embryos. In D8, Bx embryos were taken to the micromanipulator to separate ICM and TP. Total RNA was extracted from biopsies of TF and ICM individually using the Arcturus® Peak Pure RNA Isolation Kit (Life Technologies®). For cDNA synthesis was used Oligo dT primers (Invitrogen®) and SuperScript III reverse transcriptase (Invitrogen®). It was used RT-PCR-RFLP technique to detect and characterize the allele-specific expression of the MAO-A gene and 13U for digestion of restriction enzyme RsaI (Promega ®). Despite the possibility of the presence of trophoblast cells in biopsies of MCI, biallelic expression of the MAO-A gene in clone embryos (n = 25) were 32% (n = 8) in ICM and 32% (n = 8) in TP, with a prevalence of detection of G allele compared to A allele, in MCI (96% vs. 36%) and TP (100% vs 36%). We suggest that at this stage of development, the XCI is not random in ICM and TP embryo clones of cattle, or that there is an alteration induced by TN, causing an abnormal expression or some deviation in the process of XCI favoring the inactivation specific alleles, as the G allele.

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In vitro production of bovine tetraploid embryos after blastomeres fusion at two cell stage

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Keywords: blastocysts, fusion, tetraploid.

With the purpose of obtaining descendants from genetically superior animals, studies with in vitro embryo production were carried out and last generation biotechnologies have been developed, as NT. However, several anomalies have been related to animals produced by this technique. In bovines, peri-implantation losses are estimated at 50%. These losses are frequently associated with functional deficiencies that occur in early placentation. The concept of embryonic complementation consists in grouping diploid/tetraploid (2n/4n) cells with a non-random distribution of these cells. Tetraploid cells will contribute to the formation of extra-embryonic tissues while 2n cells will form the ICM. To obtain 4n embryos, 2-cell embryos can undergo blastomeres fusion or electrofusion. The aim of this study was to evaluate the use of polyethylene glycol (PEG) to fuse blastomeres of 2-cell stage embryo and subsequent embryo development toward blastocyst stage. The COCs were aspirated from slaughterhouse ovaries, selected and transferred to 100 µL droplets of TCM 199 and in vitro matured for a period of 22-24 hours. IVF was performed with semen from a single bull with proven fertility. SOF was the medium used for embryo culture. Embryos were maintained at 38.5 ° C and 5% CO2 throughout the culture. To perform cell fusion, 30-35 hours post fertilization (hpf) embryos with 2 blastomeres were selected and then inserted for 1 minute in medium containing 40% polyethylene glycol (PEG). The embryonic development was evaluated in four steps: (1) cleavage at 30-35 hpf, (2) fusion rate 3-4 hours after PEG treatment, (3) cleavage at D4 post fertilization, (4) blastocyst rate at D8 post fertilization. The experiment consisted of the following groups: G1 – non-fused 2-cell stage embryos, (G2)- fused 2-cell stage embryos after PEG treatment and G3 – non-cleaved embryos or embryos with more than 2 cells at the moment of fusion with PEG. Data were analyzed by ANOVA and the 't' test of Student. We found: (1) 66.03% of the embryos cleaved at 30-35 hpf, (2) 90.9% of embryos lost their shaft between the blastomeres, (3) at D4 the cleavage rate was 70% for G1, 81.81% for G2 and 74.32% for G3, (4) at D8 the blastocyst production rate was 50%, 63.64% and 49.61% for G1, G2 and G3 respectively. We can conclude that PEG can be used to fuse blastomeres and produce tetraploid embryos. Further studies will be carried out to analyze the embryos karyotype.