

Developmental Potential of Embryos Produced *In Vitro* by Sperm from Bulls of Contrasting *In Vivo* Fertility and Oocytes Retrieved from a Same Donor

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Abstract

Laboratory assessments of sperm traits are poor indicators of fertility. Because the variability in the quality of oocytes collected from different donors drastically influence *in vitro* embryo production, the aim of this work was to implement an *in vitro* model to compare the developmental potential of embryos produced by bulls of contrasting *in vivo* fertility and oocytes retrieved from the same donor in order to minimize the female related variability. For each trial (n=54), one pair of good quality ovaries of a same donor were split longitudinally and oocytes were recovered by slicing method. Thereafter, matured oocytes were fertilized with sperm of two bulls of low estimated relative conception rates (ERCR <-2) and high (ERCR >+2) contrasting field fertility (6 vs. 6 bulls). Cleavage and blastocyst formation rates were compared.

Sperm of high fertility bulls (ERCR+) gave also higher fertilization rates (cleavage) *in vitro* when compared to low fertility (ERCR-) bulls (odd ratio=1.23). Conversely, the embryonic development to the blastocyst stage was reduced (odd ratio=0.84) in the ERCR+ with respect to the ERCR- bulls. This paradoxical result demonstrates that *in vivo* bull fertility hardly correlates with *in vitro* blastocyst yield, but confirms that early events occurring at fertilization are better indicators of the fertility potential. Furthermore, this experimental approach indicates that differences in embryo production rates between bulls of contrasting field fertility may not be outlined *in vitro* even when bulls are compared using oocytes with variability limited to the same donor.

Keywords: Bull; Fertility; Oocyte variability

Introduction

Bull influence on embryo viability is interesting for a more comprehensive understanding of the complex parameter defined as fertility. Beside the scientific relevance, accurate prediction of bull fertility by laboratory evaluation is of great interest for semen production centers in order to rationalize the dosage of insemination straws, and for artificial insemination (AI) industry to harmonize the considerable variation reported in the field fertilizing ability of bulls. However, laboratory assessments of sperm traits only allow for a partial estimate of the level of fertility [1], and currently, the most utilized method worldwide for measuring male fertility in livestock industry is still based on expensive and time-consuming field fertility evaluation [2]. Given the limited informativeness of semen characteristics in estimating the fertility of sires, events occurring after fertilization have received increasing interest. However, attempts based on *in vitro* fertilization and embryo culture failed in identifying consistent differences among bulls of proven field fertility [1]. Because the wide variability in the quality of oocytes collected from different donors is known to influence *in vitro* embryo production [3], some authors suggested the need for reducing the variability among oocytes retrieved from offal ovaries if the paternal influence was the target of such studies [4].

The objective of the present work was to develop an *in vitro* model to compare the developmental potential of embryos produced by bulls of contrasting *in vivo* fertility and oocytes retrieved from the same donor in order to minimize the female related variability.

Materials and Methods

For each trial, oocytes were collected from a same donor, split into two groups and fertilized with sperm of two bulls of contrasting field fertility. Fertilization and blastocyst formation rates of the two groups were then compared.

Identification of Bulls

Twelve Holstein Friesian bulls were selected on the basis of their *in vivo* fertility estimated based on 56-day non-return to estrus [5] and assigned to two groups of low (estimated relative conception rates, ERCR <-2; range -2.3 to -5.4) and high (ERCR >+2; range +2.0 to +3.9) fertility. A minimum of 90% reliability (range from 90% to 99%), with number of inseminations ranging from 586 to 21,194, was used as a first criteria for selecting bulls. Six bulls of contrasting ERCR were then paired on a random base.

Oocyte Recovery and Maturation

Pairs of good quality ovaries of a same donor showing high numbers of antral follicles on the entire surface were collected from Holstein

Friesian cows/heifers in a local abattoir and transported to the laboratory in PBS supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin at 20-25°C. Ovaries of the same donor were split longitudinally into two half-portions using a scalpel blade in order to make accessible the entire population of follicles of the cortical portion. Oocytes were then recovered by slicing the half-portions in Hepes-buffered TCM 199 medium (Sigma-Aldrich) supplemented with 0.1% BSA (fraction V, Sigma-Aldrich). Oocytes were then selected in order to obtain homogeneous populations by excluding poor quality and small sized ones, and matured for 24 h in four-wells in groups of 25 oocytes per well in 500 µl of bicarbonate-buffered TCM 199 supplemented with FSH/LH (0.05 U.I/ml; Meropur, Ferring) and 10% of FBS (Sigma-Aldrich) in 5% CO₂ and 95% humidified air at 38.5°C.

***In Vitro* Fertilization and Embryo Culture**

For each trial, after thawing the straws in water bath at 20-22°C, motile sperm of two bulls of contrasting ERCR was selected by 20 min centrifugation at 500 x g on discontinuous Percoll gradients (45-90%) in HEPES buffered Ca²⁺-free TALP medium (H-TALP) (pH 7.4) and then washed by 10 min centrifugation at 5000 x g in 5 ml H-TALP supplemented with 0.6% BSA. Mature oocytes from a same donor were regrouped, cumulus mass was partially removed using hyaluronidase and, for each of the two bulls, oocytes were inseminated in 300 µl of IVF medium at a concentration of 1 x 10⁶ sperm/ml into wells, using 25 oocytes per well. At 18-20 h after insemination, the presumptive fertilized eggs were vortexed in Hepes-buffered TALP-wash medium

and cultured in 50 µl of bicarbonate-buffered SOF medium under mineral oil at 5% CO₂ and 5% O₂ in humidified air at 38.5°C. At day 4, half of the medium was renewed, and the day 6 half volume was replaced with TCM 199 supplemented with 1% BSA. Number of blastocysts forming at day 8 was recorded.

Statistical Analysis

Statistical analysis was performed by logistic regressions with cleavage and blastocyst rates (percentage of success) as the dependent variables and ERCR (low vs. high) and Oocyte-Donor, as independent variables. The coefficients returned from the logistic regression model were expressed as log odd ratios, and final odd ratio values were obtained after exponential transformation of the log odd ratio.

Results and Discussion

The experimental approach used in this study was aimed at evaluating possible differences in the developmental potential of embryos generated by bulls of contrasting field fertility and oocytes of a common donor. To this aim, pairs of ovaries were collected from 54 donor cows/heifers and sperm of 6 bulls of low and 6 bulls of high *in vivo* fertility was used to fertilize more than 4300 oocytes (range 50 to 180 oocytes retrieved per female donor, 54 sessions of *in vitro* fertilization with two bulls per session). Results of *in vitro* fertilization and embryo culture are shown in Table 1.

ID Bulls	Replicates (n)	Oocytes (n)	Oocytes undergoing cleavage n (%)	Blastocysts/cleaved n (%)
Bull A -	8	235	108 (46)	59 (52)
Bull B +	8	226	85 (38)	45 (32)
Bull C -	20	792	358 (45)	121 (34)
Bull D +	20	802	302 (38)	45 (15)
Bull E -	11	464	216 (46)	72 (33)
Bull F +	11	458	200 (44)	71 (35)
Bull G -	5	160	76 (47)	28 (37)
Bull H +	5	161	64 (40)	24 (37)
Bull I -	7	316	113 (36)	42 (37)
Bull J +	7	318	203 (64)	98 (48)
Bull K -	3	212	81 (38)	16 (20)
Bull L +	3	213	86 (40)	9 (10)

Table 1: Fertilization rates (cleavage) and embryo development of bovine oocytes collected from 54 donor cows/heifers (replicate) and fertilized with spermatozoa of 6 bulls of low (-) and 6 bulls of high (+) fertility as estimated *in vivo*. For each replicate, sperm of two bulls of contrasting fertility was used to inseminate oocytes collected from the same female donor.

The statistical model was highly efficient to explain the total variability (G Statistics; P<0.001). The variables ERCR and Oocyte-Donor showed significant effects (P<0.01). In particular, the data relative to the variability among females confirms the need of methods for controlling this parameter, which drastically influences the outcome of *in vitro* fertilization and embryo production programs and

limit the possibility of comparison among bulls in relation to their fertility. Sperm of high fertility bulls (ERCR+) gave also higher fertility rates (cleavage) *in vitro* when compared to low fertility (ERCR-) bulls with odd ratio=1.23. Conversely, in the same conditions, the embryonic development to the blastocyst stage was reduced with odd ratio=0.84 in the ERCR+ with respect to the ERCR- bulls. This

paradoxical result confirms that *in vivo* bull fertility hardly correlates with *in vitro* blastocyst yield, but confirms that early events occurring at fertilization are better indicators of fertility potential. Furthermore, this experimental approach indicates that differences in embryo production rates between bulls of contrasting field fertility may not be outlined *in vitro* even when two bulls are compared using oocytes with variability limited to one common donor.

From the literature it can be inferred that differences in gene expression under pathological conditions can be substantial when compared to healthy counterparts. However, fertility of bulls used in AI goes beyond any pathological conditions. Therefore, a further scope of this work was to develop an *in vitro* model which may be potentially used for gene expression studies on embryos aimed at highlighting possible existing faint differences of expression attributable to the male by minimizing the contribution, in terms of variability, of the female genome. In this regard, this method was sustainable for producing number of embryos adequate for gene expression studies [6], as we could obtain paired experimental groups of 5 to 15 viable embryos for each of two bulls using oocytes of a same female donor. In fact, as already mentioned, events occurring after fertilization have received increasing interest, and embryo loss, which in dairy cattle has been estimated to be in the range 35% to 50% [7], is accounted as a major cause of hypofertility. Inadequate endometrial receptivity and support to embryo growth, dysregulation of histotroph composition and immune tolerance to the embryo were all accounted as major female-related causes of failure in establishing or maintaining pregnancy as revealed by microarray analysis of endometrial transcriptome of heifers with different fertility [8,9]. More in general, physiological related causes for dairy cows to fail in conceiving have been extensively reviewed [10-12] and include the post-partum and early pregnancy period conditions, the effect of negative energy balance on immune system and on the development of female gametes which results in the ovulation of a developmentally incompetent oocytes, the duration and intensity of estrus, and the poor quality of oocyte and uterine environment from adverse metabolic environment. However, contribution of individual bulls for embryo viability has not received the same attention. In this respect, the post-fertilization period has been widely investigated as regard to gene expression patterns under potentially deleterious artificial culture conditions in order to elucidate the divergences observed between *in vivo* and *in vitro* derived embryos [13], to study the effects of maturational regimens [14,15] and embryo density on developmental characteristics [16], in animal cloning [17], and for predictive assessment of pregnancy success [18,19]. These studies have provided knowledge and the identification of panels of genes involved in embryo development throughout the preimplantation period. Since early embryonic mortality is very high during the first week of development, when activation of the embryonic genome takes place, it was hypothesized that the bull has influence on embryo apoptosis and expression of developmentally essential genes, and that this influence can be reflected by field fertility.

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