Research proposal

Mission call: 2014/2015

(Postdoctoral mission, short term 6 months)

Reference number:

Title: "Gene expression and ultrastructure evaluation of in vitro produced bovine embryos using X-sorted spermatozoa and OPU oocytes in dairy cows."

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Announcement field of research: Life sciences

Main specialization: Theriogenology

Minor specialization: Theriogenology

Gene expression and ultrastructure evaluation of in vitro produced bovine embryos using X-sorted spermatozoa and OPU oocytes in dairy cows.

Summary

Production of embryos of predetermined sex in cattle is of vital importance where female embryos can be transferred to recipients and allow greater opportunities to increase yield of replacement heifers in dairy farms. Moreover, male embryos can be transferred to beef recipients to increase beef production in beef farms. Yet, IVEP results using sex-sorted semen are lower than those to traditional semen. The process of sorting sperm might have been the underlying cause of these results. OPU oocytes are believed to have higher developmental potential than slaughterhouse-derived oocytes. Hence, I decided to compare blastocyst yields and ultrastructural features together with IGF2 and IGF2-R gene expression in blastocysts produced by the previous types of oocytes, when in vitro fertilized with traditional or sex-sorted semen. The hypothesis is that embryos produced from OPU oocytes may have greater developmental potential and better expression of developmentally important genes as IGF2 and IGF2-R. If so, then OPU oocytes may constitute a valuable source for in vitro production of sexed embryos with greater developmental potential and may result in higher conception rates to embryo transfer in recipient cows.

Research Objectives

1- Working knowledge of the OPU, IVMFC technique in cows.

2- Estimation of the effect of using OPU oocytes instead of oocytes obtained from slaughter house –in combination with sexed sperm- on the blastocyst yield (developmental potential) by comparing IVP embryos from OPU vs. slaughterhouse-derived COCs using sex-sorted or unsorted sperm.

3- Elucidation of the effect of using sex-sorted sperm for IVF of OPU vs. slaughterhouse-derived COCs on the ultra -structural features of blastocysts.

4- Comparison of IGF2 and IGF2R gene expression in blastocysts produced from IVF of OPU or slaughterhouse-derived COCs using sex-sorted or unsorted sperm by using SYBR green with Real Time Polymerase Chain Reaction (RT PCR).

Introduction

Sex diagnosis allows production of more female calves for replacements from the best cows of the herd while the average cows and heifers can act as embryo recipients or as dams for male beef calves **(Peippo et al., 2009)**. The majority of studies investigated IVEP refer to a reduced developmental potential of embryos produced in vitro using X-sorted spermatozoa **(Palma et al., 2008; Stinshoff et al., 2012).** Moreover, blastocysts produced with sex-sorted spermatozoa expressed deviations in the number and structure of organelles like mitochondria, rough endoplasmic reticulum (ER) and nuclear envelope. These morphological alterations may be responsible for compromised development observed in embryos produced with sex-sorted spermatozoa. The majority of IVF studies

using sorted-sperm have made use of bovine oocytes derived from slaughterhouse ovaries to investigate embryo development and quality **(Xu et al., 2009)**. However, intrinsic differences were found between OPU oocytes and oocytes from slaughterhouse ovaries **(Presicce et al., 2010; Plourde et al., 2012).** A combination of sex-sorted sperm and OPU-IVF techniques could optimise the use of genetic and economic resources to produce sexed embryos. However, as far as we know, the effects of sex-sorted sperm on embryo development and quality using oocytes collected by OPU, have not been extensively studied **(Presicce et al., 2010; Cebrian-serrano et al., 2013)** in Bos taurus dairy cows. The morphology classification for blastocysts selection is not an invasive method and it could predict the pregnancy rate at least for in vivo produced embryos **(Klisch et al., 1999)**. Despite the good morphology of the blastocysts produced by gamete micro-manipulation such as Somatic cell nuclear transfer (SCNT), only some of them are able to implant properly. This fact indicates that the normal shape of the ICM and trophoblast is not sufficient to predict the capability of the nucleus to be reprogrammed and to establish the normal gene expression patterns supporting embryo development to term in such embryos. In this sense, the study of gene expression pattern at blastocyst stage becomes an interesting approach to predict the reprogramming potential of a nucleus under specific conditions **(Rodriguez-Alvarez and Castro, 2010)**. Insulin-like growth factor-2 (IGF2) and insulin-like growth factor-2 receptor (IGF2 R) genes regulate cell growth and differentiation in many species **(Fagundes et al., 2011)**. Reports with SCNT embryos indicate an abnormal IGF2 and IGF2 R genes expression together with reduced developmental competence of theses embryos and attribute this to gamete manipulation which might be the case in sexed semen-derived embryos **(Pandey et al., 2009)**.

Methodology

1-Ovum pick up technique

OPU sessions will be performed according to procedures described by **Zaraza et al. (2010)** at 3 to 4 day intervals. Before each procedure, feces will be removed from the rectum and perineal area will be cleaned with water and antiseptic solution. Epidural anaesthesia will be applied to each donor cow prior to OPU (5 mL of 2% procaine hydrochloride solution). Follicles bigger than 3 mm in diameter will be aspirated using an ultrasound system with a 6.5 MHz ultrasound transducer placed in a PVC tube with a needle guide. Dulbecco' s PBS (DPBS) medium containing 1 g/L BSA, 50 UI/mL penicillin G, 50 μ g/mL streptomycin sulphate and 2.2 IU/mL sodium heparin will be used to retrieve the oocytes and flush the collection needle. A 50-mL conical tube will be used to store the aspirated fluid from each animal; the contents will be immediately passed through a 50 μ m filter and collected in a Petri dish with DPBS to be searched using a stereomicroscope and oocytes will be graded morphologically based on the cumulus investment according to **Chaubal et al. (2006)**.

2-Collection of COCs from slaughterhouse samples

This step will be carried out according to **Trigal et al. (2012).** Briefly, ovaries recovered from slaughtered cows will be placed in NaCl solution (9 mg/mL) with antibiotics (Penicillin, 100 IU/mL

and Streptomycin sulfate, 100 mg/mL) and maintained at 25 °C to 30 °C until COC collection. Follicles 3-7 mm in size will be aspirated through an 18-ga needle connected to a syringe.

3-In vitro maturation

Groups of up to 20 COCs will be washed and in vitro matured in 100 μ L drop of maturation medium consisting of TCM 199 containing 22 μ g/mL pyruvate, 2.2 mg/mL NaHCO3, 50 μ g/mL gentamycin, 10 IU/mL eCG and 5 UI/mL hCG, and 0.1% bovine serum albumin (BSA). COCs will be in vitro matured for 22 to 24 h in humidified atmosphere at 38.5° C and 5% CO2 in air.

4-In vitro fertilization

Semen straws (X-sorted sperm and unsorted sperm) of 0.25 mL commercially available from bull with proven fertility for IVF will be thawed at 30° C in a water bath for 30 s and centrifuged for 10 min at 300 × g through a gradient of 1 mL of Bovipure® Bottom Layer. The sperm pellet will be isolated and washed twice through 750 μ L Fert-TALP medium **(Parrish et al. 1988)** by centrifugation at 400 × g for 3 min. In the first washing, heparin-hypotaurine-epinephrine (HHE) will be omitted, but in the second wash HHE will be included. Following IVM, COCs will be washed thrice and co-cultured with spermatozoa in IVF medium in groups of up to 20 COCs per 35 μ L drops, for 18 to 20 h at 38.5° C in an atmosphere of 5% CO2 in humidified air.

5-In vitro culture

Presumptive zygotes will be denuded from surrounding cumulus cells in TCM-air medium, washed and transferred to 30 μ L drop of culture medium in groups of 5 to 8 embryos. Modified synthetic oviductal fluid amino acids supplemented following Holm' s recommendations **(Holm et al. 1999)** and supplemented with 4 mg/ mL BSA will be used as culture medium according to **Zaraza et al. (2010).** Culture drops will be placed in a modular incubator chamber with a gas mixture of 5% O2, 5% CO2, and 90% N2 at 39° C for a total of 9 days.

6-Evaluation of embryos

6.1-Assessment of developmental potential

Cleavage rate will be evaluated on day three (IVF = day 0). Blastocyst rate, morphological quality and timing formation of the blastocyst produced will be assessed on day 7, day 8, and day 9 **(Robertson and Nelson 1998)**. Blastocysts of grade 1 will be harvested (168 hpi) and will be fixed in McDowell and Trump fixative, and will be held at 4^0C until processing for transmission electron microscopy **(crosier et al., 2001)**

6.2-Electron Microscopy

Procedures will be carried out according to **Crosier et al. (2001)**. The determination of volume densities of cellular components will be carried out using the point-count method according to **Crosier et al. (2001)**. Evaluation of intercellular components will include intercellular spaces, blastocoele, debris within the blastocoele, extruded blastomeres, and all extruded material.

6.3-Evaluation of IGF2 and IGF2R genes expression

This step will be carried out according to **Pandey et al. (2009)** including preparation of embryo lysate, DNase digestion and reverse transcription and finally real time polymerase chain reaction (RT-PCR) analysis of gene expression.

Time table and research plan:

Project activity	**Start**	**Finish**
Prepare the lab, purchase semen and practice preliminary OPU work.	**Week 1**	**Week 6**
Practice First session of OPU and associated IVEP (first experiment, developmental potential and ultrastructure)	**Week 7**	**Week 12**
Practice Second session of OPU and associated IVEP (second experiment, gene expression profiling)	**Week 13**	**Week 18**
Analysis of data and preparation of a research article	**Week 19**	**Week 24**

Outcomes:

A better understanding of developmental potential and expression of developmentally important genes in embryos produced by OPU oocytes and sex-sorted sperm will allow us to decide targeting commercial production of sexed embryos by OPU-IVFC-ET. In addition, a highly advanced research article is expected to be obtained and published in a high impact scientific journal.

Follow up activities:

1. Establishment of an OPU-ET unit in my Department's lab.
2. Inclusion of the OPU unit as a major unit in a suggested design of an embryo research center at the university campus.
3. Complementary research based on the obtained results for further insights into sexed embryos and OPU technology.
4. Practical application of the obtained results by cooperation with private or governmental cattle farms to establish a baseline for commercial application.

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Candidate Signature

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