Use of bovine sex sorted sperm on timed artificial insemination, *in vivo* and *in vitro* embryo production programs

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The use of sex-sorted sperm in beef and dairy cattle increases the production of specific gender born calve, increasing the genetic gain progress and efficiency of beef and dairy production. The sex-sorted sperm has been routinely used in various commercial biotechnology such as artificial insemination (AI) upon estrus detection, timed artificial insemination (TAI) and in vivo (SOV) and in vitro (IVP) embryo production. Currently, it is possible to obtain acceptable conception rates (around 80% of those obtained with conventional semen) after AI with sexed semen in heifers inseminated upon estrus detection. The time of insemination in relation to the onset of estrus and/or ovulation can increase pregnancy outcomes. Greater conception rates is obtained when AI are performed 16 to 24 hours after onset of estrus (i.e. 6 to 14 hours before ovulation). In TAI programs, greater conception rate could be achieved after if the TAI is performed 10 hours before synchronized ovulation. In superstimulated cows, the use of sex-sorted sperm reduces the production of viable embryos, however the delay of 6 hours in the TAI can also increases the number of embryos collected in both *Bos indicus* and *Bos taurus* donors. Currently, IVP technique has been employed to optimize the use of sexed semen. With a single dose of sex-sorted sperm could fertilize approximately 100 oocytes with satisfactory in vitro embryo production. However, it is important to note that, regardless of biotechnologies, there is great individual variation on fertility among bulls subjected to the sorting process. These differences should be considered when the sex-sorted sperm is used for AI and embryo production programs.

Animal cloning, transgenesis and stem cell biology: powerful biotechnological tools for the good of mankind

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Keywords: animal cloning, ruminants, stem cell biology, transgenesis.

For the past three decades, the use of animal cloning by nuclear transfer, the production of transgenic animals by genetic engineering (GE), and the outcome and expansion in knowledge in stem cell biology have been envisioned as important biotechnological strategies to boost food quality, animal yield, and for the production of a wide range of bioproducts and procedures that can be used for the benefit of human and animal health. In animals, cloning by somatic cell nuclear transfer (SCNT) has been of importance for research and development, being routinely used worldwide for scientific, conservational, and/or commercial purposes, contributing to advances in many related fields, including epigenetics, genetic reprogramming, developmental biology, and even neonatology. Several applications have been associated with cloning by SCNT, as reproductive cloning, for the genetic conservation and propagation of economically important individuals and endangered species, genetic engineering and transgenesis, stem cell biology, and therapeutic cloning, which may eventually have a direct impact on human health. Next, through transgenesis, GE animals can be used to improve production traits (e.g., AquaAdvantage® salmon, AquaBounty), to reduce or minimize the impact of animal production on the environment (e.g., Enviropig™, University of Guelph), to add value to an animal product (e.g., human lysozyme goat milk, University of California at Davis and University of Fortaleza; human lactoferrin cow milk, Pharming Inc.), to promote disease resistance (e.g., chicken that do not transmit bird flu, Roslin Institute), as animal models for biomedical applications (e.g., transgenic goat models for cardiac fibrosis, Utah State University), for entertainment as *bio-art* (e.g., GloFish® fluorescent fish, Yorktown Technologies), and to produce recombinant proteins in blood, urine, semen, salivary gland, egg white or milk that can be collected, purified and used as pharmaceutical or biosimilar products (*biopharming*) or even for industrial and general use (e.g., Spider Silk transgenic goats, Nexia-Utah State University). On its turn, stem cells have been of great interest, hence being widely studied for their plasticity and potential therapeutic use. Mouse embryonic stem cells (ESC) have been extensively used both as a model for the study of cell lineage and regulation of gene expression during mammalian development and as a vehicle for genetic engineering, functional genomics, and cell therapy studies. Animal and human ESC promise to offer similar uses as in the mouse, in addition to new opportunities for use in study of early embryo development, creation of disease models, regenerative medicine, and cell and gene therapy. However, proven *bona fide* ESCs are yet to be obtained in livestock species. In addition to ESC, stem cells from fetal or adult somatic (mesenchymal, dental pulp, etc.) and germline (GC) origin, amniotic and cord blood (CB-SC) stem cells, and more recently, the induced pluripotent stem cells (iPS), have demonstrated functional potency and self-renewal capacities, with great potential to be used as tools for *in vitro* differentiation and/or cell therapy for tissue remodeling, regeneration and engineering. In fact, adult stem cells have been routinely used in medical therapies, as for example, in bone marrow transplantation used to treat leukemia in humans. In summary, cloning by SCNT, transgenesis by GE, and stem cell research have been shown as robust and viable technological strategies to aid in the resolution of problems of the modern world. However, there still exists a great deal of social, ethical, religious and scientific uncertainties surrounding research and applications in such fields. Despite some skepticism by the general public, the use of such technologies has already been translated in the development of new drugs, procedures and even therapies to the benefit of animals and humans. Some of such recent developments, potential applications, and novel understanding in biological processes associated with cloning, transgenesis, and stem cell technologies will be presented and discussed by distinguished speakers at the II Symposium of the South American Research Consortium on Cloning and Transgenesis in Ruminants.

Lentiviral-vectors as an efficient screening method to evaluate transgene expression in livestock

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Keywords: cattle, lentiviral vectors, transgenic animals.

Several methods including microinjection, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), nuclear transfer, sperm-mediated gene transfer and germ cell transfer afford relatively efficient routes to transfer transgenes to the germ line of transgenic livestock. Founder animals generated by these technologies are typically bred to produce the desired populations. Alternatively, there are certain circumstances where the transgenic studies may not need breeding populations but instead quick evaluation of gene expression or gene re-regulation/deregulation. In those circumstances, production of transgenic animals using lentiviral-mediated transgenesis is an attractive option. Lentiviruses are members of the extensive family of complex retroviruses. Lentiviral vectors have a large payload capacity for carrying DNA into cells. Typically, the DNA size is about 8-10kb, which is the DNA construct size needed for most transgenic applications. Furthermore, lentiviruses can be produced at high titers, which can increase the efficiency of transgenesis. This technology has been used to produce transgenic mice, rats, cats, swine, sheep, goats, cows and non-human primates. One advantage of lentivirus-based vectors is that the expression of the transgenes are typically sustained and not silenced as occurs in many virus-produced transgenic animals. Furthermore, transgene expression from lentiviral-transferred genes is often reflective of the transgene copy number that has integrated into the genome. The ability to analyze the relationship between transgene expression level and the phenotype in founder animals directly could represent significant cost savings for conducting transgenic studies. Recently, we have been developing lentiviral-based vectors to target proteins into the mammary gland of cattle. We have been using mammary epithelial cell lines (murine and bovine) to evaluate transgene expression before producing transgenic animals. Our lab is ultimately interested in developing the bioreactor potential of the bovine mammary gland for human and animal nutriceutical and pharmaceutical production.

Insights on bovine genetic engineering and cloning

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Keywords: bovine, induced pluripotency, somatic cell nuclear transfer, transgenic technology.

Transgenic technology has become an essential tool for the development of animal biotechnologies, and animal cloning through somatic cell nuclear transfer (SCNT) enabled the generation of genetically modified animals utilizing previously modified and selected cell lineages as nuclei donors, assuring therefore the generation of homogeneous herds expressing the desired modification. The present study aimed to discuss the use of SCNT as an important methodology for the production of transgenic herds, and also some recent insights on genetic modification of nuclei donors and possible effects of gene induction of pluripotency on SCNT.

The prevention and treatment of diarrheal illnesses using the milk from transgenic animals

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Keywords: diarrhea, goat, lysozyme, lactoferrin, transgenic animals.

Human milk contains the antimicrobial factors lysozyme and lactoferrin that help contribute to the development of a healthy intestinal tract. These key factors are lacking in the milk of common dairy animals such as the cow and goat. Transgenic goats expressing human lysozyme in their milk and transgenic cows expressing human lactoferrin in their milk are being studied as sources of milk that could help fight the diarrheal illnesses that claim the lives of more than 1.5 million children worldwide under the age of five each year. This work has progressed over the past 20 years from testing hypothesis of using transgenics to modify the properties of milk in a mouse model to the testing of the efficacy of the milk to act at the level of the intestine in a novel pig model of human health. All work to date has indicated that the milk from lysozyme transgenic goats and lactoferrin transgenic cows positively impacts gut microbiota and morphology, can mitigate intestinal damage caused by malnutrition and helps to resolve *E. coli*-induced diarrhea more quickly than control milk. Work is now focusing on the mechanism of action of these antimicrobials and moving to the rat for toxicity testing as one of the last steps before human clinical trials.

Development of transgenic goat models of cardiac fibrosis

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Keywords: animal cloning, animal model, goat, transgenic animals.

Fibrosis is a fundamental element of the damaging structural remodeling of cardiac tissue seen in a wide range of cardiac disease. In fibrosis, profibrotic factors act on cardiac cells to increase deposition of extracellular matrix. These changes alter the structure, architecture and shape of the heart affecting three cardiac functions: ventricular contractility, valvular performance and electrical conduction. Transforming growth factor β1 (TGF-β1) has a potent profibrotic function and is central to signaling cascades involved in interstitial fibrosis. We recently developed an efficient procedure for generation of transgenic goats using somatic cell nuclear transfer (Hall *et al*., *Reprod Fertil Dev*, 2012; 25:162) and produced cloned transgenic goats expressing human TGF-β1 under control of the cardiac-specific α-myosin heavy chain (α-MHC) promoter. Cardiac biopsies were obtained using a bioptome under visualization of intra-cardiac echocardiography and cardiac specific expression of hTGF-β1 was confirmed. Current status of the model characterization will be reviewed. Additionally, the presentation will discuss strategies for early non-invasive *in vivo* detection of gene expression during large animal model development and approaches for achieving reproducible gene and protein expression levels.

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Uses of mesenchymal stem cells in tissue engineered regeneration

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Keywords: animal model, pig, stem cell biology, tissue engineering.

Bone is a living polymer with substantial healing capacity. However, extensive bone loss due to disease or trauma may require tissue-engineering methods. Presently, autologous bone grafting is the gold standard for bone repair, but presents limitations including donor site morbidity, bone shape, and amount. Synthetic bone grafts such as ceramics, collagen, non-collagenous proteins, and biodegradable polymers that have been tested also have drawbacks related to poor resorbability, use of processed animal components, inferior handling characteristics, and cost. The use of stem cells appears a means to overcome such limitations. Bone marrow mesenchymal stem cells (BMSC) have been the choice, to date, for stem cell therapy for bone regeneration. Adipose-derived stem cells (ASC) are more abundant and accessible with lower donor site morbidity, making them a potentially better alternative to BMSC. Once ASC are obtained, it is critical to establish a proper animal model that closely resembles the size of human bones for their use in pre-clinical trials. Among available animal models, swine are the closest non-primate model for craniofacial configuration with two dentitions. Application of stem cells for regeneration of clinically relevant defects will require scaffolds that provide a nurturing environment, temporary function, replicate complex anatomic defects while being readily fixed to surrounding bone and be surgically implantable. The porcine animal model provides a valuable tool for scaffold use in tissue engineered bone regeneration with the use of ASC especially for complex anatomic defects in the craniofacial region.

Evaluation of risks from environmental contact with transgenic livestock

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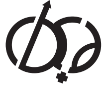
Keywords: livestock, risk assessment, transgenic animals.

Assessment of general risk posed from transgenic animals is important to their future contributions to society. Identification of potentially harmful properties of transgenic livestock is the initial step in a risk assessment. We previously developed and characterized transgenic swine containing a mammary-specific transgene, bovine α-lactalbumin, (Bα-LA) that results in increased milk production in sows. This study determined if Bα-LA is expressed in tissues of transgenic swine (T) other than the lactating mammary gland and if the transgene DNA (Tg) crosses into non-transgenic swine under various physiological and physical conditions. Specific aims were to determine whether Tg can be (1) expressed in tissues other than the mammary gland of a T sow; (2) transferred to non-T swine by direct physical association or contact; (3) transferred to non-T swine via mating; (4) transferred to non-T swine during gestation, parturition, or lactation. To address specific aim 1, T and non-T (control; C) pigs were raised to 180, 220, 250 days of age, or 112 days post-breeding and then sacrificed for tissue collection. For specific aim 2, comparable age- and weight-matched T and C pigs were housed together to allow for general contact that is normal in swine production, starting from weaning (21 days) to either 180, 220, or 250 days of age and then sacrificed for tissue collection. In experiments 1 & 2, blood, brain, jejunum, kidney, liver, lung, mammary gland, muscle, ovary, sublingual salivary gland, skin, and spleen were collected. For specific aim 3, vaginal, cervical, uterine, oviductal, and ovarian tissues were collected from C females at 2, 7, or 90 days post-mating to T males, and penis, bulbourethral gland, urethra, testis, and epididymal tissue was collected from C males 7 days after mating to Tg females. Addressing specific aim 4 was divided into 3 sub-experiments: 1) tissues from 112 day fetuses were collected from C sows bred to a C boar and T sows bred to a C boar; 2) C piglets were removed from their birth dam at parturition and before they had suckled, fostered to a lactating T sow or to a lactating C sow, and allowed to suckle for 24 or 72 h before sacrifice and tissues collection; 3) C piglets were allowed to suckle their birth dam until 3 days of age, then fostered to a lactating T sow or to a lactating C sow, and allowed to suckle for 72 or 168 h before sacrifice and tissues collection. Jejunum, liver, lung, muscle, and skin was harvested for each sub-experiment. The presence of the Tg or its expression in tissues from C and T animals was tested by PCR analyses. In total, 1,626 tissues from 295 animals were analyzed. The Tg was not expressed in tissues other than the mammary gland of a T lactating sows. The Tg was not detectable in any tissue sample C animals after co-habitation for 180, 220, or 250 days or at 2, 7, 90, or 112 days post-mating. At day 112 of gestation, all samples from C piglets whose dam was a Tg female were negative, except for the outer placental membrane, which screened positive for Tg. The latter tissue is derived from the maternal tissues and would be expected to be positive for Tg in a Tg female. Tissues from C piglets cross-fostered prior to suckling (day 0) or 3 days after birth to a lactating T sow were negative for Tg. These results strongly indicate that horizontal Tg transmission between T and C pigs does not occur during co-habitation, mating, gestation, or lactation.



Whole animal assessment of unintended effects of foreign gene products on host and non-target organisms

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Keywords: goat, human lysozyme, milk, risk assessment, transgenic animals.

Risk assessment is an important aspect for the future use of transgenic animals. While much work has been carried out determining the risks of transgenic plants and their interactions with their environments, risk analysis in transgenic animals is less defined and different parameters need to be assessed. The first goal is risk assessment in animals is to ensure that animal health and welfare are not compromised by the presence and expression of the transgene. Next, the intended function of transgene product should be verified followed by other context-specific types of analyses to determine any associated risks the transgene product may have on both the animal and consumers of the product produced from the transgenic animal. Work can also be done to assess any unintended consequences of transgene expression at the whole animal level by conducting global types of analyses to determine if a transgene product can impact aspects of an animals' physiology other than those intended by the function of the particular transgene. The Artemis line of transgenic goats that expresses the antimicrobial human lysozyme in their milk was established in 1999 and has been subjected to multiple risk assessment characterizations. The growth, reproduction and milk production of the transgenic line is in no way adversely impacted by the presence or expression of the lysozyme transgene. Unintended effects of the transgene have also been assessed using microbial and metabolite profiling techniques to investigate any effects on the physiology of the host (lactating goats) and non-target organisms (kid goats consuming the milk). The microbial profile of lactating does changed more over time than it did in response to expression of the transgene as did the metabolite profiles of kid goats consuming the milk. These types of global analyses were useful in assessing the scope of pleiotropic effects of transgenes and their products at the whole animal level. Data collected on the intended and unintended effects of transgenes is an important part of the development of a transgenic line and will contribute to the ability to make science-based decisions about the safety and future implementation of transgenic food animals.

The endometrium of cows as a source of mesenchymal stem cells

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Keywords: cattle, cell therapy, gene expression, stem cell biology.

Adult mesenchymal stem cells had been isolated from numerous tissues of different animal species; however endometrial stem cells, only from human, mice and recently form pigs, but not from cattle. It has been reported that human endometrial stem cells are more plastic than adipose or bone marrow derived (Bockeria et al., J Transl Med. 2013 Mar 5;11:56. doi: 10.1186/1479-5876-11-56). The aim of our work was to identify such cells in the bovine endometrium and to establish a model system in which to test inducers of differentiation and recruiters of stem cell niches, for potential therapeutic use in other species, such as horses. We searched for endometrial stem cells in healthy cycling cows and in cattle with clinical (C) or subclinical (SC) endometritis. For this, the uterine tracts of slaughtered cows were collected at early (days 2-5; ELF) and late luteal phases (days 11-15; LLF) of the estrus cycle of healthy cows. For endometritis diseased cattle, uterine biopsies were taken in live animals. In all cases, markers of stemness, inflammation, uterine function and housekeeping were studied both at mRNA and protein level, by RT-qPCR and Western blot/immunohistochemistry respectively. In addition, cell primary cultures were established in vitro from all the animals (n=4 for ELF, n=4 for LLF; n=4 for C and n=4 for SC). We found that the endometrium of most animals expressed embryonic stem cell markers, such as OCT4 and SOX2, but not or little NANOG, as well as CD44, c-Kit and STAT3, markers of mesenchymal stem cells. The expression profile of these markers, was not related to the stage of the estrus cycle; however there was a statistically significant reduction in the expression of embryonic stem cell markers in ill animals, being the lowest in clinically ill and intermediate in subclinal endometritis, ($P<0.05$ and Pearson´s correlation coefficient 0.92). For markers of multipotence (mesenchymal), the expression was lower in clinical endometritis ($P<0.05$). In resume, the expression profile of stem cell markers is indicative of the presence of stem cells in the bovine endometrium. At the protein level, we verified our findings for OCT4, SOX2 and CD44 using Western blot and immunohistochemistry. In general there was a concordance between mRNA and protein profiles. Inflammatory markers showed a pattern characteristic for each of the stages studied. In order to have an ultimate criterion of the presence of stem cells, we tested the differentiation potential of the isolated cell lines, upon induction to chondrogenic, osteogenic and adipogenic lineages. We found that the entire cell lines tested (n=8) displayed mesenchymal differentiation potential as demonstrated from specific stainings as well as expression of gene markers. At present, work is in progress to isolate pure stem cell populations from these primary cultures to have a deeper characterization of the cells. We showed for the first time, the presence and differentiation potential of endometrial stem cells in cattle, this can have impact on the development of new therapeutic approaches to combat uterine diseases, such as endometritis or endometrosis (in horses).

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Genetic modification of bovine embryos by lentiviral vectors

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Keywords: cattle, embryo, lentiviral vectors, transgenic animals.

Lentiviral vectors have been widely used in studies for generation of human induced pluripotent stem cells (Okita and Yamanaka, 2011. Philos Trans R Soc Lond B Biol Sci 366:2198) and for human gene therapy (Antoniou et al, 2013. Hum Gene Ther 24:363). Such vectors have also been shown to be an alternative to generate livestock. As any other transgene delivery systems, lentiviral vectors have pros and cons. Its efficiency is elevated when compared to other systems. Lilico et al. (Trans Res 20:441, 2011) generated more transgenic lambs by lentiviral vectors in 2008/2009 (32 founders with 6 different transgenes) than the previous 25 years in the Roslin Institute using other techniques. The efficiency of lentiviral vector seems to be related to its nuclear import feature and ability to integrate into the genome of non-dividing cells (Durand and Cimareli, 2011. Viruses 3:132). However, the production and manipulation of these vectors require laboratories with biosafety level two, despite the third generation of lentiviral vectors has features that increases the biosafety and reduces undesirable effects as those caused by retrovirus, as activation of proto-oncogenes (Cockrell and Kafri, 2007. Mol Biotechnol 36:184). The transfer vector size, generally smaller than 13 kb, can be a limitation, allowing inserts with up to 7.5 kb only (Al Yacoub et al., 2007. J Gene Med 9:579). Moreover, expression of lentivirus integrants may be modulated by epigenetic modification and disturbs transgene expression (Hofmann et al., 2006. Mol Therap 13:59). The usefulness of lentiviral vectors to generate transgenic cattle was reported by Hofmann et al. (Biol Reprod 71:405, 2004) by microinjecting lentiviral particles into periviteline space of matured oocytes. Microinjection into periviteline space of bovine zygotes was shown to be less efficient than of oocytes (Hofmann et al, 2004. Biol Reprod 71:405; Ewerling et al., 2006 Transgenic Res 15:447). We have also carried out studies with lentiviral vectors to delivery GFP transgene to matured bovine oocytes and zygotes. Differently from previous studies, we microinjected lentiviral particles into the periviteline space of zygotes with 6h post in vitro fertilization in an attempt to make the transgene available before syngamy. Fifty percent of the blastocysts produced had the transgene detected by PCR in contrast to 100% of blastocyts produced from matured oocytes microinjected with lentiviral vectors. In both groups, the proportion of blastocysts emitting green fluorescence was lower than that of blastocyst with the transgene detected by PCR, suggesting the silencing of GFP expression in some embryos. Eleven blastocysts produced from matured oocytes microinjected with lentiviral vectors were transferred to synchronized recipients and resulted in five pregnancies (45.4%); rate similar to that regularly reported with non-microinjected vitro-fertilized embryos. However, one fetus was lost in the 8th month of pregnancy and two out four calves died few hours before parturition without any apparent morphological alteration. The transgene was detected by PCR in umbilical cord and blood cells from one of the stillborn calves while tissues from other three calves are still under evaluations. Those results indicate that the use of lentiviral vectors by microinjection into periviteline space of bovine oocytes and zygotes still demands improvements. Nevertheless, lentiviral vectors can also be used to transduce somatic donor cells in order to generate transgenic cloned animals and may be an alternative for production of transgenic cattle (Monzani et al. 2013. Gen Mol Res 12). Despite its potential application for cattle transgenesis, lentiviral vectors may become restrict to production of transgenic cows for secretion of recombinant biopharmaceutical proteins for human and animal health purposes. As the current lentiviral vectors are based on HIV-1 nucleotide sequences, the consumer may decline to consume milk or meat produced by cattle genetically modified by those vectors. Besides, new tools to edit the genome, as meganucleases, are becoming available for livestock and may have advantages over lentiviral vectors.

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Back to the future: embryo microinjection and Meganucleases

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Keywords: embryo microinjection, meganucleases, transgenic animals.

The production of transgenic livestock between the early 1980s and the late 1990s was principally based on the technique of pronuclear microinjection, although there was some production of transgenic livestock using retroviral vector-mediated and sperm-mediated transgenesis. Pronuclear microinjection was a reliable method to produce transgenic livestock, but the technique was labor-intensive, required a reasonable amount of skill, and was limited to the random introduction of DNA sequences. With the birth of Dolly in the late 1990s, the production of transgenic livestock shifted to the use of cells genetically engineered in culture followed by somatic cell nuclear transfer-based cloning to generate transgenic animals. SCNT-based cloning has the advantage of allowing for gene targeting, and thus the production of knockouts, but still suffered from being technically demanding and low efficiencies of obtaining live born clones. While SCNT-based cloning was being developed for the production of transgenic animals other avenues were also being explored. Two of the most successful were lentiviral, instead of retroviral, vectors and transposons adapted for use in vertebrate cells. Both approaches were successful and have the advantage of high efficiency of gene transfer, but both still suffer from only being able to add genes and cannot be used for targeting. Lentiviral vectors also have the disadvantage of only being able to carry a small construct, as the amount of DNA that you can insert into the viral capsid is limited. The most recent developments affecting the production of transgenic animals is the development of the designer meganucleases. These include zinc finger nucleases (ZFN), TALENs, and most recently the CRISPR-Cas system. In each case the enzyme system can be targeted to cut at a specified location in the genome, thus providing a mechanism for gene targeting. Meganucleases and the accompanying DNA for integration can be injected into an embryo and achieve a high success of gene targeting, so we have come full circle from the initial techniques of pronuclear microinjection back to embryo injection with gene targeting potential.

Deciphering gene expression profile of early bovine embryos: insight for successful development

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Keywords: animal cloning, cattle, embryo, gene expression.

It is well known that embryos produced in vitro are less competent than their in vivo-derived counterparts. When embryos are produced or manipulated in vitro their developmental potential decreases significantly what impinges on the production of viable offspring. The efficiency and the final outcome is proportional to the complexity of these technologies, being somatic cell nucleus transfer (SCNT) the most complex and inefficient, giving no more than 10% of successful pregnancies. The lower quality of in vitro produced embryos is due to changes in their gene expression pattern as a result of the interaction with the adverse environment generated by the in vitro conditions. When embryos are produced by SCNT this scenario is even more critical since the cloned embryo is derived from the transfer of a somatic cell into an enucleated oocyte, which is responsible for reprogramming the differentiated nucleus in order to induce gene expression patterns compatible with embryonic development. More often the reprogramming process is not capable to establish an appropriate gene expression pattern what leads to a low competent embryo that probably will not develop to term. It had also seen that early embryo morphology does not correlate with an appropriate gene expression pattern, making difficult embryo selection. It seems that ideal method for embryo selection would be based on the screening of gene markers that correlate with successful pregnancy after embryo transfer. In that sense we have proposed a method to select competent embryos based on the expression of crucial genes at blastocyst stage. Based on the literature and in our own experience we hypothesize that the expression of pluripotency markers (Oct4, Sox2 and Nanog) at blastocyst stage is predictive of in vivo bovine embryo development. For that we characterised gene expression pattern of early (Day 7) bovine cloned and IVF blastocysts, with emphasis in the pluripotency markers and correlated this gene expression with embryo quality at blastocyst stage (Day 7). Firstly we found that higher percentage of development to blastocysts in culture correlates with the highest gene expression level of pluripotency markers (Oct4, Sox2, Nanog, Fgf4 and Cdx2; $P<0.05$) for both IVF and cloned embryos. This correlates as well with a higher total cell number in the blastocysts and might be of practical use for the selection of cell lines for cloning; those cells that yield higher percentage of blastocysts would probably produce more competent cloned embryos. However, we observed that embryos with a highest expression of pluripotency markers also showed greater variability of expression of these genes, suggesting than only few of them underwent a normal reprogramming process. In mouse, Oct4, Sox2 and Nanog are crucial for normal embryo development by controlling early cell fate; also Oct4 will keep an open chromatin in early embryos what is highly correlated with developmental potential of these embryos. If the same would be true for bovine embryo development, then it can be expected that high expresser embryos will produce greater successful pregnancies; however at least in our hands, the final outcome (10% of live offspring) was not improved by using cell lines that yield greater blastocyst percentage (65%) and highest level of pluripotency genes expression. In order to find out if the portrait of gene expression at Day 7 influences gene expression at elongation (Day 17; filamentous stage), we split Day 7 embryos (IVP) and transferred one half to a temporary recipient cattle, while the other half was used to analyze gene expression of several developmentally important genes. Firstly we optimize the splitting method in order to have similar hemi-embryos. More than 65 % of the split embryos generated two viable hemi-embryos with the same ability of in vitro re-expansion, similar cell number as well as homogenous gene expression (Oct4, Sox2, Nanog, Cdx2, Bcl2-l1 and BAX). Transferred hemi-embryos were recovered at day 17, classified by the elongation stage and used for gene expression analysis of the mentioned genes. From 15 embryos that were transferred, 9 (60 %) were collected with different grades of elongation (1-15 cm). A correlation analysis showed that the expression level of pluripotency markers (Oct4, Sox2 and Nanog) at blastocysts correlated with the expression level of the same genes at the elongation stage, but neither with the expression of the two trophoblastic markers analysed in the elongated embryos (Cdx2 and TP1), nor with embryo length. There are evidences suggesting a different from canonical (as for mouse and humans) role of pluripotency markers in bovine embryo development. From our work, we concluded that at least expression of Oct4 is not a good marker to predict bovine embryo quality. Functional analyses are necessary to define the actual role of the pluripotency genes in bovine embryos and their relation with early development.

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Interface between scientific and commercial applications on cloning, transgenesis and stem cell biology: a South American perspective

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Keywords: animal cloning, animal transgenics, stem cell biology.

The interaction of academia and industry is one of the major aspects related to the success of technology generation and dissemination along the productive sector. In the industrialized countries there is a very important issue related to this interaction and it is reverted in research financing. South American countries relies however to a mostly governmental investments on research and technology. This late information is however changing lately due to modifications in the research agencies and government politics. Agriculture production in South America increased the productivity in dramatic proportions within the last 5 decades, leading to be recognized as one of the major players of the food production and exportation in the world. The efficiency on agricultural production shed light to many other possibilities on the agriculture including the production of fuel and lately energy and finally ended up by increasing the price of the agricultural products with benefits the producers. In this scenario farmers were trained to believe in the technology and to rely on it to increase the productivity. In other hand, the beef cattle producers were somehow challenged by a highly efficient agriculture to increase the productivity or to move to rare new agricultural frontiers. Altogether, this leaded to an increase of the value of the bulls and semen, and especially to the genetic selection. The investment in genetics selection by cattle breeders reached a point were they needed to produce "elite" animals in order to offer to the market solutions to increase productivity. ET and than IVF came as the first and second wave of technologies related to this needs. Both, but specially IVF due to its capability to scale up the elite animals productions were very well accepted in the field and somehow responded to the breeders demand. The nuclear transfer cloning found somehow a window of opportunity on this issue, leading the IVF labs to invest on this technology. Nowadays the production of embryos are reaching a large scale proportion following the FTAI, throughout the FTET, using sexed semen, non additive genetics and indicating a great potential to produce the future. What about clones, transgenic and stem cells biology? They are part of the new coming technologies. Due to the long generation intervals, lineages as observed in pigs and chicken are not available in cattle. Animals produced by large scale cloning technologies, possibly resulted from stem cells technologies, will in a certain moment reach the ground and change the cattle production system. There are already investments in research by public and private sector with this aim. Together, in the next decades, this technologies and transgenic modification will change a paradigm of cattle production in South America and other regions in the world.

Interface between scientific and commercial applications on cloning, transgenesis and stem cell biology: a North American perspective

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Keywords: animal cloning, animal transgenics, stem cell biology.

Somatic cell nuclear transfer (SCNT), or cloning, is one of the assisted reproductive technologies (ARTs) currently used in agriculture. ARTs have a very long history, for example artificial insemination has been employed for several hundred years, and some others (embryo transfer, IVF, embryo freezing) have been extensively used in livestock breeding for decades. SCNT does not require fertilization and therefore it allows for the propagation of proven genotypes without "genetic reshuffling". Commercial applications in agriculture involve employing SCNT for the expansion of elite genetics, the propagation of lost genetics and the protection of genetics in the event of a catastrophe such as foot and mouth disease. The benefit of cloning high-quality individuals will be to increase the number of descendants of elite genotypes in the breeding population via enhanced and prolonged production of a large number of offspring. Previously population outliers (especially maternal lines) had insignificant impact on the population mean. SCNT can amplify the impact of unique genotypes on the population. The current status of SCNT utilization for agricultural and biomedical applications will be reviewed including cloning efficiencies in various livestock species and reproductive performance of clones. Regulation and development of animal biotechnologies in the U.S. will also be discussed.

Interface between scientific and commercial applications on cloning, transgenesis and stem cell biology: a dairy industry perspective

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Keywords: animal cloning, animal transgenesis, cattle, dairy industry.

Dairy cattle production was transformed by the advent of artificial insemination because bulls could be selected for desirable traits very accurately and their genetics disseminated widely. The advent of superovulation and procedures for in vitro production of embryos did not have a major impact on the dairy industry because genetically-superior females could not be identified with much accuracy and the number of offspring produced by females remains much lower than for offspring produced by bulls. Development of genome-wide selection tools such as the Illumina SNP50 and High Density chips has meant that genetically-superior females can be identified with reliabilities that approach that achieved for bulls through progeny testing. It is now possible to use genomic selection to identify genetically superior individuals as early as the preimplantation embryo stage. While genomics has leveled the playing field somewhat for genetic selection on the female vs male side, it remains true that the number of offspring produced by females remains much lower than for offspring produced by males. This situation may be changed, however, by the advent of stem cell technologies that allow differentiation of embryonic or pluripotent stem cells into oocytes. In addition, there is evidence for naturally-occurring oocyte stem cells. If real and if systems for culturing such cells can be developed, it could be possible to generate unlimited numbers of offspring from genetically-superior females. The advent of gene editing technologies, for example using since finger nucleases or TALENS, means that embryos produced in vitro could be generated with specific mutations in economically-important traits. Taken together, the development of new technologies means that the dairy industry is poised for advances in genetic selection on the female side that could rival that achieved by artificial insemination.

In vitro embryo production by Ovum Pick Up and ICSI in the horse

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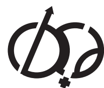
Keywords: equine, fertilization, oocyte.

In vitro embryo production in livestock, especially in bovine, has been developed to a very advanced and reproducible stage, to the point that it replaces in many instances conventional multiple ovulation and embryo transfer. In contrast, the pace of technological progress in equine has been much slower, due to the anatomical and physiological limits typical of this species, and to the inefficiency of conventional in vitro fertilization. However, in vitro embryo production associated with oocyte recovery from live donor mares (Ovum Pick Up, OPU) and ICSI (Intracytoplasmic Sperm Injection) is becoming more and more popular and finds its applications in the clinical setting not only for treating female infertility but also male infertility, overcoming limited availability of semen or poor quality semen, and even allowing out of season breeding programs also for sport performing animals, etc. The presence of functional ovaries with growing follicles is the main requirement for OPU, therefore mares of different age and reproductive status are suitable donors. Oocytes can be recovered from preovulatory follicles 24-30h after hCG or GnRH priming of a follicle larger than 35 mm to initiate in vivo maturation, or from all antral follicles present at any given time, larger than 1cm that are then subjected to a full in vitro maturation. Oocytes from preovulatory follicles have been reported to have a higher developmental competence compared to in vitro matured ones however usually there is one follicle at a time for recovery and this procedure can be performed only in cycling animals. On the contrary, collection of immature oocytes can be performed at any time, with some limitation during the deep anestrus, but being very efficient at spring or fall transition due to the presence of many medium sized follicles (1-2 cm). During the breeding season to maximize the number of medium sized follicles careful monitoring of the estrus cycle is required to avoid the dominant follicle and aim at an emerging follicular wave. Recovery rate can be 60 to 70% with an average of 10 oocytes per OPU and the procedure can be repeated at 10-15 days interval with no side effects. Oocytes are matured in vitro for 24 to 28 hours and about 60% reach the metaphase II when they are injected with a sperm. ICSI is performed with a blunt pipette driven by a piezo electric manipulator, this tool greatly facilitate the penetration of the zona pellucida and the rupture of the oolemma to inject the sperm directly into the cytoplasm with minimal damage to the oocyte. Cleavage rate after ICSI is usually in the range of 60 to 70% but with considerable individual differences between donors. After ICSI the injected oocytes are transferred to in vitro culture for 7-8 days until they reach the blastocyst stage. Only about 8% of the matured oocytes develop to the blastocyst stage and can be transferred or frozen for later transfer. After non-surgical transfer of fresh or conventionally slow frozen embryos pregnancy rate is around 60% and pregnancy losses about 20%. Important differences in developmental competence have been observed between breeds: the highest rate in Warmblood and the lowest in Arabians. On average 0.5 embryos are produced for each OPU session, ranging from 0 to 6. This technique offers a solution for treating both female and male infertility and to increase the number of foals from stallions with limited sperm available.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Metabolic Syndrome and Reproduction: a mistery to be unraveled

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Keywords: anovulatory follicles, embryo production, pregnancy.

The terminology Metabolic Syndrome was first suggested in 2002, by Johnson, in an analogy to the human Metabolic Syndrome (Johnson, 2002) and was recently described by the American College of Veterinary Internal Medicine (2010). Animals are obese, present regional adiposity, insulin resistance, hyperinsulinemia, a proinflammatory state and predisposition to laminitis as main characteristics (Frank et al., 2010; Gallantino-Homer; Engiles, 2012; Morresey, 2012). Specifically, laminitis is the most common symptom and in most occasions is the first complaint, and due to its life-threatening aspect, is the biggest concern. The exact physiopathology that explains the alterations detected in the affected animals are still far from being understood, however hormonal, genetic and nutritional aspects are intermingled (Morresey, 2012). Obesity represents an important aspect of the syndrome, as adipose tissue produces a series of adipokines that directly affect insulin metabolism. In Reproduction, several authors suggest an effect of Metabolic Syndrome on ciclicity and pregnancy, though few studies were performed and mechanisms associated with this alterations are not well established. Leptin, a hormone produced by adipocytes signaling to the hypothalamus that there is excess of energy, is normally in high levels in affected animals, maybe cause by a leptin resistant state. There were studies in which ciclicity was evaluated and seasonality was lost in hyperleptinemic individuals (Ferreira-Dias et al., 2005). Vick et al. (2006) found high levels of leptin and insulin in obese animals, along with low levels of thyroxin, reduced insulin sensitivity and estrous cycle alterations, such as increase in the interval between ovulations and longer periods with high progesterone circulating levels. It is important to highlight that several animals are misdiagnosed as hypothyroid due to low levels of thyroid hormones detected, however this characteristic is a consequence of metabolic syndrome and not the cause of alterations. In the other hand, Waller et al. (2006) evaluated high body condition score mares that were hyperleptinemic and no alterations regarding ovarian function and hormonal concentrations were found. When the pregnant status is considered, it is well know that a physiologic insulin resistance occurs in order to supply nutritional support for the fetus and placenta. However, those individuals with a preexisting insulin resistance is present, the condition is exacerbated, leading a worsening of hyperinsulinemia, and systemic, increasing the risks of developing laminitis and loosing the pregnancy (Morresey, 2012). Therefore, when facing an obese animal presenting reproductive problems and laminitis, Metabolic Syndrome should be considered as a possible diagnosis and treated accordingly to avoid worsening of the condition and life threatening situations. This research field requires attention since it has important consequences for the animal health and its reproductive efficiency.

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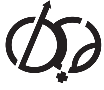

Clinical cases of reproductive endocrinopathies in the horse

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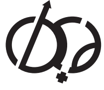
Keywords: anovulatory follicles, embryo production, pregnancy.

The most common endocrinopathy affecting reproductive performance in mares is PPID (pars pituitary intermedia dysfunction) more commonly known as "Cushing's " disease. This disease is difficult to confirm by laboratory testing as many false negatives and false positive may occur. The veterinary practitioner must learn the clinical signs of this disease and use their clinical skills to identify the presence of this endocrinopathy. Laboratory tests will confirm about 70 % of these cases. Most mares are an average of 14 years old when clinically diagnosed with PPID; however, many cases are much younger some are only 4 years of age. The important point here is that PPID takes about 14 years to completely manifest with a full array of clinical signs. Initially only one or two clinical signs may be present such as anovulation or immune suppression. Hirsuitism is the most prevalent and consistent sign associated with PPID and it is the first sign to improve when appropriate drug therapy with pergolide and /or cyproheptadine is initiated. Repeated abortion is often a result of depressed immunity caused by PPID. Equine metabolic syndrome is now more completely understood and its detrimental effects on reproductive function are also better known. Simply stated insulin resistance (IR) is the most important component of this disease and can be easily determined by a fasting serum test for Insulin or by use of an oral sugar test to assess the rise in insulin after the sugar challenge. For example, in normal horses Insulin remains below 20 mIU per ml following 15 ml of "Karo" syrup per 100 kg per os. This is a very simple and cheap test to identify IR. Other common uses of endocrine assay are to assess fetal placental function. Especially in cases of placentitis. When placental infection is present the fetus is stressed eliciting an increased secretion of fetal adrenal steroids, which are converted primarily to pregnanes (progestagens) in the peripheral circulation. When clinical signs such as vaginal discharge and or udder engorgement are present in pregnant mares that are 150 days of gestation and beyond, a single serum assay for total estrogens and progestagens can be very useful to assess the viability of the fetal placental unit. For example, when placentitis is present progestagens are elevated (10 to 40 ng/ml vs. normal of 4 to 10 ng/ml) and total estrogens are lower than normal for that stage of gestation. The level of total estrogens is perhaps the most important for assessing placental damage. The lower concentration the greater the placental damage. Exogenous estrogen therapy is often used to manage these cases along with appropriate antibiotic therapy with a positive outcome. Progesterone assays are also very useful to identify mare that have low-grade endometritis.



Improving postcryopreservation survival capacity: an embryo approach

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Keywords: cryotolerance, *in vitro* produced embryo, lipid.

The major obstacle for a greater dissemination of *in vitro* produced (IVP) bovine embryos is their high sensitivity to the cryopreservation. The modest results of IVP embryo cryopreservation impair the commercialization of embryos between countries and limit its application at the field conditions, as is done with the semen in the artificial insemination. The involvement of embryo lipids on this aspect is well documented. However, it has been recognized that is not only the amount of lipids that affects cryotolerance, the embryo survival capacity after cryopreservation is a multifactorial event. The most common action to deal with these lower results of IVP embryo cryopreservation is to vary the cryopreservation techniques and procedures. Despite this approach usually results in improvements, they often are limited, which suggests to modify the embryos themselves to make them more cryopreservable. Generally, the use of a serum-free media to reduce the lipid content and increase embryo survival after cryopreservation is the first recommended strategy. It has already been described that is possible to produce embryos in defined or semi-defined serum-free media without affecting blastocyst yield. Another approach would be the use of chemicals to modulate lipid metabolism. The addition of phenazine ethosulfate in the post-compactation period reduced the lipid accumulation and increased the postcryopreservation survival. Forskolin, a stimulator of lipase activity, has been used in the culture media to reduce lipid content and increase cryotolerance of bovine and porcine IVP embryos. More recently, L-carnitine has been describe as a chemical candidate for a non-invasive improvement of cryotolerance and developmental competence of IVP embryos because of its unique dual effects that enriches cellular lipid metabolism and provides antioxidative protection. Other strategy described in the literature is the modulation of embryo cell membrane fluidity by cholesterol or unsaturated fatty acids incorporation through its supplementation in the culture media, and oocyte/embryo donor nutritional management. While the addition of cholesterol-loaded methyl-ß-cyclodextrin to the cryopreservation media had no effect on cryopreserved IVP bovine blastocysts, it seems to have a positive effect on vitrified oocytes. In addition, the unsaturated fatty acid supplementation in the culture media improved the cryotolerance and reduced lipid content of IVP embryos. Likewise, the oocyte/embryo donor nutritional management with a diet enriched in polyunsaturated fatty acids increased the cryosurvival of ewe oocytes and porcine embryos. Therefore, the use of a serum-free media, the addition of chemicals to change lipid metabolism, and the modulation of membrane lipid composition are described as alternatives to modify the embryos themselves and to make them more cryopreservable.