Theriogenology 84 (2015) 1603–1610

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Phytohemagglutinin facilitates the aggregation of blastomere pairs from Day 5 donor embryos with Day 4 host embryos for chimeric bovine embryo multiplication

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ARTICLE INFO

Article history:
Received 11 June 2015
Received in revised form 3 August 2015
Accepted 22 August 2015

Keywords:
Chimera
Aggregation
Bovine
Lineage differentiation
Multiplication

ABSTRACT

Multiplication of bovine embryos by the production of aggregation chimeras is based on the concept that few blastomeres of a donor embryo form the inner cell mass (ICM) and thus the embryo proper, whereas cells of a host embryo preferentially contribute to the trophectoderm (TE), the progenitor cells of the embryonic part of the placenta. We aggregated two fluorescent blastomeres from enhanced green fluorescent protein (eGFP) transgenic Day 5 morulae with two Day 4 embryos that did not complete their first cleavage until 27 hours after IVF and tested the effect of phytohemagglutinin-L (PHA) on chimeric embryo formation. The resulting blastocysts were characterized by differential staining of cell lineages using the TE-specific factor CDX2 and confocal laser scanning microscopy to facilitate the precise localization of eGFP-positive cells. The proportions of blastocyst development of sandwich aggregates with ($n = 99$) and without PHA ($n = 46$) were 85.9% and 54.3% ($P < 0.05$), respectively. Epifluorescence microscopy showed that the proportion of blastocysts with eGFP-positive cells in the ICM was higher in the PHA group than in the no-PHA group (40% vs. 16%; $P < 0.05$). Confocal laser scanning microscopy revealed that the total cell numbers of blastocysts from the PHA group of aggregation chimeras ($n = 17$; 207.8 ± 67.3 [mean ± standard deviation]) were higher ($P < 0.05$) than those of embryos without ZP and exposed to PHA ($n = 30$; 159.6 ± 42.2) and of handling control embryos ($n = 19$; 176.9 ± 53.3). The same was true for ICM cell counts (56.5 ± 22.0 vs. 37.7 ± 14.2 and 38.7 ± 12.4) and TE cell counts (151.2 ± 58.0 vs. 121.9 ± 37.4 and 138.3 ± 53.0), whereas the ICM/total cell number ratio was not different between the groups. Of the 17 chimeric blastocysts analyzed by confocal laser scanning microscopy, nine had eGFP-positive cells (three of them in the ICM, three in the TE, and three in both lineages). When integration in the ICM occurred, the number of eGFP-positive cells in this compartment was 8.3 ± 2.3 (mean ± standard error of the mean). We conclude that PHA is advantageous for the formation of aggregation chimeras, but the approach tested in the present study with only two donor blastomeres and two host embryos did not result in multiplication of genetically valuable donor embryos.

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http://dx.doi.org/10.1016/j.theriogenology.2015.08.012

1. Introduction

Multiplication of superior genotypes by the production of embryo chimeras is based on the concept that pluripotent progenitor cells from a genetically valuable donor embryo form the inner cell mass (ICM) and later give rise to the embryo proper, whereas host embryos preferentially contribute to the trophectoderm (TE) and thus form extraembryonic tissues. Embryo chimeras may be produced by injection of donor cells into the blastocele cavity of a host blastocyst [1] or by aggregation of the donor cells with host embryos at an earlier developmental stage [2]. The injection into the blastocele cavity is mainly performed with embryonic stem cells and is therefore limited to species in which pluripotent stem cell lines exist. In ungulates, chimeras are mostly produced by aggregation of totipotent blastomeres or pluripotent ICM cells with host embryos at the morula stage [3–7].

Preferential differentiation of the host embryo cells into extraembryonic tissues and formation of the ICM by donor blastomeres may be achieved by asynchronous aggregation, in which the developmentally more advanced aggregation partner is more likely to contribute to the ICM [7]. Additionally, host embryos with chromosomal aberrations may be used as their cells are preferentially allocated in the TE [8]. In the mouse, the most widely used technique to produce such aggregation partners is electrofusion of the blastomeres at the two-cell stage, resulting in host embryos with a nearly uniform tetraploid state of all cells [9]. The outcome is less consistent after blastomere electrofusion of bovine two-cell embryos, resulting in a mixture of not only aneuploid, haploid, and polyploid but also diploid blastomeres in the developing embryo [7,10]. Several parameters such as timing of the first cleavage, initially determined to select the developmentally most competent embryos produced *in vitro*, can also be used to obtain host embryos with a preference to colonize the TE in aggregation chimeras as they also have a high percentage of blastomeres with an abnormal karyotype [11].

To avoid uncontrolled aggregation of the different embryos and to take advantage of the positive effects of group culture [12,13], aggregation chimeras at the morula stage can either be produced within the ZP of an aggregation partner [2] or by using a well of the well (WoW) culture dish, which allows individual culture of aggregates while maintaining positive group culture effects [14].

Phytohemagglutinin-L (PHA), a glycoprotein that binds to cellular surfaces by means of specific glycol conjugates [15], was already successfully used to facilitate blastomere aggregation in mouse chimeras [16], aggregation of SCNT-derived bovine embryos [4], and to optimize the SCNT procedure [17–19].

Here, we performed experiments to study the feasibility of multiplying selected bovine embryos by aggregating pairs of blastomeres from Day 5 morulae with two Day 4 host embryos which did not complete their first cleavage until 27 hours after IVF. We also investigated the effect of PHA on chimera formation and position of the donor cells in the chimeric blastocysts. Initial analyses by epifluorescence microscopy were validated by means of confocal laser scanning microscopy (CLSM).

2. Materials and methods

2.1. Chemicals

Unless stated differently, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. In vitro embryo production

All embryos were produced *in vitro* according to a standard procedure including IVM and IVF [20]. Briefly, follicles from ovaries collected at the local abattoir were aspirated and obtained cumulus–oocyte complexes (COCs) matured for 23 hours in modified TCM 199 (Invitrogen, Karlsruhe, Germany) supplemented with 0.0125 U/mL of LH, 0.025 U/mL of FSH (Sioux Biochemical, Sioux Center, IA, USA), and 5% estrous cow serum. All matured COCs were coincubated with 1×10^6 frozen–thawed sperm/mL from bulls with proven fertility, prepared by the swim-up method. For IVM and IVF, the COCs were incubated at 39 °C in a maximum humidified atmosphere of 5% CO_2 in air. After 20 hours of coincubation, presumptive zygotes were vortexed to remove remaining cumulus cells and transferred to synthetic oviductal fluid (SOF) supplemented with 5% estrous cow serum, 40 μL/mL of basal medium eagle's amino acids solution 50X (#B6766), 10 μL/mL of minimal essential medium nonessential amino acid solution 100X (#M7145) under mineral oil and cultured at 39 °C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

2.3. Embryo manipulation

All embryo manipulations were performed on a heated microscope plate set to 36 °C. Where not indicated differently, embryos were transferred to drops of HEPES-buffered Tyrode's lactose medium with 0.1% polyvinyl alcohol (TL-HEPES-PVA) for handling outside the incubator [21].

2.4. Zona pellucida removal

On Day 4 and 5 of embryonic development, the ZP was removed by treatment with 5 mg/mL of protease (#P5147) for 1 minute. Enzyme reaction was stopped by washing embryos in TL-HEPES-PVA supplemented with 10% fetal calf serum. Dissolved ZP was completely removed by gentle pipetting with a finely drawn glass pipet with sharp ends and an inner diameter of 80 μm.

2.5. Chimera production

Chimeras were produced from two ZP-free Day 4 host embryos (~16 cells) that did not complete their first cleavage until 27 hours after IVF and two blastomeres from a Day 5 enhanced green fluorescent protein (eGFP) transgenic embryo (donor) produced by using semen from an eGFP transgenic bull for IVF [22].

2.5.1. Sandwich technique

In the sandwich technique, first a host embryo was transferred to a depression of a 4 × 4 WoW culture dish