A279 Cloning, transgenesis, and stem cells

Comparison of cytokines concentration in conditioned medium of non-immune stimulated mesenchymal stem cells (MSCS) derived from equine amnion (AM), allantois (AL), adipose tissue (AT) and bone marrow (BM)

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Studies have shown a bidirectional interaction between MSCs and immune cells, also different sources of MSCs may release divergent paracrine factors amounts. Further, some reports suggested MSCs are not spontaneously immunosuppressive, needing to be immune-challenged to exert their immunomodulatory potential. The aim of this study was to compare the presence and concentration of pro-inflammatory (PI) INFγ, IL1α, IL2, IL4, IL08, IL15, anti-inflammatory (AI) IL1RA, IL10, MCP-1(CCL-2) cytokines and VEGF in the conditioned medium derived from AM, AL, AT and BM-MSCs, non-challenged by immune stimulus factors (LPS, IL-1α and β, TNFα ot INFγ). Cells in P3 (~5*103), previously characterized, were obtained from our cell bank, thawed, plated and cultured in medium composed of 80% DMEM/F12, 20% FBS, supplemented with antibiotics and antimycotic, at 37,5°C, 5% CO2 (Gibco-USA) and controlled humidity incubator. After reaching at least 70% of confluence, samples were washed six times with HBSS, and cultured in deprivation of FBS for 4 days. The conditioned media (CM) was then collected, centrifuged 10 min/900G, filtered in 0.22µm syringe filter and frozen at -80°C. The concentrations of cytokines were measured by quantibody array analysis, in quadruplicates, using a microarray scanner (Innopsys, model InnoScan 710). Images were analyzed by Mapix 7.0 software (Innopsys). Statistics were performed by T-Student test and one-way ANOVA, followed by Tukey's test when differences between groups were found, using Past3 Software (PAST3, 2017). Significant differences were considered when $P<0.05$. No concentrations of PI cytokines INFγ, IL2, IL4 and IL10 or AI cytokine ILRA were detected (bellow Limit of Detection) in all groups (AT, BM, AM and AL). However, IL1α low concentrations were detected, with no significant differences, for the groups AM (9,75pg/mL), AL (1,92pg/mL) and AT (1,0 pg/mL) but not in MO-CM. All groups presented IL-8 concentrations, but it was significantly higher in AL (118,02 pg/mLa) than AT and MO (21.93b and 1.53 pg/mLb), with no significant difference with AM (87,02 pg/mLab). IL15 was present only in AM (81.35 pg/mLa) and AT (70.1 pg/mLa) CM, with no differences. Also, MCP-1 (AM: 333.2 pg/mLa, AL: 341.2 pg/mLa, MO: 397.2 pg/mLa, AT: 301.8 pg/mLa) and VEGF (AM: 548.52 pg/mLa, AL: 466.6 pg/mLa, MO: 484.7pg/mLa, AT: 248.6 pg/mLa) were highly found in all MSCs-CM groups. IL8, MCP-1 and VEGF are known to be produced by MSCs in response to inflammatory stimuli and, in this study, they were found in CM of non-immune challenged MSCs from different sources, indicating their spontaneous immunomodulatory potential. Still, presence and concentrations of cytokines may vary between MSCs sources.
Acknowledgements: FAPESP.

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Mesenchymal stem cells (MSCS) derived from equine allantois (AL): Characterization and immunomodulatory potential of an alternative source

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Perinatal tissues such as umbilical cord and amniotic membrane (AM) have caught the attention of the scientific community as an advantageous source of MSCs for regenerative medicine due to their plasticity, proliferative and immunomodulatory potential, in addition to their non-invasive sampling. To obtain equine AM-MSCs, the AM and AL are detached from each other and, even though the membranes are juxtaposed by their connective tissues, ALs are often discarded. The aim of this study was to isolate, characterize and evaluate AL-MSCs proliferative, differentiation and immunomodulatory potential. Cells were obtained from 5 allanto-amniotic membranes harvested during term delivery. Membranes were manually detached, and AL portion was fragmented in smaller pieces (3cm2), excluding vessels, for enzymatic digestion with 0,1% collagenase type 1 (Sigma-Aldrich, USA) in culture medium (80% DMEM/F12, 20% FBS, antibiotics and antimycotic - Gibco, USA). Cells were cultured in the medium described, at 37°C, 5% of CO2 and controlled humidity. Immunophenotype was characterized by flow cytometry (P3) for mesenchymal and pluripotency markers. Proliferative potential was evaluated by growth curve (plated in P3, resuspended and counted every 48 hs/8 days), fibroblastic colony forming unities assay (CFU-F) and efficiency of CFC-U (EFCU-F). For that 200cells/cm2 were plated and cultured for five days and then fixed and stained with violet crystal (1%). Colonies with 20 or more cells were considered. In vitro adipogenesesis and osteogenesis differentiations were perfomed and immunomodulatory potential were analyzed by quantibody array, in quadruplicate, for IFNγ, IL1a, IL1ra, IL2, IL4, IL8, IL10, IL.15, MCP-1 (CCL2) and VEGF concentration in the conditioned medium (CM) obtained after 4 days culture in FBS deprivation. Samples displayed plastic adherence, fibroblastic morphology, good proliferation (cells/cm2 - D0: 2000; D2:3820.26; D4:14660.13; D6:25525.47; D8:40433.56), high CFU-F (189,7) and ECFU-F (10.53). High expression of mesenchymal markers (CD44: 95.6%, CD29: 97.88%), low expression of hematopoietic marker (CD34: 8.4%), low expression of MHCII: 8.26%, and low expression of epithelial cells (Cytokeratin: 9.88%) were observed. High expression of pluripotent markers SOX2: 88.76, OCT4:75.9% and NANOG: 68.3% corroborates with another fetal sources' expressions, indicating high plasticity and differentiation potential. Further, in vitro osteogenic and adipogenic differentiations were positive. No relevant concentrations of IFNγ, IL1ra, IL2, IL4, IL10 and IL15 were shown and low concentration of IL1α (1.8pg/mL) were found. However, relevant concentrations of IL8 (118pg/mL), MCP-1 (341pg/mL) and VEGF (591.6pg/mL) were found. IL8 and VEGF are known to be released by MSCs after inflammatory stimuli. These results showed AL is, indeed, a good alternative source of MSCs, releasing cytokines involved in immune response and must be further studied.

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Influence of OCT4 and SOX2 exogenous expression on imprinting maintenance at H19/IGF2 locus in cattle

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OCT4 and SOX2 are fundamental genes for the acquisition and maintenance process of cellular pluripotency and therefore for induced pluripotent cells (iPSCs) generation. Recently, it has been reported that both factors may have a huge influence on the regulation of some imprinted genes, especially at locus H19/IGF2, known to be important for the normal development of embryo and placenta. Herein we aimed to understand the possible influence of their expression, together or separately, on H19/IGF2 imprinting regulation. For that, bovine fetal fibroblasts cell lines were transduced with lentiviral vectors containing human OCT4 or SOX2 cDNAs. The fibroblasts were analyzed trough cell cytometry and positive cells were sorted. Fibroblasts expressing OCT4, SOX2, both (OCT4+SOX2), none (control) together with a non-sorted and non-transgenic control (five treatments) were investigated regarding pluripotency and imprinted gene expression as well maintenance of DNA methylation patterns at H19/IGF2 locus. Expression of OCT4, SOX2, H19 and IGF2R genes were analyzed after RT-qPCR in triplicate for each experimental group using ACTB e C2ORF29 as constitutive genes. Sodium bisulfite treatment and sequencing of the differentially methylated region (DMR) were used for imprinting analysis. The amplification of fragments from the imprinting control region (ICR, H19-ICR) and sequencing for a single nucleotide polymorphism (SNP) at IGF2/H19 locus between Bos indicus and Bos taurus allowed for the allele-specific analysis of DNA methylation. The transcript quantification by qRT-PCR showed that OCT4 and SOX2 expression increased in the respective groups, the expression of H19 gene increased in the control sorted group and IGF2R expression was not different between groups. Imprinting pattern methylation at H19/IGF2 locus showed that OCT4+SOX2 group was slightly different from others whereas the control sorted group presented a demethylation on the maternal allele: Control non-sorted (21, 42% for DMR and CTCF, respectively), control sorted (0%), OCT4+ (21, 24%), SOX2+ (2, 4%), OCT4+SOX2 (5, 95%). In conclusion, this study shows that the production of cells expressing exogenous pluripotent factors was successful, the H19 imprinted gene expression pattern was influenced by cytometry and sorting and imprinting maintenance at H19/IGF2 locus may be influenced by both cytometer/sorting procedure and pluripotency related genes overexpression. Such results are unprecedented and may greatly contribute to the understanding of the role of these pluripotency factors in acquiring and maintenance of the epigenetic patterns in reprogrammed cells in cattle. We acknowledge the São Paulo Research Foundation (FAPESP) for the funding (Grant number: 15/01407-2).

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Sirna-mediated silencing of HMTs in nuclear donor cells decreases H3K9me in cloned embryos at the 8-16 cells stage, but not at the blastocyst

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Epigenetic mechanisms are responsible for many cellular functions. Amongst them, is the epigenetic memory maintained during cell division. Consolidation of this information is made by stable chemical marks as DNA methylation and histone modification. Understanding epigenetic mechanism allows the development of several studies, such as nuclear reprogramming, which is directly influenced by those marks. Thus, H3K9 methylation has been shown as the main barrier on the rodents nuclear remodeling, it modulation showed significantly improves in both iPS generation and Somatic Cell Nuclear Transfer (SCNT). Hence, the main goal of this project was to knockdown HMTs responsible for H3K9 methylation in bovine fibroblasts preceding SCNT, and to investigate it consequences on embryo development. To achieve these modifications, histone methyltransferases enzymes EHMT2 (G9a), SUV39H1, SUV39H2, and SETDB1 were silenced by small interference RNA technique (siRNA). The H3K9me2 and H3K9me3 levels were analyzed by immunostaining. Those cells were also used as nuclear donor in SCNT to understanding their role during nuclear reprogramming. For this, cells combining siRNAs for each gene (siRNA) or not (Control) were used as nuclear donor on SCNT (N=5). IVF embryos were produced at the same oocyte collection and used as biological control. Embryos at the blastocyst stage were accounted and compared among the groups. After analyzed, our blastocyst rates were 34% (2.4), 60% (4.8), and 28% (6.3) for IVF, SCNT-Control and SCNT-siRNA, respectively. Embryos at both 8-16 cells stage and blastocysts (N=10) were collected to evaluate by immunostaining the effect of HMTs knockdown upon H3K9me2 and H3K9me3 levels. The analysis showed that, in SCNT-SIRNA group, levels of H3K9me2 were considerably lower than control and IVF at the 8-16 cells stage, but not in blastocyst. In H3K9me3 levels, this decrease at the 8-16 cells stage was less evident, but did not change on blastocysts. Changes at this stage are noteworthy since it is the same period that occur the genome activation. The recovery of H3K9 methylation levels displayed at the blastocyst stage may explain why blastocyst rates did not increase as described in mice. Studies describing the kinetics of epigenetic modifications during embryo development in bovine are needed to elucidate the divergence between species.
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A283 Cloning, transgenesis, and stem cells

Comparison of cytokines concetrations in conditioned medium derived from canine amniotic membrane and adipose tissue mesenchymal stem cells non-immune stimulated

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It is well known that the presence of cytokines is crucial for an appropriate cell communication and the stimulation of the immune system in order to restore homeostasis against an aggression. The conditioned medium (CM) is an outcome of paracrine factors secreted by the mesenchymal stem cells (MSCs) and has an essential role in inhibiting apoptosis, enhancing angiogenesis, reducing inflammation and stimulating the immune system. The purpose of this study was to compare the presence and concentration of IL2, IL6, IL8, IL10, GM-CSF, MCP-1, RAGE, SCF, TNFα e VEGF cytokines in CM derived from canine amniotic membrane (AM) and adipose tissue (AT) MSCs non immune-stimulated. The previously characterized samples, in P3, were obtained from the laboratory cell bank, thawed, plated and cultured in 80% DMEM/F12 culture medium supplemented with 20% FBS, antibiotics and antimycotic (Gibco, USA), at 37.5°C, 5% of CO2 and controlled humidity incubation. After reaching confluence of approximately 70%, the cells were washed six times with HBSS and cultured in FBS deprivation for 4 days in order to obtain the CM from both sources. Subsequently, the medium was harvested, centrifuged 10 min/900G, filtered in 0.22μm syringe filter and frozen at -80oC for future analysis. The cytokines concentrations were analyzed by quantibody array of four AM and three AT samples, in quadruplicate, using a microarray scanner (Innoppsys, model InnoScan 710). Images were analyzed by Mapix 7.0 software (Innopsys). Statistics were performed by unpaired T student test with PAST3 Software 20017. Significant differences were considered when $P<0.05$. The pro-inflammatory cytokines IL2, IL6, RAGE and TNFα were not detected in either MSC sources. IL10 (AM: 808.72 and AT: 722.02 pg/ml), GM-CSF (AM: 10.83 and AT: 9.28 pg/ml), MCP-1 (AM: 2632.89 and AT: 3914.58 pg/ml) and SCF (AM: 18.65 and AT: 33.79 pg/ml) concentrations were detected, but with no significant differences between the studied groups. IL8 concentration in the AM-CM was significantly higher than AT-CM (2049.52 pg/mla and 42.90 pg/mlb). The cytokines detected in these samples are known to be released by the MSCs after an inflammatory stimulus with LPS, IL1α and β, INFγ or TNFα. The relevant concentrations of the cytokines found in CM derived from non-immune stimulated MSCs of this experiment encourage further studies regarding its spontaneous immunomodulatory potential. Acknowledgments: CAPES e FAPESP.

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Development of an *in vitro* model to study cell-to-cell communication utilizing transgenic fibroblasts capable of secrete extracellular vesicles tagged with GFP

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Extracellular membrane vesicles, also called as extracellular vesicles (EVs), are produced and secreted from different cell types. They recently emerged as a new and important component of cellular communication. Thus, EVs can transfer different kinds of signals between the cells that produce them and the recipient cells. However, the role and tracking of the VEs during the cellular communication process is still not clear. Therefore, the development of transgenic cell lines capable of produce EVs tagged with GFP can facilitate these studies. Herein, our aim was to develop a transgenic fibroblast cell line that secretes GFP-EVs. For this purpose, bovine fibroblasts were transfected with the DNA constructs using a stable transducer with lentivirus vectors (MGH Vector Core, Boston, MA, USA). Briefly, lentiviral particles were produced by transfection of 293T cells (Invitrogen) with Lipofectamine 2000 (Invitrogen) for 12-16h. After this, the medium was recovered and centrifuged to harvest the viral particles. Fibroblasts were plated at the density of 105 cells and 50 µl of the viral concentrate plus 8ng/mL polybrene (hexamethrin bromide, Sigma) were added to the culture. The culture medium was replaced every 12h for five days. Afterwards, the cells were sorted by flow cytometry. The size, complexity and the percentage of GFP-positive cells (GFP+) were analyzed. For this, 10.000 fibroblasts from the both groups, Control (non-transfected) and GFP+, were selected by sorting of cells with GFP protein-fluorescence presence. In the control group, there were no GFP+ cells and 87.4% (n = 8.741) of the cells were retrieved. Regarding the GFP+ group, we retrieved 94.2% (n = 8.043) and all of them were expressing the fluorescent-protein. The recovered cells from both groups were re-plated at the same concentration (105 cells/mL) and after three days of culture, the medium was recovered for evaluation of the size and concentration of the produced EVs. In relation to the EVs production, the Control and GFP+ groups presented concentration of 2.86x108 and 5.95x108 particles/mL and the size average were 165nm ± 2.5 and 141nm ± 5.2, respectively. Based on the preliminary results presented here, we were able to establish and produce a transgenic cell line capable to release EVs tagged with the GFP protein. However, others evaluations are still necessary for complete characterization of these vesicles. In addition, through this in vitro model, future studies will be able to investigate the content, tracking and action of EVs in several biological models.
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Effect of stem cells application on the follicular population of bovine females (preliminary results)

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The objective of the present study was to evaluate the ovarian follicular population in bovine females after the application of allogenic (from another individual of the same species) mesenchymal stem cells (MSCs) in the ovaries. For this, 15 Nelore cows (Bos indicus) were submitted to synchronizations of the follicular growth wave to measure the amount of follicles at the beginning of the emergence of the follicular growth wave [Day-5 (D-5): insertion of an intravaginal P4 device (1.0 g) for 5 days and administrations of EB (2.0 mg im) and PGF2α (0.53 mg im, cloprostenol sodium)]. On D0, cows were divided to one of three experimental groups: CONT (n=5), MSC1 (n=5) and MSC2 (n=5), according to the amount of follicles verified in the ovaries. MSCs of adipogenic origin were isolated and cultured with IMDM culture medium with 10% SFB and 1% P/S at 37°C in 5% CO2 for cell expansion till third passage. Cells were frozen in DMSO and kept in liquid nitrogen until the day of application in the ovaries (D1). On this day, the cells were thawed and the DMSO removed. They were then maintained in IMDM culture medium until the time of application. In the CONT females were not applied MSCs. In MSC1, MSCs (3x106 cells) were applied to the cortical layer of one of the ovaries. In MSC2, MSCs (3x106 cells per ovary) were applied to the cortical layer of both ovaries. The cows were submitted to ovarian ultrasonographic (US) evaluations to quantify the follicular population on D0, D7, D14 and D21. The data were analyzed as time-repeated measure using the GLIMMIX procedure of SAS. The ovarian follicular population on days 0, 7, 14 and 21 were respectively: CONT (42.0±9.8, 44.5±9.4, 37.0±8.0 and 41.5±8.8), MSC1 (42.8±8.6, 41.8±4.3, 49.2±10.3 and 49.8±8.4) and MSC2 (40.2±6.4, 42.6±8.3, 59.2±15.3 and 48.0±8.1; Ptreatment=0.87, Ptime=0.33 and Pinteraction=0.21). Despite the numerical increase, in the present study no statistical difference was verified in the ovarian follicular population of bovine females submitted to the application of allogeneic mesenchymal stem cells in the ovaries. Further studies are needed to evaluate the long-term effect of MSCs treatment on the ovarian follicular population.

A286 Cloning, transgenesis, and stem cells

Effect of the passage number on the viability of brown brocket deer (Mazama Gouazoubira) fibroblasts

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The brown brocket deer (Mazama gouazoubira), one of the ten recognized deer of the Neotropical region, has recently been placed in a critical position due to current threats, mainly hunting and habitat loss. Several studies have shown that some endangered species may benefit from interspecific Somatic Cell Nuclear Transfer (iSCNT) due to the possibility of using fibroblasts as karyoplasts. Thus, the objective of this study was to verify the viability of M. gouazoubira fibroblasts submitted to several cell culture passages. Cells were obtained by auricular biopsy and then cultured and cryopreserved at passage 1. Cell culture was performed from this stock until the passages of interest were achieved (4, 7 and 10). Cells were cryopreserved in cryotubes (frozen/warmed group) and there was a cultured control group for each cryopreserved passage. Population Doubling Time (PDT) of each passage was determined using cultured control groups. Trypan Blue test, MTT assays and flow cytometry analysis (apoptosis and necrosis level) were performed using frozen/warmed groups with their respective cultured control groups for comparison. Statistical analysis was performed by means of ANOVA. Student's t-test and Fisher's exact test were applied when convenient. In order to detect statistical differences, a p-value of 0.05 was specified as the significance level. Fibroblastic cells were observed to grow between 12 and 14 days after the tissue explants had adhered to the culture plates. The PDT was higher ($P < 0.05$) in passage 10 (229.7 ± 3.3 h) when compared to passage 4 (89.3 ± 0.95 h) and 7 (90.8 ± 1.21 h), which were not different between them ($P > 0.05$). Cell proliferation rate of passage 10 was significantly lower ($P < 0.05$) when compared to passages 4 and 7. A high cell survival rate of more than 80% was observed in all groups, except for passage 10 in the frozen/warmed group. Regarding the MTT test, no significant difference ($P > 0.05$) was observed for the metabolic activity in the cells of passage 4 compared to passage 7. However, passage 10 exhibited low metabolic activity (62%) with respect to their control in culture. This value was significantly lower ($P < 0.05$) when compared to the passage 4 (80.5 %). Moreover, concerning the percentage of viable cells (in frozen/warmed group), it was observe that cells from passages 4 (88.6 %) and 7 (87.8 %) were statistically superior ($P < 0.05$) to passage 10 (59.1 %). Therefore, our findings clearly show the viability of M. gouazoubira fibroblasts derived from passages 4 and 7, and these cells could be used as a useful tool in conservation programs for this species.

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A287 Cloning, transgenesis, and stem cells

Effects of buffer medium and cytochalasin B on survival, development and reporter gene expression following embryo cytoplasmic microinjection with DNA

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The aim of this study was to compare the in vitro efficiency of distinct buffer media to deliver nucleic acids into bovine embryos by cytoplasmic microinjection (MI). Tris-EDTA (TE) and KCl-based (PS) buffers were compared for DNA dilution (circular GFP vector) at 30 ng/µL for the MI of bovine embryos after 5-min incubation or not in 5 µg/mL cytochalasin B (CCB) in the medium prior to MI. Bovine in vitro-matured oocytes were chemically activated in 5 µM ionomycin for 5 min, followed by incubation in 2 mM 6-DMAP for 4 h. Embryos were allocated to one of five groups: non-manipulated controls, and TE, PS, CCB+TE, and CCB+PS microinjected groups. The MI of 15 pL (1.5% of total embryo volume) was performed using a microinjector apparatus (Femtojet 4i, Eppendorf, Germany). Then, embryos from all groups were in vitro-cultured for seven days. Survival, cleavage and blastocyst rates, and proportion of GFP+ blastocysts were compared by the Chi-square test ($P<0.05$). After five replicates, 1,432 matured oocytes were chemically activated, and 1,053 microinjected. Overall, CCB-treated embryos had higher survival (82.3%, 427/519) and blastocyst (28.3%, 121/427) rates than non-treated counterparts (67.6%, 361/534; and 21.9%, 79/361, respectively), irrespective of the buffer medium, with no effect on cleavage rates (61.8%, 264/427 vs. 63.2%, 228/361, respectively), with cleavage and blastocyst rates similar to controls (70.6%, 190/269; and 26.4%, 71/269). The use of TE or PS buffers, regardless the CCB treatment, did not affect survival (73.1%, 384/525 vs. 76.5%, 404/528) or blastocyst rates between groups (28.1%, 108/384 vs. 22.8%, 92/404) and controls (26.4%, 71/269), but cleavage rate was lower using PS buffer than controls (61.1%, 247/404 vs. 70.6%, 190/269, respectively), with both being similar to the TE group (63.8%, 245/384). The TE and CCB+PS groups had similar (61.3%, 106/173; and 57.9%, 125/216) cleavage rates, but lower than controls (70.6%, 190/269), whereas the PS (64.9%, 122/188) and CCB+TE (65.9%, 139/211) groups were similar to all groups. The CCB+TE group had better blastocyst development (30.3%, 64/211) than PS (18.6%, 35/188), with both being similar to the Control (26.4%, 71/269), TE (25.4%, 44/173) and CCB+PS (26.4%, 57/216) groups. The CCB incubation did not affect GFP expressivity (50.4%, 61/121) in blastocysts when compared to no CCB exposure (51.9%, 41/79). However, DNA dilution in TE buffer improved the number of GFP+ blastocysts (62.0%, 67/108) than the PS buffer (38.0%, 35/92). A higher proportion of GFP+ embryos was seen in the CCB+TE group (63.5%, 40/64) than the PS (40.0%, 14/35) and CCB+PS (36.8%, 21/57) groups, with the TE group being similar (61.4%, 27/44) to the CCB+TE and PS groups. In summary, the embryo pre-exposure to CCB and the use of TE buffer for DNA dilution improved survival after microinjection, cleavage and blastocyst rates after culture, and proportion of GFP+ blastocysts on Day 7 of development.

A288 Cloning, transgenesis, and stem cells

Evaluation of the production of cytokines by bovine endometrial mesenchymal stem cells

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The aim of this work was to evaluate with a comercial kit the production of cytokines by bovine endometrial mesenchymal stem cells (eMSCs) challenged (treated – T) or not (control – C) with bacterial lipopolysaccharide (LPS- 1µg/ml : E. coli serotype 0111: B4, Sigma, St Louis, USA). Bovine eMSCs were collected in two phases of the estrous cycle (Phase, II N=6; Phase III N=6), and characterized. Cells on thrid passage were cultured with medium composed of DMEM high glucose/F12 (1:2), 20% fetal bovine serum (FBS), 100IU/mL penicillin, 100µg/mL streptomycin, 3µg/mL amphotericin B (Thermo Fisher Scientific®, (Walthan, USA)) and 11µg/mL amikacin (Teuto®, (Anápolis, BRA)) until 60% confluence. After confluence, cells were divided into studied groups and cultured with FBS free medium (C) or with 1µg/mL LPS (T) diluted in medium. Conditioned medium was collected after 2, 6, 12 and 24 hours of exposition, filtered through 22 µm filter and centrifuged at 2000 x g for 5 minutes. Samples were analyzed by Quantibody® Bovine Cytokine Array 1 kit (QAB-CYT-1, Raybiotech®, Norcross, USA), according to manufacturer's specifications and concentration of INFα, INFγ, IL-13, IL-1α, IL-1α, IL-F5, IL-21, MIP-1β, TNF-α on conditioned medium was analysed. The slides were scanned using a microarray scanner (Innopsys, model InnoScan 710) and the images were analyzed at Mapix 7.0 software (Innopsys). Each sample was evaluated in quadruplicate, and their data were normalized according to intra-slides positive controls. Results were achieved by eliminating the background. Sample concentration (pg/mL) was calculated based on linear regression of standard curves. Data were analyzed using the software GraphPad Prisma, version 6.01. Normality test (Shapiro Wilk) was done and groups were compared and analyzed using the non-parametric Wilcoxon test and moments at the same group were analyzed using the non-parametric Kruskal-Wallis test. For the analysis, P values < 0.05 was considered to represent a significant difference. The quantication of cytokines reveled production of all cytokines by all samples. When studied groups were compared, it was observed significant difference ($P < 0.05$) only for the cytokine TNF-α on moment 6, with greater concentration on samples of treated group (74.3 pg/mL±40.3 x 32.4 pg/mL±27.3). No difference ($P>0.05$) was observed when moments from same group were evaluated. In both experimental groups we observed the presence of pro and anti-inflammatory cytokines in the conditioned medium at all moments. The elevation of TNF- α evidences and confirms the action of LPS on the stimulated cells. We believe that future studies should be carried out with higher concentrations of LPS, mimicking pathological uterine processes, and with shorter times of collection of conditioned medium, enabling the earlier analysis of cytokine release.

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Exposure *in vitro* of endometrial bovine endometrial mesenchymal stem cells to prostaglandin E2

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The objective of this research was to evaluate the transcriptomic changes of bovine endometrial mesenchymal stem cells (MSCs) in response to different PGE2 concentrations. Primary cultures of MSCs from healthy cycling cows in late luteal phase (LLP1 and LLP4)1 were seeded at 4 x 104 cells/cm2 and maintained in DMEM-F12 with 10% FCS, supplemented with 1% AAM solution, 1 mM sodium pyruvate and 2 mM L-glutamine, and cultured in 5% CO2, 39°C and 100% humidity. The PGE2 (Caymann Chemical, Ann Arbor, Michigan, USA), was diluted in DMSO and added in concentrations of 1, 3 and 10 uM in triplicates. After 28 hours the cells were scrapped, subjected to RNA extraction using EZNA Total RNA Kit I (Omega Biotek, Santiago, Chile) and synthesis of complementary RNA with Agilent Low Input Quick Amp Labeling kit (Agilent Technologies©, Santa Clara, CA, USA). The bovine (V2) Gene Expression Microarray 4x44 (Agilent Technologies, USA) was used for differential gene expression and data obtained were analyzed using GeneSpring 12.5 extraction software (Agilent Technologies, USA). Significantly differentially expressed genes with a fold change greater than or equal 2.0 were selected and analyzed Gene Ontology (GO) using Panther software 11.1 (University of Southern California, USA), and gene interaction network was created with the GeneMania Prediction Server. To validate microarray data, the expression profile of 13 genes were selected and evaluated using qRT-PCR, and statistical analysis was conducted employing Pearson's correlation test. We found 1127 genes differentially expressed between the control group, (PGE2 concentration=0) and the rest of the doses of PGE2 considered together at p value of 0.05, and 2X fold change. The FDR was of 0.05%. The top 40 most deregulated genes demonstrated through GO that the biological processes affected the must were: cellular component organization or biogenesis and cellular and metabolic processes. Among other represented processes found were: biological regulation, development, growth and immune system. While the most represented molecular functions were: binding, catalytic, receptor, transport and structural molecule activity. GeneMania Prediction Server determined the predominant interaction was a coexpression (70.5%). A qPCR was an absolute coincidence between the microarray and qPCR data, with an average value of R = 0.89. Our findings indicate that the exposure in vitro of bovine endometrial MSCs to a mediator of inflammation such as PGE2, modifies their transcriptomic profile. So PGE2 could have a potential role in the fate of stem cell activation, migration, and, homing processes during pathological, uterine inflammation like as in endometritis and also in healthy puerperal endometrium.

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A290 Cloning, transgenesis, and stem cells

Generation of transgenic cattle for human proinsulin and human blood coagulation factor IX

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The generation of transgenic animals as bioreactors for expression of recombinant proteins is an important advance in biotechnology. Cows can produce large quantities of proteins in the milk and mammary glands are able to perform post-translation modifications in proteins, which become therefore active biologically. In this way, the mammary gland is considered one of the best tissues to produce recombinant proteins in transgenic animals. Few transgenic cows have been generated in comparison to other species due to the 1) low efficiencies of the technologies in this species, 2) to the low number of offspring generated per gestation and 3) to the long period of gestation. Mammary glands have been used for the production of biopharmaceuticals in milk and currently, only two products from the milk of transgenic animals have been approved and are being used for human health. Herein, we have generated two transgenic cows to produce recombinant proteins, one for human proinsulin and other for the human blood coagulation factor IX. Previously, two lentiviral vectors containing the genes of were constructed, with the tissue specific expression driven by β-casein promoter. The lentiviral vectors produced were used to transduce bovine fibroblasts culture. The modified cells were selected by blasticidin-resistence and used to produce transgenic embryos via somatic cell nuclear transfer methodology. Embryos at seven days of age post-fusion were transferred to recipient cows previously synchronized, the pregnancy was monitored and live born animals were obtained. The transgenic animals generated are being subjected to hormonal induction of lactation to evaluate recombinant protein expression in the milk.

A291 Cloning, transgenesis, and stem cells

Isolation and culture of stromal cells from buffalo Wharton's jelly

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Stem cells are undifferentiated cells, which can be used for the treatment of various conditions due to their immunomodulatory and therapeutic properties. The aim of the present study is to describe the isolation and culture of stromal cells (presumably mesenchymal stem cells) from buffalo Wharton´s jelly. For this purpose, approximately 30 cm of umbilical cord (n = 4) was collected in a slaughterhouse and transported to the laboratory in a solution composed of DMEM + F12, penicillin (100 IU/mL), streptomycin (100mg/mL), amphotericin B (3mg/mL) (Thermo Fisher Scientific, USA), and amikacin (11 mg/mL) (Teuto, BRA). In the laboratory, the umbilical cord was placed in 70% alcohol solution for 1 minute, followed by dissection of the umbilical vessels and collection of the surrounding tissue corresponding to Warthon´s jelly. The Wharton´s jelly was then sectioned into smaller pieces and enzymatic digestion was performed in 0.1% collagenase solution in PBS for 1.5 hours at 37.5°C. Every 10 minutes the samples were homogenized in a vortex to improve the digestion process. After digestion, the samples were filtered on 70 micron filters and centrifuged in culture medium composed of DMEM + F12 penicillin (100 IU/mL), streptomycin (100mg/mL), amphotericin B (3mg/mL) (Thermo Fisher Scientific, USA), and amikacin (11 mg/mL) (Teuto, BRA). The pellet was then resuspended and the cells cultured at 37.5°C in a humidified atmosphere, containing 5% CO2 in air. The samples showed adherence to the plastic within 24 hours and the presence of colonies with fibroblastoid morphology was observed within 72 hours. The first medium exchange was performed at 96 hours. The first passage was performed one week after the isolation and the samples were cryopreserved for later characterization. According to the morphological characteristics and adhesion to plastics, we can assume that we have isolated buffalo mesenchymal stem cells, as previously performed in other species. The characterization process is in progress to confirm this affirmation. Acknowledgment to FAPESP: Process 2015/01057-1.

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Isolation and culture of stromal cells from adipose tissue of sea turtle Caretta caretta

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The loggerhead sea turtle (Caretta caretta) is an endangered animal, according to the IUCN Red list of threatened species. Sea turtle populations have been seriously depleted worldwide due to human impacts. The TAMAR Project monitors approximately 1,100 km of beaches located on the mainland coast and oceanic islands. The project works with rehabilitation, aiming to nurse these animals back to health and release them into the sea. Isolation and culture of mesenchymal stem cells (MSC) may allows the treatment of various diseases by the use of cellular therapy and permits storing valuable genetic material. The objective of this study was to isolate and culture adipose tissue stem cells from the sea turtle Caretta caretta, comparing the efficiency of 5 protocols. The subcutaneous adipose tissue was collected from a female donor, with the authorization of the Tamar Project. The animal was submitted to general and local anesthesia and antisepsis of the inguinal region, followed by a skin incision and collection of the subcutaneous adipose tissue. The material collected was sent to OMICS Biotechnology Animal Laboratory. For transportation (6 hours), the adipose tissue fragments were placed in a solution containing penicillin, streptomycin, amphotericin and amikacin (AAS). In Group A, the fragments were placed in a solution with 0.04% of collagenase and the enzymatic digestion started during transportation. The digestion protocols were: A) Transport in collagenase solution followed by incubation in 0.04% collagenase solution in a water bath at 37.5°C for 30 minutes B) 0.04% collagenase solution for 30 min passing through the vortex every 10 min. C) 0.04% collagenase overnight. D) Collagenase 0.25% 15 min in incubator at 37°C. E) Trypsin (TrypLE Express ®) overnight. After enzymatic digestion, the solution was filtered, diluted 1: 1 in DMEM F12 with 20% fetal bovine serum (FBS), antibiotic and antifungal and centrifuged at 1500 rpm for 10 min. The cell pellet was re-suspended in two 25 cm² culture bottles/group. Culture was performed in DMEM F12 with 20% FBS, antibiotic and antifungal at 28°C and 5% CO² in air. Culture flasks were observed every 4 days to assess cell growth. After 15 days of culture fibroblastoid-like cells adherent to the plastic were observed in one bottle of Group A and one of Group B, as well as in the two bottles of Group E. After 30 days of culture the cells of group E reached confluence and were submitted to first passage with 97.5% of cell viability according to Trypan Blue staining. While those in the groups A and B, are still growing. We concluded that both, the use of trypsin overnight and 0.04% collagenase solution were efficient in isolating stromal cells in sea turtles, though the results with the use of trypsin appear to be superior. To our knowledge this is the first report of isolation and culture of adipose tissue stromal cells obtained from Caretta caretta.

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Isolation and *in vitro* culture of somatic cells derived from Jaguar (*Panthera onca*) ear tissue

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The establishment of *in vitro* culture of cells derived from skin fragments has been proposed as a useful technique for the conservation of endangered species, especially when associated with nuclear transfer (cloning). Among the species vulnerable to extinction, the jaguar (*Panthera onca*) requires strategies for the conservation of its genetic diversity. Therefore, the aim of the present work was to describe the *in vitro* culture of somatic cells derived from *P. onca* skin, using morphological analysis, trypan blue cell viability assay and metabolic activity by the 3-(4,5-Dimethylthiazol-2yl) -2,5-diphenyl tetrizoline bromide) or MTT. Thus, skin biopsy derived from peripheral ear region were recovered using surgical scissors from two males with age of 10 and 15 years, anesthetized and from zoos located in northeastern region of Brazil. Samples were transported for 3 to 5 h in minimal essential medium modified by Dulbecco (DMEM) supplemented with 2.2 g/L sodium bicarbonate, 10% fetal bovine serum and 2% penicillin and streptomycin solution, pH 7.4 at 4°C. In the laboratory, fragments (9.0 mm^3) were cultured under controlled atmosphere (38.5°C, 5% CO_2) and evaluated every 24 h. After the cells reached 70% confluency, the first cell subculture was performed to obtain the desired concentration (5.0 x 10^4 cells/mL) for the MTT assay. Additionally, a pool of trypsinized cells was evaluated for viability with trypan blue. All data were expressed as mean ± standard deviation. Thus, the total culture time was 29 days for the samples derived from both individuals and from the eight fragments submitted to the culture (four fragments per animal) all presented adherence on the first day with cell growth around the explants from the ninth day (9.0 ± 2.8). Cells reached 70% confluence forming monolayer on day 12.5 ± 2.1. In general, from the morphological analysis, all cells had fusiform morphology with a centralized oval nucleus, showing to be similar to fibroblasts. As for the trypan blue test, a viability of 99.6% ± 0.6 was obtained. In the MTT assay, the percentage of 100% ± 14.9 was obtained in D5 and in the D7 100% ± 18.1 of metabolic activity. In conclusion, skin biopsy derived from jaguar peripheral ear region allowed the isolation of viable cells similar to fibroblasts and with high metabolic functionality, providing a valuable source for the somatic cell nuclear transfer, aiming at the genetic conservation of this species.

A294 Cloning, transgenesis, and stem cells

Medium and temperature optimization for the recovery of membrane integrity of swine spermatozoa submitted to electroporation

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Electroporation (EP) is an efficient method for transfection of sperm cells. The success of EP depends, among other things, on the use of the appropriate medium during PE and on cell recovery post EP. The membrane permeabilization in the PE should only be for a sufficient internalization of the desired substance and after EP, it is ideal that the cells remain in medium and temperature that allow their membranes to regenerate. Therefore, the aim of this study was to establish for the electroporation of swine spermatozoids under conditions of low and high stress the medium and incubation temperature post EP that provide greater recovery of plasma membrane integrity (RPMI). Dilution of inseminating doses of 10 males of commercial strains were in BTS to obtain samples with 200µl and $2x10^6$spzt / ml, which composed the experimental groups. After EP in two different conditions (500 volts, 250µs and 1 pulse (EP1) or 1000 volts, 500µs and 2 pulses (EP2), the samples were recovered in four different conditions: mTBM (modified Tyrodes Buffered Medium) at 17°C or 37°C, or BTS at 17°C. Propidium iodide (PI), one membrane impermeable fluorescent dye was the reporter molecule. Each sample was electroporated into Multiporator® (Eppendorf AG, Hamburg, Germany) using BTS at room temperature (22°C). Then, centrifuging of samples were at 9100 G for 3 minutes, the supernatant was removed and 200 µl of BTS or mTBM was added and incubated for 30 minutes at 17°C or 37°C according to their respective experimental group. Analysis of samples were by flow cytometry (BD Accuri™ C6) for measuring the rate of incorporation of PI, which occurred in three moments, before EP (PI0), just after EP (PI1) and 30 minutes after recovery (PI2). The calculation of RPMI rate was by the formula: RPMI = (PI2-PI0) – (PI1-PI0). The lowest value found indicates the best RPMI. For statistical analysis, PROC MIXED (SAS, version 9.2 for Windows) was used, with comparisons using the Turkey test, analysis of interaction between variables and significance level of 5% ($P < 0.05$). There was interaction between the medium and the temperature (p=0.0008). The BTS at 17°C had a RPMI of -9.94% ± 5.91, the only combination capable of recovering membranes above pre-recovery values, being more efficient than the mTBM at 17°C (32.5% ± 5.91 ($P < 0.0001$), but with no difference with BTS at 37°C (14,04% ± 6,61%) (P=0.0525) and mTBM at 37°C (10.38% ± 6.27) (p=0.1085). Interaction between the EP and the medium used (p=0.0471) was also observed. The mTBM medium when used together with EP2 presented worse results than the other groups, presenting RPMI of 35.82% ± 6.27, being the BTS medium with EP1 was 0.49% ± 6.27 (p=0.0022) and for EP2 3.60% ± 6.27 (p=0.0056) and the mTBM and EP1 medium was 7.06% ± 5.91 (p = 0.0018). This study demonstrated that, in general, the BTS medium, mainly at 17°C, was more efficient in recovering integrity of the plasma membrane of swine spermatozoa submitted to electroporation.

A295 Cloning, transgenesis, and stem cells

Reprogramming porcine fibroblast cells into induced pluripotent stem cells (piPSCs) using lentiviral vectors and exogenous pluripotency transcription factors *in vitro*

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Lentiviral vectors are commonly used for efficient transgenes and may be used for reprogramming different types of cells into both naïve and primed pluripotency states. Recent studies have reported the production of porcine induced pluripotent stem cells (piPSCs) however, no consensus in the literature has been reported between different protocols or interspecific exogenous transcription factors and pluripotent states the iPSCs in the porcine model. This study aims to evaluate the effect of murine leukemia inhibitory factor (mLIF), human basic fibroblast growth factor (bFGF) in piPSCs during in vitro reprogramming obtained through overexpression of murine pluripotency factors OCT4, SOX2, c-MYC and KLF4 (OSMK). Porcine fibroblasts were transduced with lentiviral polycistronic excisable vector containing the murine transcription factors (STEMCCA vector). Six days after transduction the cells were replated into mouse inactivated fibroblast feeder layers (MEFs) and cultured in iPS medium: KnockOut DMEM/F-12 medium with 20% KnockOut Serum Replacement (KSR, Cat#10828028, Invitrogen, EUA), 0.5 mmol L−1 L-glutamine, 1% NEAA, 0.1mmol L-1 β-mercaptoethanol, 1% Penicillin/Streptomycin. During the reprogramming period, the cells were divided into two group: First group, the culture medium 1 (M1) was supplemented with 4 ng mL-1 basic Fibroblast Growth Factor (bFGF, Cat # 13256029, Invitrogen, EUA) and 4ng ml-1 Leukemia inhibitory factor (mLIF, Cat # ESG1107, Millipore, EUA) and second group, the culture medium 2 (M2) supplemented only LIF. piPSCs cells generation were evaluated regarding its pluripotency state through immunofluorescence analysis, phosphatase alkaline and spontaneous differentiation. The results of piPS cell colony derivation showed that first colonies at day 12 post-transduction. The clonal lines generated were positive for alkaline phosphatase and these same colonies were OCT4 positive. The immunofluorescence analysis showed that all piPSCs were positive for OCT4, SOX2, NANOG, TRA1-60 and presented normal karyotypes. piPSCs generated with M1 presented expression of SSEA1 and TRA1-80. On the other hand, piPSCs generated with M2 presented SSEA4 expression. In vitro differentiation analysis showed that cells spontaneous differentiated when the bFGF and mLIF were removed from the medium. These cells were capable of deriving embryonic bodies after 15 days in culture. In M1 condition of culture, piPSCs differentiated into the three germ layers, proven by the expression of neuro-ilament 18, actin, and alpha-fetoprotein. Although, when M2 was used, the cells were not able to differentiate in any of the germ layers. We conclude that the lentiviral delivery of murine transcription factors promotes reprogramming of porcine fibroblast when mLIF and bFGF or only mLIF are used, however, the clonal pluripotent lines derived are different regarding the pluripotency-related characteristics. These preliminary results indicated that piPSCs generation in different culture conditions may contribute to a better understanding of the pluripotency acquisition and naïve/primed balance state in swine.