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Original Research Article

# Screening of biotechnical parameters for production of bovine inter-subspecies embryonic chimeras by the aggregation of tetraploid *Bos indicus* and diploid crossbred *Bos taurus* embryos

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ABSTRACT

The aggregation of a tetraploid zebu embryo (*Bos indicus*, a thermotolerant breed) with a diploid taurine embryo (*Bos taurus*, a thermosensitive breed) should create a complete taurine fetus, whose extra-embryonic components, *e.g.*, the chorion, is derived mainly from the zebu embryo. These zebu-derived extra-embryonic components may interact positively with the taurine embryo/fetus during pregnancy in a tropical environment. We tested different parameters for the production of tetraploid Nelore (*Bos indicus*) embryos to be combined *via* aggregation with crossbred *Bos taurus* (diploid) embryos in order to produce viable chimeric blastocysts. Bovine (*Bos indicus* or crossbred *Bos taurus*) embryos were produced *in vitro* according to standard procedures. Two-cell *Bos indicus* embryos were submitted to electrofusion with varying numbers of pulses (1 or 2), voltages (0.4, 0.5, 0.75, 1.0, 1.4 and 5.0 kV/cm) and time (20, 25, 50 and 60 μs) to produce tetraploid embryos. Electrofused embryos were cultured with crossbred non-fused embryos to form chimeras that developed until the blastocyst stage. The best fusion parameter was 0.75 kV/cm for 60 μs. Four chimeric blastocysts (tetraploid Nelore with diploid crossbred Holstein) were formed after 31 attempts in 4 replicates (13%). We established an optimal procedure for the production of tetraploid *Bos indicus* (4n) embryos and embryonic chimeras by aggregation of

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crossbred *Bos taurus* (2n) with *Bos indicus* (4n) embryos. This technique would be valid in applied research, by producing exclusively taurine calves, but with placental elements from the *Bos indicus* breed, following transfer of these chimeras into recipient cows.



## 1. Introduction

The word ''chimera'' stems from Greek mythology, describing a creature composed of three distinct animals. Modern techniques of chimera production combine cells of more than one embryonic origin to make one genetically mixed embryo and animal. The first mouse embryonic chimeras were generated independently by Tarkowski and Mintz through aggregating two eight-cell embryos [1,2]. Since then, chimerism has been experimentally induced by micromanipulation of embryonic cells in many species of mammals including mice [3–6], rats [7], rabbits [8], sheep [9], goats [10], pigs [11–13] and cattle [14–16]. Interspecies mouse chimeras (*Mus musculus* and *Mus caroli*; [17]), goat–sheep chimeras [18,19] and bovine chimeras (*Bos taurus* and *Bos indicus* [20,21]) have played an important role in the study of genotype interactions and immunological factors in mother–fetus relationships [22–24].

Tetraploid cells rarely form embryonic structures, but they might complement the deficient extra-embryonic differentiation of embryonic stem (ES) cells while allowing full expression of their potential for fetal development. In this context, tetraploid embryos can be used to rescue embryonic lethality as a result of defective extra-embryonic phenotypes in mouse strains as well as a method of generating mice directly and exclusively from ES cells [25]. Spontaneous embryonic tetraploidy is rare and was first described in mice [26]. Hence, various methods have been used to induce tetraploidy in mammals either by the inhibition of cleavage at an early stage or by fusion of blastomeres. Inhibition of cleavage mitotic cycle of diploid blastomeres within two-cell embryos causes duplication of their genome (*i.e.*, tetraploidization) without occurrence of cell division, and has been performed using inhibitors of karyokinesis and cytokinesis, such as colchicine [27] and cytochalasin B [28,29], respectively. Additionally, blastomeres can be fused by polyethylene glycol [30,31], inactivated Sendai virus [32] or electric pulses [33,34]. Despite the wide variety of methods used not only for induction of tetraploidy in mammalian two-cell embryos, but also for somatic cell nuclear transfer (SCNT), alternating/direct current (AC/DC) pulse-mediated electrofusion of either blastomeres within intact 2-cell embryos [33,34] or even more frequently nuclear donor cell-ooplast couplets generated in different somatic cell cloning procedures [35–40] has developed into an effective established method.

Although tetraploid embryos can form blastocysts, their post-implantation development is impaired because of the absence of epiblast cells and the failure of embryos to survive beyond mid-gestation [41]. Tetraploid and diploid embryos can be aggregated/hybridized to make chimeras. Within this construction, tetraploid cells rarely contribute to the embryo itself (which is derived from the epiblast); rather, they contribute mainly to the hypoblast and the trophectoderm [29,42]. Therefore, chimerism of ES cells and tetraploid embryos has been used in the tetraploid complementation assay (TCA) for testing the impact of altered gene function on the lineage potency and the functional interaction between embryonic and extra-embryonic tissues [43]. Thus, TCA remains the most stringent assay for testing the pluripotency of putative pluripotent stem cells in mice.

To date, the experimental production of tetraploid embryos has yielded inconsistent results, due mainly to the variation among the parameters for pulse, voltage and time that are used for electrofusion [14,34,44–48]. Furthermore, the production of bovine chimeras by aggregation still provides poor results after transfer [15,21]. To improve our understanding of the conditions required for the electrofusion of bovine two-cell embryos, we aimed to primarily evaluate the effect of different numbers of electric pulses (1 or 2), different parameters of electric field strength (0.4, 0.5, 0.75, 1.0, 1.4 and 5.0 kV/cm) and different durations of single DC pulses (20, 25, 50 and 60 μs) on the fusion rates, cleavage activities and developmental competences to reach blastocyst stage for Nelore (*Bos indicus*) *in vitro*-produced (IVP) embryos. A second objective was to produce embryonic chimeras from these tetraploid embryos with crossbred Holstein (*Bos taurus*) and Nelore IVP embryos and assess the rates of aggregation and embryonic development.

This approach was used to develop an experimental model, in which, following pregnancy establishment, the euploid/polyploid chimeric fetus derived from tetraploid *Bos indicus* and diploid *Bos taurus* embryos is integrated with a chimeric chorion (exhibiting both DNA ploidies). The fetus and the resulting newborn singleton calf are derived exclusively from the diploid taurine embryo (*Bos taurus*, a thermosensitive breed), while the extraembryonic components, *e.g.*, the chorion, is derived mainly from the zebu embryo (thermoresistant breed). This model might allow for future research on the effects of specific placental components, *e.g.*, derived from a thermoresistant breed, which could be beneficial during the pregnancy of a European thermosensitive animal in tropical conditions, such as in South America.

## 2. Material and methods

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated.