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Effect of cryopreservation of amniotic fluid-derived goat stem cells using different cryoprotectant agents

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Amniotic fluid-derived stem cells (AFSC) are important alternative sources that can be used in cell therapy and for storage banks, however, few studies have been carried out in goats referring to the cryopreservation procedures. The goal of this study was to evaluate the viability and rate of cell proliferation of AFSC after *in vitro* culture, and the effect of DMSO and glycerol as cryoprotectant agents. Nine 3-month-old goat fetuses from multi-breed goats kept under similar nutritional and management conditions were used to obtain amniotic fluid samples obtained by laparotomy. Syringes containing 10 mL PBS and 10% fetal bovine serum (FBS) were used to aspirate 10 mL of amniotic fluid after exposure of the uterus and identification of the amniotic cavity. The suspensions containing AFSC were centrifuged at 342G for 10 min, the supernatants were discarded, and the pellets were then resuspended in 1 mL *in vitro* culture medium (DMEM + 10% FBS + 2% pen/strep + 1% amphotericin B). The *in vitro* culture was performed in cell culture dishes in an incubator at 38.5ºC, high humidity and 5% CO_2. Total exchange of the culture medium was performed every two days, and cell confluency was evaluated daily. Upon reaching 80% confluence, cells at passages 0 (P0), 1 (P1) and 2 (P2), respectively on Days 10, 15 and 17 of culture, were trypsinized and quantified in a Neubauer's chamber, with a sample evaluated for viability in 0.4% Trypan blue staining. The AFSC in P2 were centrifuged, and 20.000 cells/mL were resuspended in either DMSO (45% DMEM + 45% SFB + 10% DMSO) or GLY (45% DMEM + 45% SFB + 10% glycerol) media, loaded into 0.25mL straws, and cryopreserved in liquid nitrogen, being subsequently thawed for viability assessment. Paired t-test analysis was used to evaluate rates of cell proliferation and viability at P0, P1 and P2, with results expressed as mean ± standard error of the mean. Data on cell viability after cryopreservation were compared by ANOVA followed by a Tukey post hoc test. A significant increase in cell proliferation rate was observed from P0 (13.000 ± 2.800) to P1 (54.000 ± 10.900), remaining similar to P1 at P2 (69.000 ± 15.000). No significant differences were observed in cell viability (%) at the different *in vitro* culture passages (P0: 94.2±1.5; P1: 91.6±2.0; P2: 94.4±0.8). After AFSC cryopreservation at P2 (94.4 ± 0.8), a significant reduction in cell viability was observed in DMSO medium (82.9 ± 1.9), which was significantly higher than in GLY medium (66.8 ± 4.2). We conclude that, despite the reduction in cell viability after cryopreservation, DMSO-based medium promoted better cell survival rates than the use of the Glycerol-based medium.

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Characterization and visualization of exosomes produced *in vitro* by a transgenic bovine fibroblasts culture

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Exosomes are extracellular vesicles produced and released from different cell types. These vesicles act as a new mechanism of intercellular communication between the cells. Usually, the fluorescent dyes used for labeling exosomes are not efficient in reflect the vesicle half-life, thus hindering the correct understanding of their dynamics, visualization and even localization. Thus, we proposed to characterize and visualize by confocal microscopy (CM) and nanotracking analyses the exosomes produced *in vitro* by a transgenic bovine fibroblast culture that release exosomes labeled with the GFP protein. For this, fibroblasts of two groups, control (n=4) and GFP+ (n=4), were cultured in the same concentration ($2.5x10^3$ cells/mL) and condition (38.5°C; 5% CO_2) using culture medium exosomes-free. Fibroblasts were cultured during 72h and the culture medium (8mL) was collected for isolation of the exosomes. A set of serial centrifugations at 200*g* for 10min, 2,000*g* for 15min and 16,500*g* for 30min at 4°C were performed to eliminate cell particles and debris. Afterwards, the culture medium was filtered through a 0.22µm diameter filter. Exosomes isolation was performed by two ultracentrifugation at 90.000*g* for 70 min at 4°C. At the end, the pellet was dissolved in PBS and the size and concentration of the exosomes were analysed using NanoSight 300 (Malvern, United Kingdom), the presence of GFP protein was analysed by Western blot (WB) and the visualization of exosomes by CM. Fibroblast cell lysates were used as control for the WB. For the CM, exosomes were stained following the manufacturer's recommendations with the fluorescent dye PKH67 as a positive and for the negative control exosomes were not stained. Statistical analysis was performed using Prism version 6.0 and the t test was applied at a significance level of 5%. The exosomes concentration and size of control group ($5.73x10^8$ particles/mL; 137nm ± 8.2) were respectively similar (P>0.05) to GFP+ group ($5.06x10^8$ particles/mL; 140nm ± 9.0). On WB, the presence of GFP protein in the exosomes samples isolated from the GFP+ group was detected, whereas the samples of the control group did not present the protein. The visualization of the GFP+ exosomes was possible by CM. They were absorbed and located in the perinuclear region of the fibroblasts, similarly as the exosomes from the control+ group labeled with the PKH67 dye. Based on the results, we can conclude that transgenic fibroblast cells are producing exosomes similarly to the control group. The exosomes were labeled with GFP and was possible the visualization by CM. In addition, future studies may investigate the content and action of these exosomes in several biological models.
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Polyethyleneimine (PEI) as polyfection system for swine sperm

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For the generation of transgenic animals, the insertion of exogenous DNA into the cell or gamete is crucial. There are several methods for cell transfection, most of which are expensive, poorly effective or both. Cationic polymers, including PEI, bind to DNA, forming PEI-DNA complexes. These, bind to the cell surface, interact with the cell membrane and are internalized by endocytosis. Once inside the cells, the PEI-DNA complexes are protected by the endosome, and then migrate to the nucleus, where the DNA molecules are released. However, PEI polyplex itself has not been tested on sperm of any animal species. Thus, this study aimed to develop a protocol for transfecting swine sperm using PEI as polyfection agent. For that, seminal samples were obtained from 10 male pigs (200µL, $2x10^6$spzt/mL). Data were analyzed using the PROC MIXED (SAS, v. 9.2 for Windows) adjusted by Tukey, comparing the lsmeans and analysis of interactions between groups. To determine the effects of PEI on seminal viability, the semen samples were incubated with 0.5 mg / mL PEI for 10 min or 2 h. The flow cytometry was used to evaluate the sperm viability (plasmatic membrane damage – PMD; acrosome damage – AD; DNA fragmentation – DNAF). The incubation for 2 h led to higher PMD index (66.46±2.70%, P<0.0001) in comparison to 10 min or control group (without PEI) (19.48±2.70% and 18.27±2.70%, respectively), but no difference was found for AD and DNAF among the groups. Then, the spermatozoon viability was established with a similar experiment but using the plasmid pmhyGENIE-5 complexed with PEI (400ng/mL, PEI/VET) and the transfection efficiency was determined by FISH. Additionally, the direct plasmid incubation without PEI was also carried out as a standard protocol. The direct plasmid incubation increased the DNAF (3.67±0.03%, P<0.001) in relation to the PEI/VET (0.81±0.16%) and control group (without transfection) (0.58±0.08%). On the other hand, the PEI/VET complex improved the PMD (P<0.001) in comparison to the incubation or control (66.97±3.55%; 20.60±3.81%; 21.91±3.55%, respectively), as well as the AD (70.79±3.68%; 19.49±3.61%; 31.68±3.61%, respectively). Despite the cell damage, the spermatozoa exposed to PEI/VET presented greater transfection index (76.80±3.09%, P<0.001) than the incubation (17.80±1.07%). These results suggest that the PEI could be an efficient and low-cost transfection method for swine sperm. It is worth to point out that the cells treated with PEI/VET showed higher indexes of PMD and AD, so that it would be interesting to combine it with bio-techniques that facilitate the fecundation (i.e. FIV or ICSI) or even inclusion of antioxidant or anti-apoptotic drugs to improve the spermatozoa viability.

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Allogeneic mesenchymal stem cells therapy in dog with acute phase distemper

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Mesenchymal stem cells (MSCs) derived from adipose tissue have aroused the interest of the scientific community for their use in research and cell therapies because of their recognized proliferative, immunomodulatory potential, as well as their capacity for tissue regeneration. Because of this, they are used in the treatment of numerous diseases, both systemic and focal. Distemper is a viral disease that causes immunosuppression and multisystem involvement, without a specific guideline for its treatment. In this way, it is impossible to guarantee a clinical improvement and in many cases the disease ends up evolving to the death of the patient. The aim of the present case report was to evaluate the therapeutic success of intravenous allogeneic MSCs transplantation in patient with acute distemper and clinical signs of systemic disease. It was attended at the veterinary acupuncture and chronic pain department of the FMVZ Veterinary Hospital - UNESP Botucatu-SP, a 1-year-old male dog, non-breed, 12.4 kg weight diagnosed by PCR positive for distemper. The animal had symptoms of acute phase, this is, respiratory digestive signs, anorexia and neuroclinic signs, with evolution of 3 weeks. He received conventional support treatment that consisted in fluid therapy and antibiotics. At the general physical examination, he was apathetic and had conjunctivitis with purulent secretions. In the specific neurological examination, the animal presented non-ambulatory tetraparesis with changes in the cranial nerves (threat reflex and oculomotor diminished), dysmetria, locating the damage in the cerebellum and brainstem, encephalic multisystemic pattern typical of the distemper. The erythrogram revealed moderate anemia. It was instituted an intravenous application of MSCs, in volume of 1 ml, with a concentration of ten million allogeneic cells, isolated and produced by the Laboratory of Cell Therapy and Advanced Reproduction of the Animal Reproduction and Veterinary Radiology Department. After 5 days of transplantation, the patient had an improvement of the neurological signs and began to recover the movements of the limbs. Due to the favorable evolution of the patient's infectious and neurological condition, rehabilitation was indicated with weekly sessions until complete clinical improvement. After 1 month of transplantation, neurological examination revealed complete reestablishment of all neurological signs as well as total limb functionality, showing that intravenous MSCs therapy associated with acupuncture rehabilitation / physiotherapy was effective in the patient’s treatment. The concurrent neurological signs of distemper are difficult to handle in most cases, therefore, the use of cell therapy, coupled with adequate management, was fundamental for clinical improvement. It is important that these new treatment strategies be scientifically evaluated to elucidate these therapies in canine distemper treatment.

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Sperm Mediated Gene Transfer (SMGT). Successful gene transfection by in-vivo Artificial Insemination with carrying plasmid sperm integrated by GFP gene to bovine, ovine and rabbit embryos

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Genetic engineering in farm animals is in precarious development due to the high cost and low efficiency of the methods used. For example, male pronucleous microinjection in cattle yielded only 18 transgenic calves from 36,500 microinjected oocytes (0.0005%) and high degree of "mosaicism". Transgenic Nuclear Transfer less than 3% of GM (genetically modified) animals per trial due to "imprinting". In the other hand, by IVF+SMGT in 2010 were reported 68.5% GM pig embryos; other group by IA+SMGT, 74% GM piglets over 27 born. Our group published in 2016 51.4% GM sheep embryos by IVF+SMGT. Our goal is to standardize methods that use the sperm to produce transgenesis, either by IVF as in AI technique in different farm species. With these objectives 6 ewes (2 control; 4 treated), 4 cows (1 control; 3 treated) and 12 female rabbits (6 control; 6 treated), were hormonally stimulated to accomplish multiple ovulation. In ewes and cows heat induction and superovulation (TSOV) was performed by intra-vaginal progesterone devices associated with serial injections of FSHp. In rabbits, eCG was administered 48 hours before the planned heat, then a double dose of GnRH on the day of insemination. Treated groups were inseminated with sperm carrying a gene construct. For this purpose the commercial pEGFP N-1 that code for green fluorescent (GF) protein was used. Insemination in ewes was performed by laparoscopy, in cows by uterine trans-cervical double insemination and in rabbits, by double dose of sperm at vaginal bottom. As control group, randomly selected females of the three species inseminated with untreated sperm were considered. Embryos were collected at chronological age that allowed to find them in morula-balstocyst stage. Structures obtained were observed under fluorescence microscopy. Were considered positives those embryos that showed emission frequency compatible with GF in blastomeres, contrasting them with embryos of control groups and with unfertilized eggs. Three of the 6 donor sheep gave eggs structures (ES), 2 of treated group and 1 of control set. From them, a total of 14 ES were obtained, 5 embryos (E), 6 unfertilized ova (UO), and 3 empty zones (EZ). Five morulas from two ewes, inseminated with treated sperm, expressed GF in all blastomeres, 6 UO from the ewes inseminated with treated and untreated semen were negative to GF. All the four cows involved, treated and control group, gave 24 ES; 18 E and 6 UO were obtained. Fifteen E of 3 cows of treated group showed GF, 3 E of the control were negative to GF as well as 3 UO obtained from both categories. From 12 female rabbits involved, 10 gave 50 ES: 36 E, 9 UO and 5 EZ. Of the 50 ES, 16 E of treated group showed GF, 19 E of control set were negative to GF, the same as the 9 UO and the 6 EZ. Results showed positive transfection efficiency by sperm to the eggs since all embryos of treated groups in the three species exhibit green fluorescence in significative rate.

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Effect of long cooling periods of the ear skin at 5°C on the isolation and culture of bovine fibroblasts

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Viable somatic cell storage in cryogenic tanks is an essential step on bovine cloning achievement through nuclear transfer (NT). Cattle with high genetic potential have died suddenly without having their germplasm conserved *in vitro*. In such context, this research's objective was to verify the maximum possible time for isolation of somatic cells to be used in NT after an animal's death. For this purpose, ears of 6 Nelore females with an average age of 48 months, obtained in a local slaughterhouse, were conserved on the refrigerator at 5°C in clean ziplock bags during a 30 day period. On days 2, 4, 7, 14 and 21 after death, fibroblast isolation and culture were performed with DMEM medium supplemented with 10% bovine fetal serum. When cellular confluence was detected, cells were frozen with DMEM medium with 10% DMSO. We analised cell growth beginning (how long it took for the first cells to appear around biopsies), time of cell growth until confluence (how long it took to each animal's cells to reach confluence), cooling period in which cell isolation was possible, cell concentration on the freezing moment and contamination rate were performed. Average and Tukey tests at 5% were conducted comparing data between cooling periods. The longest period that allowed cell isolation in all animals was 14 days after the animal's death. However, the increase in cooling time significantly altered ($P<0.05$) initial cell growth, where on day 4 (D4) 4.33±1.03 days were necessary for the first cells to appear, and on day 14 (D14) 19.60±2.19 days were necessary. The time needed for cells to reach confluence was smaller on day 2 (D2) (28.00±3.10 days) in comparison with other cooling periods. Cell concentration suffered a significant decrease ($P<0.05$) only on D14 (698.125±131.203) in comparison to other periods (1.571.656±234.462). Contamination was more prevalent on cooling periods corresponding to days 14 and 21. Therefore, this study demonstrated that at 5°C, cell isolation was possible until 14 days after death. However, increase in cooling time affects cell growth pattern.

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Cellular reprogramming by defined factors of GFP expressing fetal fibroblasts in cattle: preliminary results on culture optimization

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After the emergence of cell reprogramming performed through the exogenous expression of "Yamanaka" transcription factors (Oct4, Sox2, Klf4 and cMyc - OKSM) in somatic cells, innumerous studies have been reported regarding the generation and maintenance of induced pluripotent stem cells (iPSCs) in different conditions. The success of bovine iPSCs (biPSCs) were described by Sumer et al. (2011), however, investigations on the pluripotent state of biPSCs are still needed because different protocols and characterization profiles were utilized. The aim of this study was to produce biPSCs lines which express GFP in different culture systems for better self-renewal and pluripotency maintanence. For that, three lines of bovine fetal fibroblasts (bFF1, bFF2, bFF3) were analyzed regarding chromosomal normality (cytogenetics by karyotyping), transduced with GFP lentiviral particles (FUGW vector, Bressan et al., 2013) and submitted to cell reprogramming through lentiviral transduction of OSKM transcription factors. Moreover, the cells were further cultured in reprogramming medium (DMEM/F12 KO and 20% KSR) supplemented with bFGF, LIF, bFGF+2i or LIF+2i. Cytogenetics of bFFs was analyzed by Giemsa stain and the success on GFP transduction through flow cytometry. The capacity of cell reprogramming was analyzed by colonies formation and maintenance after manually passaging and alkaline phosphatase (AP) detection. Statistical analyses were performed using the JMP Software. The cytogenetics analysis showed two normal lines (bFF1 and bFF2), however, the bFF3 was abnormal. All lines were successful regarding GFP transduction (bFF1 85.1%, bFF2 71.1% and bFF3 86.4% of GFP+ cells). Regarding reprogramming culture treatments, the three lines generated colonies in all culture systems, where 13 colonies of bFF1 (bFGF – 5, bFGF+2i – 5, LIF - 1 and LIF+2i – 3), 18 colonies of bFF2 (bFGF – 5, bFGF+2i – 5, LIF - 3 and LIF+2i – 5) and 19 colonies of bFF3 (bFGF – 5, bFGF+2i – 5, LIF - 4 and LIF+2i – 5) were manually picked. After, colonies that showed viability were passaged enzymatically. For bFF1, 80% of colonies derived from bFF1 cultured with bFGF, 75% in bFGF+2i, 100% in LIF and 100% in LIF+2i were picked for next passage. In line bFF2, 80% of colonies in bFGF were picked, 100% in bFGF+2i, 40% in LIF and 100% in LIF+2i. In line bFF3, 100% of colonies in bFGF were picked, 80% in bFGF+2i, 75% in LIF and 60% in LIF+2i ($P>0.05$). As a first pluripotency test, all colonies tested were positive for AP detection. In conclusion, lines of bFF presenting both normal and abnormal karyotypes presented success after GFP transduction and reprogramming using OSKM in all medium tested, however, further tests should be performed to analyze the pluripotency status of colonies and indicate the best culture system, in special, after several passages to confirm the maintenance of pluripotency *in vitro*.
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Viability of dog stem cells maintained at room temperature for 50 hours

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The use of mesenchymal stem cells (MSC) in the treatment of diseases has shown significant growth in veterinary medicine, since it is a therapy that can be used to accelerate wound healing, decrease inflammation and modulate the immune system, due to the release of cytokines and growth factors. MSC can be obtained from several adult tissues, however, the most commonly used source has been adipose tissue. Due to the emergency nature of some situations, as well as the need to send MSC to several Brazilian regions, an option for the immediate use of the cells in therapy emerged: the creation of cryopreservation banks of allogeneic cells. In this context, the objective of this study was to evaluate the period of time in which it is possible to maintain viable MSC at room temperature stored in a specific transport medium produced by BioCell® commercial laboratory. For this, MSC obtained from the adipose tissue of four dogs was used, each animal being considered a biological replicate. The cell line used was previously tested, by means of immunophenotyping, on the markers already described in the literature that guarantee to be a lineage of MSC. After culturing and confluence, the cells were cryopreserved and maintained in N2 until the time of the evaluations. Initially, cryopreserved samples were thawed and adjusted to $1x10^6$ / mL, protected from light and stored at room temperature in insulin syringes in the total volume of 0.5 mL of BioCell® transport medium. Samples were stained by the Alexa Fluor® 488 Annexin V / Dead Cell Apoptosis Kit (Molecular Probes) and, after 15 min incubation, the samples were evaluated in FlowSight® image flow cytometry (AMNIS, Seattle, WA). The evaluations were started 2 h after thawing and performed every 2 h for a period of 36 h, with a final evaluation at 50 h after thawing. Approximately 30,000 cells were acquired per sample and the results were analyzed using IDEAS V6.0 analysis software (AMNIS). The results were analyzed by GraphPad (Prism 6) through the analysis of variance test and the means compared by the Tukey test. It was possible to observe that, only after 30 hours in storage at room temperature, the quality of the cells changed to that observed in 2 hours of storage. To the moment of 28 hours the average of viable cells was of 78.5 ± 9.2. Based on the results, it was possible to conclude that when kept in specific transport medium at a concentration of $1x10^6$ / mL, MSC obtained from dogs can be maintained for up to 30 hours at room temperature without compromising viability.

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Integration-free induction of cell pluripotency in the porcine model as a translational model

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The generation of pluripotent stem cells (induced pluripotent stem cells, iPSCs) through forced expression of known transcription factors *in vitro* (Takahashi and Yamanaka 2006) opened a new era for translational and regenerative medicine. The swine is a very adequate species to model several conditions and syndromes due to its well-known production, maintenance and physiological similarities to humans. Initial methods of pluripotency induction relied on integration of viral vectors into host genome that, although efficient, may lead to undesired mutations and residual transgene expression possibly resulting in tumorigenicity and differentiation. Alternative methods are desired aiming the safety for future clinical use of iPSCs. Herein, we aimed to generate porcine iPSCs by use of episomal vectors due to its non-viral and non-integrative properties, that can replicate extrachromosomally and it is gradually and spontaneously eliminated from the cells. Porcine fibroblasts were nucleotransfected (Nucleofactor 2b kit, cat.#VPI-1002, Lonza) with either Addgene´s human episomal vectors containing pluripotency-related transcription factors, pCXLE-hOCT3/4-shp53-F (hOCT4, shRNA against p53, cat.#27077), pCXLE-hSK (hSOX2, hKLF4, cat.#27078) and pCXL.E-hUL (hL-MYC, hLIN28. cat.#27080) or mouse episomal vectors pEB-C5 (mOCT4, mSOX2, mKLF4, mc-MYC, mLIN28, cat.#282229) and pEB-Tg (SV 40 Large T antigen, cat.#28213). After 2 days of the nucleofection, the media was changed every other day and supplemented with 0.05mM sodium butyrate (cat.#303410, Sigma) until D12. On D6, 2x 10^4 cells were transferred into a new 10cm^2 well previously covered with Geltrex (cat. #A14133-02, Gibco) and cultured from that moment on with E8 (cat.#A15169-01, Gibco). Cells were cultured for more than 40 days in 38.5ºC and 5% CO_2. So far, morphology and presence of Alkaline Phosphatase activity with the Leukocyte Alkaline Phosphatase kit (cat.# 86R, Sigma) were analyzed. Flat, compact and clear edged colonies formed by round cells with high nucleus-cytoplasm ratio were observed on the human vector group as early as D20, at D27 they were tested and positive for AP activity. Those results suggest a successful reprogramming of the porcine cells into iPSCs by episomal vectors with the human based gene sequences. No colonies were formed on the mouse vector group until D40. It was observed a change in cellular morphology where the cells gained characteristics similar of the other species´ iPS cells. Future steps include a more complete characterization regarding gene expression and protein detection. Also, optimization of procedures in order to achieve a higher efficiency of colony formation is still need to be performed. Such results may greatly contribute to the understanding of the role of these pluripotency factors and the generation of a safe and reproducible model of study for further cellular therapies used for regenerative and translational medicine.

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Survival and developmental outcome of bovine IVP embryos following cytoplasmic microinjection for HDR gene editing at the 1-cell stage embryo

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The aim of this study was to evaluate the survival and *in vitro* development of IVP bovine embryos after cytoplasmic microinjection (MI) of donor DNA at the 1-cell stage embryo for HDR, targeting H11 and Rosa26, two safe harbor *loci* (SHL). Bovine IVP embryos were produced in 23 replications, according to Gerger *et al.* (Reprod Fertil Dev, 5:950-67, 2017). A total of 5,613 bovine *cumulus*-oocyte complexes (COC) from slaughterhouse ovaries were *in vitro*-matured (IVM) for 20 h and fertilized (IVF, D1) for either 18 h (Control intact Group n=1,071) or 8 h (treatment groups), when groups of presumptive zygotes (n=4,574) were partially denuded by pipetting 8 h post-fertilization (hpf) to facilitate MI, and then segregated into four treatment groups: Semi-denuded (Semi, n=1,039), non-MI control; MI (15 pL, 8-10 hpf) with Tris-EDTA solution (TE) and Cas9 protein (TE/Cas9, n=489); and SHL groups, targeting either the H11 (n=1,389) or Rosa26 (n=1,625) *loci*, MI with Cas9 protein (30 ng/µL), guide RNA template for each SHL (20 ng/µL), and repair oligonucleotide templates (5 ng/µL) in TE solution (15 pL, 8-10 hpf). All embryos were *in vitro*-cultured in SOF medium at 38.8°C, 5% CO_2, 5% O_2, and 90% N_2, and saturated humidity up to the blastocyst stage (D7). Post-MI survival rates (D1), and cleavage (D2) and blastocyst (D7) rates were compared by the Chi-square test ($P<0.05$). Blastocysts were individually collected for genomic studies (pending analyses). Compared to Controls (Intact), partially denuding zygotes 8 hpf (Semi) reduced cleavage (726/1,071, 67.8% *vs.* 627/1,039, 60.3%) and blastocyst (335/1,071, 31.3% *vs.* 241/1,039, 23.2%) rates, respectively. Overall post-MI survival was 84.5% (2,961/3,503), which reduced cleavage (1,738/3,503, 49.6%) and blastocyst (493/3,503, 14.1%) rates, compared to the Intact and Semi groups. However, considering only surviving embryos after MI, cleavage rates were similar between the Semi and the MI groups (TE/Cas9, 226/376, 60.1%; H11, 673/1,170, 57.5%; Rosa26, 839/1,415, 59.3%). Conversely, on a per total COC, per surviving, and per cleaved embryo basis, blastocyst rates were respectively lower in the H11 (181/1,389, 13.0%; 181/1,170, 15.5%; 181/673, 26.9%) and Rosa 26 (230/1,625, 14.2%; 230/1,415, 16.3%; 230/839, 27.4%) groups than the TE/Cas9 group (82/489, 16.8%; 82/376, 21.8%; 82/226, 36.3%), which was similar to the Semi group. In summary, denuding zygotes 8 hpf compromised further development. Approximately 15% of the zygotes degenerated after MI, with cleavage rates being similar between semi-denuded and surviving zygotes after MI, irrespective of the group. The MI with TE/Cas9 had no effect on blastocyst rates, in comparison to the semi-denuded control group, but the MI with DNA donor templates for both SHL reduced development to the blastocyst stage. Despite the slight reduction in survival and development, procedures applied in this study were technically suitable for the cytoplasmic microinjection of cattle embryos.

A251 Cloning, Transgenesis and Stem Cells

Porcine putative primordial germ cells-likes (ipPGCL) generated from induced pluripotent stem cells (piPSCs)

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Recent studies in murine model have reported that induced pluripotent stem cells (iPSCs) are able to different into primordial germ cell-like (PGCL) (Hayashi et al. 2011 Cell 146, 519-532); however, no data is already reported in domestic animal models. The porcine is an important model for translational and regenerative research due to its similar physiological, anatomical and morphological features to humans. For this reason, the generation of porcine PGCLs *in vitro* (ipPGCL) can be useful to understand genetic and epigenetic remodeling of these cells *in vitro* and *in vivo*. This study aimed the generation and characterization of porcine primordial germ cells-like (ipPGCL) *in vitro*. First, porcine induced pluripotent stem cells (piPSCs) were induced into epiblast-like cells (EpiLC) by culture in fibronectin-coated (16.7mg/ml) 6-well plates and N2B27 culture medium supplemented with 20ng/ml activin A, 12ng/ml basic fibroblast growth factor (bFGF), and 1% knockout serum replacement (KSR) for 48 h. Then, epiblast-like cells were induced to differentiation in ipPGCL by non-adherent culture (Agree well plates, StemCell Technologies, Vancouver, BC, Canada) with GK15 medium (GMEM) supplemented with 15% KSR, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 2mM L-glutamine, 1% antibiotics, 500ng/ml BMP4, 100 ng/ml SCF, 500ng/ml BMP8b, and 50ng/ml epidermal growth factor for 4 days. Lastly, putative ipPGCLs were characterized according to its morphology, alkaline phosphatase detection and expression of OCT4 and gem cells (DDX4/VASA, DAZL) markers. Our results showed that piPSCs can be induced into EpiLCs and these cells were further induced into putative ipPGCLs. In addition, ipPGCLs had typical morphological features: oval or round shape and irregular contour, were positive for alkaline phosphatase, OCT4, DDX4 (VASA) and DAZL. These preliminary data represent the first step for *in vitro* generation of porcine ipPGCLs. The ability to generate ipPGCLs from piPSCs may provide an adequate *in vitro* model to be used in the study of unanswered questions about germ cell biology and infertility.
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A252 Cloning, Transgenesis and Stem Cells

Characterization of mesenchymal stem cells of the umbilical cord of buffaloes (Bubalus bubalis) from baixada maranhense

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Several studies confirm the therapeutic efficacy of umbilical cord stem cells of many species; however, there are few studies that characterize them in buffaloes. Thus, the aim of this study was to isolate and identify stem cell populations post-mortem from the umbilical cord of buffaloes. Samples were obtained from the fetuses of pregnant buffaloes slaughtered in slaughterhouses in the city of Viana, Maranhão. For cell isolation, fragments of umbilical cord were washed with phosphate-buffered saline solution and antibiotics (100 μg/mL of streptomycin and 100 Units/mL of penicillin), cut in Petri dishes, conditioned in culture bottles containing α-MEM medium and incubated at 37ºC and 5% CO_2. For morphological analysis, the cells were evaluated every 48 hours during two weeks. Culture aliquots were frozen for further RNA extraction and, for analysis of the gene expression of the pluripotency markers CD29, CD44, SOX-2 and NANOG by real-time RT-PCR. Statistical analyses were performed using the software GraphPad Prism 5. Cells presented 80% confluence from the fifteenth day of culture, with high expansion capacity after the first pass. Morphologically, cells varied from fusiform to rounded, meeting the morphological definition of stem cells established by the International Society for Cellular Therapy. In the analysis of gene expression of the second and fourth passage cultures, it was observed that both expressed transcripts of the evaluated genes, with significant results in the fourth passage culture, because they present a larger number of cells, therefore, more homogeneous expression of the markers. According to the results, it was possible to consider that the post-mortem isolation of mesenchymal stem cells from the umbilical cord of buffaloes is feasible, since there were no changes in cell viability, and the gene expression confirms that these are, in fact, mesenchymal stem cells.

A253 Cloning, Transgenesis and Stem Cells

Characterization of mesenchymal steem cells of the bone marrow of buffaloes (*Bubalus bubalis*)

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Mesenchymal stem cells from the bone marrow are able to differentiate into several tissues, such as bone, cartilage, adipose tissue and muscles. Most stem cell research is performed on humans, rodents, felines and pigs, and there are few works with breeding animals, especially buffaloes. The aim of this study was to isolate populations of mesenchymal stem cells from the bone marrow of buffaloes post-mortem, for morphological evaluation and expression of pluripotency genes. The bone marrow was collected from the sternum of five buffaloes from the city of Boituva - SP. The samples were washed with PBS, centrifuged and, after discarding the supernatant; the pellet was resuspended in DMEM medium and deposited on Ficoll-Paque solution. After further centrifugation, a white ring of cells was formed, which was collected, resuspended in medium and plated at a density of $2x10^5$ cells/cm^2. Cells were analyzed every two days for two weeks under inverted microscope until freezing. For growth curve analysis, cells were seeded in the fourth passage in 24 well plates, at a density of $2x10^4$ cells/ well, cultured for four days and counted on the interval of 24, 48, 72 and 98h (3 wells per treatment), to determine the number of cells and the best time for population doubling. To determine the expression of the pluripotency genes CD29, CD44, SOX-2, NANOG and the constitutive gene GAPDH, real-time PCR (RT-qPCR) technique was performed. After 48 hours of isolation, was observed the adhesion of the first cells to the bottles, reaching 80% confluence after 10 days of culture. Cells exhibited high *in vitro* expansion capacity. Morphologically, the cells presented a spindle-shaped and elongated form, similar to fibroblasts. There was no expression of any of the genes analyzed in buffalo bone marrow samples. The samples were run on agarose gel and only the endogenic control was observed. The results obtained reaffirm the need for new tests to confirm or not the viability of the post-mortem isolation of the bone marrow as a source of mesenchymal stem cells.

A254 Cloning, Transgenesis and Stem Cells

In vitro development and mitochondrial gene expression of interspecific brown brocket deer (Mazama gouazoubira; Fisher, 1814) cloned embryos

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Much emphasis is currently given to the use of interspecific Somatic Cell Nuclear Transfer (iSCNT) as a potential salvage tool for endangered animals. Despite these achievements, the success rate of iSCNT is still low. Mitochondria have a broad range of critical functions in cellular energy supply and programmed cell death, suggesting that inadequate mitochondrial functions may adversely affect iSCNT success. Thus, the aim of this study was to evaluate the *in vitro* development of brown brocket deer (*Mazama gouazoubira*) cloned embryos produced by iSCNT using bovine cytoplast. Additionally, the expression levels of the mitochondrial genes (*ATP6*, *COX3* and *ND5*) in cloned embryos were verified. Cell culture was prepared from skin biopsies from a *M. gouazoubira* adult female. After 7-14 days of incubation, monolayers with fibroblast-like morphology were disaggregate and stored in liquid nitrogen. Quiescence of donor cells was induced by growth to confluence prior to the scheduled experiment. Bovine ovaries were obtained from a local abattoir and the recovered COC were submitted to IVM in supplemented TCM199 (EGF, cysteamine, pyruvate, estradiol, antibiotic/antimycotic, FSH/LH and FBS) in 5% CO_2 at 38.5°C for 20-21 h. After IVM, denuded oocytes were stained with Hoechst 33342. Enucleation was performed under inverted microscope equipped with micromanipulators (Narishige, Tokyo, Japan). Embryo reconstruction was achieved using couplets fibroblast/cytoplast: deer/bovine and bovine/bovine. The couplets were placed in a 0.2-mm micro-fusion chamber (Eppendorf, Hamburg, Germany). Membrane fusion was performed using an electroporation system (Multiporator, Eppendorf) and the activation was induced by exposure to 5 μM ionomycin in TALP-H and incubation in 1.9 mM of 6-DMAP) in SOF. A bovine IVF group was used as a control. Presumptive zygotes were cultured in SOF supplemented (FBS and BSA) and a humidified gas mixture of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. Target gene expression was normalized for *GAPDH*. Relative quantification was performed in technical triplicate using a QuantStudio 3 Real-Time PCR System (Thermo Fisher Sci., Waltham, USA). Cleavage and blastocyst rates were analyzed using Fisher's exact test. Gene expression profiles were compared using non-parametric test ($P<0.05$). There was no difference ($P>0.05$) for cleaved and blastocyst rates between deer/bovine and bovine/bovine: 72.8% vs 65.5% and 11.3% vs 5.9%, respectively. However, the IVF group showed the highest blastocyst rate (44.0%). The deer/bovine group showed increased expression ($P<0.05$) for all evaluated genes when compared to IVF group, but when compared to bovine/bovine, the only difference was verified for ATP6. This study has demonstrated that bovine cytoplasts were efficient to produce brown brocket deer cloned embryos.

A255 Cloning, Transgenesis and Stem Cells

Effect of stem cells application on the oocyte and embryo production of bovine females

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The injection of allogeneic mesenchymal stem cells (MSCs) in the ovaries can increase the number and quality of follicles and oocytes, with direct positive impact on IVEP. The aim of the present study was to evaluate the oocytes recovery rate per OPU and embryo production after the injection of MSCs in the ovarian cortical layer. In the study, 27 Nelore (*Bos indicus*) cows were submitted to synchronizations of the follicular growth wave [D -65: insertion of an intravaginal P4 device (1.0 g) and administrations of EB (2.0 mg im) and $PGF_{2\alpha}$ (0.53 mg im, sodium cloprostenol)]. Five days later (D -60), the P4 device was removed and the animals submitted to ultrasonographic evaluations and ovum pick up (OPU) followed by *in vitro* embryo production (IVEP). These procedures were repeated at 30-day intervals (D -30 and D0). On D1, cows were distributed to one of three experimental groups: CONT (there was no stem cells application; n=7), MSC1 (MSCs application – $5x10^6$ cells per ovary – on the cortical layer of one of the ovaries; n=10) and MSC2 (MSCs application – $5x10^6$ cells per ovary – on the cortical layer of both ovaries; n=10). Allogeneic MSCs from adipogenic origin were isolated and cultivated in IMDM culture medium with 20% FBS and 1% P/S, at 37 °C in 5% of CO_2 for cellular expansion until third passage. Posteriorly, MSCs were frozen in DMSO and maintained in liquid nitrogen until the day of application in the ovaries. On the injection day, the cells were thawed and DMSO removed. Followed, were maintained in IMDM culture medium until the application moment. After MSCs application, the cows of all groups were again submitted to follicular synchronizations, ultrasonographic evaluations and OPU-IVEP procedures at 30, 60, 90, 120, 150 and 180 days. The data were analyzed as time-repeated measures using the GLIMMIX procedure of SAS. After the MSCs treatment was observed interaction between treatment and time for the numeric variables: total follicles aspirated (P=0.001) total oocytes retrieved (P=0.01), viable (P=0.03) and cleaved (P=0.02), total embryos per OPU session (P=0.01) and hatched blastocysts (P=0.05). In cows receiving MSCs, the production curve of numeric variables presented less reduction in time in relation to CONT group. Interaction between treatment and time was not observed for the rates evaluated. It was verified that cows receiving MSCs had an increase in the recovery rate (CONT=62.6%^{b}, MSC1=62.8%^{b} and MSC2=67.8%^{a}; P=0.01), viable oocytes rate (CONT=63.0%^{b}, MSC1=64.3%^{ab} and MSC2=67.8%^{a}; P=0.01), cleaved rate (CONT=63.8%^{b}, MSC1=67.9%^{a} and MSC2=64.5%^{ab}; P=0.03), blastocyst rate (CONT=34.7%^{b}, MSC1=39.0%^{a} and MSC2=33.3^b%; P=0.001) and hatched blastocysts rate (CONT=26.8%^{b}, MSC1=31.5%^{a} and MSC2=26.2%^{b}; P=0.0003). It was concluded that MSCs application increased the OPU-IVEP efficiency in bovine females.

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