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Research Article

In vivo Transfer of Reconstructed Goat Chimeric Embryos Produced by Microinjection of Embryonic Stem Cells into Tetraploid IVF Embryos

Kharche SD*, Juhi Pathak, Anuj Kumar Singh Sikarwar, Chetna Gangwar, Priyadharsini R, Ravi Ranjan, Goel AK, Jindal SK, and Chauhan MS

Department of Animal Physiology and Reproduction, ICAR-CIRG, India

***Corresponding author**

Kharche SD, Department of Animal Physiology and Reproduction, ICAR-CIRG, Makhdoom P.O. Farah-28112, Mathura (UP) India, Tel: 919897987074; Fax: 915652763246; Email: kharche62@gmail.com

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Abstract

The aim of this study to observe in vivo development of a chimeric embryo (4n/2n) produced by tetraploid complementation. Cumulus oocyte complexes (COC's, 585) were collected and matured in maturation media. Out of which 524 matured oocyes were randomly divided into two groups. Group 1(n= 296) were activated with 5µM Calcium Ionophore for 5-7 minutes followed by treatment with 2.0 mM DMAP for 4 hr in mCR_2aa medium while Group 2 (n= 228) matured oocytes were used for *in vitro* fertilization. Furthermore, inner cell mass (ICM) from hatched blastocysts of parthenogenetic activated embryos were used to produce ES cell-like cells while 2 cell embryos obtained from IVF were used for their production of tetraploid embryos. Chimeric embryos were produced by microinjection of parthenogenetic ESCs in to *in-vitro* fertilized tetraploid caprine embryos. The percentage of 2 cell, 4 cell, 8-16 cell, morula, hatched blastocyst and cleavage rate of parthenogenetic goat embryos were 20.03 ± 5.09%, 20.02 ± 6.30%, 24.41 ± 4.12%, 25.61 ± 7.63%, 9.92 ± 3.97% and 72.78 ± 13.39%, respectively while the cleavage rate following IVF was 37.08 ± 4.34%. Furthermore, 75.55 ± 12.37% embryonic stem cell colonies were formed from hatched blastocysts. The IVF two cell embryo used for tetraploid embryo production, resulting in 75.09 ± 7.86% fusion rate and 86.5 ± 13.02% cleavage rate. A total of eight microinjected chimeric embryo were transfer in two naturally synchronized goat. The result indicated that caprine ES-like cells can be incorporated into IVF tetraploid embryos by microinjection so as to produce chimeric embryos. However, *in vivo* development of chimeric embryos could not achieve.

INTRODUCTION

Dairy goat is one of livestock species that provides milk and meat. It is also ideal for the transgenic production of therapeutic recombinant proteins [1]. In the last two decades there has been a great revolution in the field of biotechnology and embryo transfer technology has become a tool for enhancing lifetime productivity of livestock. *In vitro* embryo production is an 'attractive alternative' for the production of large number of embryos required for the rapid multiplication of superior germplasm of goats, upgrading a commercial or pure bred breeding programme and conservation of endangered breeds of goats [2].

Parthenogenesis is the biological phenomenon by which embryonic development is initiated without male contribution. The parthenogenetic activation of oocytes is an important tool to investigate the comparative roles of paternal and maternal genomes in controlling early embryo development [3].

Various experimental techniques have been developed over the years to maintain the full developmental potential of parthenogenetic embryos and one of the most important advances is the application of tetraploid embryos as host cells since tetraploid cells have only a limited potential in postimplantation development [4]. In earlier studies several ES cell lines were used to produce completely ES cell derived foetuses by aggregation with tetraploid morulae; however, the obtained ES foetuses died at birth [4]. Further studies demonstrated successful generation of viable and fertile ES mice which were derived exclusively from ES cells when early passage wild-type R1 [5], and TT2 [6], cells were used for aggregation with tetraploid morulae.

An alternative method, developed to reduce the damage induced by microsurgery, consisted of mechanical transfer of ESC into the embryo cytoplasm by drilling the zonapellucida (ZP).This approach includes the generation of 2n/4n chimeric embryos by microinjection of ES cells into ivf 4n embryos. Despite the

present inefficiency, the generation of viable parthenogenetic goat directly from ES cells has many advantages.

In the present study we describe a successful route towards the production of parthenogenetic goat by microinjection of ES cells into tetraploid IVF embryos. However, the goal to obtain viable parthenogenetic goat using 2n/4n complementation was not reached but this technique allows viable and fertile chimeric embryos.

MATERIALS AND METHODS

All organic and inorganic chemicals were purchased from Sigma Chemicals Co. except Research Vitro Cleave (RVCL) media from Cook Medical, Australia.

Parthenogenetic activation

Oocytes were collected from caprine ovaries obtained from an abattoir at Agra and were graded under the inverted phase contrast as per the method of [7]. Only grade A, B, C quality oocytes were selected and allowed to mature for 27 hr in humidified atmosphere of 5% CO_2 at 38.5^{0}C in a CO_2 incubator. Maturation medium consisted of TCM-199 containing L-glutamine (100 µg/ml), sodium pyruvate (0.25 mmol), gentamycin (50 µg/ml), FSH (5µg/ml), LH (10 µg/ml) and oestradiol-17β (1µg/ml), 10% FBS, 10% follicular fluid and 3 mg/ml BSA. To induce parthenogenetic activation, after removal of cumulus cells, denuded oocytes were activated with 5µM calcium Ionophore for 5-7 minutes followed by treatment with 2.0 mM DMAP for 4 hr in mCR$_2$aa medium. The embryos were then washed and cultured in embryo development medium (RVCL) followed by incubation in a CO_2 incubator at 38.5^0 C and 5% CO_2 in a humidified atmosphere. For cleavage rate, activated oocytes were observed after 48 hr under an inverted phase contrast microscope.

Embryonic stem cells production

Inner cell mass (ICM) was mechanically isolated from expanded and hatched blastocysts produced by parthenogenetic activation using microblade and were cultured on Mitomycin-C inactivated goat fetal fibroblasts feeder layer in stem cell culture medium. Passage one was performed upon primary colony formation. Subsequent colonies were passaged mechanically using a microblade every 4-5 day and media was replaced every 24 hr.

In vitro fertilization

In vitro fertilization was carried out as per the method described by [8], with slight modifications. Denuded oocytes after maturation were transferred in 50µl drops of Fert-TALP medium [9], containing 10% FBS, 8 mg/ml fatty acid free BSA and 50µg/ml heparin and the drops were inseminated with 15-20µl of the final diluted semen so as to obtain a sperm concentration of 1-2×10^6 sperm/ml. After *in vitro* insemination, the oocytes and sperm were co-incubated for 18 hr at 38.5^{0}C with 5% CO_2 in humidified atmosphere. After 18h of sperm-oocytes co-incubation, oocytes were washed and transferred in embryo development medium, Research Vitro Cleave (RVCL) media supplemented with 1% BSA for 48 hr in humidified atmosphere of 5% CO_2 at 38.5ºC in CO_2 incubator. The embryo development was observed under inverted phase contrast microscope up to 12 days.

Collection of two-cell embryos and electrofusion

In order to produce tetraploid embryos, the blastomeres of 2 cell diploid IVF embryos at the two-cell stage were fused after a short electric pulse. Briefly, two-cell embryos were equilibrated in 0.3 M mannitol solution for 5-10sec before they were placed individually between two platinum electrodes in 0.3 M mannitol. The blastomeres were fused following a short electric pulse at 1.2 kV/cm and 4µs using Electro cell manipulator BTX (ECM 2001). After the electric pulse, the fused embryos were scored and further cultured in 50 µl drops of RVCL media supplemented with 1% BSA for 48 hr in humidified atmosphere of 5% CO_2 at 38.5ºC in CO_2 incubator. The morulae were used for ESCs microinjections.

Microinjection of ES cells into tetraploid ivf embryos

The procedure was carried out as per Kharche et al. [10], with modifications on the stage of an inverted microscope (NIKON, Eclipse, TE 2000U) at 40x, 100x, and 200x magnification, using Hoffman Modulation Contrast (HMC) optics. The microscope had a camera (DXM 1200, Nikon Japan) that allowed the procedure to be followed on a computer monitor and for taking pictures to the computer. The microscope was equipped with three axis hanging joystick oil Hydraulic Micromanipulator (NT-88-V3, Narishige, Japan) and with microinjector (IM-9B, Narishige, Japan) and pneumatic injector (IM-9C, Narishige, Japan). The above micromanipulator was equipped for piezoxpert (Eppendorf AG 22331 Hamburg Germany) for smoother injection so it caused minimal damage to the zonapellucida of the embryo for optimum survival of embryos. Coarse adjustment was done at 40x magnification followed by fine adjustment at 200x magnification. 60 mm culture dish was used as a microinjection chamber. Two rows consisting of 10 µL droplets for IVF tetraploid morulae were placed right to the centre of the dish and two rows consisting of 10 µL droplets for embryonic stem cell colonies were placed on the left to the centre of the dish. These droplets were covered with mineral oil.

Briefly, an ESC like cell colony was picked up, into the injection pipette. This colony was pushed forward until it was near the tip of the pipette, the pipette was advanced quickly towards the droplet containing morula where it was held with holding pipette. The injection pipette containing ESC at the very tip of the pipette was injected in to the zonapellucida by applying piezo electric pulse.The micropipette was then pushed through the zonapellucida. When the pipette was well within the embryo, a gentle suction was applied followed by a sudden rush of cytoplasm into the pipette as the plasma membrane ruptured. Some cytoplasm was seen in the pipette and the ESC moved backwards into the pipette. The ESC and the cytoplasm were pushed back into the morula so that the ESC lies within the morula and pipette was then retracted from the morula. The injected morula was then released from the holding pipette. We routinely applied a light negative pressure to the pipette during its withdrawal. This appeared to increase the survival rate of the embryos [11]. The injected embryos were further cultured in 50 µl drops of RVCL media supplemented with 1% BSA for 48 hr in humidified atmosphere of 5% CO_2 at 38.5˚C in CO_2 incubator.

Embryo transfer

In vitro produced embryos were transferred in two non

descript Sirohi goats at naturaloestrus. Recipient was deprived of feed and water for 24 hand put under general anesthesia using xylazine (0.2 mg/kgbody weight) and ketamine (4.4 - 6.6 mg/kg body weight).The reproductive tract was exteriorized through a mid-ventralincision to allow visual confirmation of a corpus luteum/lutea on ovaries. Chimeric embryos were transferred surgically at the tip of uterine horn ipsilateral to the ovary containing corpus luteum of two naturally synchronized surrogate doe (4 embryos each).

RESULT AND DISCUSSION

Parthenogenetic activation and embryonic stem cell

The percentage of 2 cell, 4 cell, 8-16 cell, morula, hatched blastocyst and cleavage rate of parthenogenetic goat embryos were 20.03 ± 5.09%, 20.02 ± 6.30%, 24.41 ± 4.12%, 25.61 ± 7.63%, 9.92 ± 3.97% and 72.78 ± 13.39%, respectively. Furthermore, 75.55 ± 12.37% embryonic stem cell colonies were formed from hatched blastocysts.

In vitro fertilization and tetraploid embryos

The cleavage rate following IVF was 37.08 ± 4.34%. Furthermore, electrofusion of 2 cell embryo resulted in 75.09 ± 7.86% fusion rate and 86.5 ± 13.02% cleavage rate. The percentage of 2 cell, 4 cell, 8-16 cell and morula production of tetraploid embryos were 00 ± 00%, 11.33 ± 7.85%, 50.66 ± 14.54% and 38 ± 11.3%, respectively.

Microinjection of ES cells into tetraploid ivf embryos and embryo transfers

In this study 8 microinjected chimeric embryos were transferred into two goats as per the method of Kharche et al. [8]. Following transfer, none of the recipients were found pregnant at day 35th post transfer by ultrasonography. Tetraploid (4n) embryos are used to produce chimeras with embryonic stem cells (ESCs) and diploid (2n) embryos, which have been used to substitute microinjection of DNA and nuclear transfer for the creation of genetically modified animals [12,13].

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 [14]. 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 [15,16]. Therefore, chimerism of parthenogenetic ES cells and tetraploid embryos can been used in the tetraploid complementation assay (TCA) for production of parthenogenetic goat.

In mouse, ES-tetraploid chimeras were reported by Nagy et al. [4], and according to him the newborns were almost all completely ES-derived, as judged by CPI isozyme analysis, but tetraploid cells were found in the yolk sac endoderm and trophectoderm lineage. Although newborns failed to survive after birth, despite they had normal birthweight and anatomically they appeared normal. In domestic animals, chimeric calves from ES-like cells aggregated with tetraploid embryos were reported to have been born [17,18], however, ES like cells contributed to chimera formation only at a very low level.

CONCLUSION

In conclusion, the results presented here indicate that caprine ES-like cells can be incorporated into IVF tetraploid embryos by microinjection so as to produce chimeric embryos. However, *in vivo* development of chimeric embryos was not achieved.

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