Progress in genotyping in vitro-produced embryos: are we close?[2]

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Introduction

Food production efficiency has become increasingly crucial in expanding populations with limited resources. Agriculture is forced to produce more, with less input. Milk, beef and by-products have become increasingly important as a protein source, placing a major demand on cattle producers throughout the world (USDA long term projections 2015). The future of this efficiency is highly contingent upon livestock reproduction programs to optimize genetic selection, pinpoint superior genes, and insure genes are passed on to future generations. Implementation of single nucleotide polymorphisms (SNP) analysis of DNA and its relationship with production traits has led to the concept of genomically enhanced estimated breeding values (GEBVs). The values are derived from the effects of dense markers across the entire genome, potentially capturing all quantitative trait loci that contribute to variations in phenotypic expression, greatly increasing the accuracy compared to EBVs alone (Meuwissen et al., 2001). As genetic and phenotypic superior individuals have been identified, the need to generate more offspring has led a worldwide increase in the number of in vitro produced embryos (IVP) in the past 10 years, with about 500,000 IVP embryos transferred in 2013 (IETS 2014). However, production value of the resulting offspring remains unknown until the calf is born, at which time a genetic test is conducted and the individual's genetic merit is assessed. With opportunity costs of a pregnancy resulting in a genetically inferior animal or a pregnancy with offspring of undesired gender, the system becomes highly inefficient. There exists a need to reduce the generation interval, and develop a system for the genomic predilection of bovine IVP embryos prior to transfer by using procedures for preimplantation genetic diagnosis (PGD). Furthermore, for optimal commercialization and utilization of IVP embryos, not only is a reliable PGD needed but also an effective means to cryopreserve the desired embryos. The objectives of this review are 1. Discuss techniques involved in PGD including embryo biopsy techniques including technical difficulties involved with the procedures since so few cells are sampled for DNA analysis 2. Discuss cryopreservation of IVP embryos and their viability after embryo transfer.

Preimplantation Genetic Diagnosis

Cattle embryo micromanipulation for PGD, specifically gender, was described over three decades ago (Hare and Betteridge 1978). The technique involved a micro-blade and aseptic dissection of a piece of trophoblast cells without damaging the inner cell mass (ICM) of the embryo followed by DNA isolation and subsequent PCR analysis. The micro-blade technique is still successfully used with in vivo produced embryos and has been described recently for genotyping in-vivo produced embryos with pregnancy rates in the order of 50% (Shojaei Saadi et al., 2014). However, when the micro-blade technique is used with IVP embryos, the survival rate in vitro is poor (< 40% re-expansion rates) (Unpublished personal communication 2013).

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There are several methods to biopsy an embryo by using micromanipulator tools, namely a holding pipette and aspiration of cells by a fine needle. The technique involves puncture of the zona pellucida (ZP), and aspiration of the biopsied cells. The blunt force of the needle penetrating the ZP in this technique often causes distortion of the embryo, collapsing the blastocyst and allowing only a few cells to be aspirated, not enough yield for DNA genetic analysis. This technique is easier and more efficient to perform at the morula than blastocyst stage (Unpublished personal communication 2013). Therefore, zona drilling, developed as a means to limit the mechanical damage imposed on an embryo during micromanipulation, thereby increases the number of cells aspirated. One method of zona drilling involves the use of a Piezo-assisted drilling (PAD), a micromanipulation device that uses vibrations to drill through the ZP. The Piezo unit, based on the piezoelectric effect, advances the biopsy needle a very short distance (e.g., 0.5 µm) at a very high speed; the rapidness of the advancement of the needle punctures the ZP and intentionally disrupts blastomeres with minimum damage to the embryo. Piezo assisted drilling has been used with success to biopsy equine embryos for PGD of a few genetic mutations (Choi et al., 2010, 2015). Another method of embryo biopsy, based on the PAD principle is laser-assisted drilling (LAD). Laser assisted drilling utilizes a non-contact high-energy light laser to drill by melting through the ZP. A fine needle aspiration pipette can then be passed through the ZP freely to aspirate cells; the laser can then be used to cut the resulting string of cells. The size of the hole created in the ZP is contingent upon three parameters: power, pulse length, and wavelength; however high pulse rate (400µs) was found to be safe when human embryos were biopsied (Taylor et al., 2010).

A technical challenge genotyping embryos is the limited amounts of DNA obtained from the biopsied cells, if we take into account that a cell has a DNA mass of approximately 6 pg and the Illumina BovineSNP50 v2 DNA analysis Bead Chip (Illumina, San Diego CA) requires an amount in the order of 300 ng of good quality DNA for a robust genetic analysis. In addition, multiple errors in genetic analysis are due to lack of identification of an allele, known as allelic drop out. Allelic drop out occurs during amplification, and often is a result of minimal amounts of template DNA. In addition, low amounts and poor quality of starting genomic DNA may lead to poor sample call rate, defined as the fraction of identified SNPs per sample over the total number of SNPs in the data set. Increasing the number of cells biopsied increases template DNA and decreases likeliness of allelic drop out, and potentially increases call rates. Therefore, whole genomic amplification (WGA) is required to amplify the limited starting genomic DNA and must be extremely accurate with high fidelity, have no sequence bias, have high amplification rates, and be applicable to a wide array of genomic platforms (Jasmine et al., 2008). Shojaei Saadi et al., (2014) evaluated several WGA methodologies including PCR and non-PCR technologies to amplified DNA from in vivo produced bovine embryo biopsies (10-15 cells/biopsy). The study found that genomic imputation from parental or population genotypes were necessary to correct genotype data (increased call rates and decreased error rates).

Cryopreservation of In Vitro Produced Embryos

The morphology of IVP bovine embryos involves darker cytoplasm as a consequence of higher lipid accumulation than in vivo derived embryos; a phenomenon associated with impaired embryo quality and reduced cryotolerance (Batista et al., 2014). As reviewed by Seidel 2006, cryopreservation (freezing) of IVP cattle embryos resulted in a lower pregnancy rate compared to frozen in vivo produced embryos (15 to 30 vs. 55% respectively). To our knowledge, current

published methods used for embryo cryopreservation have failed to generate pregnancy rates with IVP embryos that are commercially viable or comparable with in vivo produced embryos. The high lipid content of IVP embryos interferes with water movement out of the cells during dehydration and rehydration and stability of the plasma membrane, triggering ice crystal formation, thereby exacerbating the deleterious effects of cryopreservation (Nagashima 1994).

Addressing the special needs of IVP embryos have wrought different approaches in cryopreservation, metabolic alterations, and mechanical manipulation. The most commonly used procedure for cryopreserving in vivo-produced bovine embryos consists of equilibration of embryos in cryoprotectant (ethylene glycol (EG)) for 5–10 min, seeding at around −6 °C, and cooling at about 0.5 °C/min to around −32 °C, followed by plunging into liquid nitrogen (Voelkel and Hu 1992). Another alternative is vitrification as described by Rall and Fahy (1985). This technique requires high solution viscosity, rapid cooling rates, small volumes, and the use of high concentrations of cryoprotectant solutions to bring about a physical state similar to glass. Vitrification is attractive due to the relatively rapid and inexpensive procedure, and it has been shown to be beneficial for embryos that have lower cryosurvival, such as IVP embryos (Campos-Chillon et al., 2006). Many vitrification protocols have been developed to optimize cryopreservation of IVP embryos including containers such as the open pulled straw (OPS), Cryotop, Cryolock and others. However, most vitrification techniques are difficult to implement under field conditions for strict requirements of embryo movement in timed steps under a microscope, and are time consuming (10-15 min per embryo depending on technique). Another approach has been used to modify the metabolism of the embryos to reduce lipid content (Seidel 2006). Recent studies have shown promising results when serum is removed from the culture media and metabolic regulators such as L-carnitine (Baldoceda et al., 2015, Takahashi et al., 2013), forskolin (Sanchez et al 2013), and phenazine ethosulfate (Barcelo-Fimbres and Seidel 2007) are added to the culture media. Another interesting approach has been to collapse the blastocoele of IVP embryos prior to cryopreservation. The technique has been described in successful cryopreservation of large (>300 µm) equine embryos (Choi et al., 2010, 2015) and IVP bovine embryos (Min et al., 2014) that resulted in acceptable pregnancy rates of a small subset of embryos transferred.

Materials and Methods

In vitro embryo production

Slaughter house derived embryos were obtained as previously described (Barcelo-Fimbres et al., 2015). Briefly, immature bovine cumulus oocyte complexes (COCs) were collected from ovaries of mature cows obtained from a local abattoir and transported to the laboratory at ambient temperature (approximately 22 to 25 °C). COCs were aspirated from 3- to 8-mm antral follicles. In vitro maturation took place in TCM-199 supplemented with 10 % FCS vol/vol (Hyclone; Salt Lake City, UT, USA), 0.22 % pyruvate, 0.01 units of bovine FSH (Sioux Biochem; Sioux Center, IA), for 23 h at 38.5 ºC in humidified 5 % CO_2 in air. Sperm was separated through a Sperm-Talp (Parrish et al., 1986) Percoll (Sigma P1644) gradient and coincubated ($0.5x10^6$ sperm/mL) with matured oocytes in CDM fertilization medium (De La Torre-Sanchez et al., 2006) supplemented with 0.5% FAF-BSA, 2 mM caffeine and 0.02% heparin (Sigma H9399) for 18 h at 38.5°C and 5% CO2 in air. Embryo culture (IVC) took place in BBH7 (MOFA Global, Verona, WI) at 38.5 °C in a humidified atmosphere of 5 % CO_2, 5 % O_2, and 90 % N_2 for 7 days.

Oocytes from cycling donors were obtained by standard transvaginal follicular aspiration (TVA) after ovarian stimulation as described previously (Nivet et al., 2012) with some modifications. An intramuscular injection of 100 µg of GnRH (Cystorelin®; Merial; Inselin, NJ) and an intravaginal progesterone releasing device (CIDR®; InterAg; Hamilton, New Zealand) were administered at day 0. Thirty six h later pFSH (Pluset H®, MOFA Global, Verona, WI) was injected im twice per day for three consecutive days. A total dose of 600 to 700 IU FSH was administered for cows and 450 to 575 IU FSH for heifers. The progesterone device was removed, and two prostaglandin F2α injections (Lutalyse®; Pfizer; New York, NY) of 25 mg were given 24 h before TVA (pregnant donors did not receive prostaglandin injections). The TVA was performed after a coasting period of 48 to 52 hours following the last FSH dose. The recovered COCs were searched and placed in oocyte washing medium containing 0.3 % BSA (MOFA Global, Verona WI). The COCs were then washed three times in the same medium and selected for in vitro maturation, fertilization and culture as described for slaughter house oocytes.

Embryo Biopsy

Blastocyst stage, grade one embryos were selected on day 7 (fertilization = day 0) and were biopsied using the PAD and LAD techniques. For the PAD technique, embryos were placed in 25µL drops of Syngro holding medium (SHM) (Bioniche, Pullman, WA) and held with a MPH-XLG-30 holding micropipette (Humagen Pipets Charlottesville, VA) with the embryo inner cell mass in the 9 o'clock position. An intracytoplasmic sperm injection needle, MIC-9-30 (Humagen Pipets Charlottesville, VA) was connected to Piezo-electric drill (PrimeTech PMM4G, Sutter Instrument, Novato CA). The needle was inserted in the 3 o'clock position. Pulses (speed 4, intensity 6) were applied while piercing through the ZP and a minimum of 15-20 cells were aspirated from the trophectoderm layer. Biopsies were then deposited adjacent to the embryo in the same micro drop. Using a dissection microscope 0.2µL of SHM was deposited into a 0.2mL PCR tube. Recovered biopsied cells were then washed through a minimum of two 20µL drops of SHM before being deposited into the PCR tube. Samples were then placed into a -80°C freezer until WGA was performed.

For the LAD technique, the Xyrcos laser system (Hamilton Thorne, Beverly, MA) was used and set at 300 mW of power and 400 µs of pulse. On day 6 after IVF, the ZP was pierced by applying 1-2 laser pulses to promote early trophoblast herniation. Subsequently, on day 7 after IVF the hatching blastocysts were held as in the PAD procedure but a beveled and polished biopsy micropipette (MBB-BP-SM-30, Humagen Pipets Charlottesville, VA) was used to aspirate a portion of the herniated trophoblast. The trophoblast cells (approximately 30 cells or 30% or the embryo trophoblast mass) were separated by pulling the biopsy micropipette to the opposite side of the holding pipette and aspirated at the same time. The piece of trophoblast was cut by 5-10 laser pulses and frozen as with the PAD procedure. If the ICM was herniated, another hole was made to biopsy trophoblast cells only. Biopsied embryos from both PAD and LAD techniques were allowed to recover for a minimum of two hours in the same micro drops of IVC before being subject to cryopreservation

Experiment one: embryo cryopreservation after blastocoele collapsing

Grade 1 slaughter house derived IVP blastocysts (n=350) were divided into six treatments using a 2x3 factorial design comparing intact (IB) vs collapsed blastocoele (CB)

and three cryopreservation methods: slow freezing (SF-G0.7) vs vitrification using open pulled straws (OPS) or cryotop (CT). Slow freezing embryos were equilibrated in 0.7 M glycerol and 0.1 M galactose in holding media for 5 min, held for 10 min at -6°C, seeded after 5 min, decreased to -32 °C at 0.5 °C /min and, held at -32°C for 5 min, and plunged into liquid nitrogen. Vitrified embryos were equilibrated in 1.5 M EG for 5 min, exposed to 7 M EG + 0.6 M galactose for 30 s while loaded into OPS or placed onto CT, then immediately plunged into liquid nitrogen. SF-G0.7 embryos were thawed in air for 10 s and placed in a water bath at 37°C for 45 s and then rehydrated in 1M, 0.5M and 0.25 M galactose for 5 minutes each. Vitrified embryos were warmed directly into holding medium at 37°C supplemented with 1.0 M, 0.5 M and 0.25 M galactose for 3 minutes each step. Subsequently, embryos were cultured in BBH7 and re-expansion rates were assessed at 24 and 48h post warming and data was evaluated by GLIMX. Re-expansion rates were higher for CT, than OPS, and SF-G0.7 (85 vs. 66 vs. 72% ± 0.4, respectively; $p<0.05$). Main effect means for re-expansion were higher for CB than IB (79 vs. 68% ± 0.3; $p<0.05$).

Experiment 2: embryo cryopreservation after PAD biopsy

Grade 1 slaughter house derived IVP blastocysts (n=179) were divided into six treatments using a 2x3 factorial design comparing intact embyos (control) vs PAD biopsied embryos and three cryopreservation methods: SF-G0.7 or conventional direct transfer 1.5 M EG slow freezing (EG-1.5) vs OPS . Slow freezing and vitirified embryos were equilibrated, loaded, warmed, cultured and evaluated as in experiment one. Data was evaluated by GLIMX; re-expansion rates were not different between SF-G0.7 and OPS but higher than EG-1.5 (86 vs 85 vs. 69% ± 0.5, respectively; $p<0.05$). Main effect means for re-expansion were higher for PAD biopsied than control embryos (84.6 vs. 69.4 ± 0.4; $p<0.05$).

Experiment 3: PAD vs LAD biopsy call rates

Grade 1 TVA derived IVP blastocysts were biopsied by PAD (n=40) and LAD (n=20) methods. Biopsies were amplified using the Repli-G Single Cell Kit (Qiagen Valencia, CA) following manufacturer instructions. Amplified samples were shipped overnight via dry ice to GeneSeek-Neogen, (Lincoln, NE) for SNP analysis using the Bovine Illumina 80K high density Chip. Genomic call rates were compared using T-test. The call rates for LAD were higher than PAD (0.946 ± 0.007 and 0.7923 ± 0.01 respectively) embryo biopsy SNP analysis.

Experiment 4: Pilot field study

Based on results obtained in experiments 1-3 and the difficulty of transferring OPS and SF-G0.7 in the field due to multiple step rehydration of embryos, a pilot field study was designed to compare pregnancy rates of TVA obtained fresh and LAD biopsied-frozen (EG-1.5) IVP embryos for direct transfer in a commercial setting. Grade 1 fresh (n=850) and grade 1 LAD EG-1.5 (n=76) embryos were transferred randomly into synchronized virgin heifers on day 7 of presumptive ovulations to the uterine hour ipsilateral to the ovary with the CL. Pregnancy was diagnosed at day 32-38 using the IbexPro ultrasound (E.I. Medical Imaging, Loveland CO). Data was analyzed by Chi square. Pregnancy rates were higher ($P< 0.05$) for fresh (54.9% ± 2.8) than frozen biopsied (42.1 ± 5.0) embryos.

Discussion and Concluding Remarks

Have we made progress in genotyping IVP embryos? The data presented suggests that even though IVP embryos are considerably more delicate than their in vivo counterparts, they are surprisingly plastic, and survival rates after biopsy either by the PAD or LAD methods are high. In human embryology the LAD is the preferred method for PGD leading to high pregnancy rates; however, only 5-8 cells of trophoblast cells are removed (Taylor et al., 2010). In addition, experiments 1 and 2 strongly suggest that dehydrating the blastocoele as a default of the biopsy procedure potentially reduce the effects of ice crystal formation injuries caused during normal cryopreservation and may facilitate embryo equilibration with the cryoprotectant. Furthermore, lessons learned from equine embryology suggest that expanded blastocysts >250 µm in diameter result in poor (<10%) pregnancy rates after embryo cryopreservation regardless of methodology (Eldridge-Panuska et al., 2005). However, when the blastocoele fluid of equine embryos is suctioned, acceptable pregnancy rates (50-60%) have been obtained with a small sample of large (500-800 µm) expanded blastocysts (Choi et al., 2011).

The call rates from the SNP analysis were greater for the LAD than the PAD biopsy method. We used the Repli-G Single Cell (Qiagen Valencia, CA) WGA kit following the recommendation of GeneSeek. Even though both LAD and PAD yielded around 40 µg of amplified genomic DNA, the call rates for the PAD methodology were not acceptable for reliable embryo genotyping. The amount of starting genomic DNA was almost double with the LAD (~ 20 to 30 cells) than with the PAD (~ 10 to 15 cells) biopsy method, and one can speculate that this advantage led to a higher call rate. With LAD methodology, a parental imputation is potentially not necessary. Le Bourhis et al., 2012 estimated that a calling rate of >85% seemed to be a minimum threshold value to estimate breeding values for embryos. In any case, as suggested by Shojaei Saadi et al., 2014, a subsequent SNP analysis should be conducted from the resulting calves, thereby comparing genotypes with those obtained from the embryo to corroborate validity of embryo genotyping technique.

Our data suggests that vitrification and the SF-G0.7 cryopreservation protocols result in higher re-expansion rates in vitro compared to the EG-15 for direct transfer; however, implementation of those procedures under field commercial conditions are time consuming (10-15 min/ per embryo) to rehydrate the embryos under a microscope and was clearly not desirable by practitioners. Pregnancy rates after direct transfer of IVP- LAD biopsied embryos was approximately 15% lower than fresh IVP embryos at 32-38 d of gestation. If we consider that we are only transferring desired genotyped embryos, the lower pregnancy rate may still justify the technical input required to biopsy and cryopreserve IVP embryos.

Have we made progress? Yes. Are we closer to efficient embryo genotyping? A bit. The good news is that the field of molecular biology advances at a fast pace and the technologies for SNP analysis most likely will become more sensitive and reliable. Words of caution: are we looking in the right direction when genotyping cattle and generating GEBVs? Since we are evaluating DNA, what is the role of epigenetic effects and their correlation with genotype, phenotype and individual performance? Epigenetic effects are heritable and respond to environmental factors and contribute to variability within traits expressed from the animals genome (Jaenisch and Bird, 2003). If GEBVs are calculated by estimating SNP effects from prediction equations, which are derived from a subset of animals in a population (the reference

population) that have SNP genotypes and phenotypes for traits of interest, there may be a bias if we test individuals across different geographical regions and management practices. If the bias does exist, it may result from genotype by environmental interactions; therefore, genomic evaluations should be customized to regions and specific cow families or even better, we should be looking at high-throughput sequencing of RNA to estimate breeding values in the future to account for protein expression message and its relationship to production traits.

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