Characterization of epithelial cell culture from human hydrosalpinges and effects of its conditioned medium on embryo development and sperm motility

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BACKGROUND: Recent studies have reported the negative impact of hydrosalpinx on IVF outcome. Toxic effects of hydrosalpinx fluid (HF) have been the main reason for the recommendation of functional surgery, salpingectomy, prior to IVF. The present study characterized hydrosalpinx epithelial cell culture and examined the effects of its conditioned medium (CM) on sperm motility, acrosome reaction and embryo development.

METHODS: Normal Fallopian tubes (n = 6) and hydrosalpinges (n = 9) were used to prepare epithelial cell culture and CM. Epithelial cell characterization was confirmed using electron microscopy. Sperm motility and acrosome reaction were determined using computer-aided sperm analysis and acrobead assay respectively and embryo development by mouse embryo development assay.

RESULTS: The percentage of human motile sperm incubated in hydrosalpinx CM was significantly different from those in normal Fallopian tube (NFT) CM and modified human tubal fluid medium (hTF) (control) (P < 0.05 at 3 h and P < 0.001 at 5 and 24 h), with alteration in movement characteristic, linearity, 24 h after incubation in hydrosalpinx CM (P < 0.05). However, other sperm movement characteristics remained unchanged. Reduced acrosome reaction and poor mouse embryo development were also observed in hydrosalpinx CM but not in NFT CM and hTF. CONCLUSIONS: The results suggest that hydrosalpinx epithelial cells may be producing a fluid milieu hostile to sperm and early embryo development. The established epithelial cell culture system may provide a model to further investigate the mechanisms underlying the toxic effects of HF on embryo development and the adverse effects on IVF outcomes.

Key words: acrosome reaction/conditioned medium/embryo development/hydrosalpinx/sperm motility

Introduction

Recent studies have shown that the presence of hydrosalpinx is detrimental to IVF and embryo transfer outcome (Aboulghar et al., 1998; Zeyneloglu et al., 1998; Camus et al., 1999). Adverse effects of hydrosalpinx fluid (HF) on mouse embryo development (Mukherjee et al., 1996; Beyler et al., 1997; Murray et al., 1997; Rawe et al., 1997; Hiramatsu et al., 1997; Koong et al., 1998; Spandorfer et al., 1999; Roberts et al., 1999; Carrasco et al., 2001) and fertilization (Arrighi et al., 2001) are well documented. HF is also known to be toxic to human sperm in vitro (Ng et al., 2000). However, it has not been assessed to what extent the epithelial cells of the Fallopian tube are involved in mediating the toxic effects of HF.

The role of epithelial cells in normal oviductal fluid formation and their importance in normal sperm functions and embryo development have been reported (Dickens et al., 1993, 1995, 1996; Dickens and Leese, 1994; Downing et al., 1997; Downing et al., 1999). Epithelial cells from normal Fallopian tubes have been cultured (Bongso et al., 1989; Ouhibi et al., 1989; Henriksen et al., 1990; Ménézo et al., 1990; Takeuchi et al., 1991). Co-culture of normal Fallopian tube epithelial cells with embryos has been shown to enhance in-vitro development of embryos to blastocyst stage (Bongso et al., 1989; Ménézo et al., 1990; Yeung et al., 1992), improve pregnancy rates (Bongso et al., 1992) and maintain sperm motility in vitro (Morales et al., 1996; Murray et al., 1997; Yao et al., 2000). These results suggest that Fallopian tube fluid is important for a number of reproductive events. However, epithelial cells from hydrosalpinx have not been cultured and characterized, and thus the role of epithelial cells in the hydrosalpinx.
formation of hydrosalpinx and HF has not been investigated to any significant extent (Ajonuma et al., 2002).

In an attempt to investigate the role of epithelial cells in the formation of hydrosalpinx and HF, epithelial cell cultures from human hydrosalpinx were established and characterized in the present study. The effects of hydrosalpinx epithelial cell culture conditioned medium (CM) on mouse embryo development, human sperm motility and acrosome reaction were also examined.

Materials and methods

The study was carried out at the Epithelial Biology Research Centre, Faculty of Medicine, Chinese University of Hong Kong. Ethical approval was obtained from the Ethics Committees of the Faculty of Medicine, the Chinese University of Hong Kong and the University of Hong Kong. All patients gave written informed consent prior to their participation in this study and experiments on animals were conducted strictly in accordance with the university guidelines on animal experimentation.

Epithelial cell culture and collection of conditioned medium

Normal Fallopian tubes (NFT) were obtained from healthy Chinese women within their reproductive ages and in different menstrual cycles who had bilateral salpingectomy for tubal sterilization. Patients undergoing IVF with hydrosalpinx observed during ultrasound monitoring were advised to undergo bilateral salpingectomy prior to undergoing IVF with hydrosalpinx and HF has not been investigated to any significant extent (Ajonuma et al., 2002).

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copulation plugs were killed 40–42 h post hCG administration. Recovered 2-cell embryos were placed in pre-warmed modified human tubal fluid medium supplemented with 0.5% bovine serum albumin (BSA) (hTF; Irvine Scientific, Santa Ana, CA, USA). hTF is not very different from the culture medium DMEM/F-12 used for CM preparation in that both media contain all major essential nutrients in a simple culture medium. They are also supplemented with serum, HEPES and bicarbonate at similar concentrations. Both media support embryo development and can maintain sperm motility in vitro.

Eighty-four 2-cell mouse embryos were collected after superovulation and randomly allocated: 20 to hydrosalpinx CM in duplicate (40), 24 to NFT CM, and 20 to hTF. Both NFT CM and hTF served as controls. The embryos were cultured under embryo-grade mineral oil (Sigma). Embryo development was assessed after a further 36–48 h of culture in vitro with an inverted microscope (Axiovert 25; Zeiss, Germany).

Measurement of sperm motility

Sperm collection and processing

The details of semen collection, sperm preparation, and sperm motility analysis had been previously described (Ng et al., 2000). In brief, semen samples were collected by masturbation from men attending the Assisted Reproduction Unit of The Prince of Wales Hospital, Chinese University of Hong Kong after abstinence of 3–4 days. Only the Assisted Reproduction Unit of The Prince of Wales Hospital, semen samples were collected by masturbation from men attending in vitro (Sigma). Embryo development was assessed after a further 36–48 h of controls. The embryos were cultured under embryo-grade mineral oil (Sigma). Embryo development was assessed after a further 36–48 h of culture in vitro with an inverted microscope (Axiovert 25; Zeiss, Germany).

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Sperm motility analysis

CM samples were thawed at room temperature and placed in a humidified chamber at 37°C under 5% CO₂ in air. Sperm suspensions were adjusted with modified sperm washing medium to a concentration of 20×10⁶/ml. Sperm from each sample were divided into three equal portions. The adjusted sperm suspension aliquots were centrifuged at 500 g for 5 min and the supernatants discarded as much as possible. The sperm pellets were resuspended in either 0.5 ml of NFT or hydrosalpinx CM and 0.5 ml of hTF (control).

Sperm motility and velocities were analysed at 1, 3, 5 and 24 h after incubation at 37°C under 5% CO₂ using the TOX-IVOS Sperm Analysis System (Hamilton Thorne Research, Beverly, MA, USA). TOX-IVOS Sperm Analysis System version 10.8 set-up parameters were set at ×4 objective in dark field, 30-frame shift rate at 60 Hz, minimum contrast of 80 and illumination intensity of 3200 at 0.82 magnifications. A 10 μl aliquot of the sperm suspension was transferred to a pre-warmed Hamilton Thorne Research 2X-CEL disposable semen analysis slide (Hamilton Thorne) with a chamber depth of 20 μm placed on a warmed microscope stage at 37°C. Five fields were randomly selected during evaluation and 200 sperm were analysed in each field.

The following CASA parameters were determined: percentage of motile sperm, mean curvilinear velocity (VCL, μm/s), mean straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), mean linearity (LIN; VSL/VCL), amplitude of lateral head displacement (ALH, μm), head beat cross frequency (BCF, Hz), and straightness (STR; VAP/VCL×100%).
beating cilia. There was no observed difference in the growth pattern and rate between normal and hydrosalpinx epithelial cells. Subcultures reached confluence in 2–3 days. Both the normal and hydrosalpinx cultures were stained positive for epithelial membrane antigen using specific antibodies, indicating their epithelial nature. Epithelial cells could be kept in culture for ∼10 weeks after which cells acquired a fibroblast-like morphology (seen more in hydrosalpinx samples) but still retained epithelial characteristics. The most notable difference between the two cultures was that the culture of normal Fallopian tubes had more secretory vesicles than that of hydrosalpinx epithelial cells (Figure 1A and B).

Scanning electron microscopy of normal Fallopian tube cultured epithelial cells showed cells with a cobblestone appearance, typical epithelial cell characteristics including cilia, microvilli and epithelial folds, whereas hydrosalpinx cultured epithelial cells showed epithelium devoid of cilia, few microvilli and flattening of membrane folding (Figure 1C and D).

**Effects of CM on mouse embryo development**

Osmolality and pH of both NFT and hydrosalpinx CM were within physiological ranges of 287–300 mmol/kg and 7.2–7.5 respectively and comparable with control values. In all, 3/40 (8%) mouse embryos cultured in the hydrosalpinx CM developed. Two of the three embryos developed up to 4-cell and one to 8-cell embryo stages. In NFT CM and hTF, 17/24 (71%) and 19/20 (95%) mouse embryos developed into morula and early blastocysts respectively (Figure 2). Embryo development in hydrosalpinx CM also showed poor embryo development.
development with extensive blastomere fragmentation and degeneration compared with those of NFT CM and hTF in this study.

Effects of CM on sperm motility
The percentage of motile sperm incubated in normal Fallopian tube (NFT) CM, modified human tubal fluid medium (hTF) and hydrosalpinx (HSPX) CM. Hydrosalpinx CM was significantly different from those in NFT CM and hTF (P < 0.05 at 3 h and P < 0.001 at 5 and 24 h).

Effect of CM on acrobead reaction
To determine the effect of NFT CM and hydrosalpinx CM on sperm capacitation and acrosome reaction, the acrobead assay was performed. After 24 h of incubation, hydrosalpinx CM had median acrobead score of 1 (range 0–2, n = 8) whereas the median score was 4 (range 3–4, n = 8) in NFT CM and hTF, indicating a higher percentage of sperm undergoing acrosome reaction and thus greater sperm fertilizing ability.

Discussion
To the best of our knowledge, this is the first study to characterize cultured epithelial cells from human hydrosalpinx and to demonstrate adverse effects of hydrosalpinx CM on sperm function and embryo development. Epithelial cells survive in culture for >10 weeks. Cells cultured on both coverslips and culture dishes showed some sort of polarization, as beating cilia were seen in all samples, even though this was lost after subculture. Although there was no observed difference in growth patterns between normal Fallopian tube and hydrosalpinx epithelial cells, normal Fallopian tube cells had more secreting vesicles than cultured hydrosalpinx epithelial cells. These features are confirmed by both light microscopy and scanning electron microscopy.

In the present study, CM of hydrosalpinx epithelial cell cultures demonstrated adverse effects on mouse embryo development, human sperm motility and acrosome reaction whereas CM from NTF had similar effects to that of hTF (control), suggesting that hydrosalpinx epithelial cells may be secreting substance(s) yet to be identified into the lumen. Those substances may be hostile to sperm affecting their motility, capacitation and acrosome reaction and early embryo development in post-infectious hydrosalpinx. Since hydrosalpinx yields different influences on IVF outcomes, some variations may have been possible and individual CM sample test would have provided more distinct results. However, sample size was a limiting factor in this study. Therefore, CM from all patients was pooled together prior to sperm motility and embryo development assay. Second, it would have been more clinically relevant to use human embryos, but for ethical reasons, human embryos were not used. Only two studies using human embryos in hydrosalpinx have been reported (Granot et al., 1998; Strandell et al., 1998). Granot et al. used only four samples and abnormal (three pronuclear 3PN) embryos. Small sample size may be the reason for lack of toxicity seen in their embryos.
study. On the other hand, Strandell et al. (1998) used 12 samples and had 13.9% embryo (blastocyst) development rate (range 0–24%) in their 100% HF dilution, significantly different from 50% HF (33.3% range 13–56%) and control ($P = 0.0027$).

We have suggested that abnormalities in the transepithelial ion transport across epithelia may be one of the most important factors in the pathophysiology of post-infectious hydrosalpinx formation (Ajonuma et al., 2001). Human Fallopian tube epithelial cells have been reconstituted in a polarized culture and chloride ion movement was found to be responsible for the generation of transepithelial potential difference across the cultured epithelial cells (Dicksens et al., 1996; Downing et al., 1997). These chloride fluxes were sensitive to the inhibitors of some ion channels and co-transporters (Gott et al., 1988). These transporters may include the sodium/hydrogen exchangers (NHE), anion Cl-/HCO3- exchangers (AE), sodium bicarbonate co-transporter (NBC) and sodium epithelial channels (ENaC), aquaporin water channels (AQP) and cystic fibrosis transmembrane regulator (CFTR), a cAMP-activated chloride channel. Post-infectious hydrosalpinx pathology such as atrophy of mucosal folds, marked exfoliation and loss of epithelial cells may affect epithelial membrane ions channels and transporters located on the epithelial membrane necessary for electrolyte and fluid transport, leading to abnormal fluid secretion and reabsorption. CFTR, in particular, may also play an important role in the process of HF formation, considering its multifunctional properties, i.e. a channel, regulator of other epithelial channels, and more recently, a bacterial receptor (Pier et al., 1997; Pier et al., 1998; Zaidi et al., 1999; Gercel et al., 2000; Goldberg and Pier, 2000; Ajonuma et al., 2002).

The loss of membrane polarity may lead to decreased expression of epithelial membrane transporters and ion channels. This could be responsible at least in part for the formation of HF following pelvic inflammatory disease and subsequent adverse effects (Ajonuma et al., 2002).

The electrolyte composition of CM was not analysed in this study but pH and osmolality values were within physiological ranges and comparable with the control. Therefore, reduction in the percentage of progressive motile sperm after 3 h of incubation, decreased acrosome reaction and poor embryo development in hydrosalpinx CM only were unlikely to be due to effects of pH or osmolality. Energy substrates in CM would not lead to adverse effects of hydrosalpinx CM as NFT CM prepared under the same condition exhibited CASA parameters, acrosome reaction and embryo development results comparable to hTF (control).

There are two possibilities which could account for the toxic effect of CM obtained from the epithelial cells of post-infectious hydrosalpinx. First, the hydrosalpinx epithelial cells may be producing less embryotrophic factors on which sperm motility, fertilization and early embryo development depend. Low protein values (Ng et al., 2000; Ajonuma et al., 2001) have been suggested to contribute to HF adverse effects on sperm motility. The present finding that secretory vesicles in the NFT epithelial cell culture (Figure 1) are drastically reduced, and sometimes absent, in the hydrosalpinx epithelial cells indicated possible decreased production of oviduct-specific glycoproteins in hydrosalpinges. The alternative possibility is that infected epithelial cells in hydrosalpinx may produce toxic substance(s), not yet characterized, affecting sperm functions and embryo development. For instance, higher levels of 57 kDa heat shock proteins have been observed in HF and reported to be associated with less embryo development (Beatty et al., 1993). It is also possible that hydrosalpinx epithelial cells may produce substance(s) that are hostile to sperm affecting their motility, capacitation and acrosome reaction. For example, the effect of reactive oxygen species (ROS) in HF on mouse embryo development has been reported (Bedaiwy et al., 2002). It is well recognized that infection, such as that leading to hydrosalpinx and cytokine production, can induce the expression of nitrogen oxide synthase isoflorm II (NOS2) in a variety of cells including the female reproductive tract, generating high amounts of nitrogen oxide (NO) which is toxic to both gametes and developing embryos (Hunley et al., 1995; Barroso et al., 1998). A number of other factors including cytokines may also contribute to decreased sperm motility, acrosome reaction and poor embryo development observed in the present study. David et al. (1969) suggested that macrophages, plasmocytes and other cellular elements involved in late inflammatory reaction might release cytokines, prostaglandin, leukotrienes and other compounds that could be deleterious to intrauterine ooeys. Hydrosalpinges may secrete cytokines that adversely affect pregnancy outcome (Grifo et al., 1989; Toth et al., 1992) via haematogenous and lymphatic routes. Chen et al. (2002) studied cytokines in HF and reported that their concentrations were not predictive of subsequent IVF outcomes. However, cytokine concentrations have been shown to be higher in severe pelvic adhesions (Cheong et al., 2002). Inappropriate expression of growth factor genes and integrins can interfere with blastocyst formation and implantation. Meyer et al. (1997) have demonstrated that integrins (αvβ3) associated with the window of implantation are found to be decreased in women with hydrosalpinges compared with controls. These women’s endometria were described as ‘out of phase’ endometria due to the presence of gland/stromal dys synchrony. However, 70% (14/20) of these women showed increased integrin expression after surgical correction of their hydrosalpinges. Bildirici et al. (2001) reported that surgical removal of communicating hydrosalpinges increased the expression of integrin αvβ3 and therefore may improve endometrial receptivity. Illera et al. (2000) demonstrated that blockage of integrin αvβ3 resulted in impaired implantation in the mouse. Collectively, these studies suggest that HF may contain yet unknown substance(s) that interfere with the expression of integrins. Although cytokines, NO and ROS measurements were not done in this study, further studies are required to identify the substance(s) secreted by hydrosalpinx epithelium involved in mediating the toxic effect of HF on sperm and embryo.

In summary, the present results suggest that hydrosalpinx epithelial cells may be producing a fluid milieu hostile to sperm affecting their motility, capacitation and acrosome reaction and early embryo development, thus providing evidence for salpingectomy for large hydrosalpinges prior to IVF. The reconstituted epithelial cell culture system provides a model to
further investigate the mechanism of HF formation and possible role of epithelial secretions. In-depth characterization of hydrosalpinx epithelium and its secretions is necessary. Further analysis of hydrosalpinx epithelial cell culture CM may shed light on the mechanisms underlying the toxic effects of HF on embryo development and adverse IVF outcomes.

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