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Epididymal Epithelial Cells Cultured In Vitro Prolong the Motility of Bovine Sperm

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ABSTRACT: It is well known that the epididymis is an excellent environment to maintain sperm viability. Therefore, we used different sections of bovine epididymis (caput, corpus, and cauda) to develop epithelial cell culture monolayers to identify factors that will increase sperm survival in the freezing-thawing process. Each epididymal section was dissected and treated with collagenase to obtain epithelial cell clusters. The cells were cultured in RPMI-1640 medium with 10% serum at 38.5°C. A confluent monolayer was obtained after 5–7 days in culture and preliminary characterization using cytokeratin antibody indicated that the cell culture contained 85%–95% of epithelial cells. These cellular cultures were tested for their ability to maintain motility of epididymal and frozen-thawed spermatozoa. Washed spermatozoa were added to obtain a final dilution of 1×10^6 spermatozoa/mL. The motility of frozen-thawed spermatozoa was also recorded after incubation in conditioned media. Our results show that cocultures of spermatozoa and epididymal cell monolayers for 24 and 48 hours were beneficial for maintaining epididymal and frozen-thawed sperm motility (36.0% and 20.4%) compared with spermatozoa cultured with fibroblast cells or in the absence of a cell monolayer (0%; $P < .01$). The conditioned medium provides favorable conditions for sperm motility. Results with conditioned medium on maintenance of frozen-thawed sperm motility suggest that epididymal cells in vitro secrete beneficial factors that prolong the sperm survival.

Key words: Epididymis, cryopreservation.

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Essential factors from epididymal epithelia appear to be indispensable for the maturation and the storage of mammalian spermatozoa. The lumen of the cauda epididymis in many animals has the capacity to store densely packed spermatozoa in a potentially fertilizing condition for several weeks prior to ejaculation (Moore, 1998). This epididymal attribute is under the control of androgens. In animal models, it has been estimated that sperm viability may be retained in the cauda epididymis for 2–3 weeks or even longer (Turner, 1995; Moore, 1996), compared with only 48 hours after ejaculation. The precise conditions that spermatozoa require for survival in the epididymis remain unclear (Akhondi et al, 1997). Human spermatozoa are in a quiescent state within the epididymal luminal microenvironment, but it has not yet been established which particular factors specifically act to prolong sperm viability (Moore, 1995).

Cell culture is a powerful tool for the study of cell functions, metabolic activities, and their regulation. Culture techniques were also developed from epididymal epithelia of rodents (mice, rats, hamsters; Klinefelter et al, 1982; Moore et al, 1986; Finaz et al, 1991; Byers et al, 1992; Bongso and Trounson, 1996) and humans (Cooper et al, 1990; Moore et al, 1992; Akhondi et al, 1997). Although few reports on cell culture of epididymal epithelia from domestic mammals are available (Wagley et al, 1984; Heiniger et al, 1996), Joshi (1985) has reported some success with culture of bovine cauda epididymal cells. The coculture of sperm and epididymal cells has allowed the identification of a few proteins that transfer to sperm in vitro (Tezon et al, 1985; Smith et al, 1986; Klinefelter et al, 1992). A 44-kd secretory protein that binds to rat and human sperm during coculture has been implicated in the maintenance of sperm viability and fertilizing capacity (Akhondi and Moore, 1993; Akhondi and Moore, 1994).

In this study, we investigated in vitro interactions of spermatozoa with epididymal cell cultures and established whether specific factors are beneficial in promoting bovine sperm survival.

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Materials and Methods

Testis from sexually mature bulls 10 months to 2 years of age were obtained from a slaughterhouse and kept on ice during a 2-hour transport. The epididymides were removed from testes, washed, and kept in saline containing 10 000 IU/L penicillin, 100

mg/L streptomycin, and 250 μg/L amphotericin B (Sigma, St Louis, Mo).

Preparation of Cell Cultures

Before digestion, cauda epididymal sperm were forced out by retrograde flushing with 10 mL of saline to remove them from the cell suspension. Caput, corpus, cauda epididymides were dissected to remove the connective tissues, diced, and extensively washed to remove blood and spermatozoa. Tubule fragments were incubated in 2.5 mg/mL collagenase, type II (Sigma, 371 IU/mg,) in principal cell medium (PCM) without serum for 120 minutes at 37°C in a water bath. PCM (previously described by Moore et al, 1992) consisted of RPMI-1640 medium (ICN Biomedicals, Aurora, Ohio) supplemented with 10% fetal calf serum (FCS), 200 nM hydrocortisone, 200 nM testosterone, 1 μM dihydrotestosterone, 1 mM sodium pyruvate, 100 nM insulin, 5 μg/mL transferrin, and 1 μg/mL retinol (Moore, 1998; Moore et al, 1992). The medium was supplemented with 50 μg/mL gentamycin. After enzymatic digestion, the cells were separated from tissue fragments by a 5-minute sedimentation period followed by 2 washes with fresh enzyme-free medium. The cells were centrifuged at 250 × *g* for 5 minutes, resuspended, plated in 24-well culture plates in 1 mL of PCM with 10% FCS, and incubated at 38.5°C in an atmosphere of 95% air and 5% CO_2. The culture medium was changed every 2 days.

Immunohistochemistry

A part of collagenase-dispersed cells (single and plaques) was cultured on glass coverslips until a cell monolayer formed; then the cell monolayers were rinsed 3 times with phosphate-buffered saline (PBS), fixed for 5 minutes with ethanol at −20°C, and rinsed 3 times with PBS. Cells were incubated for 90 minutes with an anticytokeratin 8 antibody (Sigma) diluted 1/5 in PBS. Anticytokeratin 8 has been shown to bind specifically to epithelial cells on crude epididymal sections (Finaz et al, 1991). Cells were rinsed 3 times for 5 minutes each with PBS and incubated for 90 minutes with a goat anti-mouse immunoglobulin G (IgG) antibody conjugated to fluorescein isothiocyanate (FITC). The coverslips were rinsed 3 times for 5 minutes with PBS and then mounted on glass slides and examined for fluorescence. Controls were incubated with the secondary antibody only. Cell nuclei were stained with Hoechst 33342 (Sigma), 10 μg/mL, for 20 minutes at 37°C.

Preparation of Fetal Fibroblast Cells

Fetal fibroblast cells were used as a negative control and were obtained from enzymatic digestion of interstitial tissue of bovine fetal tendon. Cells were cultured in 24-well culture plates in minimum essential media culture (MEM) supplemented with 5% FCS. When confluent monolayers of cells had formed, the culture medium was replaced by PCM to provide comparable conditions for spermatozoa to those that are present in epididymal epithelial cell cultures. Coincubation with spermatozoa were realized when cells were confluent after 7 days of culture.

Spermatozoa Preparation and Coincubation of Spermatozoa with Epididymal Cell Cultures

Cauda epididymal spermatozoa were gently forced out by retrograde flushing with 10 mL of saline supplemented with penicillin, streptomycin, and amphotericin. Spermatozoa were washed once with Sp-TALP media (previously described by Parrish et al, 1988), concentrated by centrifugation at 250 × *g* for 5 minutes, and resuspended in PCM. Frozen bovine semen samples prepared from a pool of semen collected from 5 bulls (Le Centre d'Insémination Artificielle du Québec, St-Hyacinthe, PQ, Canada) were used throughout the study. Frozen straws were thawed in a 35°C water bath for 1 minute and sperm were washed twice in Sp-TALP and then resuspended at a given concentration.

Epididymal and frozen-thawed spermatozoa were added to cultures of caput, corpus, and cauda epididymal cells to control fibroblast cells at a final concentration of 1×10^6 sperm/mL or to wells containing PCM without cells, and incubated at 38.5°C. Initial percentage of motile spermatozoa (number of motile spermatozoa/total motile and nonmotile spermatozoa × 100) was analyzed with a hemacytometer at a concentration of 1×10^6 spermatozoa/mL. Sperm motility was also assessed after 24 and 48 hours. This experiment was repeated 3 times in duplicate.

Assessment of Conditioned Medium Effect

PCM medium was conditioned for 48 hours with or without (control) 7-day-old caput, corpus, or cauda epididymal cell cultures. Fifty percent of fresh media was added to the conditioned media in a 500-μL/tube. Ten million frozen-thawed spermatozoa/mL prepared as described earlier were added in each treatment. Microscopic evaluation of motility was performed using a hemacytometer after 0, 3, and 6 hours. This experiment was repeated 4 times in duplicate.

Statistical Analysis

Data were expressed as means ± SEM. Fisher's protected least significant difference tests were used to compare the differences between experiments and controls for sperm motility. Values of $P \leq .01$ were considered to be statistically significant.

Results

Morphology of Epididymal Cultures

Cell clusters were obtained after enzymatic digestion. Within 12 hours in culture, epithelial cell clusters from caput, corpus, and cauda epididymides formed contiguous spheres of epithelium with the apical surface facing outward. Eventually, these vesicles attached and cells spread over the bottom of the Petri dish. Formation of a confluent monolayer was obtained after 5 to 7 days in culture. In the absence of androgen, cells show signs of degeneration, grew very slowly and, after several days, become contaminated with fibroblasts. This is in accordance with observations made by Moore et al (1992). Immunohistochemistry using anticytokeratin 8 antibodies showed that 85% to 95% of the cells in monolayers were an epithelial type (Figure 1b). When anticytokeratin antibodies were omitted in a negative control experiment, the cells remain unstained (data not shown).

Coincubation of Spermatozoa With Epididymal Cell Cultures

Initial sperm motility (Hour 0, after washing) was 92.9% for epididymal sperm and 73.9% for frozen-thawed sperm. Effects of coincubation with epididymal cells in culture on sperm motility were compared with fetal fibroblast cells and PCM alone. Results are shown in Figure 2a and b. At 24 hours, the percentages of motile epididymal and frozen-thawed sperm incubated with caput, corpus, and cauda epididymal cells were 55.1% to 39.8%, 56.4% to 37.2%, and 57.2% to 40.4%, respectively. These values were higher than those obtained when coincubation was performed with fibroblast cells (26.8% to 15.2%) and into defined medium alone (3.8% to 0.5%).

At 48 hours, the percentage of motile spermatozoa coincubated with caput, corpus, or cauda epididymal cells were 31.3% to 22.2%, 35.6% to 18.2%, and 41.2% to 20.7%, respectively. In control conditions, with fibroblasts cells and into medium alone, no motility was observed.

Assessment of the Effect of Conditioned Medium

The presence of epididymal cells to condition the culture media had a significant effect on sperm motility. Initial sperm motility of frozen-thawed sperm was 74.9%. Beneficial effects of conditioned medium recovered from epididymal cell cultures are shown in Figure 3. At 3 and 6 hours, the percentage of motile sperm incubated in PCM medium conditioned by caput, corpus, and cauda epididymal cell cultures were 55.5% to 46.8%, 65.0% to 47.0%, and 59.9% to 48.8%, respectively. In contrast, motility evaluation of frozen-thawed spermatozoa was recorded at 38.8% to 32.3% in nonconditioned PCM for 3 and 6 hours, respectively. Sperm motility was not maintained after 24 hours of incubation in conditioned medium.

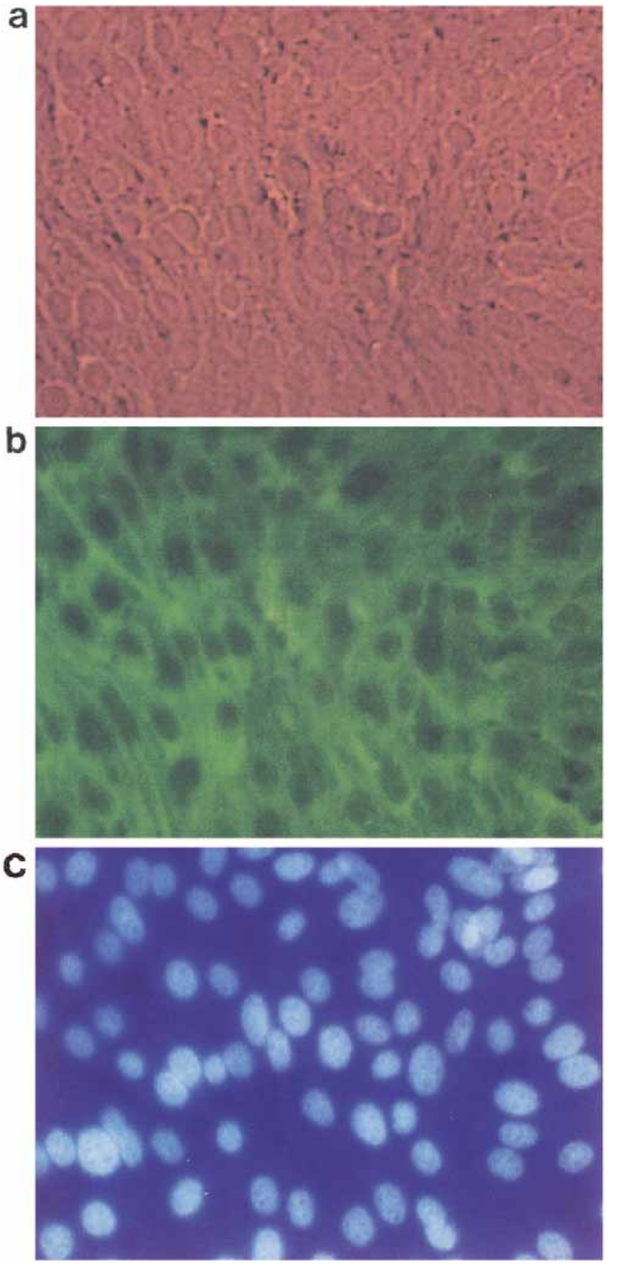

Figure 1. Immunohistological detection of cytokeratin-containing intermediate filaments of epididymal cells in culture. **(a)** Light micrograph of cauda epididymal cells in culture. **(b)** Fluorescence photomicrograph of cauda epididymal cells in culture probed with anticytokeratin 8 antibody. **(c)** Fluorescence photomicrograph of a cauda epididymal cell nucleus stained with Hoechst 33342 (400×).

Discussion

The present study demonstrates that a functional culture system was obtained for epithelial cells of the 3 segments (caput, corpus, and cauda) of bull epididymis. Functional culture systems were developed with hamster and human epididymides (Moore et al, 1986, 1992). Epithelial principal cells retained their characteristic morphology, their polarity, and some microvilli; however, enzymatic digestion of cells from the cauda region of human epididymis failed to produce functional cells because of the large amount of connective tissue surrounding the epididymal tubule (Akhondi et al, 1997; Moore, 1998). A method to obtain a purified principal cell culture from the cauda of bull epididymis has been established by perfusion of the lumen with a collagenase solution (Joshi, 1985). Moore (1998) suggests, however, that because the epithelial architecture was disrupted, purified principal cells rapidly became dedifferentiated in culture and lost their function. Thus, we developed a simple and rapid method that permits the establishment of primary cell cultures of all 3 sections of epididymis. This method allowed us to obtain a large quantity of cells from small pieces of tissue.

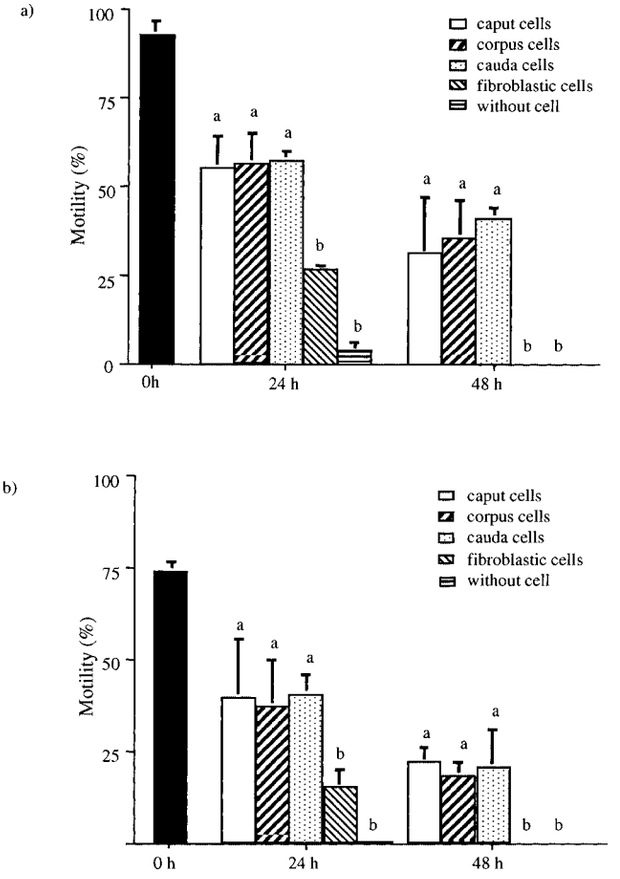

Figure 2. **(a)** Percentage of motile epididymal spermatozoa or **(b)** frozen-thawed after incubation with fibroblast cells or caput, corpus, cauda epididymal cell monolayers. Free culture medium was used as a control. Different superscripts denote significant differences ($P \leq .01$).

Considering that the primary cultures of epithelial cells may be contaminated by surrounding fibroblasts, endothelial cells, or both, we sought to confirm the epithelial origin and to evaluate the epithelial cell content of our epididymal cells cultured in vitro. Cytokeratins are members of 5 distinct subgroups of intermediate filament proteins. Cytokeratin 8, which is similar to other cytokeratins, has been shown to be restricted to true epithelia (Franke et al, 1978; Denk et al, 1983; Finaz et al, 1991). In our studies, we used anticytokeratin 8 antibodies to confirm the epithelial cell origin of our cell cultures. As judged by the results shown in Figure 1, nonepithelial cell contamination was absent or present at a very low level; however, the relative degree of contamination may vary from one cell preparation to another. Thus, our different immunohistochemistry evaluations using anticytokeratin 8 antibodies showed that 85% to >95% of the cells that formed monolayers were of epithelial cell origin. These findings were comparable with those obtained by Finaz and coworkers (1991). By extracting these cells with an enzymatic digestion and sedimentation of crude cell suspension, the majority of rat primary epididymal cell cultures exhibited positive staining with cytokeratin 8 antiserum (>95%). With this method, cell contamination corresponded to that of myoblasts, in which less than 5% of cells became positive after staining with antidesmin antibody. It has been shown that the percentage of cytokeratin-positive cells does not vary over 7 days of culture (Akhondi et al, 1997).

Coculture of bovine spermatozoa and epididymal cell monolayers maintains epididymal and frozen-thawed sperm motility and, therefore, their survival, compared with fibroblast cells or in the absence of cell monolayers (Figure 2). This positive effect on the maintenance of frozen-thawed sperm motility was also observed when conditioned media were used (Figure 3). The positive effect of conditioned media on sperm motility suggests that epididymal cells probably secrete one or more beneficial compounds, which prolongs sperm viability; however, direct contact between sperm cells and epididymal cells in culture appears to be more efficient in sperm survival. Unexpectedly, cells that originated from the 3 parts of the epididymis had the same effect on sperm motility. Because the 3 epididymal segments showed different patterns of protein production (Vreeburg et al, 1991; Syntin et al, 1996), the mechanisms by which these cells maintain sperm viability must be a common factor that is produced by all the epithelial cells in the epididymis.

How epididymal cells maintain sperm viability in vitro remains unknown. Oviductal epithelial cells in vitro also maintain sperm motility and viability (Chian et al, 1995; Smith and Nothnick, 1997; Lapointe and Sirard, 1998) and were nearly as beneficial as epididymal cells in doing so. Mechanisms that can be implicated in this seem to be different between oviductal and epididymal cells. It has been suggested that oviductal cells secrete a catalase that binds to sperm surfaces and protects them from oxidative damage (Lapointe et al, 1998). An antibody against catalase did not neutralize the beneficial effect of epididymal cell secretions on sperm motility as it did with oviductal cell secretions (unpublished data). Previous studies have shown that human and hamster sperm viability were enhanced by epididymal epithelial cells (Smith et al, 1986; Akhondi et al, 1997). Moore (1998) suggests that intimate sperm cell interactions may result from a need for membrane exchanges of glycosylphosphatidylinositol (GPI)-anchored moieties (Kirchhoff and Hale, 1996) and the labile nature of epididymal secretions.

A few hypothesis may explain why epididymal cell cultures have a beneficial effect on the survival of frozen-thawed spermatozoa. The first is that cells have the capacity to restore the homeostasis of the sperm membrane, which is disturbed during the process of cryopreservation (Bailey and Buhr, 1994). A second possibility is that epididymal cells reduce the metabolism of the sperm. It is

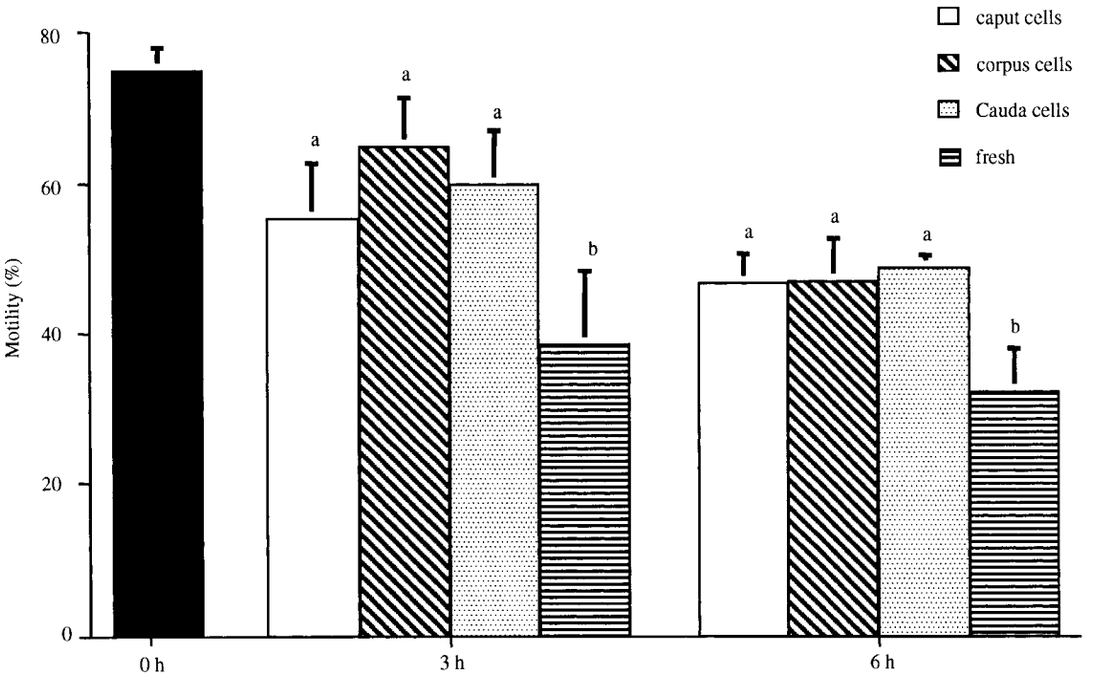

Figure 3. Percentage of motile frozen-thawed spermatozoa after different incubation periods with media conditioned by caput, corpus, or cauda epididymal cell monolayers. Different superscripts denote significant differences ($P \leq .01$).

well known that in vivo, epididymal cells can preserve sperm in a metabolic quiescent state (Usselman and Cone, 1983; Turner, 1995; Jones and Murdoch, 1996; Cooper, 1998). Another hypothesis is that epididymal cells produce factors such as decapacitation factor, which prevent rapid capacitation of cryopreserved sperm (Moore, 1996; Cormier et al, 1997; Cooper, 1998).

The epididymal cell culture system presented here appears to be a powerful instrument to understand epididymal functions in cows. In vitro cell culture techniques may be used to investigate the molecular interactions between sperm surface and epididymal epithelium. Coculture of spermatozoa with epididymal cell cultures may yield important information on epididymal sperm maturation and storage.

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