Prolonged survival of human spermatozoa when co-incubated with epididymal cell cultures

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Human epididymal tissue was recovered from 11 patients undergoing orchidectomy without anti-androgen treatment. Everted epithelial fragments from the caput and corpus epididymis of six patients were successfully cultured in a modified RPMI 1640 medium supplemented with HEPES and androgens for up to 110 days (mean 56 ± 28) in 5% CO2 in air at 37°C. Epithelial cells from human oviduct and non-reproductive tract cells (breast epithelial cells, fibroblasts) were also cultured for comparison. The proportion of epididymal epithelial cells in primary cultures assessed by immunofluorescent localization using a cytokeratin monoclonal antibody was shown to be >70% for the first 6–8 weeks of culture. Light and electron microscopy indicated that epithelial cells maintained polarity and some normal morphology during the culture period. Washed epididymal or ejaculated spermatozoa prepared by a ‘swim-up’ procedure were co-incubated (i) directly with epididymal cells in culture wells, (ii) in 12 mm Millicell® inserts within culture wells, thereby preventing contact of spermatozoa with culture cells; and (iii) in culture medium alone. A significant proportion of spermatozoa in direct contact with culture cells or in Millicell inserts were viable after 6 days of co-incubation (30–45%) and exhibited progressive motility, while all spermatozoa in medium alone were non-motile by 3 days. Using computer-assisted sperm analysis it was shown that the progressive motility of viable spermatozoa decreased gradually for the first 5 days in culture and then remained constant (~30 µm/s, average path velocity). After 12 days of co-incubation, 15 ± 4% of spermatozoa in direct contact with epithelial cells remained motile; in one experiment, a few spermatozoa (<1%) were motile at 17 days. Light and electron microscope observations indicated that prolonged sperm survival was associated with close apposition of spermatozoa (by equatorial segment) to the apical membrane of epithelial cells. Oviductal epithelial cells were also beneficial for sperm survival, but other cell types had no effect.

Key words: CASA/co-culture/epididymis/sperm maturation

Introduction

In normal fertile men, the majority of epididymal spermatozoa acquire the potential to fertilize, as assessed by sperm function assays, on passage into the corpus and cauda regions of the epididymis (Mooney et al., 1972; Hinrichsen and Blaquier, 1980; Moore et al., 1983; Dacheux et al., 1987). When the epididymal tubule is blocked or absent, a small proportion of spermatozoa may acquire fertilizing potential in the more proximal region of the excurrent duct (see Bedford, 1994; Turner, 1995; Moore, 1996; Moore and Akhondi, 1996). These observations suggest that the maturation and storage of human spermatozoa may not be as closely dependent on passage through the caput and proximal corpus regions of the epididymis as in laboratory species. Nevertheless, secretions of the epididymal epithelium do seem to be important for sperm maturation and survival, although the exact nature of these factors has yet to be determined (Moore et al., 1992; Akhondi and Moore, 1993; Boué et al., 1994; Boué and Sullivan, 1996). In a fertile individual, spermatozoa must be maintained in a viable condition during epididymal transit and during storage in the distal cauda epididymis and vas deferens. Investigations of human semen quality in relation to the interval between ejaculations have not indicated any consistent decline in semen quality after 10 days sexual abstinence (Blackwell and Zaneveld, 1992; Cooper et al., 1993), but the precise conditions that spermatozoa require to survive in the epididymis remain unclear. 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). However, compared with most mammals, men are endowed with a poorly differentiated cauda epididymis and have a relatively small sperm storage capacity (Moore and Pryor, 1981). Amann (1981) has estimated that the normal storage capacity for human spermatozoa within the cauda epididymis is only 3.7 days, while the entire transit time for spermatozoa from the testis to ejaculation is estimated to be 12 days (Rowley et al., 1970). It is apparent that changes in androgen concentrations (Orgebin-Crist et al., 1975) or elevated cauda temperatures (Esponda and Bedford, 1986) alter the capacity of the epididymis to store spermatozoa and affect the ion and protein profiles of fluid from the cauda epididymal lumen. Temperature-dependent secretory factors from the epithelium may also act to stabilize membranes to maintain sperm viability (Esponda and Bedford, 1986). Human spermatozoa are in a quiescent state within the epididymal luminal micro-environment, but it has not been established whether particular ions or proteins act specifically to prolong sperm viability (Moore, 1995). Furthermore, underwear may cause elevated scrotal temperatures so that spermatozoa are often stored at near body temperature and still maintain fertilizing potential. Thus, human spermatozoa may be relatively insensitive to temperatures which in other species would compromise cell survival.
Specific secretions from epididymal principal cells associate with spermatozoa during maturation and storage (Moore, 1996). At present, there is a lack of evidence that such factors play a direct role in the development and maintenance of human sperm fertilizing capacity, although a 34 kDa human epididymal sperm protein involved in zona pellucida interactions (Boué et al., 1994) has been associated with cases of male infertility (Boué and Sullivan, 1996). When epididymal spermatozoa are incubated with epididymal cell cultures they can undergo maturation which, in some instances, leads to the development of sperm fertilizing capacity (Moore and Hartman, 1986; Klinefelter et al., 1992; Moore et al., 1992) and may also influence the developmental competence of epididymal spermatozoa (Bongso and Trounson, 1996). A feature of in-vitro maturation is an increase in progressive sperm motility and enhanced sperm survival (Smith et al., 1986; Moore et al., 1992; Bongso and Trounson, 1996). Human oviductal cells may also have a beneficial effect on washed ejaculated human spermatozoa in terms of viability, the synergistic induction of capacitation and the induction of hyperactivated motility (Chian and Sirard, 1995; Kervancioglu et al., 1995; Pacey et al., 1995). These effects seems to be more specific than with other epithelial cell lines (Kervancioglu et al., 1995). The aim of this study was to investigate the interactions of spermatozoa in vitro with epididymal cell cultures and to establish whether specific factors are beneficial in promoting human sperm survival.

Materials and methods

Source and preparation of epididymal epithelium for tissue culture

Epididymal tissue was obtained over a 6 month period from 11 men (mean age 54 ± 12 years) undergoing orchidectomy without anti-androgen treatment. Full ethical permission had been granted. The samples were transferred to pre-warmed RPMI 1640 medium during the operation and brought to the laboratory within 30 min. On arrival in the laboratory, tissue was placed in a vertical laminar flow hood, dissected free of fat and washed extensively to remove blood. Small segments of epididymides from various regions were then placed in principal cell medium (PCM) supplemented with antibiotics, as described previously (Moore et al., 1992), but also containing 25 mM HEPES, and minced using scissors. Human epididymal tubules were always surrounded by a thick layer of connective tissue. Depending on the thickness of the connective elements, the tissue was incubated in PCM containing 2.5 mg/ml collagenase (type II) at 37°C in 5% CO₂ in air for between 8 and 12 h. After digestion, tubule fragments were dissected free of surrounding collagen with a needle, washed with fresh medium and fragmented by shaking. About 30–50 fragments of tubule were cultured in PCM in 5% CO₂ in air and 100% humidity at 37°C. Cell cultures were established from epididymal epithelium from the caput and corpus epididymidis of all 11 patients. Cauda epididymal tubules could not be digested adequately with collagenase to establish long-term cultures. Tissue from only six patients was subsequently used for co-incubation experiments. Cultures from four samples displayed poor viability and failed between 10 and 20 days after initial preparation. In one tissue sample, the cultures became infected with yeast after 8 days of culture. Individual wells of the remaining primary cultures were maintained for between 4 and 12 weeks before they failed or were overgrown with fibroblasts.

Spermatozoa were co-incubated with epididymal cultures at various times during this period (Table I).

Maintenance and assessment of epididymal cell cultures

Culture medium was changed every other day. For the first 2–3 days, while cells were becoming attached to the bottom of the culture well, about half of the medium was replaced. Once the cells were attached and not free in the medium, it was then possible to replace most of the medium (90%) without loss of tissue. The cells were examined by phase-contrast microscopy every day using an inverted microscope (IMT-2; Olympus, London, UK) and the appropriate photography was performed. For electron microscopy, epididymal epithelium was recovered at various stages of culture and fixed in 2.5% glutaraldehyde (25% Aqueous solution; Sigma Ltd, Poole, UK) in phosphate-buffered saline for 2 h, before preparation for electron microscopy. Sections were cut on a Reichert Ultracut E ultramicrotome (Leica, Cambridge, UK) and examined using a Philips (Eindhoven, The Netherlands) CM10 transmission electron microscope.

At various periods, epididymal cultures were probed with antibody against cytokeratin to establish the proportion of epithelial cells (Henriksen et al., 1990). Two methods were used. When cells had plated out on the plastic, it was possible to probe the entire culture dish or well. This was achieved by washing the cells in fresh medium and then adding monoclonal antibody against cytokeratin at a dilution of 1:500. Cells were incubated with primary antibody for 1 h, washed three times and then incubated with fluorescein-conjugated goat anti-mouse immunoglobulin G for a further 1 h. The cells were then washed three times with fresh medium and examined using fluorescence microscopy. Cells were also recovered from the culture dish by vigorous pipetting. They were placed in a small Eppendorf tube (1.0 ml) and incubated in a similar manner with primary and secondary antibody. At the end of the incubations, cells were transferred to a slide and examined for fluorescence.

Oviductal epithelium and other epithelial cell lines

Fragments of human oviductal epithelium were obtained from a woman undergoing surgery (provided by Dr Allan Pacey) and prepared as described elsewhere (Pacey et al., 1995). Between 10 and 20 fragments were placed in each well of a 24-well culture plate in 1 ml PCM (without testosterone and dihydrotestosterone). For co-incubation experiments, spermatozoa were introduced after 3 days of culture. Three different human fibroblast cell lines (171 BR, MRC5, MRCV1) and one human epithelial cell line (breast epithelial cells, Hs 578T) were obtained from the Institute for Cancer Research (University of Sheffield, Sheffield, UK). Cells were passaged in 24-well tissue culture plates in their appropriate medium. When confluent monolayers of cells had formed, their normal medium was replaced with PCM to provide comparable conditions for spermatozoa to those present in epididymal or oviductal epithelial cultures. Co-incubation experiments with spermatozoa were carried out when cells were confluent after 5 days of culture.

Epididymal spermatozoa

Sufficient motile spermatozoa were found in four of the 11 epididymal samples for use in co-incubation experiments with previously established epithelial cell cultures. Following tissue dissection, as described above, small segments of epididymides were placed in PCM, minced using scissors and incubated for 10–20 min to release the spermatozoa. The sperm suspension was then separated from the diced tissue and concentrated by centrifugation at 350 g for 10 min. The sperm pellet was resuspended in PCM to give a final dilution of ~5×10⁶ spermatozoa/ml.
Table I. The survival of epididymal spermatozoa incubated with epididymal cell cultures derived from the caput and corpus epididymis or in medium alone

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>Length of epididymal cell culture (days)</th>
<th>Maximum sperm survival (days)</th>
<th>With culture</th>
<th>Inserts</th>
<th>Medium alone</th>
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<tr>
<td>1</td>
<td>59</td>
<td>10</td>
<td>8</td>
<td>2</td>
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<td>4</td>
<td>19</td>
<td>8</td>
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Spermatozoa were incubated directly with epididymal cells or within Millicell inserts.

Table II. The survival of washed ejaculated spermatozoa incubated with epididymal cell cultures derived from the caput or corpus epididymis or in medium alone

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>Length of epididymal cell culture (days)</th>
<th>Maximum sperm survival (days)</th>
<th>With culture</th>
<th>Inserts</th>
<th>Medium alone</th>
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<tr>
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<td>9</td>
<td>12</td>
<td>9</td>
<td>2</td>
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</table>

Spermatozoa were incubated directly with cells or within Millicell inserts.

Ejaculated spermatozoa

Human semen samples were obtained by masturbation from donors attending the Andrology Laboratory, Department of Obstetrics and Gynaecology, Jessop Hospital for Women (Sheffield, UK). All donors were free of any detectable pathology, including hepatitis and human immunodeficiency virus infection. Each semen sample was allowed to liquefy for at least 30 min before a routine semen analysis was undertaken to ensure that it could be classified as exhibiting a normozoospermic semen profile according to the criteria laid down by the World Health Organization (WHO, 1992). These criteria were: volume >2 ml, concentration >20×10⁶/ml, motility >50% with forward progressive movement and morphology >30% normal. Semen samples (1 ml) of good quality were placed in a sterile 15 ml conical-bottomed, plastic tube (Falcon; Becton Dickinson and Co., Plymouth, UK) and brought to the laboratory within 1 h. Spermatozoa were separated from seminal plasma by centrifugation at 350 g for 10 min and then either washed three times in minimal essential medium by further centrifugation or left alone. To separate the motile sperm population, a ‘swim-up’ procedure was performed as described elsewhere (Brewis et al., 1996).

Co-incubation of human spermatozoa with human epididymal cell cultures and other preparations

Epididymal and ejaculated spermatozoa prepared as described above were added to human epididymal cell cultures or other cell cultures at a final concentration of 2–5×10⁶ spermatozoa/ml in the culture well. Spermatozoa were co-incubated in the following ways: (i) directly with epithelial epididymal cell culture; (ii) in transparent 12 mm Millicell® CM inserts (Millipore, Watford, UK) within the culture well, thereby preventing contact of spermatozoa with epithelial cells; or (iii) in PCM alone. Every day approximately two-thirds of the culture medium was exchanged for pre-warmed fresh medium. When Millicell inserts were present, the medium was exchanged in the well rather than in the insert. Three culture wells from the same initial preparation were used for each co-incubation experiment.

Evaluation of spermatozoa during co-incubations

The motility of spermatozoa in co-incubations was examined every day using a phase-contrast inverted microscope (×10 magnification objective lens) fitted with a thermostatically controlled stage (37°C). Because of the height of the insert in the well, spermatozoa in Millicell inserts could not be visualized by the ×10 objective lens and were observed instead by a ×4 objective which gave a greater depth of focus. Alternatively, a small amount (30 µl) of medium from the insert was placed on a microscope slide and spermatozoa were assessed using the ×10 objective. Video recordings of sperm motility and the interaction of spermatozoa with epithelial cells were also carried out each day.

Sperm motility was analysed by computer-assisted sperm analysis (CASA) using the Hobson Sperm Tracker (Hobson Tracking Systems Ltd, Sheffield, UK). The settings for human spermatozoa were as described elsewhere (Mohammad et al., 1996). Briefly they were as follows: search radius = 9.19 µm, predict = off, aspect = 1.49, refresh rate = 6 s, threshold = +16–25, filter weightings 1 = –2, 2 = –2, 3 = 0, 4 = 0. The tracker was used at an image capture rate of 25 frames/s. CASA was performed at the time of co-incubation and every 24 h after co-incubation. For this study, only the main velocity measurements of the spermatozoa [curvilinear velocity (VCL), average path velocity (VAP) and straightline velocity (VSL)] were analysed. Aliquots (30 µl) of medium from the different co-incubations were transferred into wells of a 24-well tissue culture plate, covered with oil and analysed immediately on an inverted microscope with a thermostatically controlled stage at 37°C. The attachment of human spermatozoa to epithelial cells and the difficulty...
Sperm survival with epididymal epithelium in vitro

Figure 1. (a) Phase-contrast micrograph of human epididymal epithelial fragments from the corpus region before culture. Bar = 500 µm. (b) Phase-contrast micrograph of epididymal fragment as in (a) after culture for 5 days. Epithelial cells have migrated from the main fragment and plated out on the bottom of the culture well. Bar = 100 µm.

Figure 2. Proportion of cytokeratin-positive (epithelial) cells after various lengths of culture (n = 12).

of detaching these spermatozoa without damage precluded the quantitative analysis of sperm movement after 8 days. Individual tracks of spermatozoa were obtained using the ‘Trail-draw’ facility of the Hobson Sperm Tracker.

Sperm–epithelial interactions were analysed from video recordings using Image 1.52 software. This facility could display and animate images of spermatozoa during attachment and detachment to epithelial cells.

Statistical analysis
A $\chi^2$ analysis was used to compare differences in significance between the treatment groups for sperm motility and survival.

Results
Preparation and maintenance of epididymal cell cultures
Plaques of human epididymal epithelium were prepared as shown in Figure 1a. These preparations everted overnight to form spheres of tissue which subsequently started to attach to the bottom of the well by 3–5 days in culture to form nodes of epithelium. Epithelial cells migrated from these nodes of tissue and plated out in a characteristic ‘paving stone’ arrangement. During the subsequent culture period, these areas of epithelial cells became larger as more cells migrated from the original tissue (Figure 1b). Epididymal cells from the caput and corpus region were cultured for a mean of 56 ± 28 days (Tables I and II). The preparation of cells from the cauda region proved difficult and inconsistent. Fragments of tissue failed to evert in culture because of inadequate digestion by

Figure 3. Electron micrograph of epididymal epithelium after 42 days in culture. Principal cells are reduced in height but have maintained polarity and display a morphology consistent with secretory and absorptive activity. Bar = 5 µm.
Table III. Survival of epididymal and washed ejaculated spermatozoa incubated with oviductal epithelial cultures or medium alone

<table>
<thead>
<tr>
<th>Number and type of sperm sample</th>
<th>Length of oviductal cell culture (days)</th>
<th>Maximum sperm survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With culture</td>
<td>Inserts</td>
</tr>
<tr>
<td>Epididymal (n = 1)</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Ejaculated (n = 2)</td>
<td>10–14</td>
<td>12</td>
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</table>

Spermatozoa were incubated directly with epithelial cells or within Millicell inserts.

Figure 4. Mean proportion (%) of motile ejaculated spermatozoa after incubation with epididymal cell cultures or with medium alone (n = 12). (•) ~0.1%, one sample only. (**) Significantly different from medium alone (P < 0.05). After this time spermatozoa in medium alone were non-motile. Bars = ±SEM.

Collagenase and did not attach to the bottom of the culture wells. These preparations were not used for subsequent experiments.

Epididymal epithelial cells were identified by immunofluorescence using a monoclonal antibody (Sigma) against the epithelial marker, cytokeratin. Bright immunofluorescence was detected on cells with the morphological characteristics of epithelial cells. The proportion of cytokeratin-positive cells at various times in culture is shown in Figure 2.

As assessed by electron microscopy, epithelial principal cells retained their characteristic morphology when they remained attached or were close to the original basal lamina. An electron micrograph of human epididymal cells cultured for 42 days is shown in Figure 3. Epithelial cells remained in tight contact with each other by intracellular junctions and the basal lamina was still present. Although the cells were reduced in height, they maintained polarity and some microvilli.

Morphology of non-epididymal epithelial cells and fibroblasts in culture

As assessed by light microscopy, human oviductal epithelial cells showed the same characteristics in culture as epididymal epithelial cells and attached to the bottom of the culture well after 2–3 days. The new passage of a human breast epithelial cell and three different fibroblast cell lines led to monolayer confluency in ~2–4 days.

Survival of epididymal and ejaculated spermatozoa co-incubated with epididymal cell cultures, oviductal cell cultures and other cell lines

In four experiments, human epididymal spermatozoa from the cauda region were co-incubated with epididymal cell cultures. Epithelial cultures were beneficial for sperm survival compared with medium alone (Table I). When in direct contact with epithelial cells, 40 ± 15% of spermatozoa were motile after 6 days. Maximal sperm survival was 10.8 ± 2.5 days (range 8–14), as assessed by motility. In Millicell inserts, 32 ± 12% of spermatozoa were motile after 5 days and maximal survival was 8.5 ± 1.3 days (range 7–10). In medium alone, spermatozoa survived for 2.0 ± 0.5 days (Table I), although after 24 h there was good sperm survival (60%).

Washed ejaculated swim-up spermatozoa exhibited a similar profile as epididymal spermatozoa. When incubated directly with epididymal cells, 45 ± 10% of spermatozoa were motile after 6 days and 15 ± 9% were motile after 12 days. The mean maximal survival time was 12.5 ± 1.6 days, with some spermatozoa surviving for 17 days in one experiment (Table II). There was a reduction in the survival times for spermatozoa incubated within inserts, with 28 ± 12% of spermatozoa motile after 6 days and a mean maximal survival time of 9.3 ± 1.1 days. In contrast, in medium alone or when ejaculated spermatozoa were not washed by centrifugation before their swim-up, mean maximal survival was 2 days even when epithelial cells were present (Table II).

Human epididymal or ejaculated spermatozoa were also co-incubated with oviductal epithelial cultures. In one experiment with cauda epididymal spermatozoa, 40 ± 10% of spermatozoa survived for 6 days when co-incubated directly with cultured epithelial cells; the maximum survival time was 10 days. Spermatozoa incubated within inserts survived for 7 days, while those in medium alone survived for 2 days (Table III). Two experiments were carried out with ejaculated washed spermatozoa (Table III). After 6 days of co-incubation directly with cells, 36 ± 10% of spermatozoa were still motile and the mean maximal sperm survival was 11.5 ± 0.5 days. Within inserts, 21 ± 12% of spermatozoa were motile after 6 days, and the mean maximal survival time was 11 days. In contrast, co-incubation of human spermatozoa with a human epithelial cell line or with various fibroblast cell lines of non-
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Figure 5. Mean sperm velocity measurements [curvilinear (VCL), average path (VAP) and straightline (VSL)] for washed ejaculated spermatozoa in direct co-culture with corpus epididymal epithelial culture or in medium alone. (*) Significantly different from medium alone at this time and later \( (P < 0.05) \). Bars = ±SEM.

The mean progressive motility of viable spermatozoa decreased gradually over the first 5 days and then remained constant (~30 μm/s for VAP). Although some spermatozoa remained motile after this period, there were too few to measure accurately using CASA. In comparison, the mean velocity parameters of spermatozoa in medium alone decreased rapidly; within 2–3 days all spermatozoa were immotile (Figure 5).

Sperm–epithelial interaction during co-incubation

Without inserts, spermatozoa bound to epididymal epithelial cells during co-incubations. At the beginning this was for short periods (~30 s), after which spermatozoa detached. During the later stages of co-incubation (i.e. 8 days), sperm–epithelial attachments were firm and extended up to 1 h.

Figure 6. Electron micrograph of a spermatozoon bound to the apical surface of an epididymal principal cell after co-culture for 14 days and motile before fixation. Electron dense material lies between the equatorial region and the principal cell (arrowed). The spermatozoon has an intact acrosome. Bar = 500 nm.

reproductive origin led to no significant increase in sperm survival compared with medium alone (2–3 days).

Motility of ejaculated spermatozoa co-incubated with epididymal cell cultures (without insert)

In direct co-incubation at 37°C, 50 ± 10% of spermatozoa displayed progressive motility after 6 days. There was a 25% survival of washed ejaculated spermatozoa in culture after 10 days with progressive motility. After 12 days, 15% of spermatozoa were motile, and in one experiment a few spermatozoa (~1%) still displayed progressive motility after 17 days. However, ejaculated spermatozoa that were not washed by centrifugation failed to survive in co-incubations longer than in medium alone (Figure 4).

By CASA, it was possible to analyse the velocity parameters (VCL, VAP and VSL) of spermatozoa in co-incubations for the first 8 days of co-incubation (Figure 5). By CASA, it was possible to analyse the velocity parameters (VCL, VAP and VSL) of spermatozoa in co-incubations for the first 8 days of co-incubation (Figure 5).

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Discussion

An important aspect of co-culture experiments is that close interactions between spermatozoa and epithelial cells can be examined in detail. Clearly, these systems do not represent the conditions prevailing in the epididymis. Spermatozoa are motile, and at a far lower concentration \( (×10^3) \) than in the lumen of the tubule; therefore careful interpretation of the results is required. The main conclusion to be drawn from our study is that human epididymal epithelial cells maintained in culture are highly beneficial for the long-term...
survival of human spermatozoa in vitro. For co-incubations carried out at 37°C, ~50% of spermatozoa were motile after 8 days; in one case, a few spermatozoa survived with progressive motility for up to 17 days. This indicates that human spermatozoa may be resilient to body temperature under the appropriate conditions. Indeed, the condition of post-vasectomy ejaculates closely parallels our in-vitro results. After vasectomy in men, spermatozoa retained in the vas deferens distal to the ligation will be stored close to, or at, core body temperature. There is a progressive decline in sperm viability during this time, which has been shown to result in complete loss of motility in 84% of semen samples by 13 days post-vasectomy (Bedford and Zelikosky, 1979) and in 100% of samples by 15 days post-vasectomy (Jouannet and David, 1978). Richardson et al. (1984) were able to construct a regression equation for this decline in the viability of post-vasectomy spermatozoa, and calculated a complete loss of motility at ~16 days. This corresponds closely with the 17 days maximum survival of spermatozoa in our co-incubations, and suggests that the inherent maximal survival time of human spermatozoa is at about this length of time. The ability of spermatozoa to penetrate zona-free hamster eggs was still apparent in some individuals 8 days after vasectomy (Richardson et al., 1984), indicating that at this stage a reasonable proportion of cells were functionally competent. This time period also corresponds to the proportion (50%) of spermatozoa still displaying progressive motility during co-incubations.

Oviductal epithelial cultures were nearly as beneficial as those from the epididymis in maintaining sperm viability. In contrast, other cell lines (breast epithelial cells, fibroblast cells) had no effect. This may indicate that only epithelium of the reproductive tract is advantageous for spermatozoa. Certainly, human oviductal cells in culture have a beneficial effect on washed ejaculated human spermatozoa, and these effects seem to be more specific than with other epithelial cell lines, e.g. Vero cells (Kervancioglu et al., 1995). This action may be species-specific because cultures of rat epididymal epithium have been shown to be ineffective for enhancing human sperm survival times (M.A.Akhondi and H.D.M.Moore, unpublished data). However, further studies with epithelium from other species need to be made.

Human epididymal epithelial cells were maintained for longer periods than reported previously by this laboratory (Moore et al., 1992) and still retained some biological activity. Other investigators (Byers et al., 1986; Cooper et al., 1990; Klinefelter, 1992; Raczek et al., 1994, 1995) have cultured human epididymal epithelium in primary culture for extended periods (i.e. 6 weeks), and have also demonstrated biological activity consistent with epithelial function (principal cell function). The PCM was modified in our study to include 25 mM HEPES for greater buffering capacity. This addition would have maintained a more consistent pH when examining cultures outside the incubator. Furthermore, after attachment of the epithelium to the culture wells, more than half the medium was replaced each day. The most important requirement for obtaining a reasonable epithelial preparation for culture was collagenase digestion of the thick layer of connective tissue surrounding the tubules. Overnight incubation with collagenase was found to be most beneficial in this respect. Electron microscopy of epididymal epithelium after 6 weeks in culture (Figure 3) clearly indicated that principal cells had retained polarity and differentiation. Although epithelial cells displayed little proliferative activity, once they were established on the surface of the dish they spread out from the original plaque of tissue and quickly formed a confluent mass, from which fibroblasts and other non-epithelial cells were excluded (as determined by cytokeratin immunolocalization). Surprisingly, in the most viable cultures, fibroblast overgrowth did not occur for many weeks, suggesting that epithelial cells actively inhibited their proliferation. In contrast to epithelium from the caput and corpus region, cultures of epithelial cells from the cauda region were less successful, probably because the basal lamina of connective tissue and smooth muscle was much thicker and could not be adequately digested with collagenase. In addition, spermatozoa were bound to tissue fragments, making it difficult to obtain samples completely free of epididymal spermatozoa.

A number of experiments were carried out with epididymal spermatozoa, but it was often not possible to obtain these cells at the same time that epididymal cell cultures had been established. Therefore ejaculated spermatozoa were also used to examine sperm survival. An important finding was that the procedure for the initial preparation of ejaculated spermatozoa was crucial for their long-term survival in the presence of epididymal epithelial cultures. Only spermatozoa that had been washed by centrifugation prior to a ‘swim-up’ protocol maintained good survival during the subsequent co-incubation. The reason for this is unknown. One can speculate that thorough washing may remove seminal plasma components that are detrimental to spermatozoa or would otherwise interfere with beneficial factors from the epididymal epithelium.

Incubating spermatozoa in Millicell inserts was carried out to prevent direct contact with the epithelial culture. As sperm survival was enhanced in these preparations, it would suggest that the factors responsible were soluble and could diffuse through the Millicell membrane. The pore size for these membranes is 0.4 µm, and therefore macromolecules would pass across freely. However, the longest sperm survival was obtained when there was direct contact between the epididymal epithelial cells and spermatozoa. This suggests that, in vitro at least, an intimate sperm–epithelial interaction occurs. This could be a result of the need for membrane exchange of glycosylphosphatidylinositol-anchored moieties (Kirchhoff and Hale, 1996) and the labile nature of epididymal secretions. Attachment of spermatozoa by the equatorial segment to the apical surface of epithelial cells seemed to be particularly advantageous for sperm survival. Generally, those spermatozoa attached to epithelial cells remained motile and viable and were retained in the incubation (rather than being lost during medium exchange each day). Previous studies in the hamster had also noted that sperm viability was enhanced by epithelial interaction (Smith et al., 1986). The integrity of the sperm acrosome
was also maintained by spermatozoa bound to epididymal epithelial cultures. A similar stabilization of the acrosome has been noted for spermatozoa interacting with oviductal cells in vitro (Morales et al., 1996). In contrast, spermatozoa failed to bind to the monolayers of the various cell lines during co-incubation, and these cells were not conducive for good sperm survival.

How epididymal cells maintain sperm viability in vitro remains unknown. Secretions from principal cells might prevent lytic enzymes, released from the acrosomes of degenerating spermatozoa, from acting upon other cells. A major mRNA of the epithelium encodes a protease inhibitor (Kirchhoff et al., 1991), and a serine protease inhibitor similar to or the same as α2-antitrypsin is also secreted by principal cells in culture (Akhondi and Moore, 1993; Moore and Akhondi, 1996). The epididymis may also prevent oxidative stress in spermatozoa. In the rat, at least, it has been shown that γ-glutamyl transpeptidase is present in the lumen particularly in the proximal region (Hinton et al., 1991). This enzyme regulates the level of glutathione, which is a potent anti-oxidant that can protect against free oxygen radicals that might otherwise attack sperm membranes.

Acknowledgements

This study was supported by a grant from the MRC. M.A. was supported by a studentship from the Iranian Government.

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Received on September 4, 1996; accepted on December 6, 1996