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Quantitative mRNA expression in ovine blastocysts produced from X- and Y-chromosome bearing sperm, both *in vitro* and *in vivo*

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Abstract

Artificial insemination (AI) of sex-sorted sperm results in decreased fertility, compared with non-sorted sperm, in most species. However, this has not been the case in sheep, where the low-dose AI of sex-sorted ram sperm produced similar, if not superior, fertility to non-sorted controls. The aim of the present study was to determine the impact of sex-sorting technology on ovine embryo gene expression following embryo production *in vivo* and *in vitro*. After semen collection, ejaculates were split and either sex-sorted by flow cytometry and frozen, or diluted and frozen. Embryos were produced *in vivo* by inseminating superovulated ewes with either X- or Y-chromosome enriched sperm, or non-sorted control sperm, and collected by uterine flushing on Day 6 after AI. Embryos were produced *in vitro* using the same sperm treatments and cultured *in vitro* for 6 d. The relative abundance of selected gene transcripts was measured in high-grade blastocysts, defined by morphological assessment, using RT-qPCR. The mRNA expression of DNMT3A and SUV39H1 was upregulated in embryos cultured *in vitro*, compared to those cultured *in vivo* (DNMT3A: 3.61 ± 1.08 vs 1.99 ± 0.15; SUV39H1: 1.88 ± 0.11 vs 0.88 ± 0.07; mean ± SEM; $P < 0.05$). Both G6PD and SLC2A3 transcripts were reduced in embryos produced from sex-sorted sperm, *in vivo* (SLC2A3: 0.23 ± 0.03 vs 0.64 ± 0.10; G6PD: 0.32 ± 0.04 vs 1.01 ± 0.16; $P < 0.05$). The expression of DNMT3A was up-regulated in male (3.85 ± 0.31), compared to female embryos (2.34 ± 0.15; $P < 0.05$). This study contributes to the growing body of evidence citing aberrant patterns of gene expression resulting from *in vitro* culture. Whereas the process of sex-sorting altered the expression of several of the genes examined, no effect on embryo development was detected.

Keywords: mRNA expression; Gene expression; Sex-sorted sperm; IVF; Sheep

1. Introduction

Sperm that withstand the process of flow-cytometric sorting, and subsequent cryopreservation steps, are frequently reported to produce fewer pregnancies, regardless of species, following AI [1,2]. In addition, embryo production rates are recurrently cited as lower following the insemination of sex-sorted, rather than non-sorted sperm, both *in vitro* [3–6] and *in vivo* [7–9]. However, refined sperm sorting and associated processing techniques have vastly improved the performance of sex-sorted sperm in recent years. With specific reference to sheep, the *in vivo* fertility of sex-sorted sperm, once reported to be very poor [1], has recently been shown to be superior compared to that of non-sorted sperm [10,11]. Similarly, *in vitro* sperm characteristics,

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including motility, viability, mitochondrial activity, and acrosome integrity, are no longer reduced after modified flow cytometry [12].

Whereas the *in vitro* production of embryos using sex-sorted sperm has been described as both quantitatively and qualitatively similar to that using non-sorted sperm in sheep [13–15], for other economically important species, this is not always the case. The developmental capacity of bovine embryos has been reported to be lower in response to insemination with sex-sorted compared to non-sorted sperm treatments [6]. More specifically, rates of fertilization [16,17], cleavage [2,18], blastocyst formation [3,19,20], and pregnancy [16] have all been shown to be significantly reduced after the insemination of sex-sorted sperm in cattle. Additionally, aberrant timing of development [2], and reduced embryo production efficiency [5] have been identified in sex-sorted sperm treatments.

The differential expression of developmentally important mRNA transcripts in bovine blastocysts derived from sex-sorted and non-sorted sperm may partially explain the aforementioned reduction in embryo production efficiency [5]. Despite sheep and cattle being closely related species, ovine embryos were more tolerant or adaptive to changing culture conditions, as quantified through morphology, cryotolerance, and relative mRNA abundance [21]. Maintenance of cytosine methylation in the male pronucleus, and lack of passive demethylation throughout the preimplantation period until the blastocyst stage, is unique to sheep [22], and may influence the sensitivity of ovine gametes and embryos to epigenetic change. As such, molecular differences in ovine blastocysts produced from sex-sorted sperm may provide some insight into the efficacious application of sorting technology in sheep.

The objective of the current study was to compare the relative abundance of several developmentally important gene transcripts in ovine blastocysts produced using sex-sorted X- and Y-chromosome bearing sperm, with those produced using non-sorted sperm. Embryos derived *in vivo* were used as a physiological standard for those produced *in vitro*. More specifically, the relative quantification of DNA methyltransferase 3a (DNMT3A), and suppressor of variegation 3-9 homolog 1 (SUV39H1) were considered to assess possible differences in epigenetic methylation between treatments. In addition, heat shock protein 70 (HSP70), associated with cellular stress, glucose-6-phosphate dehydrogenase (G6PD), related to oxidative stress, and glucose transporter-3 (SLC2A3), involved in cellular metabolism, were investigated as indicators of general cellular function. By contrasting previous observations in bovine mRNA expression [5] with the current ovine model, we aimed to highlight molecular mechanisms that may be responsible for the robust performance of sex-sorted sperm in sheep.

2. Materials and methods

The procedures herein were approved by The University of Sydney's Animal Ethics Committee.

2.1. Experimental design

Embryos were generated, both *in vitro* and *in vivo*, from sex-sorted X-chromosome enriched (*in vitro*: $n = 12$; *in vivo:* $n = 15$) and Y-chromosome enriched (*in vitro:* $n = 13$; *in vivo:* $n = 13$), and non-sorted (*in vitro*: $n = 40$; *in vivo:* $n = 14$) sperm. The same rams (n = 2) and ejaculates were used for both *in vitro* and *in vivo* embryo production. Each embryo treatment group was defined by ram, sperm type (sorted or non-sorted), sex (confirmed using PCR) and means of production (*in vivo/vitro*). Blastocyst cell number, cleavage rate at 48 h and blastocyst formation rate on Day 6 were recorded for each group. Messenger RNA expression was measured using quantitative real time polymerase chain reaction (RT-qPCR), with the relative abundance of transcripts calculated using the $^{\Delta\Delta}$CT method with efficiency correction [23,24], and the subsequent fold difference in expression used for comparison between groups.

2.2. Collection, sex-sorting and cryopreservation of sperm

Semen was collected by artificial vagina from Merino rams (n = 2) housed at the University of Sydney, Camperdown, NSW, Australia, between March and September, 2008 (during the breeding season). The concentration of each ejaculate was determined by haemocytometer and motility assessed subjectively [25]. Only ejaculates with a wave motion score of greater or equal to "4" (on a scale of 1–5) were used [25]. Semen allocated to control groups was diluted 1:4 (semen: diluent, v/v) using a tris-citrate-glucose cryoprotective diluent containing 15% egg yolk and 5% glycerol (CRYO; Salamon's freezing diluent) and frozen using the pellet method [25].

Sperm to be sorted were diluted to a concentration of 400×10^6/mL with a Tris-buffered medium (TRIS) [25], supplemented with 0.3% (w/v) bovine serum albumin (BSA). Each 1 mL sample was incubated with 270-293 μM of Hoechst 33342 (H33342; Sort Ensure,