



Development of caprine chimeric embryos reconstructed through ES-tetraploid complementation assay

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The first mouse embryonic chimeras were generated independently by Tarkowski through aggregating 2 eight-cell embryos (Tarkowski 1961). Since then, chimerism has been experimentally induced in many species of mammals including sheep (Butler *et al.* 1987), goats (Jia *et al.* 2008), bovine (Razza *et al.* 2016), pigs (Nagashima *et al.* 2004) and cattle (Saito *et al.* 2003).

Attempts to improve chimeric embryo production by ES-tetraploid complementation have met with little success. As RVCL and mCR2aa are widely used for embryo culture media for parthenogenetically activated and IVF derived embryos (Kharche *et al.* 2011, Kharche *et al.* 2016, Pathak *et al.* 2017), this prompted us to evaluate the developmental potency of chimeric embryos in RVCL and mCR2aa +10% FBS. To the best of our knowledge, the effects of readymade/complex culture medium and chemically defined medium on developmental potency of chimeric goat embryos have not been reported.

Collection of ovaries, recovery of oocytes and *in vitro* maturation (IVM) was done as per the method of Kharche *et al.* (2016). *In vitro* fertilization was carried out as per the method described by Kharche *et al.* (2011) with slight modifications. For tetraploid embryo production, fertilized two cell embryos (609) were equilibrated in fusion buffer (0.3 M mannitol solution containing 0.1 mM MgSO₄·7H₂O, 0.05 mM CaCl₂·2H₂O, 0.5 mM HEPES and 1 mg/ml BSA) for 5–10 sec before they were placed into a 0.5 mm gap BTX micro slide fusion chamber (BTX, ECM 2001) filled with fusion buffer in such a manner that the inter-blastomeric axis was positioned in parallel to the electrodes. Two-cell embryos were aligned between electrodes using an AC field of 5V and 5s, and fusion of blastomeres was induced at 38.5°C by a single DC pulse for 1.2 kV/cm for 4µs with 3s post fusion AC. After the electric pulse, the fused embryos were scored and cultured in RVCL media supplemented with 1% BSA for 48 h in humidified atmosphere of 5% CO₂ at 38.5°C in CO₂ incubator.

Collection of ovaries, recovery of oocytes and *in vitro*

maturation (IVM) was done as per method described for the production of tetraploid fertilized embryos. After 27 h of *in vitro* maturation, denuded oocytes (n=2628) were then chemically activated with 5 µM calcium ionophore for 5 to 7 min followed by 4 h horticulture in embryo development medium (RVCL) containing 2 mM DMAP. After 4 h of culture, the oocytes were washed and cultured in RVCL. These embryos were developed up to expanded to hatched blastocyst stage in embryo development medium in humidified atmosphere at 38.5°C with 5% CO₂ in CO₂ incubator.

For stem cell production, inner cell mass (ICM) was mechanically isolated from hatched blastocysts and were cultured on Mitomycin-C inactivated goat fetal fibroblasts feeder layer in stem cell culture medium. Subsequent colonies were passaged every 4–5 day and media was replaced after every 24 h.

A pair of zona-free tetraploid embryos and one clump of pESCs at passage 2 and 3 were randomly distributed for aggregation in different culture media. In Gr 1 the aggregates were cultured on granulosa cumulus cells monolayer (GC) in RVCL in humidified atmosphere at 38.5°C with 5% CO₂ in CO₂ incubator while in Gr 2 aggregates were cultured on granulosa cumulus cells monolayer (GC) in mCR₂aa medium (10% FBS) in humidified atmosphere at 38.5°C with 5% CO₂ in CO₂ incubator.

A total of 609 cleaved two-cell caprine IVF embryos were subjected to electrofusion at 1.2 kV/cm for 4µs to produce tetraploid embryos. The percentage of fusion was 85.26±1.55% and fused embryos were further developed upto 4-cell stage as 4-cell embryos formed were utilized for chimeric embryo production. Cleavage rate was 57.89±2.60% and the percentage of 2-cell and 4-cell embryo were 23.27±3.28 and 71.31±3.78, respectively.

The embryo development among RVCL (Gr 1) and mCR2aa medium (Gr 2) was compared. In Gr 1, the percentage of aggregation efficiency, aggregates at 8–16 cell, morula and blastocyst were 83.33±6.00%, 42.24±7.71%, 29.59±7.64% and 11.49±5.36%, respectively while in Gr 2, the percentage of aggregation efficiency, aggregates at 8–16 cell, morula and blastocyst (Fig. 1A-F) were 91.66±4.32%, 54.76±7.75, 22.32±5.75 and 14.58±5.18% (Table 1).

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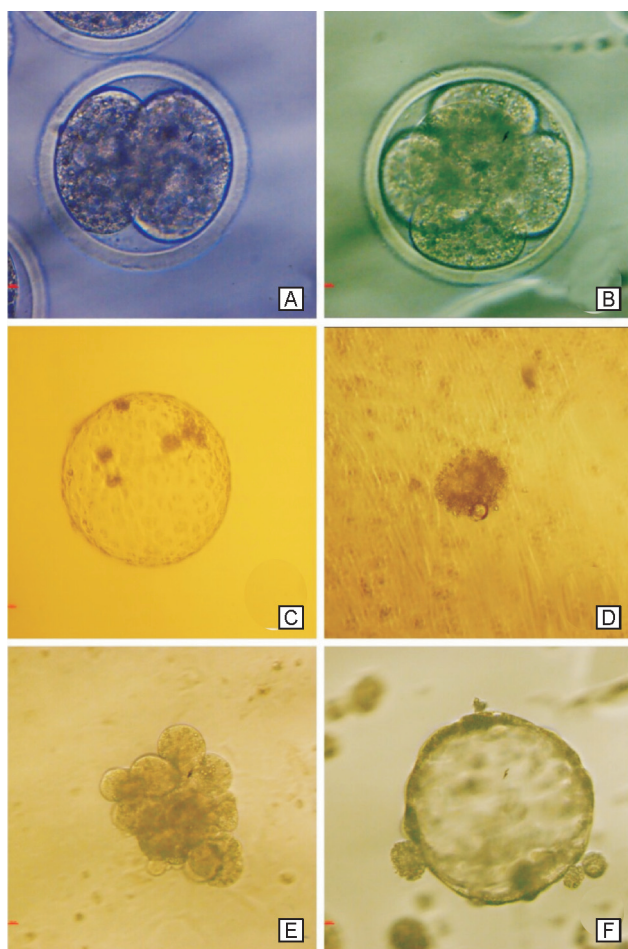


Fig. 1(A-F). Generation of chimeric embryos. **A.** *In vitro* fertilized 2-cell embryos (2n), **B.** Tetraploid morula (4n), **C.** Parthenogenetic hatched blastocyst (2n), **D.** Parthenogenetic pESCs (2n), **E.** Aggregate of pESCs (2n) and tetraploid morula (4n), **F.** Chimeric blastocyst.

The composition of the embryo culture medium can directly influence embryo developmental capacity and blastocyst gene expression. Readymade/complex culture medium *in vitro* embryo production systems are believed to provide necessary growth factors and (or) metabolize embryo toxic factors inhibitory to development while for defined medium, stimulatory effects of serum supplementation (Gutierrez-Adan *et al.* 2001), essential and non-essential amino acids on rates of embryonic development are well described. The supplementation of basal culture media with animal serum of different origins is essential for cell growth, metabolism, and to stimulate proliferation. Fetal bovine serum (FBS) is a cocktail of most of the factors required for cell attachment, growth and

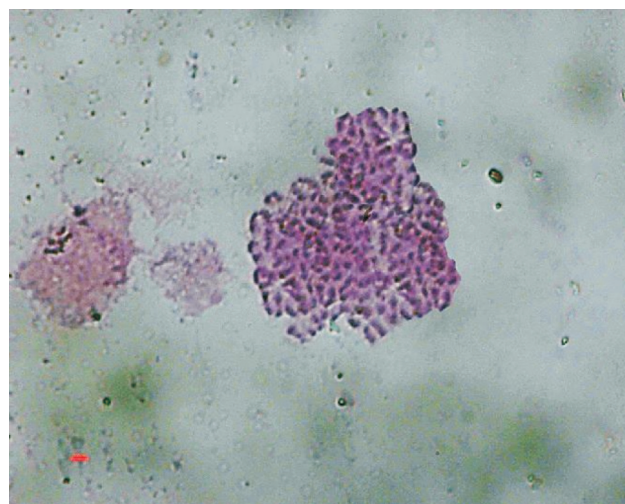


Fig. 2. Tetraploid chromosome.

proliferation and is thus used as an almost universal growth supplement effective for most types of human and animal cells.

Our results indicate that the aggregate efficiency, 8–16 cell stage and blastocyst formed in mCR₂aa + 10% FBS were comparatively higher than in RVCL media while morula formation in mCR₂aa + 10% FBS was comparatively lower than in RVCL media. Higher number of morula in RVCL media might be due to the fact that more number of embryos got arrested at morula stage and did not develop to blastocyst while in mCR₂aa + 10% FBS more number of embryos cleared embryonic arrest at morula stage and developed to blastocysts. It can be concluded that both the media support the development of chimeric embryos up to blastocyst stage.

SUMMARY

The aim of the present study was to evaluate the development of caprine chimeric embryos in different culture media, viz. RVCL and mCR₂aa + 10% FBS. Inner cell mass (ICM) from hatched blastocysts of parthenogenetic activated embryos were used to produce ES cell-like cells (103) while 4-cell embryos obtained from IVF were utilized to produce tetraploid embryos (231). The aggregates prepared were randomly divided into 2 groups, viz. Gr 1 (RVCL) (53) and Gr 2 (mCR₂aa + 10% FBS) (50) followed by culture in humidified atmosphere of 5% CO₂ at 38.5°C in a CO₂ incubator. Outcome measures were aggregation, 8–16 cell, morula and blastocyst formation. The percentage of aggregation, 8–16 cell, morula and blastocyst in Gr 1 (RVCL) was 83.33±6.00%, 42.24±7.71%,

Table 1. Es-tetraploid complementation assay in different culture media

Group	No. of tetraploid embryos	No. of aggregates	Aggregation efficiency (%)	Aggregates at 8–16 cell stage (%)	Morula (%)	Blastocyst (%)
RVCL	113	53	43 ^a (83.33±6.00%)	21 ^a (42.24±7.71%)	16 ^a (29.59±7.64%)	6 ^a (11.49±5.36%)
mCR ₂ aa	118	50	46 ^a (91.66±4.32%)	27 ^a (54.76±7.75%)	12 ^a (22.32±5.75%)	7 ^a (14.58±5.18%)

Values within different superscripts in the same column are significantly different (P<0.05).

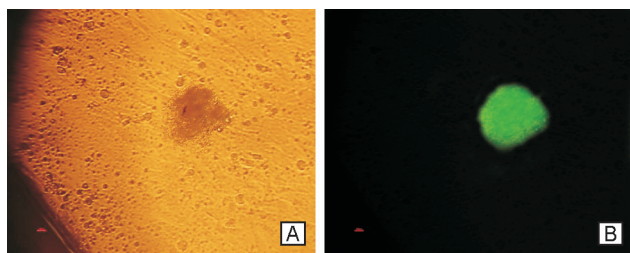


Fig. 3 (A-B). A. Parthenogenetic embryonic stem cell colony, B. Immunofluorescence of pESCs with surface marker SSEA-4.

29.59±7.64% and 11.49±5.36%, respectively while the percentage of aggregation, 8–16 cell, morula and blastocyst in Gr 2 (mCR2aa +10% FBS) was 91.66±4.32%, 54.76±7.75%, 22.32±5.75% and 14.58±5.18%, respectively. In conclusion, both the media supported the development of chimeric embryos up to blastocyst.

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