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Short communication

# Assessment of parthenogenetic embryo production by activation of in vitro matured caprine oocytes with different concentrations of ethanol

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ABSTRACT

The objective of this study was to compare the effectiveness of different concentrations of ethanol treatment for activation of oocytes and their developmental potency in vitro. Ovaries were collected from a local abattoir and transported within 4 h to the laboratory in warm saline (37 °C) containing 100 IU penicillin-G and 100 μg streptomycin sulphate per ml. A total of 2680 cumulus oocyte complexes (COCs) were collected from 899 ovaries. Oocytes were matured in TCM-199 medium containing FSH (5 μg/ml), LH (10 μg/ml), supplemented with 20% fetal bovine serum at 38.5 °C and 5% $CO_2$ in an incubator under humidified air for 27 h. After 27 h of IVM, oocytes were denuded, washed and randomly divided into five groups. Group 1 consisted of in vitro matured oocytes ($n = 403$) as control which were washed with KSOM medium without ethanol. Group 2 consisted of in vitro matured oocytes ($n = 412$) activated with 1% ethanol for 5 min in KSOM medium. Group 3 was comprised of in vitro matured oocytes ($n = 362$), activated with 1% ethanol for 5 min in KSOM medium. Group 4 was comprised of in vitro matured oocytes ($n = 564$) activated with 5% ethanol for 5 min in KSOM medium. Group 5 consisted of in vitro matured oocytes ($n = 634$) activated with 7% ethanol for 5 min in KSOM medium. Group 6 consisted of in vitro matured oocytes ($n = 305$) activated with 9% ethanol for 5 min in KSOM medium. After 5 min activation, the oocytes were washed 5–10 times in the culture medium (KSOM) and cultured in 50 μl drop of KSOM. Development of activated oocytes was observed at every 24 h till day 10 post insemination under inverted phase contrast microscope (200×, Nikon, Japan). The percentage of cleavage and morula production in groups 1, 2, 3, 4, 5 and 6 were 0.00% and 0.00%, 0.00% and 0.00%, 8.28% and 6.66%, 10.43% and 26.31%, 33.19% and 29.26%, 40.32% and 14.63%, respectively. These results suggested that the activation of in vitro matured oocytes by 7% ethanol for 5 min in KSOM is most favorable for parthenogenetic caprine embryos production.

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## 1. Introduction

Parthenogenetic activation represents a valid tool to mimic the fertilization Ca2+ transients and oscillation in nucleus transplantation experiments and to investigate the comparative roles of paternal and maternal genomes in controlling early embryonic development (Hou et al., 2009). Furthermore, parthenogenetic activation is relevant to cloning research, because artificial activation of oocytes is an essential component of nuclear transfer protocols (Kim et al., 1996). When a spermatozoon activates an oocyte, it promotes multiple and periodic oscillations of intracellular free calcium (Yang et al., 1994). These

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pulses provoke a cortical reaction, resumption of meiosis, maternal mRNA recruitment, pronuclear development and mitotic cleavage (Ducibella et al., 2002). Although sperm supply the natural stimulus for oocyte activation, oocytes may also be activated parthenogenetically, without any contribution from sperm, by a variety of physical and chemical stimuli. Artificial activation of oocytes aims to mimic the action of sperm cells during fertilization (Nakada and Mizuno, 1998).

Several methods have been developed for induction of parthenogenetic activation, promote an increase in intracellular free calcium concentrations by the release of calcium from cytoplasmic stores, including calcium ionophore (Funahashi et al., 1994), electrical shock (Kim et al., 1996), $CaCl_2$ (Machaty et al., 1996), Ca-EDTA (Lee et al., 2007), protein kinase inhibitors (Mayes et al., 1995), G protein stimulation (Machaty et al., 1996), cycloheximide (CH) (Nussbaum and Prather, 1995), ionomycin (Loi et al., 1998), ultrasound (Sato et al., 2005), ethanol (Loi et al., 1998), strontium (Meo et al., 2004) and magnetic field (Max et al., 2007). These treatments are commonly associated with protein synthesis inhibitors, that prevent cyclin synthesis, such as cycloheximide, and phosphorylation inhibitors, which prevent MPF activation, such as 6-dimethylaminopurine (Susko-Parrish et al., 1994; Loi et al., 1998). One common, universal method or activation agents has not been developed for all species because the process is highly specific for each species. Similarly concentration as well as incubation time of the activation agents are also species specific and need to be optimized.

However, the literature on activation protocols for goat oocytes is limited, creating the need for effective oocyte activation protocols that can be used during in vitro parthenogenetic embryo production. Therefore the objective of this study was to standardize the activation protocol for caprine oocyte with single activation agents viz. ethanol and assessment of the embryos development following activation.

## 2. Materials and methods

All chemicals used in this study were from Sigma–Aldrich (St. Louis, MO, USA), except where otherwise indicated.

### 2.1. Collection of ovaries

Goat ovaries (899) were collected from the abattoir at Agra, located about 35 km from the Central Institute for Research on Goats, Makhdoom, Farah, Mathura. Ovaries were collected just after the slaughter in normal saline containing antibiotics maintained at 35–37 °C and brought to the laboratory within 4 h of slaughter to avoid any detrimental effect. Maintenance at 35–37 °C minimizes the risk of abnormal culture conditions. Prior to oocytes retrieval, surplus tissue on the ovaries was removed and the ovaries were washed several times in sterile normal saline containing antibiotics to avoid associated environmental contamination.

### 2.2. Recovery of oocytes and in vitro maturation (IVM)

The oocytes were collected from each ovary in a Petri dish containing oocyte collection media (OCM) (Dulbecco's phosphate-buffered saline with 1 mg/ml BSA, 50 μg/ml streptomycin and 60 μg/ml penicillin) by follicle puncture method using 18-G needle. Only grade A and B oocytes (Kharche et al., 2008) were chosen as they have evenly granulated cytoplasm which represents their active physiological state with having bunch of compact cumulus cell mass around them. Selected oocytes (2680) were washed two or three times in Oocyte Holding Medium (OHM) containing (TCM-199 with Hepes modification, EGS 10%, Sodium Pyruvate 0.25 mM, gentamicin 50 μg/ml, Glutamine 100 μg/ml, BSA 3 mg/ml) and subsequently two three times in oocyte maturation media (TCM-199 with 10% FBS, sodium pyruvate 0.25 mM, glutamine 100 μg/ml, LH 5 μg/ml, FSH 5 μg/ml, BSA 3 mg/ml and gentamicin 50 μg/ml) and allowed for maturation in 50 μl drop of IVM medium in 35 mm x 10 mm Petri dishes for 27 h in a $CO_2$ incubator maintained at 38.5 °C, 5% $CO_2$ and 90% humidity.

### 2.3. Activation of oocytes

After maturation for 24–27 h, oocytes were stripped off their cumulus cells by treatment with 0.1% hyaluronidase and gentle pipetting for 5 min in KSOM handling medium (potassium simplex optimized medium). These oocytes were randomly divided into five treatment groups. Activation media for various treatments was prepared by adding absolute ethanol in KSOM medium (Lawitts and Biggers, 1993) with various concentrations ranging 0, 1, 3, 5, 7 and 9% ethanol. In each group, all the matured oocytes were subjected to parthenogenetic activation treatment using different concentrations of ethanol.

Group 1 consisted of in vitro matured oocytes ($n = 403$) as control washed in KSOM medium without ethanol.
Group 2 consisted of in vitro matured oocytes ($n = 412$) activated with 1% ethanol for 5 min in KSOM medium.
Group 3 was comprised of in vitro matured oocytes ($n = 362$), activated with 3% ethanol for 5 min in KSOM medium.
Group 4 was comprised of in vitro matured oocytes ($n = 564$) activated with 5% ethanol for 5 min in KSOM medium.
Group 5 consisted of in vitro matured oocytes ($n = 634$) activated with 7% ethanol for 5 min in KSOM medium.
Group 6 consisted of in vitro matured oocytes ($n = 305$) activated with 9% ethanol for 5 min in KSOM medium.

After 5 min activation, the oocytes were washed 5–10 times in the culture medium (KSOM) and cultured in 50 μl drop of KSOM.

### 2.4. In vitro culture of activated oocytes

After 48 h of parthenogenetic activation treatment, caprine oocytes were examined for cleavage. Development of parthenogenetic embryos was observed at every 48 h till day 10 post activation under inverted phase contrast microscope (200×, Nikon, Japan). The culture media was replaced with freshly prepared embryo culture media after every 48 h and observations were made for subsequent embryos development.

### 2.5. Statistical analysis

The maturation stage of oocytes was calculated as a percentage. Cleavage rates among the different treatment groups were compared using the Chi-square test. The level of significance was recorded at the 1% level of confidence (Snedecor and Cochran, 1989).

## 3. Results

The average recovery of good quality oocytes for IVM was 2.98 per ovary. The oocytes recorded a 86.0% maturation rate based on the cumulus cell expansion after 27 h of culture at 38.5 °C in 5% $CO_2$ of humidified air. The percentage of cleavage and morula production after parthenogenetic activation of oocytes in groups 1, 2, 3, 4, 5 and 6 were 0.00% and 0.00%, 0.00% and 0.00%, 8.28% and 6.66%, 10.43% and 26.31%, 33.19% and 29.26%, 40.32% and 14.63%, respectively. Significant differences ($P < 0.05$) were recorded among treatments for these variables. The influence of different ethanol concentrations on cleavage rate and morulae production from in vitro matured goat oocytes are summarized in Table 1. The ethanol increases the cleavage rate of the in vitro matured caprine oocytes in a dose dependant manner. However the morula production increases as the concentration of ethanol for activation is

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