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Developmental Competence of Embryos Derived from Reciprocal *In Vitro* Fertilization between Yak (*Bos grunniens*) and Cattle (*Bos taurus*)

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Abstract. The purpose of this study was to investigate fertilization ability and embryo development to the blastocyst stage after reciprocal *in vitro* fertilization (IVF) between yak and cattle in an attempt to clarify the problem of low conception rate after mating yak females with cattle bulls. *In vitro*-matured (IVM) cattle and yak oocytes were inseminated with either Holstein or yak spermatozoa, and after an 18-h of coincubation period, a proportion of the oocytes was fixed and examined for sperm penetration, polyspermy and male pronuclear formation. The remaining oocytes were cultured *in vitro* and evaluated for cleavage and blastocyst formation rates. The percentage of IVM oocytes penetrated by spermatozoa ranged from 78.5 to 90.5%, and the formation of one or two pronuclei and the incidence of polyspermy did not differ among the different combinations. The cleavage and blastocyst rates were not affected by the species of the sperm, but they were affected by the species of the oocytes ($P < 0.05$), with cattle oocytes having a higher ($P < 0.05$) cleavage and blastocyst rates (69.9 and 31.3%) than yak oocytes (62.7 and 11.5%). The blastocyst formation rate was calculated from the cleaved zygotes. The interaction between sire and oocytes species ($P < 0.05$) influenced blastocyst formation rate, with the highest blastocyst rate occurring in cattle oocytes fertilized with yak spermatozoa (36.5%) and the lowest rate occurring in yak oocytes fertilized with yak spermatozoa (9.4%). The effect of heterosis was apparent at the blastocyst stage, but there was a large reciprocal difference in blastocyst production between crosses. It was concluded that the low conception rate that results from crossing yaks with cattle is not due to either a species-specific block of fertilization or the developmental competence of the early stage embryo.

Key words: Cattle, Early development, Embryos, *In vitro* fertilization (IVF), Yak

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The yak is one of the world's most remarkable domestic animals — a herbivore living on the “roof of the world”, in and around the Himalayas and further north at altitudes ranging from 2500 to 5500 m with no frost-free period and mostly above the tree line. They are very important to local people for meat, milk and draft power given that few other animals survive in these areas. However, their production traits are inferior to those of improved cattle (*Bos taurus*) breeds [1, 2].

Interest in the commercial use of local cattle × yak hybrids has existed for at least 3000 years. During this time, attempts have been made to improve meat and milk production (quoted from Wiener *et al.* [1]). A number of systematic studies carried out from 1950s to 1990s have examined the meat and milk performance of hybrids of yak derived from both natural mating and artificial insemination using semen from exotic breeds of cattle such as the Holstein, Simmental, Hereford and Shorthorn. The results have shown that F1 hybrids from dairy cattle breeds produce 100–300% more milk than the yak [3, 4]. However, the cost-effectiveness of such hybridization remains debatable for several reasons. First, there is a marked difference between the pregnancy rates of purebred yaks (>70%) compared with hybrid animals (<30%) [3, 5], but the causes of this difference have not been well studied at the gamete and embryo levels. Second, the mean birth weight of hybrid yaks is

much higher than that of purebred yak calves, and this results in a higher incidence of dystocia in the former group [1]. Third, sterility of F1 males prevents successful *inter-se* matings. F1 hybrid females can be mated back to yak or cattle bulls, but the poor performance of progeny (both meat and milk) makes backcross generations commercially unattractive. Since F1 females have a larger body size and a normal or improved reproductive performance compared with the purebred yak, they should be ideal recipients of hybrid F1 embryos. In this way, the F1 hybrid females would not only give additional milk, but also produce valuable offspring (F1).

During the past decades, embryo transfer in farm animals has continued to make major strides and now includes transfer of *in vitro* produced (IVP) embryos [6–8]. However, the efficiency of this procedure in the yak has been relatively poor [9–11]. Therefore, the objective of this study was to investigate fertilization ability and embryo development to the blastocyst stage after reciprocal *in vitro* fertilization (IVF) between yak and cattle in order to understand the low conception rate after mating yak females with cattle bulls.

Materials and Methods

Materials

Dulbecco's phosphate-buffered saline (DPBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT, USA), Folltropin

Table 1. Fertilization status of putative zygotes produced *in vitro* from different combinations of yak and cattle gametes

Species combination	No. oocytes	No. (%) 1 PN*	No. (%) 2 PN	No. (%) >2 PN	No. (%) penetrated
Yak ♀ × yak ♂	79	1 ^a (1.3)	52 ^a (65.8)	12 ^a (15.2)	65 ^{a,b} (82.3)
Yak ♀ × cattle ♂	93	1 ^a (1.1)	64 ^a (66.8)	8 ^a (8.6)	73 ^a (78.5)
Cattle ♀ × yak ♂	84	0 ^a (0.0)	64 ^a (76.2)	12 ^a (14.3)	76 ^b (90.5)
Cattle ♀ × cattle ♂	83	1 ^a (1.2)	59 ^a (71.1)	10 ^a (12.1)	70 ^{a,b} (84.3)

* PN = Pronucleus. ^{a,b} Within a column, values without a common superscript are different (P<0.05).

(FSH) was purchased from Bioniche Life Sciences Inc. (Belleville, Ontario, Canada) and fetal calf serum (FCS) was purchased from Gibco (Grand Island, NY, USA). All other chemicals and reagents used for *in vitro* maturation (IVM), IVF and *in vitro* culture (IVC) were cell-culture tested and obtained from Sigma-Aldrich (St. Louis, MO, USA). Synthetic oviductal fluid (SOF) medium without glucose was prepared according to the formula of Tervit *et al.* [12]. All media were filtered through a 0.2 µm Millipore filter (Carrigtwohill, Cork, Ireland) and placed in an incubator (Forma Series II, Waltham, MA, USA) to equilibrate for 4 to 6 h in an atmosphere of 5% CO₂ in air.

Oocyte collection and IVM

Collection and IVM of oocytes were as previously described [11, 13]. Briefly, ovaries were collected from cattle and yaks at local slaughterhouses from October to December and transported, within 3 h, to the laboratory in DPBS (29–33 °C). Cumulus-oocyte complexes (COCs) were aspirated from follicles (2–8 mm diameter) using a hand-held 10-ml syringe connected to an 18-gauge needle. COCs were collected in DPBS supplemented with 6 mg/ml BSA under a low-power (20×) stereomicroscope (Leica MZ16, Germany). The COCs were rinsed three times in DPBS containing 5% (v/v) FCS and twice in TCM 199 supplemented with 20% (v/v) FCS, 5 µg/ml FSH, 50 IU/ml LH, 1 µg/ml estradiol-17β, 100 U/ml penicillin and 100 µg/ml streptomycin (maturation medium). Only COCs having one or more layers of cumulus cells and evenly granulated ooplasm were selected for IVM. Up to 30 COCs were placed in each culture well (Nunc, Naperville IL, USA) containing 600 µl of maturation medium covered with mineral oil. COCs were allowed to mature for approximately 24 h at 38.6 °C in an atmosphere of 5% CO₂ in humidified air.

Sperm preparation, IVF and embryo culture

Sperm preparation for cattle and yak bulls and IVF of oocytes were conducted according to previously described procedures [11, 13] with some modifications. Frozen semen was thawed and washed by centrifugation through a Percoll gradient (30%/45%) containing Hepes-buffered SOF medium supplemented with 5 mg/ml BSA, 50 µg/ml caffeine, 30 µg/ml glutathione and 20 µg/ml heparin (sperm medium) and centrifuged at 500 × g for 10 min to separate motile sperm. After centrifugation, 120 µl of the concentrated sperm fraction was removed, placed into 200 µl of sperm medium and incubated at 38.6 °C for 15 min.

After maturation, excess cumulus cells were partially removed by gently swirling the COCs in a 36-mm Petri dish containing SOF with 6 mg/ml BSA (IVF medium). The COCs were further washed

twice in IVF medium before being transferred into 4-well plates (up to 30 per well) containing 500 µl IVF medium covered with mineral oil per well. Sperm were added to produce a final concentration of approximately 1 × 10⁶ motile sperm/ml. Coincubation was carried out for 24–26 h at 38.6 °C in an atmosphere of 5% CO₂. Remnant cumulus cells were removed from the putative zygotes by gentle pipetting at 26 h of coincubation. The putative zygotes were then washed three times in SOF medium. Groups of 25 embryos were cultured in 600 µl of SOF medium supplemented with 6 mg/ml BSA, 0.5 mg/ml myoinositol, 3% (v/v) essential amino acids, 1% (v/v) nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml L-glutamine (culture medium) under mineral oil in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 38.5 °C [14, 15]. The number of zygotes that cleaved was recorded at 48 h post insemination (hpi). The culture medium was changed at 96 hpi, and blastocyst development was determined on Days 7 to 9 (Day 0: day of insemination).

The presumptive zygotes were randomly examined for evidence of fertilization according to previously described methods [11, 16]. Briefly, at 18 hpi, the ova were removed from the culture dish. The cumulus cells and any remaining sperm were completely removed by repeated pipetting in culture medium containing 150 U/ml of hyaluronidase. The presumptive zygotes were then mounted and placed in fixative (1:3; acetic acid:ethanol) at room temperature for 48–72 h. They were then stained with 1% orcein in 45% acetic acid, cleared with acetyl-glycerol and examined under a phase-contrast microscope at 200× and 400× magnification. Oocytes were considered penetrated when one or more pronuclei were observed in the ooplasm.

Statistics

Penetration, polyspermy and pronuclear formation were analyzed by Chi-square analysis. Percentage data of cleavage and blastocyst rates were subjected to arcsine transformation before analysis by ANOVA, with oocyte species, sperm species and the interaction in the model. Tukey's hsd test was used to determine differences between means. Each experiment was replicated five to seven times.

Results

Fertilization

Oocytes from cattle (n=167) and yak (n=172) inseminated with either cattle or yak sperm were examined to determine pronuclear status (Table 1). The formation of one pronucleus, the formation of two pronuclei (normal fertilization) and the incidence of

Table 2. *In vitro* development of yak and cattle oocytes inseminated with either yak or cattle spermatozoa*

Species combination	No. oocytes examined	No. cleaved embryos (%; mean $\pm$ SEM)	No. blastocysts (%; mean $\pm$ SEM)
Yak oocytes inseminated with			
Yak spermatozoa	356	233 (65.0 $\pm$ 2.4) ^{a,b}	22 (9.4 $\pm$ 1.8) ^a
Cattle spermatozoa	381	226 (61.1 $\pm$ 2.9) ^a	31 (13.1 $\pm$ 1.5) ^a
Combined	737	459 (62.7 $\pm$ 2.0) ^d	53 (11.5 $\pm$ 1.2) ^d
Cattle oocytes inseminated with			
Yak spermatozoa	442	318 (72.4 $\pm$ 2.2) ^b	109 (36.5 $\pm$ 3.6) ^b
Cattle spermatozoa	470	312 (67.0 $\pm$ 1.9) ^{a,b}	78 (25.2 $\pm$ 4.1) ^c
Combined	912	630 (69.9 $\pm$ 1.6) ^e	187 (31.3 $\pm$ 3.1) ^e

* Five to seven replicated trials were carried out, and blastocysts were harvested on Day 9. Blastocyst formation rate was calculated from the cleaved zygotes. ^{a-c} Within a column, values without a common superscript are different ($P < 0.05$). ^{d,e} Values for main effect least-squares means without common superscripts differ ($P < 0.05$).

polyspermy (>2 pronuclei) did not differ among the four combinations of fertilization. The percentage of IVM oocytes penetrated by spermatozoa ranged from 78.5 to 90.5% across the treatment groups. The percentage of cattle oocytes penetrated by spermatozoa from yak was greater ($P < 0.05$) than that of yak oocytes penetrated by cattle spermatozoa, but the percentage of yak oocytes penetrated by spermatozoa from yak did not differ compared with those penetrated by cattle spermatozoa.

Cleavage and early embryo development

Cleavage at 48 hpi was not affected by the species of the sperm (Table 2), but it was clearly affected by the species of the oocytes ($P < 0.05$), with cattle oocytes (69.9%) having a higher ($P < 0.05$) cleavage rate than yak oocytes (62.7%). There was no sire species $\times$ oocyte species interaction. The percentage of yak oocytes cleaving after insemination with cattle spermatozoa (61.1%) was similar to that of yak oocytes inseminated with yak spermatozoa (65.0%).

The overall blastocyst production rate was not affected by the species of the sperm, but it was clearly affected by the species of the oocytes ($P < 0.05$; Table 2), with cattle oocytes (31.3%) having a greater ($P < 0.05$) blastocyst yield than yak oocytes (11.5%). The blastocyst yield was higher ($P < 0.05$) for purebred cattle embryos than for purebred yak embryos (25.2 vs. 9.4%). Furthermore, there was a sire species $\times$ oocyte species interaction ($P < 0.05$), with the highest blastocyst yield being obtained for cattle oocytes fertilized with yak spermatozoa (36.5%) and the lowest yield being obtained for the homologous yak combination (9.4%). The critical comparison was that of the purebred versus the crossbred embryos — the blastocyst rate was 7.5% higher in the latter (9.4 and 25.2% vs. 13.1 and 36.5%).

Discussion

Many studies have shown that pregnancy rates are above 70% if female yak are mated with yak bulls [1, 5, 17, 18]. However, when the yak female is inseminated (naturally or artificially) with semen from *Bos taurus* or *Bos indicus*, the pregnancy rate falls [3, 5]. It has long been suspected that this reduction results, at least partly, from species-specific sperm-egg interactions that ultimately affect heterologous fertilization and early embryo development. How-

ever, in this study, the rate of fertilization as assessed by the formation of pronuclei did not differ when yak and cattle oocytes were inseminated with either yak or cattle spermatozoa. These results are in agreement with those reported in previous studies showing that cattle oocytes can be fertilized with sperm from the oryx [19, 20], yak [9, 11], bison, banteng and gaur [9], suggesting little cross-species specificity with regard to gamete interaction among Bovidae. Thus, penetration failure and/or abnormal fertilization are not the main factors that contribute to poor conception rates when female yaks are crossed with cattle.

Oocytes obtained from cattle were more likely to cleave and develop to the blastocyst stage after *in vitro* maturation and fertilization than oocytes from yak. The intrinsic quality of the oocytes is thought to be of critical importance in determining the developmental outcome [21–25]; any deficiency in yak oocytes might reflect coincidental changes in cytoplasmic and/or nuclear components that are related to the evolving adaptability of the yak in their unique environment. We cannot dismiss the involvement of extrinsic factors in the results. The yak oocytes in the present study were obtained at the end of the breeding season, and seasonal conditions may have impaired oocyte competence. The lower development of yak embryos might also be related to the culture system, which has been developed specifically for use in cattle. Oliveira-Filho *et al.* [26] and Fischer *et al.* [27] attributed lower blastocyst formation rates in *B. indicus* to the greater compatibility of the culture system with *B. taurus* gametes. Also, the blastocyst rates of yak hybrid embryos observed in this study were much greater than those reported in previous studies [9, 10].

The most significant finding in this study was that the cleavage rates for heterologous IVF of yak and cattle gametes were comparable to those achieved with homologous yak IVF, and the blastocyst rate for yak oocytes fertilized with cattle spermatozoa was not significantly different from that for homologous yak IVF. Thus, it is likely that the lower conception rates that occur when female yaks are crossed with cattle are not due to the developmental competence of early stage embryos. To our knowledge, this is the first time this has been reported in this field.

Interestingly, hybrid embryos of the yak and cattle had a significant heterosis for blastocyst yield with a large difference occurring between reciprocal crosses. Individual bulls effects in IVF might

have contributed to these differences [15, 24], but our previous work also showed that the blastocyst yield of cattle oocytes fertilized with yak spermatozoa was greater than for homologous cattle IVF [11]. Such heterosis has also been reported in gilts [28], *B. taurus* × *B. indicus* [29] and *B. taurus* [30, 31], although in another study [27], no evidence of heterosis was found for crosses within *B. taurus* breeds or between *B. taurus* and *B. indicus*. The cause of this discrepancy is not clear, but it is possible that breed maternal effects can influence outcome [32]. The results of the present study also indicate that this reciprocal difference occurs at fertilization and during early cleavage development *in vitro*.

In conclusion, the cleavage rates of heterologous and homologous IVF were not significantly different. The blastocysts derived from yak oocytes fertilized either with yak or cattle spermatozoa were also not significantly different. The blastocyst rate obtained from cattle oocytes fertilized with either cattle or yak spermatozoa was significantly higher than those from yak oocytes fertilized with either yak or cattle spermatozoa. The results from this study showed that the low conception rate after mating yak females with cattle bulls does not correlate with the species specific block of fertilization or developmental competence of the early stage embryos.

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