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A comparative study of parthenogenetic activation and in vitro fertilization of in vitro matured caprine oocytes

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Summary

The aim of the study was to compare the parthenogenetic activation and in vitro fertilization (IVF) of in vitro matured caprine oocytes. A total of 881 cumulus-oocyte complexes (COC’s) were collected from 243 ovaries. Oocytes were matured in TCM-199 medium containing eCG (20 IU/ml), ICG (20 IU/µl), oestradiol-17β (1 µg/ml), BSA embryo tested (3 mg/ml) supplemented with 10% fetal bovine serum at 38.5°C and 5% CO₂ in an incubator under humidified air for 27 h. Based on cumulus expansion, the maturation rate was 86.86%. Morphological matured oocytes (n=749) were selected, denuded and randomly divided into two groups. Group 1 (n=223) in vitro matured oocytes activated with 5 µm calcium ionophore for 5 min and cultured in mCR-aa medium containing 5 mM DMAP for 4 h. After 4 h of DMAP treatment, the presumptive zygotes were washed and cultured in the embryo culture medium. Group 2 (n=526) in vitro matured oocytes processed for IVF in mTALP using fresh semen of a fertile pure bred adult Sirohi buck and in vitro culture in mCR-aa medium. Development of putative zygotes was observed every 24 h till day 9 post activation or fertilization under inverted phase contrast microscope. The cleavage rate, morula and blastocyst percentage in groups 1 and 2 were 67.36%, 23.07% and 9.23%, and 30.99%, 19.63% and 9.82%, respectively. The results indicated that the cleavage rate was comparatively higher following parthenogenetic activation with ionomycin/6-DMAP than IVF.

Key words: Goat, Maturation, Oocytes, IVF, Parthenotes

Introduction

Recent advances in knowledge of oocyte maturation and research on different culture conditions have led to substantial progress in in vitro embryo production (IVEP) systems (Freitas and Melo, 2010) for small ruminants. The process of IVEP consists of 3 main steps: 1) Oocyte collection and in vitro maturation (IVM) of cumulus-oocyte complexes (COCs), 2) In vitro fertilization (IVF) and 3) in vitro culture of embryos (Garcia-Garcia et al., 2007). In the provision of in vitro produced embryos, the processes of IVM of oocytes and IVF are almost inextricably linked (Kane, 2003). Natural activation of oocytes by sperm following fertilization and artificial activation of recipient oocytes following somatic cell nuclear transfer is necessary for the further development of oocyte to embryo. Before fertilization, the first step is to select the most motile and viable spermatozoa from the whole fresh ejaculate or the frozen-thawed sperm. The principal techniques used to select spermatozoa are swim-up and centrifugation in Percoll or Ficoll density gradient. Cognie et al. (2003) reported capacitation of frozen-thawed sperm using 10% (v/v) estrus sheep serum in SOF medium and 0.5 g/ml of heparin during 1 h. Paramio (2010) after fresh sperm selection by swim-up, the supernatant recovered and capacitated in mDM with 50 g/ml heparin for 45 min. After oocyte maturation and sperm capacitation, oocytes are transferred to microdrops of modified Tyrode’s medium (TALP) as described by Parrish et al. (1986), supplemented with hypotaurine and glutathione. Different fertilization media have been used by different authors: BO (Ongeri et al., 2001), SOF (Rho et al., 2001) and TALP-fert medium (Katsa-Ksiazkiewicz et al., 2004). Mammalian oocytes of most species arrest at the metaphase II (MII) stage immediately prior to ovulation from ovarian follicles. Resumption of meiosis of an MII oocyte is inhibited by maturation promoting factor (MPF), which is abundant in the mature oocyte (Shen et al., 2008). When a spermatozoa penetrates the zona pellucid and attaches to the vitelline membrane of the MII oocyte, oscillations of intracellular calcium (Ca²⁺) occur (Whitaker and Patel, 1990). The elevation of Ca²⁺ triggered by sperm penetration inhibits dephosphorylation of P³⁴⁺⁺Ca²⁺ kinase, which in turn reduces MPF activity and activates the oocytes (Shen et al., 2008).

Although sperm supply the natural stimulus for oocyte activation, oocytes may also be activated parthenogenetically, without any contribution from sperm (Mishra et al., 2008). In traditional method for production of cloned goat embryos, electric pulses were applied for activation of reconstructed oocytes (Melican et al., 2005; Shen et al., 2006). Oocyte activation by electrical pulse is initiated by an elevation of intracellular Ca²⁺. Immediately after electrical stimulation, there is an influx of extracellular Ca²⁺ which in turn triggers an increase of intracellular Ca²⁺ (Cheong et al., 2002). Chemical treatment can also induce oocyte activation via...
elevation of ooplasmic Ca\textsuperscript{2+} concentration to down-regulate MPF activity and activate oocytes parthenogenetically. Various chemicals like Ca ionophore, ethanol and strontium have been used for activation of oocytes (Varga et al., 2008; Pathak et al., 2013).

To our knowledge, standard IVF protocol compared to \textit{in vitro} parthenogenetic activation of oocytes matured with PMSG and hCG has not been previously investigated in detail. The present study was designed to activate \textit{in vitro} matured caprine oocytes parthenogenetically and compared them with the standard IVF protocol for embryo developmental rates.

**Materials and Methods**

**Chemicals**

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where otherwise indicated.

**Harvest of oocytes**

Ovaries (n=243) were obtained from various breed of goats (non-descript, Sirohi, Boer, Barbari) at Agra slaughterhouse from June to September during the non-breeding season and transported in 3-4 h to the laboratory in a thermos containing physiological saline solution (0.9% w/v NaCl) supplemented with 100 IU penicillin-G and 100 \( \mu \)g streptomycin sulphate per ml at 30 to 35°C. Oocytes were harvested by slicing (Pawshe et al., 1994) with a surgical blade and the oocyte collection medium consisted of Dulbecco’s phosphate-buffered saline (D-5773) with 3 mg/ml BSA. Only oocytes (n=881) surrounded by compact, dense cumulus cell layers, grade A and B (Kharche et al., 2008) were selected for the maturation under stereomicroscope.

**In vitro maturation**

Selected COCs (n=881) for \textit{in vitro} maturation were washed 10-14 times in oocyte-holding medium (OHM) containing TCMP-199 (M-7528), Sodium pyruvate (0.25 mM), gentamycin (50 \( \mu \)g/ml), glutamine (100 \( \mu \)g/ml), BSA embryo-tested (3 mg/ml). The medium used for maturation of COCs was OHM supplemented with 20 IU/ml eCG Folligon\textsuperscript{®} from Intervet, International B. V., Boxmeer-Holland, 20 IU/ml hCG Pregnyl\textsuperscript{®} (Kouamo and Kharche, 2014) from Nile Co. for Pharmaceutical and Chemical Industries A.R.E., 1 \( \mu \)g/ml estradiol 17-\( \beta \) and 10% FBS (fetal bovine serum, Cat N° A15-104 from PAA Laboratories GmbH, The Cell Culture Company Austria) under 400 \( \mu \)L of mineral oil. The pH was adjusted to 7.4 and the osmolality was adjusted to 280 mOsm/kg. Oocytes were incubated for 24 h at 38.5°C in 5% \( \text{CO}_2 \) of humidified air in a \( \text{CO}_2 \) incubator. Assessment of maturation was done by the degree of expansion of cumulus cell mass. During IVM process, 132 oocytes were lost.

**Parthenogenetic activation**

Cumulus cells were removed from oocytes (n=223) after \textit{in vitro} maturation (27 h) using 50 \( \mu \)g hyaluronidase (H-4272) and repeated pipetting. The oocytes were washed 5 times in a mCR\textsubscript{aa} medium. The unfertilized oocytes were exposed to 5 \( \mu \)M Ca ionophore in mCR\textsubscript{aa} for 5 min at room temperature followed by 5 mM 6-DMAP for 4 h (Kharche et al., 2012). Activated oocytes (n=193) were then washed three times in mCR\textsubscript{aa} and were transferred to the drops with the culture medium (10-15 oocytes per drop) and incubated for 9 days at 38.5°C in 5% \( \text{CO}_2 \) of humidified air.

**In vitro fertilization**

After 27 h of culture, matured oocytes (n=526) with expanded cumulus cells were separated from the cumulus cells by treating the complex with 0.1% hyaluronidase enzyme and passing it repeatedly through a fine pipette. Fresh semen samples were collected using the artificial vagina from a fertile pure bred adult Sirohi buck. The first and second seminal ejaculates were examined for volume, colour, consistency and gross sperm and progressive motility. The sperm concentration was counted by hemocytometer and checked for acceptable motility (at least 80% progressively motile). A sample of 100 \( \mu \)L fresh semen was diluted with 5 ml of sperm-TALP medium (100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO\textsubscript{3}, 0.3 mM NaH\textsubscript{2}PO\textsubscript{4}, 21.6 mM Lactate, 2 mM CaCl\textsubscript{2}, 0.4 mM MgCl\textsubscript{2}, 10 mM Hepes, 1 mM pyruvate, 50 mg ml\textsuperscript{-1} Gentamycin, 10 \( \mu \)g ml\textsuperscript{-1} Heparin and 10% FBS) for the respective treatment groups 1 and 2 and washed by centrifugation at 1200 × g for 5 min. The supernatant was discarded and the pellet was diluted with 5 ml of medium and kept for capacitation in a \( \text{CO}_2 \) incubator at 38.5°C for 1 h (Cognie et al., 2003). After incubation, the sperm suspension was centrifuged and the supernatant again discarded and 100 \( \mu \)L of the sperm pellet was diluted in 750 \( \mu \)L Fert-TALP medium (Sperm-TALP medium without Hepes + 4 mg ml\textsuperscript{-1} BSA Embryo tested). An insemination drop containing the oocytes was inseminated with 25-50 \( \mu \)L of the final diluted semen so as to obtain a sperm concentration of 1 × 10\textsuperscript{5} sperm/ml. After \textit{in vitro} insemination, the oocytes and sperm were co-incubated for 18 h at 38.5°C in 5% \( \text{CO}_2 \) incubator in humidified air.

**In vitro culture**

The culture of parthenogenetic and IVF embryos was performed with 100 \( \mu \)L mCR\textsubscript{aa} medium culture droplets (10-15 embryo/drop) under mineral oil. The culture environment consisted of 5% CO\textsubscript{2}, 5% O\textsubscript{2} and 90% N\textsubscript{2} in humidified air at 38.5°C. Cleavage, morula and blastocyst development rates were evaluated from the presumptive zygotes on nine days after each 24 h after parthenogenetic activation or IVF under an inverted phase contrast microscope.

**Statistical analysis**

Cleavage rate, morula, blastocyst stages in parthenogenetic activation and IVF groups were analyzed using the Chi-square test. The level of significance was recorded at the 5% level of confidence (Snedecor and Cochran, 1989).
Results

Recovery of oocytes and maturation rate
The overall COCs recovered per ovary was 3.57 ± 0.21 and 86.86% were matured, done by the expansion of cumulus cell mass.

In vitro fertilization and parthenogenetic activation of in vitro matured oocytes
Data on cleavage, morula and blastocyst/cleavage rate of parthenogenetically activated and IVF embryos are presented in the Table 1. Figs. 1A, B, C, D, E and F show different stages of embryos from parthenogenetic activation and IVF. The cleavage rate is comparatively higher following parthenogenetic activation. Parthenogenetically activated embryos also cleaved earlier than IVF-derived embryos.

Discussion
The activation of oocyte is induced by an increase in the oocyte’s intracellular free calcium concentration (De et al., 2012). In normal fertilization, sperms trigger release of calcium from the endoplasmic reticulum and this act as a signal for oocyte activation (Whitaker, 2006). Combined treatments with different chemicals for parthenogenetic activation have been widely used for reconstructed oocytes (sheep: Schnieke et al., 1997; cattle: Cibelli et al., 1998; goat: Keefer et al., 2001, 2002) to increase intracellular Ca\(^{2+}\) concentration (such as EP, ethanol, calcium ionophore or ionomycin) and inhibit protein synthesis (e.g., CHX) or MPF activity (e.g., 6-DMAP). In this study, cleavage rates following parthenogenetic activation (67.36) and IVF (30.99) revealed significant difference (P=0.00, Table 1). This is in accordance with Pablo et al. (2008). The latter hypothesized that because all oocytes used for parthenogenetic activation were denuded prior to activation, which allowed for selection based on the presence of a polar body and evenly granulated cytoplasm, a higher proportion of developmentally competent oocytes might have been selected for these procedures. The combination of calcium ionophores with 6-DMAP induces high rates of activation, pronucleus

<table>
<thead>
<tr>
<th>Experiments</th>
<th>No. of treated oocytes</th>
<th>Total cleaved (%)</th>
<th>2C (%)</th>
<th>4C (%)</th>
<th>8C (%)</th>
<th>16C (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parthenogenetic activation</td>
<td>193</td>
<td>130 (67.36)</td>
<td>22 (16.92)</td>
<td>19 (48.2)</td>
<td>35 (26.92)</td>
<td>12 (9.23)</td>
<td>30 (23.07)</td>
<td>12 (9.23)</td>
</tr>
<tr>
<td>IVF</td>
<td>526</td>
<td>163 (30.99)</td>
<td>9 (5.52)</td>
<td>41 (25.15)</td>
<td>43 (26.38)</td>
<td>22 (13.49)</td>
<td>32 (19.61)</td>
<td>16 (9.82)</td>
</tr>
</tbody>
</table>

\(^a,b^\) Values in the same column with different letter are different at P<0.05. C: Cells, and No.: Number

Fig. 1: Different stages of embryos. (A) 2 cells, (B) 4 cells, (C) 8 cells, (D) 16 cells, (E) Morula, and (F) Blastocyst

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formation and development to blastocyst stage like in sheep (Loi et al., 1998) and cattle (Liu et al., 1998). In contrast, Onger et al. (2001) and Çevik et al. (2009) found that cleavage rate had no significant difference following the two treatments. The cleavage rate of activated oocytes and their potential for further embryonic development depends on several factors, like species, source and quality of oocytes, IVM conditions, type and composition of culture media and activating agent (Çevik et al., 2009).

The efficiency of the culture system may be expressed in terms of the percentage of embryos that cleaved and reached more than the 8-cell stage (Keskintepe and Brackett, 1996). In our study, the blastocyst development rates revealed no significant difference between the two treatments. In cattle, where the IVF system is better developed, percent blastocyst development obtained from oocytes that were activated with a calcium ionophore followed by 6-DMAP was similar to that of IVF (Liu et al., 1998). So, all competent goat oocytes activated or coinubated with spermatzoa could potentially develop to blastocyst. In both cases, the blastocyst rates were similar to those reported by Kharche et al. (2012) and lower than those reported by Dinnyës et al. (2000), Arat et al. (2006) and Çevik et al. (2009). The difference might be due to factors such as culture system (Çevik et al., 2009), media, quality of oocytes and quality of sperm or capacitation. The low percentage of blastocystcs developing from IVF may also be attributed in part to the polyspermy (De Smedt et al., 1996; Onger et al., 2001). In this study, embryos activated with ionomycin/DMAP cleaved earlier than IVF-derived embryos. With the parthenogenetic procedures, the time of activation is synchronized by ionomycin treatment while sperm entry during IVF takes place within a period of 6 h (Pablo et al., 2008).

The present study indicated that matured caprine oocytes activated with ionomycin/6-DMAP exhibit higher cleavage rate than IVF procedure, however, the developmental competence of embryos or parthenotes to blastocyst stage grow similarly.

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خلاصهی مقالات به زبان فارسی

استفاده از ترکیبات مشتق شده از گیاهان در درمان لیشمانیاسیس

اهمد عربان

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(دریافت مقاله: ۲۵ آبان ۱۳۹۳، پذیرش نهایی: ۹ اسفند ۱۳۹۳

لیشمانیاسیس یک مطلب بیشمار عوامل تأثیرگذار است که در طول زندگی انسان با پیش‌های خانگی مواجه می‌شوند. تهیه‌نامه‌های فیبری کشورهای در حال توسعه و تولید قرار می‌دهد. ارگانیسم مربوط به پیش‌های خانگی به عنوان یکی از تیمارهای هوشمند شیمی درمانی، همچنین بر اساس استفاده از ترکیبات آنتی‌میکولار، نتیجه‌گیری می‌شود که این گیاهان به جهت بهبود وضعیت بهبودی جسمانی و ساده‌تر استفاده‌گونه شدند. درک بهتری از این موضوع بهبودی جسمانی افراد مبتلا به لیشمانیاسیس می‌تواند به بهبود وضعیت بهبودی جسمانی این مبتلایان کمک کند.

واژه‌کلیدی: لیشمانیاسیس، درمان، انتخاب‌های بیوت‌محیطی، عصاره‌ها، گیاهی

مطالعه مقایسه‌ای فعال سازی پارتنوژنتیک و پاروسازی آزمایشگاه‌های گیاهی برای بلوغ یافته در محیط زندگی

جاسوس کوامو و سورس دیگر خارجه

گروه جراحی و آزمایش‌های پزشکی، دانشکده دامپزشکی و علوم دامپزشکی دانشگاه تهران، تهران، کشور؛ ایش فیزیولوژی تولید می‌شود. (CIRG)، مخدوم، فرهاد-۲۱۹۱۱۱۲۳، مانوژا، یوترا بروندی، ۲۱۹۹۱۲۲۸ (دریافت مقاله: ۱۳ آذر ۱۳۹۳، پذیرش نهایی: ۲۵ مرداد ۱۳۹۳

هدف از این مطالعه مقایسه‌ای فعال سازی پارتنوژنتیک و پاروسازی آزمایشگاه‌های گیاهی برای بلوغ یافته در محیط زندگی بود. تعداد ۸۸۱
معادله 1:

\[
\text{DAMP} = \frac{1}{T} \text{CFU} \times \frac{1}{T} \text{CFU} - \frac{1}{T} \text{CFU} \times \frac{1}{T} \text{CFU}
\]

معادله 2:

\[
\text{IFN-\gamma} = \frac{1}{T} \text{IFN-\gamma} \times \frac{1}{T} \text{IFN-\gamma} - \frac{1}{T} \text{IFN-\gamma} \times \frac{1}{T} \text{IFN-\gamma}
\]

معادله 3:

\[
\text{IL-10} = \frac{1}{T} \text{IL-10} \times \frac{1}{T} \text{IL-10} - \frac{1}{T} \text{IL-10} \times \frac{1}{T} \text{IL-10}
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