Creation of Parthenogenetic Sheep Embryos: Preliminary Study

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The monoparental embryo (in other words, with only the maternal genome, termed a parthenogenote, or with only the paternal genome, termed an androgenote) is a powerful model to study the imprinting status of developmentally regulated genes – that is, genes that are expressed (turned on) only when inherited from one parent. Therefore, to use monoparental embryos for future study of placental development in normal and compromised pregnancies, the objective of this study was to test, validate and optimize the methodologies necessary to create parthenogenetic sheep embryos. Cumulus oocyte complexes (COC) were collected from nonpregnant and pregnant ewes and matured overnight in maturation medium. The oocytes were activated using ionomycin (a calcium ionophore) and 6-dimethylaminopurine (DMAP; a protein kinase inhibitor); further, a portion of the oocytes was activated in medium containing serum and another portion was activated in serum-free medium. After activation of oocytes in medium containing serum, 70 to 83% of the oocytes had cleaved (indicating activation of embryonic development) and 14 to 33% of the cleaved oocytes developed to the blastocyst stage. Activation of oocytes in serum-free medium resulted in minimal cleavage rates. Thus, parthenogenetic embryos should be created using activation medium containing serum. This study demonstrated that creation of parthenogenotes is feasible and that parthenogenetic embryos can be used in the future to study parentally imprinted genes.

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Introduction

The term parthenogenesis is defined as the production of an embryo from a female gamete without any contribution from a male gamete, with or without the eventual development into an adult. In fact, spontaneous parthenogenesis occurs in many species (Rougier and Werb, 2001). The use of monoparental embryos is a powerful model to study the imprinting status of developmentally regulated genes (Dean et al., 2001), but its use is limited due to nuclear non-equivalency. That is, mammalian chromosomes of both maternal and paternal origin are required for development to term. The monoparental embryo phenotype exhibits poor development of one derivate (embryonic or extraembryonic tissue), and overdevelopment of its counterpart, and therefore constitutes a unique model for studying defects in placentation and the epigenetic status of genes regulating placental function (Dean et al., 2001; Reik et al., 2003). Monoparental embryos can be experimentally produced by artificial activation of the egg (parthenogenotes, containing only maternal genes) or by micromanipulation of the sperm or male pronucleus (androgenotes, containing only paternal genes), and both have been successfully established in several species including sheep (Loi et al., 1998; Alexander et al., 2006; Ptak et al., 2006), cows (Lagutina et al., 2004; Matsushita et al., 2004), pigs (Prather, 2001), mice, primates, and various others (Yamano et al., 2000; Rougier and Werb, 2001; Edwards, 2007).

Several protocols have been used to successfully create parthenogenetic sheep embryos (Loi et al., 1998; Bogliolo et al., 2000; Alexander at al., 2006; Ptak et al., 2006). However, sheep parthenogenetic embryos have not been created in any American laboratory for further study. We hypothesized that in our experimental conditions we would be able to create ovine parthnogenotes using two types of activation medium. Therefore, the aim of this study was to test, validate, and optimize a protocol to obtain parthenogenetic ovine embryos for future studies of placental development in normal and compromised pregnancies.

Material and Methods

Oocyte collection and in vitro maturation

Ovaries were collected at slaughter from mature nonpregnant or pregnant Western range-type ewes (n = 20) of mixed breeds (predominantly Targhee x Rambouillet) or Romanov breed (n=3). Ovaries were transported to the laboratory in an incubator at 39°C. Cumulus oocyte complexes (COC) were isolated by opening each visible follicle with a scalpel blade and flushing it with oocyte collection medium [medium TCM199 with 2% fetal bovine serum (FBS), heparin and penicillin/streptomycin (P/S)]. Under a stereomicroscope, COC were recovered from the collection dish and transferred to a petri dish containing fresh collection medium without heparin. Cumulus oocyte complexes were then washed three times in maturation medium [TCM-199 containing 10% fetal bovine serum, ovine FSH (5 μg/mL; oFSH-RP-1; NIAMDD-NIH, Bethesda, MD, USA), ovine LH (5 μg/mL; oLH-26; NIADDK-NIH),

estradiol -17β (1 µg/mL; Sigma St. Louis, MO, USA), glutamine (2 mM; Sigma), sodium pyruvate (0.25 mM; Sigma), epidermal growth factor (10 ng/mL; Sigma), and P/S (Gibco, Grand Island, NY, USA); Grazul-Bilska et al., 2003, 2006; Luther et al., 2005; Borowczyk et al., 2006]. Three separate cultures performed at three different time points (October to December) were carried out. Oocytes were matured in vitro in maturation medium for 24 h at 39°C in 5% CO_2 and 95% air followed by cumulus cell removal using 1% (wt/vol) hyaluronidase (Type I, Sigma) in PBS. Oocytes were then transferred to equilibrated activation medium (TCM199 with P/S and with or without 2% FBS).

In vitro activation

A portion of the oocytes was activated in activation medium with 2% FBS and another portion was activated in activation medium without serum; both in the presence of ionomycin (5 µM; Sigma) for 5 min at 37 C. For washing, all oocytes were then transferred to culture medium consisting of synthetic oviductal fluid (SOF; Stenbak et al., 2001) containing bovine serum albumin, glutamine, MEM, and BME amino acids and P/S; the washing was followed by a 3 h incubation in culture medium containing 2 mM 6-dimethylaminopurine (DMAP, a protein kinase inhibitor; Sigma). Oocyte treatment with ionomycin and DMAP was performed in 95% air and 5% CO_2. After treatment, the oocytes were washed in culture medium twice.

Embryo culture

All activated oocytes were incubated at 37 C in 5% CO_2; 5% O_2; 90% N_2. After 24 h of incubation, cleaved oocytes (indicating embryonic development) were transferred to culture medium with glucose (1.5 mM), and incubated for 8 days at 37 C in 5% CO_2; 5% O_2; 90% N_2. Every second day the stage of embryonic development was evaluated and embryos were transferred to fresh culture medium with glucose.

Results

In three separate experiments, activation of oocytes in medium containing serum resulted in the creation of parthenogenetic embryos (Fig. 1). The rate of cleavage was 70% in culture 1, 73% in culture 2, and 86% in culture 3. The rate of cleavage of oocytes activated in medium without serum was less than 5%. The rate of blastocyst formation after activation in medium containing serum was 14% in culture 1, 22% in culture 2, and 33% in culture 3. None of oocytes activated in medium without serum developed to the blastocyst stage. Fig. 1 shows parthenogenetic embryos from the 8-cell to the blastocyst stage, from day 4 to 8 after activation.

Discussion

The present study demonstrated that creation of parthenogenetic embryos from oocytes obtained from North Dakota Western type and Romanov sheep is possible. Furthermore, our study has shown that activation medium should be supplemented with serum obtain high rates of embryonic development. Other researchers also obtained similar results, with high rates of oocyte activation manifested by cleavage and parthenogenetic embryonic development to the blastocyst stage in sheep (Loi et al., 1998; Bogliolo et al., 2000; Alexander at al., 2006; Ptak et al., 2006). By using a protocol similar to ours, the rates of cleavage ranged from 65 to 83% and blastocyst formation rates ranged from 27 to 58% (Loi et al., 1998; Ptak et al., 2006). When ionomycin treatment or electric stimulation was used followed by incubation with DMAP or cycloheximide, the rates of cleavage and blastocyst formation were also quite high (81 to 83 and 15 to 21%, respectively; Loi et al., 1998; Ptak et al., 2006). However, replacement of ionomycin with ethanol treatment resulted in decreased blastocyst formation (from 58% to 19%, respectively) but not cleavage rates (83 and 81%, respectively; Loi et al., 1998). Unfortunately, it is unclear if these researchers supplemented activation medium with serum (Loi et al., 1998; Ptak et al., 2006). Thus, addition of serum to the activation medium seems to be critical to obtain high yields of parthenogenetic sheep embryos in our laboratory.

In cows, activation of oocytes using ionomycin and DMAP or cycloheximide plus cytochalasin B resulted in 93.6% or 77.5% cleavage, and 14% or 6% blastocyst formation rates, respectively (Lagutina et al., 2004). In addition, replacement of ionomycin with calcium ionophore A23187 followed by DMAP treatment resulted in 83% cleavage and 29% development of blastocyst stage, and these rates of development were similar to obtained after in vitro fertilization (Gougoulidis

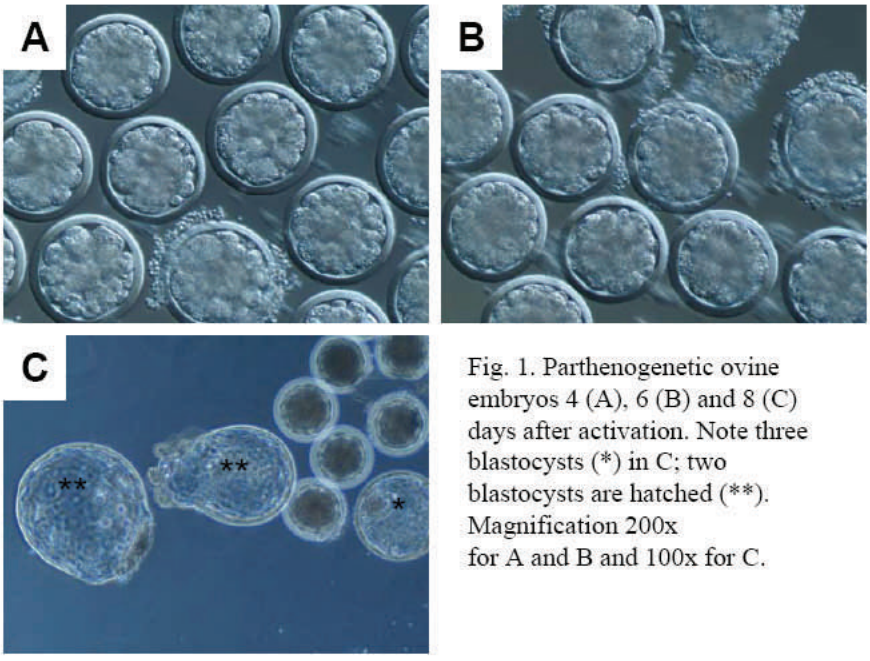

Fig. 1. Parthenogenetic ovine embryos 4 (A), 6 (B) and 8 (C) days after activation. Note three blastocysts (*) in C; two blastocysts are hatched (**). Magnification 200x for A and B and 100x for C.

et al., 19999). In another study, the rate of blastocyst formation was 25% after oocyte stimulation with direct current pulses and treatment with cycloheximide plus cytochalasin B (Matsushita et al., 2004). Thus, the activation protocol may have a profound effect on success of the oocyte activation to obtain parthenogenetic embryos.

In addition to the above mentioned oocyte activation factors, several other factors can activate oocytes to induce parthenogenetic development, including chilling or warming, exposure to colchicine, exposure to electric pulses in the presence of GlutaMAX-I, pricking, certain anesthetics, and factors disturbing the balance between free calcium and the state of the cycloskeletal system (Yamano et al., 2000; Pivko et al., 2004; Edwards, 2007).

The parthenogenetic embryo is a powerful tool to study gene imprinting (Lyle, 1997; Rougier and Werb, 2001; Okamura and Ito, 2006; Edwards, 2007). Genomic imprinting is a functional specialization of the paternal and maternal genomes, and leads to the expression or repression of genes solely on the basis of the parent from which they were inherited (Rougier and Werb, 2001). In fact, with imprinted genes a maternally inherited gene is not equivalent to a paternally inherited one; whereas the paternal genome seems essential for the normal development of extraembryonic tissues, including the placenta, the maternal genome may be essential for some stages of embryogenesis (Rougier and Werb, 2001). Furthermore, parthenogenentic embryos can be used to create stem cells for further research and/or for therapeutic cloning (Rougier and Werb, 2001; Fangerau, 2005).

In summary, parthenogenetic embryos were created in our laboratory using activation medium containing serum and ionomycin followed by treatment with DMAP. In the future, such parthenogenetic embryos will be used to study the role of imprinted genes during placental development.

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