CAPRINE BLASTOCYST DEVELOPMENT AFTER IN VITRO FERTILIZATION WITH SPERMATOZOA FROZEN IN DIFFERENT EXTENDERS

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ABSTRACT

The feasibility of using frozen-thawed semen in caprine IVF outside the breeding season was investigated. Electroejaculated spermatozoa from a Nubian buck were washed twice and then frozen in skim milk- or in egg yolk-based extenders. Goat oocytes were matured and inseminated by frozen-thawed spermatozoa selected by swim-up. In vitro fertilization was performed in a modified defined medium (mDM), altered experimentally, for 24 h. Embryos were cultured in 50 µL of c-SOF+NEA for 9 d. The percentages of oocytes exposed to heparin-capacitated spermatozoa, (previously cryopreserved in skim milk-based extender) that cleaved, reached morula, blastocyst and expanded blastocyst stages were 82.8, 57.1, 35.7 and 30.0%, respectively. Without heparin treatment the rates for cleavage, morula, blastocyst and expanded blastocyst stages were 44.3, 31.4, 18.6 and 8.6%, respectively. Therefore, heparin treatment was included in sperm capacitation. Use of spermatozoa with BSA in the IVF medium yielded no cleavage. Although extenders containing 8 to 20% egg yolk enabled good sperm motility after cryopreservation, in vitro fertilizing ability was compromised under our conditions. By contrast, semen commercially processed in season in an egg yolk-based diluent remained effective for IVF. The highest proportion of blastocysts resulted from the use of spermatozoa diluted in a skim milk extender, heparin capacitation, and insemination in medium containing lamb serum.

Key words: caprine, blastocyst, sperm cryopreservation, extenders, IVM/IVF/IVC

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INTRODUCTION

In vitro maturation, fertilization, and culture (IVM/IVF/IVC) of caprine oocytes (5,14,25,26,37) has received wide interest with the successful demonstration of the goat as an animal model for transgenesis (15). Current zygote microinjection approaches require large numbers of pronuclear ova for increasing the likelihood of successful genetic manipulation, since only a small percentage of injected foreign genes actually become incorporated into the chromosomes and are expressed (32).

Conditions for caprine oocyte maturation, fertilization and culture in vitro have been investigated by several groups (14,25,26,28,30,33), and protocols for these procedures are adequate to assure reasonable results. One of the main obstacles remaining to the production of caprine embryos in vitro is that of immediate availability of spermatozoa. Semen collection via an artificial vagina or electro-ejaculation becomes difficult with increasing day length, outside the normal breeding season. Doe goats normally have estrous cycles with shortening day length, i.e., fall or short-day seasonal breeders, and fertility patterns of bucks tend to follow those of the females.

Different semen extenders such as skim milk-glycerol, lactose-egg yolk-glycerol, or Tris (hydroxymethyl) aminomethane-citric acid-egg yolk-glycerol and different freezing protocols have been described for buck semen (12,13,18,31). Even though there has been a growing interest in artificial insemination of goats with frozen semen, the conditions for IVF with cryopreserved semen have not been adequately described. The present research was undertaken to investigate the feasibility of using frozen-thawed semen in caprine IVF outside the breeding season.

MATERIALS AND METHODS

Experiments were carried out January through June 1996, after the normal fall breeding season in our locality.

Oocyte Retrieval and Maturation

Ovary collection and oocyte aspiration were carried out in a similar manner as described earlier (26). Briefly, ovaries were obtained at slaughter, and follicles 3 to 6 mm in diameter were aspirated with a 3-cc syringe attached to a 20-gauge needle. Oocytes having 2 to 3 layers of surrounding cumulus cells and a homogeneous-appearing cytoplasm were washed twice with Tyrode's medium, including 0.4 mL polyvinyl alcohol (PVA) and 0.4 mL sodium pyruvate, and then transferred into 1 mL of maturation medium which contained Hepes-TCM 199, 10 µg each oFSH and bLH (NHP, NIDDK, NICHD, USDA), 20% fetal bovine serum (FBS; cat. no. 230-6140P1, Gibco Laboratories, Grand Island, NY, USA), 3.6 mL sodium bicarbonate, 112 µg/mL pyruvate, and 50 µg/mL gentamicin in a thermos (38.5°C) for 4.5 h during transport to the laboratory. Then oocytes were transferred into 75 µl of freshly prepared maturation medium under paraffin oil and 5% CO₂, 5% O₂, 90% N₂ for an additional 23 h of incubation at 38.5°C prior to insemination.
Sperm Collection and Freezing

Cryopreserved Nubian buck semen from 2 different sources (commercial and our laboratory) was employed. Semen collected within the breeding season and processed in an egg yolk-based extender (10^8 sperm/0.5 mL straw) was purchased from Magnum Semen Works (Hampstead, MD, USA) and was used as the standard in our experiments. In our laboratory semen was collected from a Nubian buck by electroejaculation, outside the breeding season, and was processed in both skim milk- and egg yolk-based diluents. After electroejaculation, the semen was held in a 37°C water-filled thermos during the 30-min transit to the laboratory. Sperm washing was carried out twice as described by Machado and Simplicio (29). Sperm diluents containing skim milk (35) or egg yolk (18) were divided into 2 parts, one with glycerol and the other without glycerol. The diluent without glycerol was added to spermatozoa before the sperm cells were taken into a cold room (5 to 6°C). The sperm-containing tubes were placed in a 250-mL beaker containing 150 mL of 25°C water. After 1.5 h in the cold room, when water temperature in the beaker reached 5 to 6°C, the diluent with glycerol was added to the sperm cells in three 10-min intervals. Spermatozoa were loaded into 0.5-mL French straws to yield 10^6 cells/straw. The straws were then held 4 to 5 cm above 12 cm liquid nitrogen for 8 min and plunged into liquid nitrogen for cryopreservation.

Sperm Preparation and In Vitro Insemination

After thawing in a 37°C water bath for 30 sec, sperm selection was effected by layering 50 µL of thawed semen under 500 µL of TALP medium (3) containing 3 mL essentially fatty acid free BSA and 0.5 mg/mL caffeine. Each of several small tissue culture tubes (12 x 75 mm; Fisher Scientific, Pittsburgh, PA) prepared in this way was held at a 45° angle for 45 min at 38.5°C. Then the top 450 µL from each tube were removed, pooled in a sterile 15-mL centrifuge tube, centrifuged (320 x g) for 10 min, and the supernatant discarded. The resulting sperm pellet was resuspended to 200 µL in TALP without or with heparin (200 µg/mL, sodium salt, H-7005; Sigma) depending on the experiment. Alternatively, thawed spermatozoa were treated as previously described (14,26) and found to be effective for goat IVM/IVF/IVC (26); this approach included elevation of calcium lactate during the insemination interval.

After 27 h of maturation, 15 oocytes were placed in 90 µL of defined medium (4) modified as described for bovine IVF (24) but with 10% (v/v) FCS or 10% (v/v) prepuberal lamb serum replacing PVA. Then 10 µL of previously cryopreserved and processed spermatozoa were added for a final concentration of 2 x 10^6 sperm/mL. Oocytes and spermatozoa were co-cultured under paraffin oil and the physical conditions described above for 18 h. Then ova were freed of loosely attached cells (i.e., denuded) by vigorous pipetting with a finely drawn Pasteur pipette, approximately 150 µm in diameter. After denuding the ova were transferred into culture medium.

In Vitro Culture

Culture of presumptive zygotes was initiated in 50 µL of BSA-free synthetic oviduct fluid (SOF; 36) with addition of 3.0 mg/mL PVA, 0.5 mM citrate (sodium citrate-trisodium salt, S-4641; Sigma), and nonessential amino acids, (abbreviated as c-SOF + NEA; 24). Embryos were cultured under paraffin oil and a humidified 5% CO2, 5% O2, 90% N2 atmosphere at 38.5°C.
Embryonic development was monitored, and 50% of the culture volume was replaced with fresh medium at 24-h intervals. Ova that did not progress to the next cleavage stage were removed from drops containing developing embryos at the time of each change of medium. Proportions of inseminated oocytes reaching the 2- to 4-cell stage were recorded at 48 h; morulae were recorded at 120 h, blastocysts at 168 h, and expanded blastocysts at 216 h post insemination.

Experimental Design

Results of IVM/IVF/IVC after the use of frozen-thawed spermatozoa subjected to different capacitation conditions, sperm freezing extenders and IVF-medium supplements were compared in 3 different experiments. These experiments, each involving a total of 80 to 100 oocytes, were repeated at least 3 times. In Experiment 1, spermatozoa cryopreserved in skim milk-based extender were compared for fertilizing abilities in medium (24) with no PVA but lamb serum supplementation after capacitation with heparin (HC), without heparin (WH), and in calcium lactate-containing medium (CLC:14). The most promising condition from the first experiment (heparin-capacitation) was employed for insemination in subsequent experiments. In Experiment 2, caprine oocytes were inseminated (in modified defined medium with lamb serum added; 24) with frozen-thawed spermatozoa after cryopreservation in either skim milk-based or egg yolk-based diluents. Resulting proportions of oocytes undergoing fertilization and embryo development were compared with those inseminated with frozen-thawed spermatozoa that had been processed commercially within the breeding season with an egg yolk-based diluent. In Experiment 3, effects of sera collected from prepuberal lambs (6 mo old) or fetal calf blood used to supplement the insemination medium (10% v/v) were investigated. In this experiment, spermatozoa previously cryopreserved in our laboratory in a skim milk-based extender and those commercially cryopreserved in an egg yolk-based extender were studied.

Statistical Analysis

Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package. One-way repeated measures ANOVA was applied to assess statistical differences. The Bonferroni t-test was used to determine differences among the groups. Differences of P<0.05 were considered to be significant.

RESULTS

Experiment 1

Semen was collected outside of the breeding season by electroejaculation and was processed in skim milk-based diluent before cryopreservation. The results afforded by heparin capacitation of the frozen-thawed spermatozoa were superior to those obtained with spermatozoa treated in the same manner but without heparin and to those of calcium lactate treatment (Figure 1). Thus, more oocytes were fertilized (87.8% cleaved by 48 h) and reached the morula stage (57.1% by 120 h), blastocyst stage (35.7% by 168 h), and expanded blastocyst stage (30.0% by 216 h) in the group inseminated with heparin capacitated spermatozoa (Figure 1).
Experiment 2

Skim milk- and egg yolk-based diluents for sperm cryopreservation were evaluated for their influences on sperm fertilizing ability and sperm-initiated development to 2- and 4-cell, morula, blastocyst, and expanded blastocyst stages (Table 1). Despite observations of good post-thaw motility (> 50% total) of spermatozoa cryopreserved in egg yolk based extender (either processed commercially elsewhere or processed out-of-season in our lab), lower percentages of fertilization resulted (Table 1). Microscopic evaluation of frozen-thawed sperm cell morphology revealed no obvious physical damage. Similarly, the developmental rates of oocytes obtained after insemination with sperm cells processed in skim milk-based diluent were significantly higher (P < 0.05) than those after insemination with spermatozoa that had been cryopreserved in egg yolk-based diluents (Table 1).

Experiment 3

Supplementation of insemination medium with BSA led to no fertilization by cryopreserved spermatozoa in these conditions (data not shown). Influences of FCS and addition of prepuberal lamb serum to the IVF medium were evaluated; the results are shown in Figure 2. Addition of lamb serum during the insemination interval enabled 48.5% of inseminated oocytes to reach morula, 29.3% to reach blastocyst, and 22.5% to reach expanded blastocyst stages after use of spermatozoa previously cryopreserved in skim milk-based extender.
Table 1. Improved caprine IVF results with spermatozoa cryopreserved in skim milk-based extender

<table>
<thead>
<tr>
<th>Freezing extender</th>
<th>No. of oocytes inseminated</th>
<th>48 hours</th>
<th>120 hours</th>
<th>168 hours</th>
<th>216 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim Milk</td>
<td>80</td>
<td>64 (80.0)</td>
<td>38 (47.5)</td>
<td>24 (30.0)</td>
<td>16 (20.0)</td>
</tr>
<tr>
<td>Egg Yolk C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80</td>
<td>48 (60.0)</td>
<td>28 (35.0)</td>
<td>14 (17.5)</td>
<td>9 (11.3)</td>
</tr>
<tr>
<td>Egg Yolk C</td>
<td>80</td>
<td>20 (25.0)</td>
<td>13 (16.3)</td>
<td>9 (11.3)</td>
<td>3 (3.8)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Spermatozoa processed commercially in season in commercially prepared egg yolk-based extender; the other freezing extenders were prepared and used for processing semen out of season in our laboratory.

<sup>b</sup>-<sup>c</sup> Different superscripts in the same column denote significant differences (P < 0.05).

Figure 2. Effects of sperm processing medium and serum supplementation of the IVF medium on early embryonic initiation by cryopreserved caprine spermatozoa. Results are reported as percentages of oocytes that developed to the 2- and 4-cell (C), morula (M), blastocyst (B), and expanded blastocyst (EB) stages at indicated intervals after insemination. Treatment groups consisted of sperm processed in skim milk-based extender, IVF medium supplemented with lamb serum (SLS); sperm processed in skim milk-based extender, IVF medium supplemented with FCS (SFCFS); sperm processed in egg yolk-based extender, IVF medium supplemented with lamb serum (ECLS); sperm processed in egg yolk-based extender, and IVF medium supplemented with FCS (ECFCFS). Different superscripts denote significant differences (P < 0.05) within developmental stages.
DISCUSSION

Various sperm capacitation conditions that are effective for achieving fertilization in vitro with frozen-thawed spermatozoa have been reported. Kusunoki et al. (27) demonstrated that ejaculated goat spermatozoa displayed acrosome reaction in a chemically defined medium when they were preincubated in an anaerobic environment and in substrate-free defined medium. Anand et al. (2) indicated that incubation timing for epididymal and ejaculated spermatozoa was critical for capacitation and acrosome reaction to occur. Also, they showed that calcium in the capacitation medium was essential for sperm motility maintenance and/or activation as well as for the initiation of acrosome reaction. Our present results of IVM/IVF/IVC (Experiment 1) demonstrated that brief heparin treatment (15 min) of frozen-thawed spermatozoa before insemination was superior to that of calcium lactate, or to no capacitation treatment at all (Figure 1). The significance of calcium for capacitation of freshly ejaculated spermatozoa has been well documented (6,14,24). An elevated calcium concentration (10 mM) supported stabilization of in vitro fertilization rate in sheep (6,19). Differences between fresh and frozen-thawed buck spermatozoa under in vitro conditions might be due to extender and/or freezing on sperm membranes. Acrosomal damage after equilibration was more pronounced after freezing in egg yolk-citrate-glycerol extender (11). Ijaz et al. (22) found that when ejaculated bovine spermatozoa were incubated in egg yolk TEST semen extender for 8 h, they became capacitated. Capacitating activity was attributed to Tris (22), and TEST-yolk processing enabled bull spermatozoa to fertilize cow oocytes in vitro (21). In contrast to exposure of spermatozoa to egg yolk-Tris-glycerol at 23°C, cooling bull spermatozoa in this egg yolk-based extender to 4°C was shown to promote capacitation (7). Recent work with human spermatozoa implicated phosphatidyl choline as the dominant factor in TEST-yolk buffer found to enhance zona binding and acrosome reaction (16). Capacitated spermatozoa have lowered longevity, and, in our present work, premature capacitation of buck sperm cells may have resulted from certain treatments, e.g., calcium lactate, egg yolk-based extenders, which, in turn, resulted in impaired in vitro fertilizing ability (Figures 1 and 2, Table 1).

Caprine spermatozoa frozen in different extenders performed at different levels when assessed by IVF (Table 1). Skim milk-glycerol extender enabled higher fertilization rates than egg yolk-glycerol-citrate extender. Egg yolk and/or milk increase sperm survival during freezing or rapid cooling in a number of species (17). Egg yolk may have a detrimental effect on buck spermatozoa when used in an extender, because phospholipase produced by accessory sex glands of the buck catalyzes the hydrolysis of lecithins in the egg yolk to fatty acids and lysolecithins (34), which are toxic to spermatozoa (1,23). Washing spermatozoa prior to dilution with the egg yolk-based extenders improved motility, with a significantly higher percentage of spermatozoa with normal acrosomes being found after washing (12,31). Machado and Simplicio (29) demonstrated that the toxic effect of phospholipase could be negated by washing spermatozoa with Ringer’s solution. Sperm motility and acrosome morphology have been depressed in the presence of seminal plasma in milk extender (31). Although we washed the spermatozoa twice before freezing, our results indicated that cryopreservation in the egg yolk extender yielded inferior sperm fertilizing ability after thawing, leading to compromised blastocyst development when compared with caprine spermatozoa processed in skim milk extender (Table 1).
Additions such as serum to IVF medium have been shown to influence embryo development in an in vitro system (6, 9, 10). Estrous sheep serum was found to be an effective supplement for caprine IVF (8, 9, 10). In earlier work (26) when caprine oocytes were inseminated with ejaculated spermatozoa incubated in modified defined medium with 20% fetal bovine serum and capacitated with calcium lactate, more oocytes reached the blastocyst stage (P < 0.05) than after other sperm treatments. However, the addition of heat-treated lamb serum during the insemination interval in our present work effectively enhanced the proportion of oocytes undergoing cleavage and development to blastocysts (Figure 2). These results were similar to those of Crozet et al. (9), who used 20% estrous sheep serum in the capacitation and fertilization media. Whether lamb serum is as effective as estrous sheep serum (20) in supporting cholesterol efflux and hence sperm capacitation remains to be studied.

In conclusion, the data (Table 1, Figures 1 and 2) in our study demonstrated that caprine embryos can be produced in vitro from oocytes collected outside the normal breeding season and from spermatozoa likewise collected and cryopreserved outside the breeding season. Processing caprine semen with skim milk-based extender improved the fertilization results and subsequent embryonic development compared with processing semen in egg yolk-based extender. Heparin provided better capacitation of previously cryopreserved spermatozoa than no heparin treatment or calcium lactate treatment, as determined by in vitro fertilization results. Supplementation of the IVF medium with lamb serum yielded optimal fertilization results.

REFERENCES


