

Effect of hypoxia on production of caprine chimeric blastocyst

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Received: 2 September 2020; Accepted: 29 August 2022

Keywords: Caprine, Chimeric embryo, Embryonic stem cells, Hypoxia, *In vitro* fertilization, Tetraploid embryo

Parthenogenesis is common in invertebrates, but has been also reported in about 70 lower vertebrate species that produce offspring. It can be a viable alternative method of producing the identical female and thus faster multiplication of a few superior female animals can be achieved. Parthenogenetic blastocysts have been implanted in caprine (Kharche *et al.* 2014), but no live offspring is obtained till date as whole parthenogenetic blastocyst is not able to develop to full term fetus due to paternal gene deficiency. Reports in mouse showed that when parthenogenetic embryonic stem cells are complemented with fertilized tetraploid embryos, the chimeric embryo developed with pES genome. However, parthenogenetic goat chimeras constructed by fertilized tetraploid embryos, and parthenogenetic embryonic stem (pES) cells and tetraploid embryos have apparently not been reported.

The effects of hypoxia on embryo development have been the subject of many reviews, but its effect on chimeric embryo development biology has received no attention. To the best of our knowledge, we describe for the first time, the influence of hypoxia on development of chimeric embryos formed from tetraploid complementation assay. So, our primary aim was to evaluate the effect of the two different O₂ concentrations on the chimeric embryo development from tetraploid complementation assay in terms of aggregation efficiency, and blastocyst formation.

Tetraploid fertilized embryos production: 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 (IVF) was carried out as per the method described by Kharche *et al.* (2011) with slight modifications.

For tetraploid embryo production, fertilized two cell embryos (644) (Fig. 1A) 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 5 V and 5 sec, and fusion of blastomeres was induced at 38.5°C by a single DC pulse for 60 V for 4 µs with 3 sec post fusion AC. After the electric pulse, the fused embryos were scored and further cultured in RVCL media supplemented with 1% BSA for 48 h in humidified atmosphere of 5% CO₂ at 38.5°C in CO₂ incubator.

Putative parthenogenetic embryonic stem cell production: Collection of ovaries, recovery of oocytes and IVM was done as per method described for the production of tetraploid fertilized embryos.

After 27 h of IVM, COCs with expanded cumulus were treated with 0.1% hyaluronidase enzyme for removal of cumulus cells. Denuded oocytes were then selected and chemically activated with activation agent 5 µM calcium ionophore for 5 to 7 min followed by 4 h culture 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 blastocyst stage in embryo development medium in humidified atmosphere at 38.5°C with 5% CO₂ in CO₂ incubator.

For production of ESCs, inner cell mass (ICM) was mechanically isolated from hatched parthenogenetic blastocysts (Fig. 1C) produced and were cultured on Mitomycin-C inactivated goat fetal fibroblasts feeder layer in stem cell culture medium. Subsequent colonies (Fig. 1D) were passaged every 4–5 day and media was replaced after every 24 h. Characterization of goat ES-like cells colonies was performed with alkaline phosphatase staining kit (Sigma Chemical Co., USA). The expression of surface marker SSEA-3 and intracellular marker (OCT4) was examined by carrying out immunofluorescence staining with ES cells marker sample kit (Merck Life Science Pvt Ltd., India).

Tetraploid complementation: A pair of zona-free tetraploid embryos and one clump of pESCs at passage 2 and 3 were randomly distributed for aggregation in two different O₂ concentrations.

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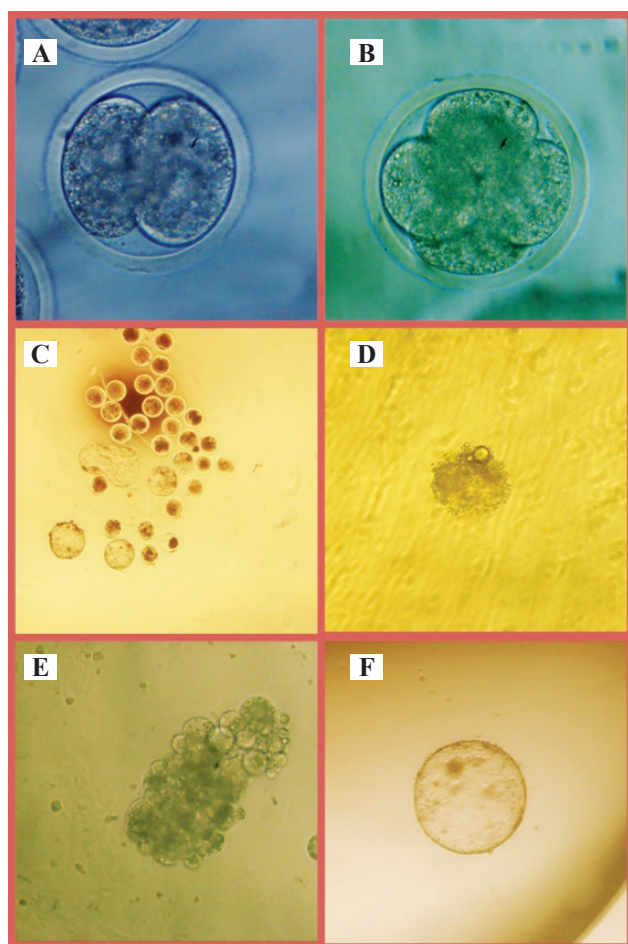


Fig. 1. Production of chimeric embryos. **A.** *In vitro* fertilized 2 cell embryos (2n), **B.** *In vitro* fertilized 4 cell (4n), **C.** Parthenogenetic hatched blastocysts (2n), **D.** Parthenogenetic pESCs (2n), **E.** Aggregate (Morula) and **F.** Chimeric blastocyst.

Group 1 (control): Clump of tetraploid embryos and pESCs for production of chimeric embryos through tetraploid complementation were cultured on granulosa cells monolayer in mCR2aa medium (10% FBS) in humidified atmosphere at 38.5°C with 5% CO₂ and 21% O₂ in CO₂ incubator.

Group 2 (5% O₂): Clump of tetraploid embryos and pESCs for production of chimeric embryos through tetraploid complementation were cultured on granulosa cells monolayer in mCR2aa medium (10% FBS) in humidified atmosphere at 38.5°C with 5% CO₂ and 5% O₂ in CO₂ incubator.

After morula formation (Fig. 1E), embryos were cultured in 50 µl drops RVCL covered with mineral oil for blastocyst formation (Fig. 1F).

Statistical analysis: Aggregation, 8-16 cell, morula and blastocyst rates between different treatment groups were compared using ANOVA method with SPSS software (16.1 version). The inter-group differences with $p < 0.05$ were considered to be statistically significant.

A total of 644 cleaved two-cell caprine IVF embryos were subjected to electrofusion at 1.2 kV/cm for 4 µs to

produce tetraploid embryos (Pathak *et al.* 2020). The percentage of fusion recorded was $91.44 \pm 1.22\%$ and fused embryos were further developed upto 4 cell stage as 4 cell embryos formed were utilized for chimeric embryo production. Cleavage rate calculated was $61.48 \pm 3.68\%$ and the percentage of 2 cell and 4 cell embryo were $30.11 \pm 2.85\%$ and $69.88 \pm 2.85\%$, respectively.

The aggregation efficiency, percentage of aggregates at 8-16 cell, morula and blastocyst stage among Group 1 (control), Group 2 (5% O₂) were compared. Aggregation efficiency observed in Group 1 and Group 2 was $91.66 \pm 4.04\%$ and $96.59 \pm 2.83\%$, respectively. Furthermore, percentage of aggregates that reached 8-16 cell stage in Group 1 and Group 2 was $49.07 \pm 5.08\%$ and $51.89 \pm 9.14\%$, respectively while percentage of aggregates that reached morula stage in Group 1 and Group 2 was $25.92 \pm 6.05\%$ and $16.28 \pm 5.52\%$, respectively. Blastocyst formation in Group 1 and Group 2 was $16.66 \pm 4.95\%$ and $28.40 \pm 8.10\%$, respectively (Table 1).

Embryo development depends on a variety of factors, including culture media, incubation volume, embryo density and reduced oxygen tension (Gardner 2008). The interaction between culture medium and oxygen concentration is not surprising, likely attributable to redox status and altered metabolic activity.

An aim of chimeric embryos is to overcome the challenges in successful birth of parthenogenetic offspring, and existing evidence suggests that cells cultured in 21% oxygen can change its properties when transferred into the low pO₂ environment in the surrogate mother. Equally, if the aim is to understand the *in vivo* behaviour of chimeric embryos, then experiments carried out at low, physiological pO₂ are likely to be necessary. In any case, it is desirable that reporting of how oxygen is provided to chimeric embryos in culture should be more detailed.

A better blastocyst outcome and a significant improvement in pregnancy and birth rates were found with low oxygen concentrations in various animal species, e.g. pig (Berthelot and Terqui 1996), mouse (Feil *et al.* 2006), bovine (Gaspar *et al.* 2015), human (Kirkegaard *et al.* 2013), rabbit (Li and Foote 1993) and goat (Nasar and Rahman 2012) compared with 20% oxygen conditions. These data challenged us to investigate the impact of low oxygen concentrations on chimeric embryo development. We monitored all the stages, from aggregate formation until blastocyst formation with each of the two oxygen concentrations.

We describe comparatively higher aggregation efficiency and aggregate formation at 8-16 stage with culture in 5% O₂ as compared to 21% O₂ while morula formation with 5% O₂ was comparatively lower than 21% O₂. Significantly more surplus embryos reached the blastocyst stage when cultured in 5% O₂ compared to 21% O₂ concentration. Higher number of morula with 21% O₂ might be due to the reason that more number of embryos got arrest at morula stage and did not develop to blastocyst while with 5% O₂ more number of embryos cleared embryonic arrest at morula stage and developed to blastocysts (Table 1).

Table 1. Effect of oxygen concentration on production of chimeric embryo

Group	No. of tetraploid embryos	No. of aggregates (n)	Aggregation efficiency n (%)	Aggregates at 8-16 cell stage n (%)	Morula n (%)	Blastocyst n (%)
Group 1 (21% O ₂)	116	54	50 ^a (91.66±4.04%)	28 ^a (49.07±5.08%)	12 ^a (25.92±6.05%)	10 ^a (16.66±4.95%)
Group 2 (5% O ₂)	106	50	48 ^a (96.59±2.83%)	23 ^a (51.89±9.14%)	10 ^a (16.28±5.52%)	15 ^b (28.40±8.10%)

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

Unfortunately, accurately controlling pericellular pO₂ is technically challenging. The first problem is the time required for equilibrating a cell-culture medium previously exposed to atmospheric pO₂ with a gas phase at a lower pO₂. Routine laboratory practice is to change the medium and to passage the cells in a laminar flow hood under atmospheric pO₂. The gas trapped inside the culture vessel is initially at the atmospheric pO₂, and equilibration with the low pO₂ gas phase can take several hours. Most of this problem can be overcome by equilibrating the culture medium at the desired pO₂ before adding it to the cells. If these precautions are not taken, not only is there an 'oxygen shock' associated with changing the medium but also the time during which the cells were actually exposed to the low oxygen tension is shorter than the time they were in the incubator.

In conclusion, the use of low oxygen concentration (5%) in the chimeric embryo culture results in better aggregation efficiency and blastocyst production.

SUMMARY

This study was conducted to evaluate the effect of 5% and 21% oxygen concentrations on chimeric embryo development. Inner cell mass (ICM) from parthenogenetic activated blastocyst were used to produce ES cell-like cells. A pair of zona-free tetraploid embryos and one clump of pESCs at passage 2 and 3 were aggregated on granulosa monolayer in such a manner that the clump of pESCs was sandwiched between the tetraploid embryos. The aggregates prepared were randomly divided into two groups viz. Group 1 (21% O₂) (n=54) and Group 2 (5% O₂) (n=50) followed by culture in humidified atmosphere of 5% CO₂ at 38.5°C in a CO₂ incubator. Aggregation rate and embryo quality was similar in both the groups. The percentage of aggregation, 8-16 cell, morula and blastocyst Group 1 (21% O₂) was 91.66±4.04%, 49.07±5.08%, 25.92±6.05% and 16.66±4.95%, respectively while the percentage of aggregation, 8-16 cell, morula and blastocyst Group 2 (5% O₂) was 96.59±2.83%, 51.89±9.14%, 16.28±5.52% and 28.40±8.10%, respectively. The aggregates and 8-16 cell stage embryos formed in Group 2 (5% O₂) were comparatively higher than in Group 1 (21% O₂) while morula formation in Group 2 (5% O₂) was comparatively lower than in Group 1 (21% O₂). Also, Group 2 (5% O₂) had significantly more blastocysts as compared to Group 1 (21% O₂). To conclude, culture under 5% O₂ leads to improved aggregation and also enhances the production of blastocysts rather than with atmospheric oxygen concentrations (21%) during embryo incubation.

ACKNOWLEDGEMENT

The authors are very thankful to the ADG, National Agricultural Science Fund, New Delhi for funding and the Director, ICAR-CIRG for providing necessary facilities to carry out this study.

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