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In vitro production of cattle–water buffalo (*Bos taurus – Bubalus bubalis*) hybrid embryos

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Summary

Interspecific hybrid embryos are useful models for the study of maternal–fetal interactions, transmission pattern of species-specific markers and parental contributions to growth and developmental potential of pre-attachment embryos. In an attempt to investigate the possibility of producing hybrid embryos of domestic cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*), cattle oocytes were exposed to buffalo sperm and buffalo oocytes were exposed to cattle sperm and the cleavage rate and the post-fertilisation features of hybrid embryos up to the blastocyst stage were compared with those of buffalo and cattle embryos. The cleavage rate in buffalo oocytes exposed to cattle sperm was low (40.8%), with only 8.8% of these hybrid embryos reaching the blastocyst stage. Cattle oocytes exposed to buffalo sperm showed 86.3% cleavage, while 25.9% of these attained the blastocyst stage. The speed of development of both types of hybrids was intermediate between that of cattle and buffalo embryos, with hatching occurring on day 7.5 in hybrid embryos, day 8–9 in cattle and day 7 in buffalo. The proportions of cells contributing to the trophectoderm and the inner cell mass were closer to those of the maternal species in both types of hybrid embryos. Our results indicate that cattle–water buffalo hybrid embryos produced using interspecies gametes are capable of developing to advanced blastocyst stages and that their *in vitro* fate, and developmental potential, are influenced by the origin of the oocyte.

Keywords: Embryos, Interspecific hybrids, Maternal influence, Reciprocal fertilisation, Water buffalo

Introduction

Interspecies hybrids are important resources for agriculture and research (Gray, 1972; Anderson, 1988). However, interspecies mating is successful only between a few closely related species due to natural barriers or isolating mechanisms (McGovern, 1976). Such barriers include anatomical differences, incompatible physiology and/or geographic isolation of the species concerned. Among the domestic bovids, there are several examples of hybrids resulting from interspecies breeding. European domestic cattle (*Bos taurus*) successfully interbreed with zebu cattle (*Bos indicus*), and American bison (*Bison bison*) has been hybridised with European bison (*Bison bonasus*) and domestic cattle (Basrur, 1986). The domestic goat (*Capra hircus*) is able to hybridise with Barbary sheep (*Ammotragous cerviae*) albeit at a low frequency (Anderson, 1988). Goats bred to sheep conceive but the hybrid pregnancies are generally lost by week 6 of gestation (Kelk *et al*., 1997*a*), although a few live-born goat–sheep hybrids have been documented (Bunch *et al*., 1976; Basrur, 1986; Anderson, 1988). Sheep bred to goats do not conceive although sheep oocytes exposed to capacitated buck sperm are fertilised *in vitro* and establish preg-

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nancies when transferred to recipient sheep (Kelk, 1997). As with the sheep–goat crosses, these hybrid pregnancies fail between weeks 6 and 8 of gestation (Kelk, 1997). When cattle are inseminated with semen from sheep, or sheep inseminated with semen from cattle, fertilisation does not occur. Yet, cattle and sheep gametes undergo fertilisation and the resulting hybrid embryos develop to the 8-cell stage (Kelk, 1997; Slavik *et al.*, 1997).

Water buffaloes (*Bubalus bubalis*) are co-reared with domestic cattle in many countries. In spite of this there are no reports of cattle–buffalo hybrids. Attempts to inseminate buffalo cows with cattle semen and vice versa have not resulted in offspring or pregnancies (Drost, 1984). The present study was designed to investigate the feasibility of producing cattle–buffalo hybrid embryos by *in vitro* fertilisation to evaluate their developmental potential. To accomplish this, cattle oocytes matured *in vitro* and exposed to capcitated buffalo sperm were analysed and compared with buffalo oocytes matured *in vitro* and exposed to capacitated domestic cattle sperm for investigating the cleavage rate and the *in vitro* growth potential of hybrid embryos.

Materials and methods

Unless specified, all chemicals used were cell culture/embryo tested from Sigma (Sigma Chemical Co., St Louis, MO).

In vitro production of buffalo (B × B) and buffalo–cattle hybrid (B × C) embryos

Maturation of buffalo oocytes

The procedure of *in vitro* maturation and fertilisation of buffalo oocytes was as previously described by Totey *et al.* (1993). Briefly, buffalo ovaries were collected from a slaughterhouse (New Delhi, India), and were transported to the Embryo Biotechnology Lab, National Institute of Immunology, New Delhi, in normal saline (30 °C) within 2 h of slaughter. Ovaries were washed thoroughly in warm water, rinsed in warm normal saline and sliced with a surgical blade to release the cumulus–oocyte complexes (COCs) in Hepes-buffered Tyrode lactate medium (TL-Hepes). The COCs were washed three times in TL-Hepes and twice in maturation medium consisting of Ham's F10 supplemented with 10% fetal calf serum (Gibco Life Technologies, Grand Island, NY), 5 μg of oLH/ml, 0.5 μg oFSH/ml (National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Programme (NIDDK-NHPP), MD) and 1 μg 17β-estradiol/ml (Sigma). The COCs were cultured in 100 μl drops of maturation medium covered with sterile mineral oil at 39 °C in a humidified atmosphere of 5% CO_2 in air, for 24 h.

Sperm preparation

Frozen semen from a Murrah buffalo bull previously tested to yield >70% cleavage in *in vitro* trials (S.M. Totey, unpublished data) was used. Motile spermatozoa were obtained using Percoll (Sigma) gradient separation (Totey *et al.*, 1993) of frozen semen (from 0.5 ml straws) thawed in a 35 °C water bath. The thawed semen was layered on top of the Percoll gradients and centrifuged at 800 *g* for 15 min. The top layers were discarded and the pelleted spermatozoa were resuspended in sperm-TALP supplemented with 6 mg/ml bovine serum albumin (BSA) prior to repeating centrifugation. The supernatant was removed to retain 100 μl of sperm suspension and the sperm concentration was calculated and adjusted to the volume needed to fertilise the oocytes. Domestic cattle bull sperm of known fertility was processed in the same manner (from 0.5 ml frozen straws) prior to *in vitro* fertilisation. Both types of semen were processed simultaneously for each replicate in the experiment.

In vitro fertilisation was carried out as previously described by Totey *et al.* (1992, 1993). In brief, oocytes incubated for maturation were washed twice in TL-hepes followed by washing again with Tyrode's fertilisation medium containing 6 mg/ml of BSA (fatty acid free) and 0.25 mM pyruvate. Mature oocytes surrounded by expanded cumulus cells were randomly allocated to two groups and were added to 50 μl drops of fertilisation medium containing 10 μg/ml of heparin under mineral oil (10–15 oocytes per drop). The sperm suspension was added to the oocytes to yield a final concentration of 2×10^6 spermatozoa/ml. The oocytes and spermatozoa were co-incubated for 20 h at 39 °C, under 5% CO_2 in air.

Presumptive zygotes, washed free of sperm and cumulus cells, were transferred to 100 μl droplets of culture medium and co-cultured with buffalo oviductal epithelial cells (BOEC), under oil. On days 2 and 4 of culture, 25 μl of fresh equilibrated culture medium was added to the culture drops. Cleavage rates were determined after 48 h of culture and the rate of growth (proportion of cleaved embryos reaching the blastocyst stage) was determined on day 8 of culture.

In vitro production of cattle (C × C) and cattle–buffalo hybrid (C × B) embryos

Maturation of cattle oocytes

Cattle ovaries obtained from a slaughterhouse in Milan, Italy were transported to the laboratory of Instituto di Anatomia degli animali Domestici, Milan, Italy within 2–3 h of slaughter. Ovaries were washed

thoroughly in water and in normal saline prior to aspirating the COCs from the follicles on the surface of the ovaries with a vacuum pump attached to a tube and 18 G needle. The COCs, released in collection medium consisting of TCM-199 (Sigma) with 0.4% BSA (Sigma), 25 mM Hepes (Sigma) and 10 µg/ml of heparin (Sigma), were examined and those with at least three or four layers of cumulus and homogeneous oocyte cytoplasm were selected, washed twice in maturation medium (TCM-199 with 10% fetal calf serum supplemented with 0.1 g/l glutamine (Sigma), 0.1 µl/ml FSH/LH, 1.0 µg/ml estradiol (Sigma) and 10 µl/ml of insulin transferrin selinite–ITS (Sigma). The COCs were cultured in maturation medium in a 4-well plate (300 µl per well) for a period of 24 h at 39 °C in 5% CO_2 in air.

Sperm preparation and *in vitro* fertilisation

The procedure used for *in vitro* fertilisation of the matured cattle oocytes was as described by Gandolfi *et al*. (1997). Briefly, frozen sperm from a cattle bull and a buffalo bull, known for high *in vitro* fertility, were subjected to Percoll gradient separation to obtain the sperm-rich pellet as described above. The sperm suspension was adjusted to obtain a final concentration of 1×10^6 spermatozoa/ml. The fertilisation medium consisted of IVF-TALP supplemented with 1 µl of 100× PHE (penicillinase–hypotaurine–epinephrine) solution/100 µl of IVF medium. The COCs, incubated for 24 h for maturation, were washed twice in Hepes-TALP followed by two rinses in IVF-TALP before transferring them in groups of 20–25 oocytes to the 300 µl wells of fertilisation medium supplemented with PHE. Sperm were added to the fertilisation well and allowed to co-incubate with oocytes for a period of 18 h.

In vitro *culture*

The presumptive zygotes were washed free of the sperm and cumulus cells at the termination of incubation and washed twice in Hepes-TALP and twice in culture medium (IVC), made up of TCM-199 + 10% fetal calf serum supplemented with 0./1 g/l glutamine (Sigma), 2.1 G/l $NaHCO_3$ (Sigma). The zygotes were cultured in 50 µl drops of IVC supplemented with BOEC in 35 mm dishes overlaid with mineral oil. Cleavage rates were determined at 48 h of culture and blastocysts were harvested on day 8 of culture. On days 3 and 5, 25 µl of medium was added to the drops and, after pipetting and rinsing the embryos, the same amount was withdrawn without disturbing the embryos.

Penetration assay

The presumptive zygotes were removed from the fertilisation medium and fixed in 3:1 methanol:acetic acid overnight and then stained with aceto-lacmoid stain and examined under a phase-contrast microscope. The zygotes exhibiting two pronuclei including one with a sperm tail, were considered to be fertilised and normal while those with one pronucleus or three or more pronuclei were considered as unfertilised or polyspermic, respectively.

Cytogenetic analysis of embryos

The chromosome spreads from blastocysts were prepared as described previously (King *et al*., 1979). In brief, embryos were placed in 500 µl wells of culture medium containing 0.5 µl/ml of colcemid (Gibco BRL) and incubated for 5 h at 39 °C in a humidified atmosphere of 5% CO_2 in air. Each embryo was treated with 1.0% (w/v) sodium citrate and fixed on a glass slide. Slides were stained with 4% Giemsa for 4 min, air-dried and the chromosome spreads examined at ×1000.

Differential staining

At the end of the culture period the blastocysts, categorised according to stage of development (blastocysts, expanded blastocysts and hatched blastocysts), were harvested and differentially stained according to the procedure described by De la Fuente & King (1997) to distinguish the cells of the inner cell mass and the trophectoderm. Blastocysts were placed in 0.1% pronase in Ham's F10 to remove the zona pellucida prior to exposing them to 100 µg/ml of fluorescein isothiocyanate (FITC)-labelled wheat germ agglutinin (Sigma) in phosphate-buffered saline (PBS) for 20 min at 39 °C. Blastocysts were fixed at 4.0 °C in absolute ethanol for 10 min, before transferring them to PBT buffer (PBS (pH 7.4) + 0.4% BSA (w/v; Sigma) and 0.1% Tween-20 (Bio Rad, Hercules, CA) supplemented with 10 µg/ml of propidium iodide (Sigma) for 10 min. Embryos were rinsed in FITC solution for 30 s and fixed overnight in 100% ethanol at 4 °C. The fixed embryos were mounted with an anti-fading medium (DABCO, Aldrich Chemical, WI) on a clean glass slide, compressed under a coverslip and examined under a fluorescence microscope (Leitz-Aristoplan, AB0078) equipped with a dual rhodamine-FITC filter at 560 nm wavelength.

Statistical analysis

The cleavage and development rate were analysed by general linear mode analysis using SAS (1990). One-way ANOVA was used to evaluate the differences in the cleavage, development rates and the differences in the cell count of different embryonic cell lineages (Statistix 1.0, Analytical Software, 1996).

Table 1 Cleavage and blastocyst rates of buffalo and cattle control and cattle and water buffalo hybrid embryos

Source of oocyte	Source of sperm	Total no. of oocytes	No. of oocytes cleaved	Cleavage %	No. of Blastocysts	Blastocyst %
Buffalo (B)	Buffalo (B)	1703	991	58.2 (9.13[a])	294	29.6 (5.73[a])
Buffalo (B)	Cattle (C)	1302	531	40.8 (6.18[b])	47	8.8 (5.03[b])
Cattle (C)	Buffalo (B)	483	417	86.3 (8.70[c])	82/364	22.5 (9.23[a])
Cattle (C)	Cattle (C)	121	112	92.5 (2.87[c])	25	20.6 (3.39[a])

[a,b,c]Different superscripts in the same column combination with the same oocyte source indicate significant differences ($p<0.05$).
Values are expressed as mean (standard error).

Results

Buffalo oocytes fertilised with buffalo or cattle sperm

The pronuclear assay used to test for evidence of sperm penetration and fertilisation status of the presumptive zygotes was carried out on three random replicates at 18 h post-insemination (hpi). Thirty-three of the 52 (64%) oocytes evaluated exhibited two pronuclei with a sperm tail associated with one of the pronuclei in the buffalo control group (buffalo × buffalo), while 20 of 44 oocytes evaluated (44%) showed this feature in the hybrid group.

The cleavage and development rates, determined from 18 replicates of control and 14 replicates of hybrid embryos, are summarised in Table 1. The buffalo oocytes exposed to buffalo sperm (B × B) resulted in a significantly higher cleavage rate (58.2%) compared with buffalo oocytes exposed to cattle sperm (40.8%). Embryos cultured up to 8 days post-insemination, to determine the blastocyst rate, revealed that a significantly higher proportion of B × B embryos (29.6%) reached blastocyst stage while among the B × C hybrid group only 8.8% reached this stage. No indication of parthenogenetic development was observed in either group, and the fragmentation rate (after incubating oocytes without sperm) revealed less than 2% activation.

The chronology of development of hybrid and control embryos post-insemination is summarised in Fig. 1. The first three cleavage divisions occurred at comparable times (hpi) in control and hybrid embryos. Compaction was observed on day 5 (120 hpi) in B × B and at 5.5 days (126 hpi) in B × C embryos. Blastocysts were first observed on day 6 (144 hpi) in the B × B group and day 6.7 (162 hpi) in B × C embryos. Blastocyst hatching occurred early on day 7 in B × B while most of the blastocysts in the B × C group hatched late on day 7, or early on day 8 in culture. Morphologically B × B and B × C embryos were indistinguishable from each other at all times of development (Fig. 2).

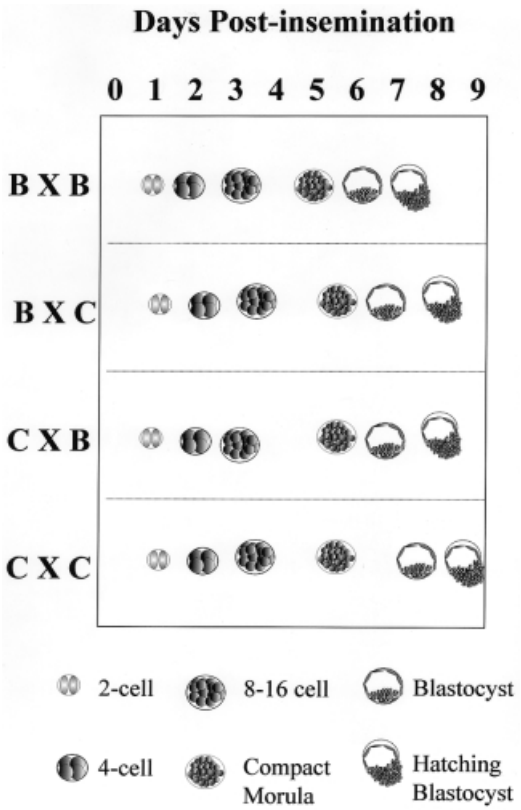

Figure 1 The chronology of development of buffalo (B × B) and cattle (C × C) and cattle–buffalo hybrids (B × C and C × B). The landmark stages from 2-cell to hatching reached by each embryo type are indicated for the 9 days of observation. Numbers (above) indicate days in culture.

Cytogenetic analysis of metaphase spreads of B × C blastocysts revealed 55 chromosomes with 3 to 5 bi-armed chromosomes in each spread. The metaphase plate of B × B embryos revealed 50 chromosomes indicating 5 pairs of bi-armed autosomes characteristic of river buffaloes (Basrur *et al.*, 1988). The presence of more than one bi-armed chromosome, confirming their hybrid status, was detected in all B × C embryos which reached the blastocyst stage (Fig. 3).

The number of embryos differentially stained to evaluate the distribution of cells in trophectoderm (TE)

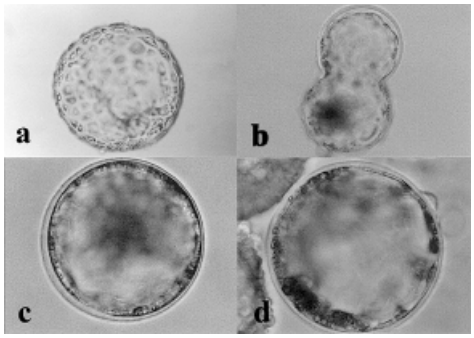

Figure 2 Representative images of buffalo, cattle and cattle–buffalo hybrid blastocysts: (*a*) buffalo × buffalo blastocyst; (*b*) buffalo × cattle hatching blastocyst; (*c*) cattle × buffalo blastocyst; (*d*) cattle × cattle blastocyst.

and inner cell mass (ICM) were different in the two groups. Since there were more buffalo (B × B) blastocysts, they could be divided into early, expanded and hatched blastocysts. The B × C embryos, on the other hand, were less abundant (n = 24) and hence were harvested and stained on day 8 without grouping them into subsets of blastocysts. Table 2 summarises the distribution of cells to inner cell mass and to trophectoderm in buffalo, cattle and hybrid embryos. Although the hybrid embryos reached the blastocyst stage almost 18–24 h later than the B × B embryos, the two cell lineages were readily identifiable in both types of hybrids. The buffalo (B × B) embryos showed a statistically significant difference (p<0.05) in the total cell number (TCN), depending on the stage of blastocyst development (expanded or hatched blastocyst). However, the ratio ICM:TCN in B × B embryos did not show any significant difference (p<0.05) according to blastocyst substage. The ICM:TCN ratio was higher, however, in B × B than in B × C hybrid embryos (0.39 vs 0.30).

Cattle oocytes fertilised with cattle or buffalo sperm

The cleavage rate for the hybrid group (C × B) was 86.3%, 25.94% of which developed to the blastocyst stage. In the group considered as control for this experiment (C × C), the penetration and cleavage rate were 94.0% and 92.5% respectively, with 22.32% of the embryos reaching the blastocyst stage (Table 1). Developmental landmarks reached by the C × B embryos were temporally similar to those of B × C embryos, with the first four cleavage divisions observed in 72–84 h post-insemination and compaction evident by day 5.5. Blastocysts were observed on day 6.7 and the hatching was completed by day 7 or 8 post-insemination (Fig. 1). On the other hand, the C × C group showed compaction on day 5, blastocysts by day 7 and hatching by day 9. Differential staining of C × B blastocysts on day 7 revealed TCN as 89.65 ± 23.7 and the ICM as 21.86 ± 6.9. The ICM:TCN ratio for C × B was 0.24 (Table 2).

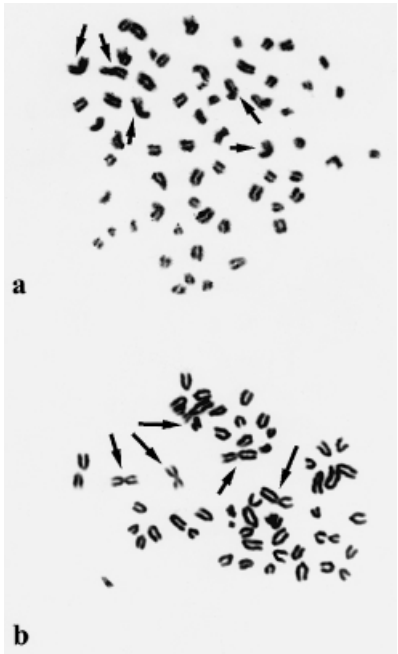

Figure 3 Chromosome spread of a buffalo × cattle hybrid blastocyst (*a*) and cattle × buffalo blastocyst (*b*) showing 5 bi-armed chromosomes (arrows).

Table 2 Differential cell count of hybrid embryos and their controls, at the blastocyst stage of development

Embryos	Stage of embryo	No. of embryos	TCN	ICM	TE	ICM:TCN ratio
B × C	BL	19	62.4 ± 10.3^{a}	19.1 ± 3.5^{a}	43.3 ± 8.2^{a}	0.30
C × B	BL	23	89.6 ± 23.7^{a,b}	24.9 ± 6.9^{a}	67.2 ± 15.4^{a,b}	0.24
B × B	BL	40	65.1 ± 9.1^{a}	25.5 ± 4.7^{a}	39.5 ± 6.5^{a}	0.39
C × C	BL	25	102 ± 12.6^{a,b}	21.8 ± 3.6^{a}	80.2 ± 9.9^{a,b}	0.21

TCN, total cell number; ICM, inner cell mass; TE, trophectoderm; B × C, buffalo × cattle; C × B, cattle × buffalo; B × B, buffalo × buffalo; C × C, cattle × cattle; BL, blastocyst.

^{a,b,c}Different superscripts in the same column indicate significant differences amongst each other (p<0.05).

Values are expressed as mean ± standard error.

Discussion

Fertilisation, cleavage and *in vitro* development of embryos up to the blastocyst stage were achieved with cattle oocytes fertilised with buffalo sperm (C × B) and buffalo oocytes fertilised with cattle sperm (B × C). However, the embryos displayed differences in the parameters examined depending upon their gametic origin.

Cleavage rates and development of buffalo (B × B) and cattle (C × C) embryos

In vitro fertilisation procedures are used routinely to produce 'transferable'-quality buffalo embryos (Madan *et al*., 1991; Totey *et al*., 1992, 1996); however, as with cattle, the rate of fertilisation and subsequent development vary considerably between attempts, probably depending on the quality of oocytes, maturation procedures and the medium used for fertilisation (Gordon & Lu, 1990; Totey *et al*., 1992, 1993). In studies using buffalo ovaries from the abattoir, cleavage rates range from 60% to 70% with subsequent rate of development to the blastocyst stage ranging from 20% to 30% (Suzuki *et al*., 1991; Totey *et al*., 1993). In our study, the rates of penetration, cleavage and development of embryos up to the blastocyst stage for buffalo oocytes exposed to buffalo sperm (B × B) were 65.1%, 58.2% and 29.6%, respectively. These results are remarkably similar to those previously reported (Madan *et al*., 1991; Totey *et al*., 1993), in spite of the fact that a rigorous selection of the source of oocytes was not possible in the present study due to the limited availability of buffalo ovaries. Penetration rate, cleavage and the proportion of embryos reaching the blastocyst stage for cattle oocytes under similar *in vitro* fertilisation conditions were 94.0%, 92.3% and 22.5% respectively (Gandolfi *et al*., 1997; Kochhar & King, 1998), suggesting that although these experiments were conducted in different laboratories using material from animals reared under different conditions, the culture system used in the present study for buffalo and cattle *in vitro* fertilisation was capable of supporting development at least up to the blastocyst stage at a rate comparable to that reported by other investigators (Gordon & Lu, 1990; Totey *et al*., 1993; Gandolfi *et al*., 1997; Kochhar & King, 1998).

Cleavage rates and development of hybrid embryos

Fertilisation and cleavage occurred in both types of hybridisation schemes. However, due to the availability of cattle and buffalo ovaries and the legal restrictions on the importation of semen, the same cattle and buffalo sperm and ovary pools could not be used in both experiments, thus preventing the direct comparison of the two hybrid types. The cleavage rate in C × B embryos (86.3%) was not significantly different from that in the simultaneously run (C × C) cattle controls (92.6%). However, in the B × C hybridisation scheme the cleavage rate obtained (40.8%) was significantly lower than that in the buffalo (B × B) controls run in parallel (58.2%). The proportion of cleaved embryos reaching the blastocyst stage was significantly different in the two schemes of hybridisation as well. Among the C × B hybrids 22.3% reached the blastocyst stage, whereas among the B × C hybrids only 8.8% reached that stage at the corresponding time. The percentage of B × C embryos reaching the blastocyst stage was also significantly lower than that in B × B embryos (85% vs 29.6%). In all these cases the hybrid status of the zygotes and embryos was confirmed at the pronuclear stage by virtue of a sperm tail attached to one of the pronuclei, and at later stages of development by the presence of 3 to 5 bi-armed chromosomes in hybrid blastocysts (bi-armed chromosomes in excess of one in the embryo spreads serve as unequivocal proof of their status as hybrids).

Developmental arrest at specific post-fertilisation stages has been detected as a consistent feature of cattle–sheep hybrid embryos (Kelk, 1997; Slavik *et al*., 1997). In the present study, 40.5% of B × C embryos were arrested at the morula stage while the proportion of other embryos arrested at this stage was lower (20.3%, 22.8% and 26.2%, respectively, for C × B, C × C and B × B embryos). It has been demonstrated that the arrest of cattle embryos at the 8-cell stage may be an indication of the inability of the culture environment to support the development of embryos through the time of activation of the embryonic genome (Eyestone & First, 1991). More recently, transition from the morula to the blastocyst stage has been achieved by the modification of culture medium: e.g. serum or glucose supplementation (Thompson, 1997). The culture conditions used for B × C hybridisation in the present study were those optimised for water buffalo (B × B) embryo development. It is conceivable that this medium was inadequate for releasing the B × C hybrid embryos from the block that retained them at the morula stage. However, preliminary efforts to supplement serum at the morula stage did not increase the proportion of B × C embryos reaching the blastocyst stage (data not shown), suggesting that the failure to reach subsequent stages of development may be due to factors other than deficient culture medium. It is interesting in this regard that the penetration and cleavage rates obtained with sheep oocytes exposed to goat sperm *in vitro* can be augmented by the addition of 20% oestrous sheep serum and 7.75 mM calcium lactate (Kelk, 1997). However, serum and calcium lactate supplementation did not improve the reciprocal scheme (Kelk, 1997). Similarly, Slavik *et al*. (1997) found that *in vivo* culture

of 2-cell stage cattle–sheep hybrid embryos was not able to overcome the developmental block in such embryos. These observations would suggest that the large proportion of B × C embryos blocked at the morula stage probably reflects their inherent inability to overcome this developmental block rather than an *in vitro* induced developmental failure.

Some of the B × C embryos (and their C × B counterparts) survived the block, reached the blastocyst stage on 7 day post-insemination and hatched by day 8. However, they were different from the C × C and B × B control group in these aspects. The hybrid embryos were temporally intermediate to cattle and buffalo embryos in reaching the blastocyst stage (i.e. delayed compared with buffalo controls and accelerated compared with cattle embryos).

The lower rates of cleavage in buffalo oocytes exposed to cattle sperm and the slower development of B × C embryos to the blastocyst stage compared with C × B and C × C embryos suggest a polarity in hybridisation and developmental potential. Differences in the success rate and a polarity in growth pattern among the reciprocal crosses are seen in interspecies hybridisations which are successful in one combination but achieve only limited success in the reciprocal scheme. Sheep and goat hybrids exhibit polarity both *in vivo* and *in vitro* (Kelk *et al*., 1997*a*; Kelk, 1997). When sheep oocytes were exposed to goat sperm *in vitro*, fertilisation rates were higher than in the reciprocal scheme and the cleavage and blastocyst rates were 67.9% and 45.0% respectively (Kelk, 1997). However, in goat × sheep hybridisation the corresponding values were much lower (46.1% and 16.2%) as in our B × C hybrid embryos. These differences in hybridisation potential are manifested early during the post-fertilisation process and at the morula stage when blastocoele development and/or cavitation of compacted morula are initiated. On the other hand, C × B hybrid embryos do not appear to experience the impediments to the same degree, either at the time of fertilisation or during post-fertilisation development.

Cell counts and differentiation of embryonic lineages

Success of *in vitro* fertilisation and viability of early embryos is generally gauged by the proportion of embryos reaching the blastocyst stage, which represents the initiation of embryonic differentiation and in many species the stage of pre-implantation (or elongation) changes (Petters, 1992). The inner cell mass (ICM) and trophectoderm (TE) represent the two distinct cell lineages which can be assessed to evaluate whether the contribution to ICM is adequate to initiate the pregnancy (Bavister, 1996; van Soom *et al*., 1997). Embryos lacking an ICM or with low ICM cell counts do not survive implantation or attachment (Bavister, 1996). Using a protocol developed in our laboratory (De la Fuente & King, 1997) for differential staining of ICM and TE cells we were able to assess the ICM:TCN ratio in the control (B × B) blastocysts categorised into substages of development (blastocyst, expanded blastocyst and hatched blastocyst). The TCN obtained for the early B × B blastocysts (65.1 ± 9.14) is consistent with the 52–65 cells at blastocyst stage in previous reports (Totey *et al*., 1996; Narula *et al*., 1996). However, the ICM:TCN ratio in our study (0.33) varied, being 0.39 in early blastocysts, 0.29 in expanded blastocysts and 0.32 in hatched blastocysts. Narula *et al*. (1996) reported a higher proportion of ICM cells in early buffalo blastocysts with a decrease in subsequent stages. Our observations would suggest that the proportion of cells devoted to the trophectoderm fluctuates as buffalo blastocyst development progresses. On the other hand, cattle blastocysts show a higher total cell count (average 102 cells) with a lower ICM:TCN ratio (0.21) compared with those of buffaloes (De la Fuente & King, 1997; Kochhar & King, 1998). Furthermore, the proportions of cells allocated to ICM in cattle blastocysts, unlike those in their buffalo counterparts, do not change between different substages.

The TCN of B × C embryos was comparable to that of the B × B (control) embryos at the early blastocyst stage. However, their ICM:TCN ratio was significantly ($p<0.05$) lower (0.30 vs 0.39). The reciprocal hybrids (C × B) showed a higher TCN value (89.65 ± 23.7) which was closer to that of the C × C embryos (TCN = 102.0 ± 12.6) at a comparable stage. The ICM:TCN ratio of C × B hybrid embryos (0.24) was also similar to that of the C × C embryos (0.21). In other words, while the hybrid blastocysts showed clearly established contributions to TE and ICM, they differed in total cell counts, which in C × B hybrids were similar to that of the cattle (C × C) embryos. It would appear that the TCN in the hybrids is influenced by the source of oocytes and that the similarity of B × C hybrids to B × B blastocysts and C × B hybrids to C × C blastocysts may be due to an oocyte-encoded factor. It is not known whether this is a unique feature of bovid hybrids or a feature of all *in vitro* derived hybrids since, to our knowledge, no similar studies have been carried out with other interspecies hybrids.

In conclusion, our studies have shown that interspecies fertilisation using cattle and water buffalo gametes can successfully be carried out *in vitro*. The resulting hybrid embryos develop to the blastocyst stage and their rate of growth and overall cell number reach a level intermediate between that of cattle and buffalo controls. Our observations further indicate that cleavage rate, viability of embryos post-fertilisation, and the overall growth of the embryos evidenced by total cell number in the blastocysts, are influenced by

the source of oocytes rather than the embryonic genome. Although this study did not evaluate the fate of hybrid embryos beyond the hatched blastocyst stage, the parameters examined, including the presence of cells in adequate number in the inner cell mass of embryos that survived to the blastocyst stage, point to the viability of these pre-attachment hybrid embryos.

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