



A202 Cloning, Transgenesis and Stem Cells

Obtaining transgenic bovine embryos using crotonamine as a foreign DNA carrier molecule

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Keywords: crotonamine, embryo, transgenesis.

Currently, several methods are used for generation of transgenic animals, such as pronuclear microinjection, nuclear transfer and others. Despite the great advances, animal transgenesis still presents a low efficiency and, consequently, other methodologies have been developed. Kerkis et al. (2004, FASEB J, 18, 1407-9) and Nascimento et al. (2007, J Biol Chem 282, 21349-60) verified crotonamine action – a cationic peptide from the venom of South American rattlesnake (*Crotalus durissus terrificus*) – as a penetration and transfection agent in several murine cell types. Therefore, the aim of this work was to evaluate crotonamine as a DNA carrier molecule for the production of transgenic bovine embryos. Crotonamine-plasmid DNA complex (Cr-pEGFP) was used to transfect *in vitro* produced bovine embryos according to Vichera et al. (2011, Reprod Dom Anim 46, 214-20). Two groups of *in vitro*-fertilized intact oocytes were then constituted: one group, incubated for 24 h in the absence (Cr0), and another, in the presence of Cr-pEGFP complex (Cr1) at a final concentration of 50 ng.µL⁻¹ crotonamine per µg of DNA. After this period, the embryos were cultured in CR2 medium for seven days at 38.5°C and 5% CO₂. Following several repetitions (n=7), embryonic development and transfection rates were evaluated by fluorescence microscopy on successive days (D2, D7 and D8) of *in vitro* culture. Cleavage, blastocyst, hatching and transfection rates were analyzed using ANOVA in combination with Tukey test (P<0.05). The cleavage rates of Cr0 and Cr1, evaluated on D2, were 63.8% (127/199) and 63.2% (134/212), respectively. The blastocyst rate on D7, calculated in relation to the total number of oocytes in culture, was 25.1% (50/199) and 24.1% (51/212) for the Cr0 and Cr1 groups, respectively. On D8, the blastocyst rates in Cr0 and Cr1 were 29.6% (59/199) and 26.4% (56/212), respectively. Also on D8, the hatching rates in Cr0 and Cr1 were 45.8% (27/59) and 46.4% (26/56), respectively. In all embryonic stages, crotonamine did not affect the viability of bovine embryos, as confirmed by cleavage and blastocyst rates (P>0.05). Interestingly, concerning the transfection rate, 9.8% (5/51) and 17.9% (10/56) of blastocysts expressed EGFP on D7 and D8, respectively. Neither embryos on D2, nor oocytes in Cr0 group, showed EGFP transgene expression. Altogether, these results present evidences about the competence of crotonamine to mediate bovine embryo transfection, without the removal of the zona pellucida or the use of DNA microinjection. In conclusion, crotonamine-mediated transfection appears as a promising alternative for the production of transgenic bovine embryos. Additional studies are being undertaken to improve the knowledge about this new methodology and to make possible its application for animal transgenesis.



A203 Cloning, Transgenesis and Stem Cells

Effects of *in vitro* maturation environment on bovine oocytes enucleation for somatic cells nuclear transfer

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Keywords: handmade cloning (HMC), *in vitro* maturation (IVM), oocytes.

Cloning by somatic cells nuclear transfer (SCNT) using manual removal of oocyte nucleus (Handmade Cloning – HMC) is a technique that still presents very low efficiency. *In vitro* maturation (IVM) of oocytes is a critical step of the process, since it is when the cytoplasm of the oocyte is prepared to host and reprogram the donor cell nucleus. Hence, the pursuit for an efficient and optimized maturation environment seems to be essential for the success of this technique. The serum, used in most IVM systems, contains undefined components in variable amounts, which might impair the standardization of results and even embryonic/fetal development. Previous studies performed in our laboratory with IVP revealed that oocytes matured in serum-free medium and low O₂ tension yielded embryos of better quality compared to conventional IVM system (Pereira et al., *Reprod. Fertil. and Develop.* 22:1074-1082, 2010). The present study was aimed to evaluate the efficiency of enucleation of recipient oocytes under different IVM conditions. Oocytes obtained from slaughterhouse ovaries were distributed into two treatments: T1 (n=116)-oocytes matured with 10% SFB in 20% O₂ atmosphere and T2 (n=124) – oocytes matured with 0.1% PVA in a 5% O₂ atmosphere. The basic maturation medium was TCM 199 (Invitrogen, Carlsberg, USA) supplemented with FSH (20 µg/mL), EGF (10 ng/mL), 0.1% antibiotics and incubation at 38.5 °C, 5%CO₂, saturated humidity. After 19h of IVM, oocytes were stripped of cumulus cells in 0.1% hyaluronidase, and matured oocytes were selected based on expulsion of the 1st polar body (PB). Matured oocytes were treated with demecolcine (0.5 µg/mL) for 2 h to induce the formation of the cone (protrusion) on cytoplasmic membrane (indicative of nucleus location). Afterwards, the zona pellucida was removed in a pronase solution (2 mg/mL) and then oocytes were manually enucleated by using microblades (Bioniche, Pullman, USA). The 1st PB extrusion and cone formation rates were evaluated for both treatments and data were analysed by chi-square test. There was no significant difference (P>0.05) on 1st PB extrusion rate (74% and 77%, for T1 and T2, respectively) nor on conical protrusion formation rate (70% and 58%, for T1 and T2, respectively) between treatments. These results suggest that the 5% O₂ atmosphere (low O₂ tension) and serum-free maturation environment may be used for recipient oocytes in HMC cloning with no detrimental effects on 1st CP extrusion and/or the metaphasic cone formation.

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***In vitro* differentiated mouse germ cells from ESC are able to sustain initial embryonic development when injected into the cytoplasm of oocytes**

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Keywords: differentiation, germ cells, mouse.

Different groups and experiments have shown the *in vitro* differentiation of male and female germ cells (GC) from mouse embryonic stem cells (ESC). Our group has demonstrated that both gametes can be produced under similar conditions, but in different intervals, without genetic manipulation. However, the functionality of these "artificial gametes" is discussed and requires further studies. The aim of this project was to evaluate the functionality of USP-1 cells *in vitro* differentiated immature germ cells obtained under the same conditions of Kerkis *et al.* (Cloning and Stem Cells. December 2007, 9 (4): 535-548), using the protocol of Kimura and Yanagimachi (Development, 121 (8): 2397-405, 1995). Briefly, for *in vitro* differentiation, cultured ESC were isolated with trypsin, washed and the cell concentration was determined and adjusted to 1250 cells in 25 ul. Droplets were formed and maintained in "hanging-drops" for 48 hours to form embryoid bodies (EB). After this period, around 20 EB were transferred to non-treated 35 mm dishes, containing 2 ml of neurobasal medium supplemented with B27 and retinoic acid (RA). Every 48 hours the medium was changed and the RA was maintained for 8 days. To check the functionality, oocytes were collected from BL6D2F1 superovulated females 13h after treatment with hCG. Cumulus cells were removed with hyaluronidase and oocytes were activated with strontium. With a micromanipulator and a piezo-electric, immature GCs at different stages of differentiation were selected and injected into the cytoplasm of the oocytes. Out of the oocytes that survived microinjection, 42.47% (271/638) cleaved after 24 hours of culture in CZB medium at 37 ° C and 5% CO₂ and high humidity. From two-cell embryos, 203 were transferred surgically to the oviducts of 13 pseudo-pregnant females. After 19 days, recipients were sacrificed and 14 implantation sites were found in the uterus of one female, and three sites and one uterine dilation of around 0.6 cm, compatible with an embryo that failed to develop, were found in another female. Our results suggest that immature GC, *in vitro* differentiated, were able to support early embryonic development. To better answer the questions, DNA was isolated from implantation sites and embryo to confirm ES origin. Also material was collected for epigenetic and histopathological analysis. Upcoming experiments will give more information about functionality of immature GC.

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A205 Cloning, Transgenesis and Stem Cells

Use of amniotic fluid cells and wharton jelly cells for production of bovine embryos by nuclear transfer (cloning)

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Keywords: biotechnology, blastocyst, micromanipulation.

Nuclear transfer (NT) is a technique that has been used commercially for production of bovine and also in studies of transgenic animal production, however, many parameters remain as limiting factors to increase the production of healthy individuals. An important pre-requisite to improve the efficiency of this technique is the availability of cells capable of undergo nucleus reprogramming and support the embryo and fetal development after NT (Mastromonaco et al., 2006, BMC Developmental Biology 6, 1-13). The objective of this study was to evaluate embryo production with the use of two cell types as nucleus donors: 1) Amniotic fluid cells (AFC) collected from the uterus of a 73-day pregnant cow, by means of intravaginal ultrasound procedure, cultured in Amniomax® medium (Gibco, USA) and utilized between P3 and P9; and 2) Wharton jelly cells (WJC), from the umbilical cord collected at birth of a calf, cultured in DMEM (Dubelcco's Modified Eagle Medium, Invitrogen, USA) and utilized between P2 and P5. Both cell types were cryopreserved in 10% dimethylsulfoxide (DMSO) solution. Cumulus-oocyte complexes recovered from slaughterhouse ovaries were matured for 18h at 38.5°C and 5% CO₂. The NT procedure was performed using micromanipulators according to Kuroiwa et al. (2002, Nature 20, 889-894), with modifications, to compare the efficiency of both cell types. Parthenogenetically activated oocytes were used as the control treatment. The rates of electrofusion and blastocyst production in relation to the number of fused structures (B/F) were analyzed by t test ($p < 0.05$), and the rates of cleavage and blastocyst production in relation to the number of structures cleaved (B/C) were evaluated by One Way ANOVA and Tukey tests ($p < 0.05$). The electrofusion rates were $54.38 \pm 8.2\%$ when using the AFC and $71.76 \pm 8.18\%$ for the WJC. These results differ significantly and can be explained by the larger size of the WJC than AFC, making the procedure easier. Cleavage rates did not differ between cell types ($40.64 \pm 18.77\%$ and $38.46 \pm 5.7\%$, AFC and WJC, respectively), but were significantly lower than the rate of the control group ($77.29 \pm 12.31\%$). In relation to B/F rates, there was a significant difference between cell types ($14.95 \pm 7.36\%$ and $27.26 \pm 9.34\%$, AFC and WJC, respectively). By analyzing the B/C rate, it was observed that the WJC also showed embryo development rate ($68.68 \pm 14.04\%$) significantly higher than the AFC ($37.82 \pm 16.58\%$). Both treatments did not differ in embryo development rate from control group ($52.82 \pm 20.46\%$). Therefore, we conclude that it is possible to obtain viable cells from amniotic fluid and umbilical cord of live animals without damaging them and produce embryos efficiently using the technique of NT.



A206 Cloning, Transgenesis and Stem Cells

Isolation, culture and cryopreservation of umbilical cord cells and amniotic fluid cells from bovine fetuses

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Keywords: biotechnology, mesenchymal stem cells, nuclear transfer.

Cryopreservation and storage of animal gametes and embryos are the keys to germplasm banks. However, gametes and embryos are finite and must be replenished when used regularly. Therefore, cryopreservation of viable cells with high capacity to undergo the nuclear reprogramming assume an important role, since they may be propagated in culture and used in nuclear transfer (NT). In this context, this study had the following objectives: a) evaluate the possibility of isolation and cultivation of umbilical cord cells (UCC) and amniotic fluid cells (AFC) compared to fibroblast ear cells (FEC), which is a classical model and b) test the effect of 3 cryoprotectant solutions containing 10% dimethylsulfoxide (DMSO), 5% dimethylformamide (DMF) and 7% glycerol (Gly). Therefore, 3 gravid uteri, aged 70-90 days, were collected at a local slaughterhouse, the amniotic fluid was collected and centrifuged at 500xg for obtaining cells. The pellet was resuspended with Amniomax® (Gibco, USA) medium and cultured in cell culture flasks. Ear and umbilical cord were biopsied from the fetuses and incubated in DMEM (Dubecco's Modified Eagle Medium, Invitrogen, USA) medium containing 10% fetal bovine serum and antibiotics, in an incubator with 5% CO₂ at 38.5 °C. After the third passage of *in vitro* culture, the cells were cryopreserved in each cryoprotectant solution: T1-10% DMSO, T2-5% DMF and T3- 7% Gly diluted in culture media of each cell type. The diluted cells were stored in straws (0,25mL), kept at -80°C for 24 hours and stored in liquid nitrogen. Cell viability was evaluated with 0,4% Trypan Blue stain. Treatments were compared using the One Way ANOVA and Tukey (P <0.05) tests. It was possible to isolate AFC and UCC and establish the *in vitro* culture for 20 passages. The average times to achieve confluence were 5.7 days, 7.4 days and 12.5 days, respectively for FEC, UCC and AFC. The cryoprotector solutions with 10% DMSO assured cellular viability of 90.33±5,58%; 90.56±4,40% and 81.90±3,31%, respectively for FEC, UCC and AFC, being significantly more efficient and presenting less variation on the preservation when compared to the other treatments. The solutions with 5% DMF showed cell viability of 66.5±22.37%, 28.86 ±13.31% and 35.48±17.55%, respectively for FEC, UCC and AFC and solutions with 7% Gly showed cell viability of 56.56±14.70%, 44.38±13.15% e 43.20±14.89%, respectively for FEC, UCC and AFC. There were no statistical differences between cryopreservation media with 5% DMF and 7% Gly. Therefore, we conclude that it was possible to efficiently isolate, cultivate and cryopreserve AFC and UCC, in order to compose a cryobank of cells with the purpose of their use as donor cells in nuclear transfer. The cryoprotectant solution with 10% DMSO is most indicated for cryopreservation of the cell types studied.



A207 Cloning, Transgenesis and Stem Cells

Bovine embryo production through nuclear reprogramming of somatic cells expressing the exogenous pluripotency factors HOCT4 and HSOX2

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Keywords: cellular reprogramming, ips, nuclear transfer

Studies on cell reprogramming, differentiation and proliferation have revealed that transcription factors, such as, OCT4, SOX2 and NANOG, act together promoting cell commitment or pluripotency. Mechanisms of induced reprogramming by pluripotency-related transcription factors or nuclear transfer (NT) seem to be mediated by the same pathways, eliciting nuclear remodeling and modulating gene expression. However, abnormal chromatin conformation, often resulting in disrupted imprinting and atypical gene expression patterns are frequently observed on *in vitro* reprogramming. The combination of both reprogramming techniques – induced reprogramming and nuclear transfer, is hypothesized to result in better reprogramming and may also help to study the mechanisms of gene expression that are responsible for pluripotency. To test such hypothesis, fetal fibroblasts expressing either human OCT4 (hOCT4) or hSOX2 combined with the fluorescent reporters vexGFP or mCitrine, respectively, were produced through lentiviral transduction. Transgenic lineages were analyzed for OCT4 and SOX2 gene expression through qPCR and flow cytometry analysis, which enabled the sorting of positive cells used as donor cells for somatic cell nuclear transfer procedures. Briefly, bovine oocytes obtained from slaughterhouses were *in vitro* matured for 18h, enucleated and reconstructed with fibroblasts expressing OCT4-vexGFP (n=182, in four replicates), SOX2-mCitrine (n=203, in four replicates) or control cells (non-transduced, n=178 and n=149, in four replicates as a control of OCT4 or SOX2 expressing cells each). After reconstruction, embryos were activated with ionomycin (5µM, 5 minutes) and 6-DMAP (2mM, 3h) and *in vitro* cultured until blastocyst stage (seven days) in SOF medium supplemented with 2.5% FBS and 3mg/mL BSA. Results were compared by ANOVA at 5% significance. Fusion rates were 60.0 x 64.95% and 70.53 x 67.24% for SOX2 x control and OCT4 x control groups, respectively; cleavage rates (48h after activation) were 66.66 x 81.68 and 86.47 and 85.18%, respectively; and blastocyst rates (192h after activation) were 33.05 x 44.15 and 52.06 x 44.78, respectively. In conclusion, we report here that the production of embryos by NT of hSOX2 or hOCT4 expressing donor cells resulted in similar rates of *in vitro* developmental competence when compared to control cells regardless different profiles of pluripotency-related gene expression presented by donor cells. A better understanding of the contribution of each reprogramming factor used in induced reprogramming will result in the establishment of strategies aiming to enhance *in vitro* reprogramming performance. Such knowledge will contribute to *in vitro* animal production by increasing the cloning efficiency at term and regenerative medicine through the derivation and adequate culture of reprogrammed embryonic stem cells.



A208 Cloning, Transgenesis and Stem Cells

Cloning as a tool to rescue the genotype of a freemartin flemish cow

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Keywords: biodiversity, cattle, nuclear transfer.

The Rouge Flamande (Flemish) is a dual-purpose breed of cattle that produces milk with high protein content and good quality meat. The population of Flemish cattle in the Brazilian highland plateau of Santa Catarina (SC) state was considerably reduced in the last decades due to replacement by modern specialized breeds, which threatens Flemish breed with imminent risk of extinction. There are currently about fifty remaining animals at the Epagri experimental research center in Lages, SC. One animal is an 18 years old freemartin cow, which due to sterility conserves the genotype free of inbreeding with the remainder cows of the herd. If an offspring could be produced from this cow, this would represent an opportunity for increasing the genetic diversity in this Flemish herd. However, there are no reports showing the use of freemartins as cell donors for nuclear transfer. Therefore, the aim of this study was to produce a normal cloned calf using somatic cells from a freemartin Flemish cow. Somatic cells were obtained through explantation of an ear skin biopsy, and embryos were produced by handmade cloning (Mezzalira et al. 2011Cell. Reprog, 13, 65-76). Cleavage, blastocyst and pregnancy rates (at 60 days) were respectively 87% (204/233), 34% (80/233), and 75% (9/12). However, only 1 recipient cow remained pregnant after 120 days of pregnancy. This cow initiated labor at day 252, probably due to hydro-allantois, and underwent an emergency caesarean section. The calf was born weighing 39 Kg, and despite the administration of surfactant and O₂ she died about 10 minutes after birth, with clinical signs of a heart stroke. Necropsy findings include several abnormalities such as limb joints hyper-contraction, moderate anasarca and thyroid gland hyperplasia. Cardiopulmonary abnormalities found included fibrin deposition in the pericardium, focal hemorrhage in the tricuspid valve and lung with petechial hemorrhages. Kidneys presented congestion and multifocal cysts. The abnormalities found were incompatible with calf survival. However, genital organs had normal morphology. The length of the vaginal canal was about 12 cm. The uterine body and horns, as well as the cervix were normal. Both ovaries had normal morphology and size of approximately 1.0 x 0.7 cm. Histological evaluation showed normal endometrium with normal chorion and glandular epithelium. The ovaries had normal stroma and contain late antral follicles and oocytes. These findings suggest that it is possible to rescue the genotype of freemartin cows using somatic cell nuclear transfer.



A209 Cloning, Transgenesis and Stem Cells

Induction of positive early remodeling of endometrial tissue microenvironment following transplantation of allogeneic mesenchymal stem cells in mares suffering from endometrosis

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Keywords: equine endometrosis, positive remodeling of endometrial tissue, stem cells.

Mesenchymal stem cells (MSC), due to their self-renewal potential and differentiation capacity, appear as a tool for tissue regeneration. More recently, immunomodulatory and trophic properties of MSC have been demonstrated, suggesting their use as medicinal signaling cells. Equine endometrosis is a progressive, degenerative disease responsible for endometrial fibrosis including glandular alterations, which cause infertility. Currently, no effective treatment is available for this disease. Herein, we analyzed the capacity of allogeneic equine adipose tissue-derived MSC (eAT-MSCs) to induce early endometrial tissue remodeling in mares with atypical morphological and functional differentiation of glandular and periglandular endometrial stromal cells. Morpho-functional features of endometrosis using, both conventional histopathological and immunohistochemical methods, were evaluated before and after eAT-MSCs infusion into the uteri of mares during the breeding season. We used standardized population of eAT-MSCs of high quality, which were previously isolated and characterized (Mambelli et al., 2009). eAT-MSC were labeled with Vybrant®, a fluorescent-nanocrystal dye, and infused into mares uterus (n=4). Control animals (n=2) received only saline solution. After 7, 21 and 60 days uterine biopsies were collected and conventional histopathology, as well as immunohistochemical methods for the detection of proteins, such as laminin, vimentin, Ki-67-antigen, α -smooth muscle actin (α -SMA) and cytokeratin 18 (CK18) were performed. Changes, which occurred in expression pattern of endometrial proteins in mares suffering from endometrosis and treated with the cells, suggest that allogeneic eAT-MSCs are able to contribute positively to the early remodeling of endometrial tissue microenvironment. These changes did not occur in the uterus of control animals, which were not treated with cells. Therefore, we demonstrated, for the first time, that eAT-MSCs were able to induce early remodeling of mares' endometrium suffering from endometrosis. We showed that these cells act through multiple mechanisms, such as eAT-MSCs homing in fibrotic periglandular and glandular space, modulation of expression pattern of secretor proteins and induction of glandular epithelia cell proliferation. The results of the present work are very promising, however logic rationality suggests that the combination between local and systemic stem cell therapies may provide more efficient tools to combat endometrosis, the major cause for equine infertility.



A210 Cloning, Transgenesis and Stem Cells

Establishment of transgenic goat fibroblast cell lines by nucleofection

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Keywords: fibroblast, goat, transfection.

The delivery of DNA into cells has been useful for studies on functional genomics, gene therapy, transient gene expression, and for the generation of cell lineages for the production of transgenic animals through somatic cell nuclear transfer. The transfection of mammalian primary culture cells, mainly fibroblasts, usually results in low transfection rates and low cell viability. Recently, DNA delivery into the cell nucleus through nucleofection procedures has been developed to improve DNA transfection and transgene integration efficiencies. The aim of this study was to evaluate the performance of a new generation electroporation device (Amaxa™ 4D-Nucleofector™, Lonza, Germany), developed to use fewer cells and lower DNA amount. Due to the lack of data on goat cell nucleofection, seven programs (CA-137, CM-138, DS-150, EH-100, EN-150, EO-114, FF-113) for use in distinct cell types (fibroblasts, epithelial cells, tumor cells, stem cells) from different species (mouse, human, pig) were tested for the transfection of goat fetal fibroblast cells with a linearized green fluorescent protein (GFP) reporter plasmid (pEGFP-N1, Clontech, USA). Fibroblast cells were isolated from a Day-35 goat fetus and grown in DMEM supplemented with 10% fetal calf serum. Sixteen transfection rounds were performed using 1×10^5 cells and 2 μ g DNA. Cell confluence and transfection rates were estimated 24 h after nucleofection. Then, transfected cells were subjected to 400- μ g/mL antibiotic selection (Geneticin®, Gibco, USA) for 20 days to determine transgene integration efficiency. Cell confluence and transfection rates after 24 h, and number of colonies after antibiotic selection were analyzed by the Tukey test ($P < 0.05$). Mean cell confluences ($78.0 \pm 4.3\%$) and transfection rates ($27.9 \pm 0.1\%$) 24 h after nucleofection were similar between groups. However, transfection rate did not correlate with integration rate, based on the number of selected colonies for each program. Interestingly, program EN-150, a program that was recommended for a number of cell types, including mammalian fibroblasts, and for the three species, rendered a high transfection rate ($40.0 \pm 14.1\%$) and a significant higher number of colonies after antibiotic selection (11.0 ± 1.4) than the other groups, resulting in one GFP-selected cell for every 9,100 transfected cells (1:9,100). All the other programs resulted in selected colonies, but at a significantly lower efficiency, ranging from 1:18,000 to 1:100,000 cells, for transfection rates that varied from 12 to 45%. Therefore, our preliminary results indicate that the 4D-nucleofection technology may be an efficient approach for the establishment of transgenic goat fibroblast cell lines, providing that the nucleofection conditions are optimized to each specific cell type, gene construct, and nucleoporation parameters.



A211 Cloning, Transgenesis and Stem Cells

Effect of the cytoplasm source on the *in vivo* development of cloned goat embryos transgenic for human lysozyme

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Keywords: cloning, goat, transgenic.

Cloning by somatic cell nuclear transfer (SCNT) is a valuable tool for the production of transgenic animals. However, the availability of good quality oocytes (cytoplasts) is a limiting factor in many species, including the goat. The aim of this study was to compare the effect of the cytoplasm source (*in vitro*- vs. *in vivo*-matured) on the *in vivo* development of transgenic cloned goat embryos. *In vitro*- or *in vivo*-matured oocytes were obtained either post-mortem by ovary slicing (68 females) or *in vivo* by a semi-laparoscopic follicular aspiration of superovulated females (n=36) prior to ovulation. In the *in vitro*-matured group (*in vitro* group), viable oocytes (705/1,583) were *in vitro*-matured (IVM) for 20 h (Feltrin et al., 2012, *Reprod Fertil Dev* 24:127). In the *in vivo*-matured group (*in vivo* group), most recovered oocytes were viable (360/372). To assess maturation rates, denuded oocytes were examined for the presence of the first polar body (PB). For the *in vitro* and *in vivo* groups, respectively, matured oocytes (183/360 and 360/705) were enucleated by micromanipulation procedures in 5 µg/mL cytochalasin B + 10 µg/mL Hoechst 33342 solution. Transgenic somatic cell lines for the human lysozyme gene (adult and fetal fibroblasts, adult mesenchymal stem cells) were used for embryo reconstruction and membrane fusion by two 20-µs long, 2.0-kV/cm DC pulses (281/360 and 124/183). Fused structures (248/281 and 98/124) were activated in 5 µM ionomycin for 5 min and in 2 mM 6-DMAP for 3.5 h. Cloned embryos (231/248 and 85/98) were *in vitro*-cultured for 18 h in SOF medium + 10% FCS prior to the semi-laparoscopic transfer (206/231 and 79/85) into the oviducts of 19 and eight synchronous recipient females (8-12 embryos/female). Pregnancy diagnoses were performed by ultrasonography on Day 30 of development. Data were analyzed by the t and by the χ^2 tests (P<0.05). After five replications, a higher number of oocytes were recovered from the *in vitro* (23.3 ± 8.3) compared to the *in vivo* (10.1 ± 5.3) group, which in turn had a higher percentage of viable oocytes (96.8%) than the *in vitro* group (44.5%), with no differences observed in the number of viable oocytes per female (10.0 ± 3.6 vs. 10.4 ± 3.1), maturation rates (51.1% vs. 50.8%), or the number of matured oocytes per female (5.1 ± 2.8 vs. 5.3 ± 2.4), respectively. Even though oocytes from the *in vitro* group rendered more fused embryos (88.3%; 3.6 ± 1.4) than the *in vivo* group (79.0%, 2.7 ± 1.2), pregnancy rates on Day 30 were similar between the *in vitro* (14/19; 73.7%) and the *in vivo* (6/8; 75.0%) groups. Currently, two ongoing pregnancies from the *in vitro* group are underway. Under our experimental conditions, due to a more practical, cost effective, and less technically demanding recovery procedure, *in vitro*-matured oocytes were proven as a more efficient cytoplasm source for the establishment of pregnancies with transgenic cloned goat embryos.

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A212 Cloning, Transgenesis and Stem Cells

DNA co-transfection efficiency for the establishment of bovine double transgenic cell lines

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Keywords: bovine, co-transfection, transgenic.

The establishment of transgenic cell lines is one of the critical steps in the process of producing genetically modified animals by somatic cell nuclear transfer. The integration of the transgene of interest typically accompanies an antibiotic resistance gene, which is propagated to the animal produced. Up to date, there is limited information on the efficiency of co-transfection methods, in which two different DNA constructs are transfected simultaneously. This method creates the possibility of inserting different DNA constructs into different chromosomes, allowing the elimination of one of them, as for example the antibiotic resistance cassette, by breeding. The objective of this study was to evaluate the efficiency of the method of co-transfection by electroporation in bovine fetal fibroblasts (bFF) obtained from a Day-45 fetus, and a newborn bovine fibroblasts (nBF), obtained from a 4 days old female calf. Cells were expanded and cryopreserved at passage 2 and co-transfected at passage 3. Co-transfections were carried out by adding 4 µg of a DNA construct of 27 Kb containing the promoter region for the bovine α s1-casein, and another 1 µg of a 1.8 kb DNA construct containing an expression cassette for neomycin resistance (Neo-R) in 1×10^6 cells in suspension by electroporation (Nucleofector Amaxa™, Lonza®), using program U-012. Twenty-four hours after co-transfection, cells were diluted and 24 h later the antibiotic selection was started with 400 µg/mL of Geneticin (Invitrogen®) in M199 medium (Sigma-Aldrich®) supplemented with 15% FCS, for 12 days. The isolated colonies were individually removed and cultured in 96 well plates and further expanded. In each passage, cell samples from each colony were stored for the diagnosis of integration events of transgenes by PCR. The average number of colonies was statistically different ($P < 0.05$) between cell types; 18 colonies were obtained from bFF and 48 colonies for nBF. The total number of colonies obtained after three repetitions were significantly different ($P < 0.05$); 54 for bFF and 146 for bFN. The probability of obtaining the number of colonies in relation to the number of co-transfected cells was 2.7 times higher for bNF (1:20.548) than bFF (1:55.000). It was possible to isolate and expand 93% (50/54) of colonies from bFF and 64% (93/146) of the colonies from bBF. For bFN isolated colonies, 16% (15/93) were positive for both DNA constructs α s1-casein and Neo-R, meaning that for every 6.2 isolated colonies, one was positive for both transgenes. For the bFF, none of the isolated colonies were positive for both constructs simultaneously. The results show that the co-transfection method can be used to establish genetically modified cell lines, despite the low efficiency. In addition, it was found that bNF cells showed higher integration, especially for α s1-casein construct, in relation to bFF cells.

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A213 Cloning, Transgenesis and Stem Cells

A novel strategy of mesenchymal stem cells delivery in the uterus of mares suffering from endometriosis

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Keywords: endometriosis, mesenchymal stem cells, paracrine effects.

The body contains so-called stem cells that are non-specialized and can renew themselves, as well as give rise to specialized cells like muscle, bone, cartilage and others. These adult stem cells are “spare parts” of the organism, which are activated in case of injury, disease or trauma. The discovery of isolation methods and *in vitro* cell cultivations have provided a qualitative leap in stem cell technologies, which became more efficient through employment of mesenchymal stem cells (MSC). These cells can be readily harvested from bone marrow, fat tissue, and dental pulp among others. Currently, many cell therapies use allogeneic MSC based on their differentiation capacity and trophic properties, which are achieved by secreting factors that aid wound healing. Equine endometriosis is an age-associated, degenerative alteration of the uterine glands and the surrounding stroma, directly related to fertility problems in mares. MSC, due to their properties, appear as a promising tool for the treatment of this disease. The success of stem cell therapies depends, in part, on methods of cell delivery, which should guarantee a wide cell distribution at the injured site. On the other hand, such methods should not be complicated, in order to allow for a possible introduction into the practice of endometriosis treatment. Therefore, the aim of the present study was to develop a novel strategy of MSC delivery into the uterus of mares suffering from endometriosis. Previously, our group isolated and fully characterized multipotent MSC from equine adipose tissue (eAT-MSC) (Mambelli et al., 2009). Six mares suffering from endometriosis were used. The disease was confirmed by histopathological and protein (laminin, vimentin, smooth-muscle- α -actin, Ki-67 antigen and estrogen receptor) secretion analysis. The procedure of MSC application was performed on the 8th day of the cycle (ovulation = day zero) following routine protocol of estrus synchronization in mares. In order to trace MSC in equine uterus after transplantation, the cells were stained with fluorescent dye. Cells were inoculated into uterus using a disposable pipette connected to a syringe through a sterile connector rubber containing the cells (1×10^6 cells diluted in 20 ml of sodium chloride 0.9%). Following this simple technique, similar to the procedure for artificial insemination in mares, seven days after transplantation, the efficient homing of eAT-MSC in glandular and periglandular space was observed in all treated animals. Confocal microscopy of endometrial biopsy demonstrated the presence of these cells in the uterine body and in both uterine horns. This method is minimally invasive and can be easily learned by horses’ owners and veterinarians, opening new avenues for the future treatment of endometriosis in mares using MSC.



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Use of polyethyleneimine and lipofectamine 2000 for transfection of bovine fibroblasts cultured *in vitro*

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Keywords: fibroblast, GFP, transfection.

The use of transgenic somatic cells as nucleus donors in Nuclear Transfer (NT) has opened up several possibilities for production of genetically modified animals. However, the production of transgenic cell lineage still has difficulties, which includes the establishment of efficient methods for transgene delivery. Among the alternatives available for transgene delivery are cationic lipids and polymers carriers. The aim of this study was to compare the efficiency of LipofectamineTM 2000 (Invitrogen, Carlsbad, USA), a cationic lipid, and polyethyleneimine (PEI; St. Lois, USA), a polymer, for transfection of transgenic bovine fibroblasts with plasmidial DNA (pDNA; pLL 3.7, Addgene, Cambridge, USA) carrying the green fluorescent protein (GFP) gene. The cells were taken from the ear of a seven years-old Gir cow and cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with SFB (10%) and antibiotic for two passages before being frozen in 10% DMSO. After thawing, cells were cultured in four well plates for approximately 48h until reaching 80% of confluence. Solution of pDNA:carrier complexes was produced in DMEM without FCS (LipofectamineTM carrier) or 5% glucose solution (PEI carrier in a 18mM solution), both in the proportion of 1µg pDNA for 1µL carrier (1 µg:1 µL ratio of pDNA:carrier). Transfection occurred for 5h in 400 µL DMEM without SFB and antibiotic added with 100 µL of solution with pDNA:carrier complexes, totalizing 2µg of pDNA in 500 µL medium. The culture medium was totally replaced by DMEM supplemented with SFB and antibiotic after the end of transfection. After 48h, the fibroblasts were trypsinized and microdrops of 1µL were exposed to UV light (450-490nm) in an epifluorescent microscopy. The percentage of transfected cells in the whole population was calculated by the proportion of fluorescent cells based on the total cell number. Two repetitions with four replicates each were performed per treatment. Data were submitted to ANOVA. The values are shown as mean±SEM. Greater proportion (P<0.05) of cells positive for GFP were found with LipofectamineTM (8.76±1.48%) than PEI (2.66±0.65%). In the condition of the present study, LipofectamineTM was more efficient for transfection of bovine fibroblast at the pDNA:carrier ratio tested. However, studies are required to confirm such results, including different ratios of pDNA:carrier and cell types.

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215 Cloning, Transgenesis and Stem Cells

***In vitro* development of bovine zona-free clones reconstituted from over-expressing Oct4 or c-Myc somatic cells**

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Keywords: c-myc, cloning, oct4.

Differentiated cells are able to reactivate most of the genes required for normal development in a standard SCNT process. In a similar way, it was recently demonstrated that Oct4, Sox2, Klf4 and c-Myc are able to reprogramme a somatic murine nuclei when they are over-expressed together, maintaining pluripotency (iPS cells; Takahashi and Yamada, Cell 126:663, 2006). For these reasons, our objective was to compare the *in vitro* development of bovine cloned embryos from cells that are over-expressing the genes Oct4 or c-Myc individually. To this aim, bovine COCs were *in vitro* matured in standard conditions for 22 h and then zona free (ZF) oocytes were enucleated. Fetal fibroblasts were transduced with lentiviruses containing exogenous human Oct4 or c-Myc bicistronically linked to fluorescent reporters vaxGFP or mCerulean respectively. Fluorescent cells overexpressing Oct4 or c-Myc were analyzed and recovered through flow cytometry and cell sorting, and then induced into quiescence and used as donor cells. Non transduced cells were used as control. A standard cloning protocol was carried out and 2 h after fusion, ZF reconstituted embryos were activated with ionomycin and 6-DMAP. A slightly modified well of well system was used to culture the clones. Cleavage and blastocyst rates were assessed 2 and 7 days after activation respectively. Data were analyzed by Fisher's test ($p < 0.05$). Blastocysts obtained were classified according to their diameter. Cleavage rates were 84% (82/97) for Oct4 group, 88% (75/85) for c-Myc and 87% (87/100) for the control. Acceptable blastocyst percentages were obtained in all groups: 19% (n=18) for Oct4, 12% (n=10) for c-Myc and 15% (n=15) for the control. According to blastocyst diameters, 50, 70 and 78% of blastocysts from Oct4, c-Myc and control respectively were 80 to 170 μm size. The rest of them (50, 30 and 22% respectively) were bigger than 170 μm . No significant differences were found between groups, neither in cleavage, nor in number or quality of the blastocysts produced. However, there is a tendency to the improvement of embryo quality in Oct4 clones according to diameter parameters. In conclusion, we demonstrated that the transfected fibroblast with Oct4 and c-Myc individually have no effect in the cleavage or blastocyst production in ZF-cloning. Further experiments are necessary to compare the overexpression of others factors individually and combined to improve the reprogramming and then the survival of cloned embryos.



216 Cloning, Transgenesis and Stem Cells

Establishment of embryonic stem cells colonies from blastocysts (IVP) of different developmental stages

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Keywords: bovine blastocyst, development, embryonic stem cells.

Introduction: Stem cells are undifferentiated cells that can be defined by two unique properties: self-renewal and differentiation potential. According to their differentiation ability the stem cells can be classified in totipotent, pluripotent and multipotent. The pluripotent cells have the capacity to generate cells of three germ layers (ectoderm, mesoderm and endoderm). The greatest example of pluripotent stem cells are the cells of the inner cell mass (ICM) of the blastocyst, also called embryonic stem cells (ESC). Several studies have been conducted to find out what is the best developmental stage of bovine blastocysts for the establishment of ESCbov. To confirm the pluripotency of the cells, markers of the families SSEA and TRA1, Oct-4 and Nanog are used. **Objective:** Thus, the objective of this study was to analyze the different ages of bovine blastocysts in the establishment of colonies ESCbov. **Material and methods:** For this, bovine blastocysts (IVP) were cultured in basal medium with the addition of 2.5% (Group 1) or 10% FCS (group 2). The ICM of blastocysts with 7, 8 and 9 days of development were removed mechanically with the aid of two insulin needles. The ICM cells were plated in six wells, in a monolayer of mitotically inactivated bovine fibroblasts (20×10^4 cels/ 1.9cm^2 per well), containing specific medium for ESC. The blastocysts and the colonies were fixed and labeled for ESC markers, according to an immunofluorescence protocol to detect the following proteins: OCT-4 NANOG, SSEA-1, SSEA-3, SSEA-4-TRA 1-60, TRA-1-81. After the adherence of the ICM to the monolayer the media was changed every 48 hours. For statistics we used analysis of variance (ANOVA), $p < 0,05$. **Results:** No difference on blastocyst production was observed among groups. The only ICM that showed adherence to the monolayer were the ones obtained from blastocysts and expanded blastocysts ($n=160$), independently of the serum concentration used during embryo culture. Colony growth began 2 days after seeding. None of the ICMs obtained from early blastocysts ($n=300$) showed growth during culture. On the other hand, the ICM from hatched blastocysts ($n = 45$) presented a tendency of forming cystic embrioid bodies. Embryos from all ages, and colonies, after 7 days in culture were positively marked for Oct-4, NANOG, SSEA-3 and TRA-1-81. There was no detection of SSEA-1, SSEA-4 and TRA 1-60. The negative control showed no fluorescence for any protein. **Conclusion:** We conclude, therefore, that blastocysts and expanded blastocysts are more viable for establishment and culture of bovine embryonic stem cells.

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217 Cloning, Transgenesis and Stem Cells

Reversibility of cell cycle inhibition induced by extracts of *azadirachta indica* in bovine fibroblasts

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Keywords: cellular inhibition, gene reprogramming, SCNT.

Among the numerous challenges found in the technique of SCNT, gene reprogramming is a critical point of the process, because the nucleus must assume a gene expression pattern of a newly fertilized embryo. The use of nuclei donor cells stationed in G0/G1 stages of cell cycle is important to a correct reprogramming. Extracts of the *Azadirachta indica* (Neem) have a potential to inhibit the cell cycle of bovine fibroblasts; however, the reversibility of such inhibition is needed to resume the mitosis of zygotes reconstructed by SCNT. The objective of this study was to evaluate the reversibility of the inhibition effect caused by the extracts of this plant. Bovine fibroblast were cultured and exposed to concentrations of the extracts that have high inhibitory potential, previously evaluated (Rabelo et al., 2011. *Acta Sci. Vet.* 39[Supl.]:S338), as follow: 50µg/mL, 100µg/mL and 200µg/mL for 24h, for ethanol extract, and 50µg/mL, 100µg/mL and 200µg/mL for 12 h and 50µg/mL, 100µg/mL for 24 h, for hexane extract. These repetitions were performed in triplicate for each treatment. Simultaneously, a serum-starvation group was used as a control (absence of extract, cells cultured with 0,5% serum for three days). The reversibility of inhibition of the cell cycle was evaluated by Flow Cytometry (Facs Callibur, Becton Dickinson, San Jose, CA, USA) through determining the percentage of cells in each stage of the cycle, 0, 12, 24 and 36 h after removal of the extracts. Resumption of the cell cycle was considered when the proportion of cells stationed at G0/G1 stage was reduced after removing the extracts. The histograms were evaluated using the WinMDI software to determine the percentage of cells in each stage of the cell cycle (G0/G1, S and G2). Statistical analysis was performed by analysis of variance, and the averages were compared by Student Newman Keuls test. P<0.05 values were considered significant. There was a reduction in the proportion of cells in G0/G1 stage 12h after removing them from ethanol extract at a concentration of 100µg/mL, showing resumption of the cell cycle. The serum-starvation also showed a decrease in the proportion of cells in G0/G1, however, this happened 24 h after removing this condition. The other treatments, including those with hexane extract, did not affect the percentage of cells in G0/G1 at 0, 12, 24 and 36 h after removing the extracts. In conclusion, the inhibition of cell cycle with 100µg/mL ethanolic extract of Neem can be reversed at 12 h after removing the extract, which is faster than serum-starvation, and, therefore, it is the concentration indicated among those tested for bovine fibroblasts.



218 Cloning, Transgenesis and Stem Cells

Simple and efficient method for the production of transgenic bovine embryos

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Keywords: embryos, IVF, transgenic.

Techniques for producing transgenic bovine embryos such as pronuclear microinjection, cloning, lentiviral perivitelline injection or ICSI, require specific tools and methodologies, limiting the use of this technology to well equipped laboratories and maintaining a very high cost. Herein, we present a simple and efficient methodology for the production of transgenic bovine embryos, based on the production of embryos by IVF, followed by zygote transduction with lentivirus. The lentiviruses were produced in HEK 293 cells by lipofection, using lentiviral vectors ViraPower Packaging Mix kit, the FUGW and pLenti-p β cas5-hINS. The latter vector was constructed by modifying the pLenti6.2-GW/EmGFP, whose promoter PCMV had been replaced by the bovine β -casein promoter, containing 5.335-kbp, and the human preproinsulin gene was introduced in place of the EmGFP gene. After lentiviral production, the lentiviruses were concentrated by PEG precipitation. Ovaries from slaughterhouses were aspirated, matured and fertilized *in vitro* following standard protocol. After 18 hours of IVF, the cumulus cells were removed with hyaluronidase and zygotes separated into three groups. In the first group, the pellucid zone (ZP) was completely digested with pronase; in the second, ZP was partially digested and; in the third, ZP was maintained. The embryos were cultured in the well of the well system in SOF with lentiviral solution (5% of total drop) for 7 days. The blastocysts obtained were evaluated for the fluorescence emission of GFP (FUGW vector) and showed 100% efficiency of transgenesis for embryos of the three groups. The group without the ZP showed higher fluorescence intensity, suggesting greater integration of the transgene, but had embryos with poor quality. Groups with ZP and partial digestion of the ZP showed no visual differences on the fluorescence emission. The selection of transgenic embryos transduced with lentivirus containing the insulin gene was performed by adding blasticidin (6 μ g/mL) on the third day of development. The blastocysts were evaluated individually by PCR for the blasticidin resistance gene and showed 100% efficiency in the three different groups. This methodology was efficient for the production of transgenic bovine embryos and it has shown advantages over the methods currently used. Further studies will be performed to determine the number of copies inserted and for assessing the establishment of pregnancy.



A219 Cloning, Transgenesis and Stem Cells

Detection of transgene signals and quantification of transgene copy number for meganuclease mediated transgenesis in bovine embryos

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Keywords: bovine, meganuclease, transgenesis.

Transgenesis methods in mammals remain quite inefficient. Recently, a simple and highly efficient transgenesis procedure for transgenic animal generation using I-SceI meganuclease was described in *Xenopus*. The method consists on the cytoplasmic injection into fertilized eggs of I-SceI enzyme with a plasmid DNA carrying the meganuclease recognition sites. In this work, transgene signals following meganuclease transgenesis were measured by FISH and quantity of transgene present at different embryo stages was detected by qPCR technique. Briefly, *in vitro*-produced embryos by IVF were divided in two groups: one group, injected with pIS CAGegfp (50 ng/ul) associated with 0.5 IU I-SceI in 1x I-SceI buffer (pIS+Mega), and another, injected with pIS CAGegfp alone (50 ng/ul) (pIS). For FISH, day 2 and day 3 embryos were synchronized, fixed and subjected to hybridization with a pCX-EGFP plasmid probe, rhodamine labeled, and with a bovine chromosome 19 pericentromeric probe, FITC labeled as a control. For qPCR, the amount of transgene was measured at 0.5h, 22h and D7 of embryonic development (blastocysts) post-injection. No difference was observed between groups regarding number of embryos showing transgene signals in at least one of their blastomeres, calculated over total embryos (24/25 vs 21/29; pIS+Mega vs pIS, $P>0.05$). However, the pIS+Mega group presented a higher proportion of embryos with transgene signals in all of their blastomeres, calculated over total embryos analyzed (11/25, 44.0% vs 2/29, 6.9%, pIS+Mega vs pIS, $P<0.05$). The number of transgene signals in embryo blastomeres ranged from one to four. The quantity of transgene present at the different embryo stages did not statistically differ between groups as determined by qPCR ($P>0.05$). However, at 30 min post-injection and in blastocysts, the pIS+Mega group tended to show higher transgene copy number (transgene copy number+ standard error: 25620+8940; 2250+525 and 6540+3345, pIS+Mega group vs 4320+960; 1185+360 and 2250+870, pIS group in 0.5 h, at 22h and blastocysts). These results show that cytoplasmic injection into presumptive zygotes with pIS plus I-SceI results in increased presence of transgene signals than injection with DNA alone. In addition, the presence of I-SceI appears to stabilize the transgene, preventing its early degradation. In conclusion, I-SceI transgenesis constitutes a promising alternative for transgenic domestic animals production.



A220 Cloning, Transgenesis and Stem Cells

Pre- and post-implantation development of bovine cloned embryos derived from fibroblasts treated with histone deacetylase inhibitor

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Keywords: bovine, histone deacetylase inhibitor, SCNT.

Abnormal epigenetic reprogramming of nuclei after somatic cell nuclear transfer (SCNT) can be considered a major reason for the low efficiency in the number of live births in cloned cattle. Histone deacetylase inhibitors (HDACi) are being used in an attempt to improve nuclear reprogramming efficiency and consequently, bovine cloned embryo quality. The aim of this study was to use valproic acid (VA), a HDACi, in fibroblast cells, used as donor nuclei, observing the effects on embryo development pre- and post-implantation. Bovine fibroblasts were collected from a male Gir that was approximately 14 years old. The samples were frozen and stored in liquid nitrogen. For this experiment, a fibroblast vial was thawed and the culture was seeded in two separate petri dishes, one for the control group (CG) and another for the treatment group (TG). Both CG and TG were placed in serum starvation for 72 hours. Twenty-four hours before SCNT, 2 mM of VA was added to the TG medium. Bovine oocytes were denuded after 18 hours of *in vitro* maturation and at this time, the 1st polar body (PB) was identified. These selected oocytes were enucleated and randomly separated in two groups for reconstruction. One group was reconstructed with CG fibroblasts and the other used TG fibroblasts. The oocyte-cell complexes were fused and fusion rate was determined 1 hour after fusion. Twenty-six hours after the initiation of oocyte maturation, fused oocytes were chemically activated with ionomycin (5 µM) for 5 minutes and then transferred to culture medium supplemented with 6-DMAP (6-Dimethylaminopurine) (2mM) for 3 hours. Following the 3-hour incubation with 6-DMAP, the oocytes were transferred into medium for *in vitro* culture. Four replicates were produced and observed for: fusion rate, blastocyst rate on the seventh day of culture (7D) and pregnancy on the thirtieth day of gestation (30D). Statistical analysis was performed using BIOSTATS v.4.0 software and the variables were analyzed by Chi-square test at the 5% significance level. This experiment used 876 oocytes. From those oocytes, 590 had the 1st PB (67.35%) and 64 blastocysts were produced, 32 from each group. These were then transferred to recipient cows that were previously synchronized. There were no statistically significant differences between the CG and TG, for: fusion rates CG n=139; 77.22% and TG n= 115; 68.45%, blastocysts rates CG n= 44; 31.65% and TG n=38; 33.04% and pregnancy rates on 30D CG n=6; 18.75% and TG n=7; 21.87%, respectively. In conclusion, VA can be used in somatic cell culture for SCNT, since it did not have adverse effects on the development of bovine cloned embryos pre- and post-implantation. However, it is necessary to observe the gestations to term, to evaluate the effects of VA use at the later stages of gestation and development.



A221 Cloning, Transgenesis and Stem Cells

Effects of 5-aza-2/-deoxycytidine, valproic acid and PD0325901 in the *in vitro* development of porcine parthenogenetic embryos

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The drugs 5-aza-2/-deoxycytidine (5-aza-dC), Valproic acid (VA) and PD0325901 (PD) change the pattern of DNA methylation, acetylation, and inhibit MEK in the MAPK pathways, respectively. They induce a more undifferentiated state of the cells, which could be important to improve the reprogramming of reconstructed SCNT embryos. The objective of this work was to study the effect of these drugs on *in vitro* development of parthenogenetic pig embryos, to establish the best conditions for SCNT. To this aim, oocytes recovered from slaughterhouse ovaries were *in vitro* matured in modified TCM199 medium for 42-48 h at 39°C and 5% CO₂. Then, matured oocytes were subjected to electrical activation in activation medium (0.3 M mannitol, 1.0 mM CaCl₂, 0.1 mM MgCl₂ and 0.01% PVA) by one DC pulse of 1.2 kV/cm for 80 μsec, followed by incubation in 2 mM of 6-DMAP for 3 h. All the embryos were cultured in SOF medium supplemented with the different drugs. Two concentrations were tested for each drug (1 μM - 2 μM 5-aza-dC, 2 mM - 4 mM VA, and 0.5 μM - 1 μM PD). In addition, the different drugs were combined at the concentrations that resulted in the higher blastocyst rates (2 μM 5-aza-dC + 2 mM VA; 2 mM VA + 1 μM PD and 2 μM 5-aza-dC + 1 μM PD). After three days, the agents were removed and culture was continued till day 7 in SOF medium. A parthenogenetic control group was cultured under standard conditions. Statistical differences were determined by Fisher test. Blastocyst rates did not differ between groups incubated in 5-aza-dC (1 μM and 2 μM) and the control group [29/119 (24.4%) and 60/281 (21.4%) vs. 57/205 (27.8%) respectively, (p<0.005)]. VA had a detrimental effect on blastocyst development, showing statistical differences respect to the control, in both concentrations assayed (2 mM and 4 mM) [10/120 (8.3%) and 10/201 (4.9%) respectively, p<0.05]. In addition, the combination of 5-aza-dC 2 μM with 2 mM VA reduced blastocyst rates [12/81 (14.8%), p<0.05]. The lower concentration tested of PD (0.5 μM) showed significant differences respect to the control, [35/191 (18.3%)] however the highest concentration (1 μM) did not significantly differ [55/256 (21.5%)]. Also, the combination of 2 mM VA with 1 μM PD resulted in lower blastocyst rates, 26/138 (18.8%). The combination of 2 μM 5-aza-dC with 1 μM PD tended to produce higher blastocyst rates than the control [68/219 (31.1%)]. In summary, this last combination seems to be beneficial for embryo development. Further experiments should be performed to evaluate the effect of these drugs for the generation of SCNT embryos.