Vascularization and VEGF expression altered in bovine yolk sacs from IVF and NT technologies

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Reproductive technologies are widely used in cattle, although many are associated with high embryonic mortality, especially during early gestation, when the yolk sac undergoes macroscopic changes in structure. We hypothesized that vasculogenesis and angiogenesis are affected, thereby affecting embryonic and placental differentiation. To test this, we studied yolk sac development and gene expression of the vascular endothelial growth factor system (*VEGF-A*, *VEGFR-1/Flt-1*, *VEGFR-2/KDR*). Samples from days 25 to 40/41 of pregnancy from control cattle (n = 8) and from pregnancies established with *in vitro* fertilization (IVF, n = 7) or somatic cell nuclear transfer/clones (NT, n = 5) were examined by histology, immunohistochemistry and qRT-PCR. Yolk sacs in IVF- and NT-derived pregnancies were immature. Development of villi was sparse in IVF yolk sacs, whereas vascularization was barely formed in clones, and was associated, in part, with thin or interrupted endothelium. Transcript levels of the genes characterized exceed minimum detection limits for all groups, except in the mentioned clone with interrupted endothelium. Levels of mRNA for *VEGF-A* and *VEGFR-2* were significantly higher in IVF yolk sacs. Clones had substantial individual variation in gene expression (both up- and down-regulation). Our data confirmed the broad range in expression of *VEGF* genes. Furthermore, over-expression in IVF yolk sacs may compensate for an immature yolk sac structure, whereas in clones, patchy expression may cause structural alterations of blood vessels. In conclusion, we inferred that disturbances of yolk sac vasculature contributed to increased early embryonic mortality of bovine pregnancies established with reproductive technologies.

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Transcriptomic analyses of buffalo oocytes and blastocysts using RNAseq

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The analysis of the whole transcripts of the gametes and embryos can generate information about metabolism, cell-signalling pathways and cell cycle regulation (Graf et al., PNAS, 2014, 11:4139) and these information can be useful to improve in vitro embryo production protocols. Here we show partial results of the first whole mRNA transcriptome of mature oocytes and blastocysts of buffalo using RNAseq approach. Buffalo oocytes obtained from abattoir-derived ovaries were in vitro matured in TCM199 media supplemented with 0.5µg/ml FSH, 50µg/ml LH, 50µg/ml gentamycin, 10mg/mL pyruvate and 10% BFS, for 19h at 38.5°C in a 5% CO_2 incubator with humidified air. For in vitro fertilization, frozen sperm from one bull was thawed, processed by Percoll gradient and $2x10^6$ sperm/mL of spermatozoa were incubated in 80µL droplets containing 20 oocytes for 24 hours. After fertilization, presumptive zygotes were cultivated in 100µL droplets of modified synthetic oviduct fluid medium supplemented with 5 mg/mL BSA, 10% FBS, 0.2 mM pyruvate, and 50 mg/mL gentamycin for 6 days in a 38.5°C and 5% CO_2 incubator with humidified air. Matured oocytes (showing first polar body) and embryos (blastocysts) were frozen in RNAlater® solution and kept at -80°C until mRNA extraction, which was performed using Dynabeads© mRNA Direct Micro Kit. The mRNA libraries were construct using 35 buffalo embryos and 205 in vitro matured oocytes. The resulting libraries were amplified and then quantified on Qubit® 2.0. Barcoded libraries were sequence on an Ion Proton™ system. Sequencing reads of low quality (Phred < 20) were filtered using 'fastQC' tool. Just filtered reads were mapped to both Btau_4.6.1 (NCBI annotation) and UMD3.1 (Ensemble annotation) assembly using CLC Genomics Workbench 4.7.2 software. As result, 13ng and 17ng of amplified mRNA were used to construct the oocyte and blastocyst sequencing libraries, respectively. After sequencing, a total of 7.677.937 and 21.626.473 reads were generate from buffalo mature oocytes and blastocysts, respectively. The UMD3.1 genome reference resulted in higher proportion of mapping reads in comparison to the BTAU_4.6.1 for both oocytes (97.9 *versus* 94.9%, respectively) and blastocysts (97.6 *versus* 94.7%, respectively). That find was expected and, according literature, it was possibly related to the different proportions of embryonically expressed genes in these annotations (Chitwood et al., BMC Genomics, 2013, 14:350). Considering the UMD3.1 mapping, we were able to find among protein coding genes the percentage of "éxon / éxon-éxon junction" and "intron / intergenic regions" mapped reads in oocytes (66.1 *versus* 33.9%, respectively) and embryos (53.2 *versus* 46.8%, respectively). Proportionally, oocytes presented higher percentage of "éxon / éxon-éxon junction" than embryos, we think that it can be explained by the fact that oocytes storages many mature transcripts in cytoplasm to support embryo development and embryos needs to undergo mitosis and for that transcription machinery is very active producing many immature transcripts. This project is currently in development and further results are expected to be publish soon.

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Ovarian Function Restoration in Fresh and Cryopreserved Cat Ovarian Tissue Autografted to Subcutaneous Tissue

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This study aimed to evaluate the efficacy of fresh and frozen-thawed cat ovarian tissue autotransplantation to a peripheral site (CEUA protocol 76940/2012). Seven queens were subjected to ovariohysterectomy. Ovaries of three queens were fragmented and immediately autografted to the subcutaneous tissue of the dorsal neck region (Fresh group). For the other four animals, the ovaries were cut and cryopreserved by slow freezing (Cryo group). After one week, they were thawed and autografted to the same region. The grafts were removed after 7, 14, 28, 49 and 63 days and fixed for histological evaluation. One ungrafted sample (Day 0) from each group was also fixed. The percentage of morphologically normal (MN) primordial and growing follicles on Day 0 were 97.7% and 88.6% for the Fresh group, and 73.7% and 96.7% for the Cryo group (immediately after thawing). In the Fresh group, the percentage of MN primordial follicles was always higher than 70% after grafting, and although the percentage of MN growing follicles decreased (varying from 2.3% to 15.6%) their proportion increased after grafting. In the Cryo group, primordial follicles were mostly degenerated (over 70%) and growing follicles were hardly seen after grafting. No MN follicles were found after 49 days of transplantation. Moreover, all animals from Fresh group presented antral follicles in one or more of the grafts on Days 28, 49 and 63 after rafting. All the animals demonstrated at least three estradiol peaks that matched the antral follicles found, and one animal showed estrous behavior on three occasions. Although there was a great follicular loss after cryopreserved ovarian tissue transplantation, two antral follicles were found on day 28 post-transplantation in one of the queens from Cryo group and they were alive (as shown by TUNEL essay) and proliferative (as demonstrated by Ki67 immunohistochemistry). In conclusion, fresh cat ovary autotransplantation to the subcutaneous tissue restored ovarian function and estrous cyclicity. However, follicular survival after cryopreservation and grafting was low, probably due to the combination of injuries caused by cryopreservation with the damages caused by ischemia-reperfusion period in the early days of the tissue transplant. Thus, the techniques of cryopreservation and transplantation must be optimized.

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Deep impact: effect of GnRH immunization in the epididymal proteome

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Epididymal proteome studies have shown the diversity of its fluid and maturating spermatozoa, with several hundreds proteins described in several species in a wide range of abundance. Regional differentiation is established progressively with aging and is dependent of several mechanisms such as the level of testosterone, and the action of factors originating either from the testis or from the epididymis. Previous results based on 2D SDS-PAGE studies showed that approximately 48% of all the proteins secreted in the boar epididymis are dependent on the presence of androgens. This figure suggests a significant modification in the epididymal milieu when alterations in androgens concentrations occur. The development of more sensitive proteomic approaches made possible a broader understanding of protein composition of biological materials. The aim of our study was to verify if GnRH-immunization has an influence on the proteome of cauda epididymal fluid and spermatozoa in boars by means of multidimensional protein identification technology (MudPIT). Twenty adult boars (Large White and Duroc breeds) between 10 and 18 months old were used for this study. Experimental groups were assigned as follows: GnRH-Immunized Group: ten boars were immunized with Vivax® (Pfizer) 2 months before slaughter, according to the manufacturer's guidelines and epididymides were collected after slaughter; Control Group: epididymides from 10 healthy boars with no reproductive disease were obtained after orchiectomy. Collection of cauda epididymal fluid (CEF) was performed by dissection of the cauda region and perfusion of the epididymal tubule. Epididymal fluid and spermatozoa were separated by centrifugation. Protein extracts were prepared and submitted to MudPIT analysis. Also, differential expression of proteins between groups was observed in both epididymal fluid and spermatozoa. A total of 1355 proteins were identified in spermatozoa and 718 in the epididymal fluid. Spermatozoa obtained from GnRH immunized boars presented the up-regulation of 53 proteins and a down-regulation of 50 proteins. The impact of immunization in the epididymal fluid was even higher, with 417 proteins being present only in vaccinated animals. Gene ontology analysis showed a significant change in proteins associated to cellular stress, which is induced by the depletion of serum and testicular testosterone and increase of seminiferous tubuli apoptosis. This study provides a major insight into endocrine regulation on epididymal physiology and possible impacts on sperm quality and male fertility.

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Site-directed transgene insertion with CRISPR/Cas9 in somatic cells for the generation of human lactoferrin transgenic goats by SCNT

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The use of DNA editing tools to introduce targeted double-strand breaks allows for site-directed insertions of the transgene to generate specific transgenic animal models, safeguarding the integrity of endogenous genes and also the transgene expression. The aim of this study was to compare the overall efficiency of the transgene insertion either into a safe harbour *locus* (Rosa26) or a chosen gene sequence (PRNP gene) to produce transgenic cloned animals. For that, the hLF gene sequence was inserted into the pBC1 vector under the β-casein promoter (pBC-hLF), along with the neomycin resistance gene. CRISPR/Cas9 systems were designed for both the Rosa26 *locus* (Rosa26-Cas9) and the prion gene (PRNP-Cas9). Goat skin fibroblast cells from a male neonate were *in vitro*-cultured and co-transfected either with the pBC-hLF + PRNP-Cas9 (Group 1) or with the pBC-hLF + Rosa26-Cas9 (Group 2), using 1x10 cells per group in the Neon® Transfection System set at 1.8 KV, for 20 ms (1 pulse). Following cell selection with 400 µg/mL G418 for 12 days after transfection, cell colonies were picked and cultured in 48-well plates. Allele insertion of the hLF transgene was evaluated by PCR and positive colonies were expanded and frozen for use as nucleus donors. Afterwards one positive cell colony from each treatment group was chosen for cloning. A total of 224 and 222 cloned embryos were produced using cells from Groups 1 and 2 were transferred to 20 (11 embryos/recipent) and 17 (13 embryos/recipient) recipients, respectively. Pregnancy diagnosis was performed by ultrasonography on Days 23 (D23), with weekly evaluations of pregnancy losses up to Day 50 (D50) after transfer. Transfection rate, colony selection efficiency, proportion of positive colonies and mono- or bi-allelic insertions, and pregnancy rates and losses after cloning were evaluated by the Chi-square or Fisher Exact test. Overall, no differences were observed between Groups 1 and 2 in the percentage of viable cells after co-transfection (42.0% *vs.* 58.0%), colony selection efficiency (63/4.2x10^5 cells, 0.015% *vs.* 89/5.8x10^5 cells, 0.015%), colony survival after isolation (29/63, 46.0% *vs.* 32/89, 35.9%), hLF positive cells (12/29, 41.3% *vs.* 10/32, 31.2%), monoallelic (3/12, 25.0% *vs.* 2/10, 20.0%,) and bi-allelic (1/12, 8.3% *vs.* 1/10, 10.0%) hLF-directed insertion, overall hLF insertion (4/12, 33.3% *vs.* 3/10, 30.0%), respectively. In addition, no differences were observed in pregnancy rates on D23 (5/20, 25.0% *vs.* 6/17, 35.2%), and in pregnancy losses up to D50 (2/5, 40.0% *vs.* 5/6, 83.3%) between between the 2 *loci*. In conclusion, the use of the Rosa26 *locus* as a safe harbor and the knockout (KO) of the PRNP gene for the insertion of a transgene into the goat genome resulted in similar efficiencies up to Day 50 of pregnancy. The use of Rosa26 *locus* in the goat to achieve ubiquitous and persistent transgene expression still need to be proven, whereas the PRNP gene KO offers an additional advantage to the transgene integration, contribuing to the development of transgenic cloned goats with better potential for mammary gland-specific recombinant protein production.

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Estrous Synchronization Followed by Artificial Insemination in Collared Peccaries (*Pecari tajacu*) - The First Attempt

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Due to the ecological and economical importance of collared peccaries (*Pecari tajacu*), the interest on development of assisted reproductive techniques for their conservation and multiplication has increased in last decade. In this context, the implementation of protocols for pharmacological reproductive control associated to the offspring production would represent a welcome and major technological advance for the species. This study presents the first attempt of estrus synchronization associated to artificial insemination (AI) in collared peccaries. Ten primiparous females aging 2.5 ± 0.5 years were used. To facilitate the experimental managements, they were anesthetized with intravenous administration of 5 mg/kg propofol in bolus. For estrus synchronization, females received a single dose (5-mL, as recommended by the manufacturer) of a commercial gonadotropin association containing 400 IU eCG and 200 IU hCG (PG-600®, Merck and Co., NJ, USA). From this moment, they were monitored every three days for estrus external signs, and measurement of blood concentrations of estrogen and progesterone. When estrus was detected, each female was submitted to two AI at a 2-day interval, using a fresh ejaculate collected by electroejaculation from stud males at each occasion. For AI, females were positioned in dorsal recumbence, and semen was deposited in the cranial vagina at using a pipette conventionally used for AI in bovines. After 30 and 60 days, females were restrained for conduction of pregnancy diagnosis by ultrasonography and blood collection for hormone measurement. Results were analyzed through descriptive analysis. In the day of drugs administration for estrus synchronization, all females were in luteal phase, presenting ~6.4 ng/ml and ~33.7 pg/ml for estrogen and progesterone, respectively. After six days, all females synchronized and presented classic estrus external signs as open, swollen and hyperemic vulva, and the presence of vaginal mucus, as well as high estrogen levels (~ 221.6 pg/ml). Ejaculates used for AI presented a volume of 4.0 ± 2.2 ml, with a concentration of 397.9 ±180.5 x 10^6 sperm/mL, 86.4 ± 5.2% motile sperm with vigor 4.5 ± 0.5, 85.6 ± 4.5% viable sperm, 91.2 ± 2.7% functional membrane integrity and 86.1 ± 9.1% normal sperm. At the ultrasonography conducted after 30 days, no embryo was visualized, but seven females (7/10) presented high progesterone values (~67.1 ng/ml), and such values remained high even on day 60 (~73.4 ng/ml). During this time, 60 days, these females did not present any external signs of estrus. Two of these females presented an increased uterine volume at day 60, with thickening walls (0.9 cm) with discreet presence of anechoic liquid in lumen. In this occasion, all females presented estrogen concentrations lower than 6.4 pg/ml; but exactly the two females presenting increased uterus also showed the highest progesterone values, 98.1 and 93.7 ng/ml. Nevertheless, we can not discard the occurrence of a pregnancy followed by embryo resorption with presence of persistent corpus luteum, since the luteal phase in natural estrous of peccary usually lasts 15 days on average. In fact, it is reported that high concentrations of gonadotropins could decrease prostaglandin synthesis in porcine oviducts, causing disruption of the oviduct environment and, in turn, perturbing development of embryos and their transport to the uterus. Based on these statements, we believe that other lower gonadotropins doses should be tested for estrus synchronization in collared peccaries. Moreover, the occurrence of stress, mainly derived from the recurrent physical and chemical restraint necessary to facilitate peccaries' management, may also have contributed for embryonic loss. In conclusion, we demonstrated that the gonadotropins association is effective for estrus synchronization in collared peccaries; however, it was not effective for producing offspring derived from artificial insemination. These are important and valuable information to be used in the improvement of protocols for assisted reproductive techniques applied for the peccaries' management.

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Small interfering RNA-mediated knockdown of aquaporin 3 and reduces antrum formation of sheep secondary follicles cultured in vitro

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Aquaporins (AQPs) are water channel proteins responsible for water homeostasis, and are involved in many physiological functions in different tissues including ovarian follicles. Genes silencing technologies have been applied to investigate the role of aquaporins during folliculogenesis. The objectives in this study were to (i) develop an effective protocol for transfection of ovine secondary follicles, and (ii) assess the effect of attenuating aquaporin 3 (AQP3) using a small interfering RNA (siRNA-AQP3) on antrum formation and follicular growth in vitro. For the first objective, various combinations of Lipofectamine® volumes (0.5, 0.75 or 1.0 µL), fluorescent oligonucleotídio (BLOCK-iT ™) concentrations (3.18, 27.12 or 36.16 nM) and exposure times (12, 14, 16, 18 or 20 h) were tested. For the second objective, the BLOCK-iT ™ was replaced by siRNA-AQP3 in the transfection complex. Ovine secondary follicles were isolated and cultured in vitro for 6 days using standard protocols. Follicles were transfected on day 0, 3 or both days (0 and 3) and then cultured for additional 3 or 6 days. As revealed by the fluorescence signal, the Lipofectamine®/BLOCK-iT ™ complex (0.75 µL + 27.12 nM by 12 h of incubation) crossed the basement membrane and granulosa cell and reached the oocytes. Although AQP3 protein was immunodetected in both transfected and control follicles, mRNA levels were lower in Lipofectamine® / siRNA-AQP3 transfected compared to control follicles. Follicular growth was also decreased in transfected compared to controls. In general, the rate of intact follicles was higher and the rate of antrum formation was lower in transfected compared to control follicles. Our study showed that ovine secondary follicles can be successfully transfected during in vitro culture, and that siRNA-mediated attenuation of *AQP3* gene reduced antrum formation of secondary follicles cultured in vitro.

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High concentrations of resveratrol in boar insemination doses is harmful to total motility, plasma and acrosomal membranes integrity and mitochondrial membrane potential

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The cellular metabolism generates reactive oxygen species (ROSs) which are responsible for causing oxidative stress; which, in turn is a deleterious processes resulting from an imbalance between the excessive formation of ROS and limited antioxidant defenses. Oxidative stress impairs sperm motility and integrity of plasma and acrosomal membrane. In this context, Resveratrol (RVT) polyphenol compost with an intense capacity to inhibit ROS formation has been used to improve sperm motility of different animals species. The current study was performed to evaluate whether the addition of RVT would improve sperm motility, simultaneous integrity of plasma and acrosomal membranes and mitochondrial membrane potential of boar sperm cooled at 17˚C for 72 h. Thus, six ejaculates from six boars (n=36) were collected. After raw semen analysis, samples were extended in BTS medium and RVT was added, giving rise to the following treatments: T0 (BTS + RVT 0 mM) without RVT, T1 (BTS + RVT 0.01 mM), T2 (BTS + RVT 0.1 mM) and T3 (BTS + RVT 1 mM). Treatments were cooled at 17ºC and were evaluated at 0, 24, 48 and 72 h to total motility (SCA- Microptic, Microptic S.L., Barcelona, Spain), membrane mitochondrial membrane potential and simultaneous integrity of plasma and acrosomal membranes by flow cytometry. There was interaction between time and treatment ($p < 0.05$) to all variables analyzed. Addition of 1 mM of RVT in boar insemination doses was deleterious for the total motility in all times of evaluation (78.17 ± 1.50^{a}; 77.50 ± 1.57^{a}; 75.90 ± 1.50^{a}; 5.19 ± 0.82; percent of motile sperm for T0, T1, T2 and T3, respectively), and to mitochondrial membrane potential, the highest concentration of RVT (1mM) decrease JC-1 fluorescence intensity only after 24 and 48 hours of incubations at 17ºC (0 hours: 29.50 ± 1.70; 30.65 ± 1.37; 31.44 ± 1.66; 33.30 ± 1.41 / 24 hours: 29.61 ± 1.39; 32.85 ± 1.53; 29.49 ± 1.42; 22.46 ± 1.11 / 48 hours: 28.57 ± 1.12; 30.31 ± 0.99; 27.15 ± 0.77; 21.76 ± 1.17 / 72 hours: 31.09 ± 1.43; 32.66 ± 1.38; 28.43 ± 1.68; 27.10 ± 1.84; mean fluorescence intensity $x10^{3}$ [arbitrary units] for T0, T1, T2 and T3, respectively for all times assessed). The highest concentration of RVT (1mM) was not deleterious ($p < 0.05$) to plasma and acrosomal membranes integrity compared to absence of RVT at 0 hours of incubation, but it was harmful to this characteristics compared to lower concentrations (85.98 ± 1.78^{ab}; 88.26 ± 1.64^{a}; 88.35 ± 1.66; 80.86 ± 1.88^{b}; percentage of membrane integrity for T0, T1, T2 and T3, respectively). Insemination doses with 1 mM of RVT showed lower percentages of simultaneous plasma and acrosomal membrane integrity in the others three times of incubation, compared with others treatments (24 hours: 86.69 ± 1.58^{a}; 88.54 ± 1.42^{a}; 88.65 ± 1.44^{a}; 70.13 ± 2.58^{b}; T0, T1, T2 and T3, respectively / 48 hours: 87.25 ± 1.61^{a}; 88.24 ± 1.37^{a}; 87.79 ± 1.63^{a}; 56.08 ± 2.64^{b}; T0, T1, T2 and T3, respectively / 72 hours: 85.17 ± 1.71^{a}; 85.87 ± 1.73^{a}; 86.75 ± 1.53^{a}; 59.73 ± 3.56^{b}; T0, T1, T2 and T3, respectively). In conclusion, concentrations higher than 1mM of RVT in boar insemination doses was deleterious to mitochondrial membrane potential resulting in decrease of total motility, and also reduce integrity of plasma and acrosomal membrane. However, additional studies with RVT in boar insemination doses are necessary to understand the effect of lower concentrations of RVT.

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