Relationship between oxidative stress and embryotoxicity of hydrosalpingeal fluid

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BACKGROUND: Oxidative stress mechanisms are involved in the pathophysiology of many reproductive disorders. The objective of this study was to characterize oxidative stress parameters in hydrosalpingeal fluid (HSF) and examine their possible role in early embryo development. METHODS AND RESULTS: HSF was aspirated at laparoscopic salpingectomy in 11 infertile women. Reactive oxygen species (ROS), total (non-enzymatic) antioxidant capacity (TAC) and lipid peroxidation (LPO) were assayed. Two-cell mouse embryos were incubated with 25, 50 or 75% HSF and the blastocyst development rate was observed. ROS was detected in five of 11 (45%) HSF samples with a mean of $4.2 \times 10^4$ c.p.m. LPO was detected in all samples at a mean (±SD) value of $5575.4 \pm 6091.9 \mu$mol/l malonaldehyde. The mean blastocyst development rate at 25, 50 and 75% HSF and in the control group was 88.9 ± 9.4, 65.7 ± 19.1, 45.7 ± 5.7 and 96.7% respectively ($P < 0.0001$). The blastocyst development rate was positively correlated to ROS concentrations ($P < 0.02$) but was not significantly related to LPO.

CONCLUSIONS: The blastocyst development rate decreased with increasing concentrations of HSF. For the first time, the presence of ROS, LPO and TAC activity in human HSF was characterized. A possible role of oxidative stress in the embryotoxicity of HSF is suggested.

Key words: embryotoxicity/hydrosalpinx/lipid peroxidation/reactive oxygen species/total antioxidant capacity

Introduction

Lower pregnancy and implantation rates have been reported by many investigators in patients with hydrosalpinges undergoing IVF and embryo transfer. The adverse effect of hydrosalpinges was reversible by salpingectomy prior to IVF (Vandromme et al., 1995; Murray et al., 1998; Nackley and Muasher, 1998; Bredkjaer et al., 1999; Strandell et al., 1999). Three theories have been postulated to explain this phenomenon. The flow of hydrosalpingeal fluid (HSF) into the endometrial cavity may lead to mechanical flushing of the embryos from the uterus (Sharara and McClamrock, 1997). The tubal epithelium may secrete cytokines, leukotrienes or prostaglandins into the sequestered fluid that could directly alter endometrial function (Barmat et al., 1999). Meyer et al. have shown that HSF reduced endometrial integrins (Meyer et al., 1997), which may facilitate implantation. These integrins were restored to normal following the excision of the hydrosalpinges. It has also been speculated that the negative influence of HSF may be due to embryotoxic substances in the fluid (Mukherjee et al., 1996; Meyer et al., 1997; Rawe et al., 1997; Sachdev et al., 1997; Roberts et al., 1999). Others have reported no adverse effect of hydrosalpinges on IVF and embryo transfer (Beyler et al., 1997).

The study of oxidative stress in reproductive diseases is a growing science. Recently, the presence of various oxidative/antioxidant systems in a number of reproductive tissues has sparked an interest in studying the relation of oxidative stress parameters with different female and male aspects of infertility (Murphy et al., 1997). The presence of reactive oxygen species (ROS) in reproductive tissues, peritoneal fluid in endometriosis patients and follicular fluid of patients undergoing IVF, has been documented (Murphy et al., 1997; Attaran et al., 2000). Because the exact mechanism by which HSF induces its embryotoxic effect is unknown, we hypothesized that an oxidative stress-mediated mechanism may be involved in this phenomenon. This study was designed to examine the presence of ROS, lipid peroxidation (LPO) and non-enzymatic total antioxidant capacity (TAC) in HSF and to correlate these parameters to HSF induced embryotoxicity using a mouse embryo model.

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Materials and methods

Following the approval of the Institutional Review Board, 11 infertile women with known hydrosalpinges undergoing laparoscopic salpingectomy before IVF were enrolled. At the time of laparoscopy, hydrosalpingeal fluid was obtained from each patient by needle aspiration before the tube was excised. All patients were diagnosed with bilateral hydrosalpinges, and the fluid was pooled into one sample. An aliquot from each sample was processed immediately for measurement of ROS by the chemiluminescence assay. The remaining fluid was centrifuged at 300 g for 7 min to remove the cellular debris then stored at −70°C pending the analysis for LPO, TAC and mouse embryotoxicity.

Reactive oxygen species measurement

ROS measurements were made using a Berthold luminometer (Autolumat LB 953, Wallac Inc., Gaithersburg, MD, USA). Aliquots of 400 µl of unprocessed specimens were prepared in duplicate along with the blank and control. Concentrations of ROS were determined by the chemiluminescence assay using luminol (5-amino-2,3-dehydro-1,4 phthalalazine; Sigma Chemical Co., St Louis, MO, USA) as the probe (Attaran et al., 2000). After the addition of 10 µl of luminol (5 mmol/l) prepared in dimethyl sulphoxide (Sigma), measurements were recorded for 15 min in integration mode and results were expressed as × 10⁴ counted photons per minute (c.p.m.).

Total antioxidant capacity measurement

Non-enzymatic total antioxidant capacity was measured in the HSF using the enhanced chemiluminescence assay (Attaran et al., 2000). Aliquots of the HSF that had been stored at −70°C were thawed at room temperature and immediately assessed for antioxidant capacity. HSF was diluted 1:10 with deionized water (dH₂O) and filtered through a 0.2 µm millipore filter (Allegiance Health Care Corporation, McGaw Park, IL, USA). Signal reagent was prepared with the chemiluminescence kit (Amersham Life Science, Buckinghamshire, UK). Aliquots of 20 µl of horseradish peroxidase-linked immunglobulin (HRP) (Amersham Life Science) was added to 4.98 ml of dH₂O. This was further diluted 1:1 to give a working solution with the desired luminescence output (3×10⁷ c.p.m.). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble α-tocopherol analogue, was used as a standard (25–75 µmol/l). With the luminometer in the kinetic mode, 100 µl of signal reagent and 100 µl of HRP were added to 700 µl of dH₂O and mixed. The solution was equilibrated to the desired level of chemiluminescence output (between 2 and 3×10⁷ c.p.m.) for 100 s. Aliquots of 100 µl of the prepared HSF were added immediately to the signal reagent and HRP, and the chemiluminescence was measured. Suppression of chemiluminescence and the time from the addition of HSF to 10% recovery of the initial chemiluminescence were recorded. TAC was expressed as molar Trolox equivalents.

Lipid peroxidation measurement

The frozen aliquots of HSF were thawed at room temperature. LPO concentrations were measured using the thiobarbituric acid method (Wang et al., 1997a). Ferrous sulphate and sodium ascorbate (Sigma) were used to promote lipid peroxidation. Aliquots of HSF were incubated with 125 µl each of ferrous sulphate (2.5 mmol/l) and sodium ascorbate (12.5 mmol/l) for 1 h in a 37°C water bath. Controls did not contain ferrous sulphate and sodium ascorbate during the incubation period. To precipitate proteins after incubation, 250 µl 40% ice cold trichloroacetic acid (Sigma) was added. The sample was centrifuged at 300 g for 12 min, 500 µl of clear supernatant was collected, and 250 µl of 2% thiobarbituric acid (Sigma) in 0.2 mol NaOH (Sigma) was added to the supernatant. The test tubes were boiled for exactly 15 min. The sample was cooled in crushed ice and the optical density of the pink colour product was measured at 534 nm. The malonaldehyde (MDA) concentration was calculated by comparing the optical density of the sample with that of a malonaldehyde standard (malonaldehyde bis(dimethyl acetal)). The lipid peroxidation concentration was expressed as µmol/l MDA of HSF (Wang et al., 1997a).

Effect of hydrosalpingeal fluid on mouse embryo development

A two-cell mouse embryo culture system was used to evaluate the embryotoxicity of each HSF specimen. Cryopreserved embryos (Embryotech, Wilmington, MA, USA) were grown in gradually increasing concentrations of HSF diluted with human tubal fluid (HTF) media (Irvine Scientific, Santa Ana, CA, USA) containing 0.5% bovine serum albumin (BSA) (Irvine Scientific) to a final volume of 1 ml. The culture dishes containing 1 ml HTF with 0.5% BSA were incubated at 37°C in 5% CO₂ overnight to allow equilibration. The HSF was equilibrated for 2 h at 37°C and 5% CO₂ before exposure to the embryos. A total of 30–40 embryos were cultured in 0, 25, 50 and 75% concentrations of HSF in a final volume of 1 ml in an atmosphere of 5% CO₂ at 37°C for each of the 11 HSF samples. The embryos were examined after 72 h incubation and the number of embryos progressing to the blastocyst stage was recorded. The blastocyst development rate was calculated by dividing the number of blastocysts by the total number of embryos incubated.

Statistical analysis

The effect of various factors such as concentration of HSF, ROS, TAC and LPO on rates of embryotoxicity were assessed using multivariate logistic regression analysis. All analyses were based on individuals, with embryo results ‘clustered’ within each individual. Each embryo’s development was classified as a binary outcome (yes/no), but because each experiment utilizes several embryos, logistic regression using generalized estimating equations (GEE) was used to account for this clustering of outcomes. GEE methodology adjusts for the correlation within embryos incubated together. A secondary analysis used presence or absence of ROS as a binary independent variable rather than as continuous variables. Logistic regression was also used to calculate odds ratios (OR) and their 95% confidence intervals (CI). Based on the final results of parameter estimates and standard errors from GEE logistic regression, a post-hoc power calculation was performed to determine the degree of effect that could have been detected with the sample size that was utilized. The sample size in this study had 90% power to detect increased odds of blastocyst development rate by 2 times or reducing odds of blastocyst development rate by one-half (odds ratio = 0.05), with two-tailed significance tests using P < 0.05. The significance level for statistical tests was P < 0.05, and all calculations were performed with SAS version 8 (SAS Institute Inc., Cary, NC, USA).

Results

Reactive oxygen species

Reactive oxygen species were detected in five of 11 (45%) HSF samples, with a mean ± SD of 4.2 ± 11.9×10⁴ c.p.m. Increasing ROS concentrations were significantly related to increasing blastocyst development rate (P < 0.02, OR 1.8 and 95% CI 1.09–2.98).
TAC concentrations were detected in Total antioxidant capacity

The LPO concentrations were not signifi-

The blastocyst development rate of the mouse embryos development rate.

of 12.5 samples. These two samples demonstrated a TAC concentration –

\[-0.02, 1.80, 1.09\]

\(\text{Table I. Relationship of hydrosalpingeal fluid concentration, reactive oxygen species and lipid peroxidation concentrations with blastocyst development rate. HSF = hydrosalpingeal fluid; ROS = reactive oxygen species; LPO = lipid peroxidation}

<table>
<thead>
<tr>
<th>Variable</th>
<th>P-value</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSF concentration</td>
<td>&lt;0.0001</td>
<td>0.28</td>
<td>0.17–0.46</td>
</tr>
<tr>
<td>ROS</td>
<td>0.02</td>
<td>1.80</td>
<td>1.09–2.98</td>
</tr>
<tr>
<td>LPO</td>
<td>&lt;0.2</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

\(P < 0.05\) was considered significant.

Lipid peroxidation concentrations

The mean \(\pm\) SD of LPO was 5575.4 \(\pm\) 6091.9 \(\mu\)mol/l MDA. The LPO concentrations were not significantly related to the blastocyst development rate of the mouse embryos (Table I).

Total antioxidant capacity

TAC concentrations were detected in <20% (2/11) of HSF samples. These two samples demonstrated a TAC concentration of 12.5 \(\mu\)T of Trolox equivalent. It is unlikely that the extremely low concentrations of TAC would directly affect the blastocyst development rate.

Blastocyst development rate of mouse embryos

The blastocyst development rate of the mouse embryos (mean \(\pm\) SD) was 88.9 \(\pm\) 9.4%, 65.7 \(\pm\) 19.1% and 45.7 \(\pm\) 15.7% at HSF concentrations of 25, 50 and 75% respectively. The blastocyst development rate of the control group was 96.7%. There was a negative correlation between increasing HSF concentrations and decreasing blastocyst development rate \((P < 0.0001, \text{ OR } 0.28 \text{ with } 95\% \text{ CI of } 0.17–0.49)\) (Figure 1).

Discussion

Oxidative stress may play a role in the pathophysiology of HSF. ROS is a normal by-product of cellular metabolism. ROS at certain concentrations are detrimental to living cells. The most vulnerable cellular component to ROS effects is the cell membrane lipids. Lipid peroxidation is a process that involves interaction of ROS with the cell membrane lipids generating the lipid peroxy radical, which can propagate further oxidation. Antioxidants scavenge the damaging effects of ROS. It has been shown that ROS generation in a mouse IVF model impedes preimplantation embryo development and that supplementation with antioxidants reverses this effect (Legge and Sellons, 1991; Goto et al., 1992, 1993).

We demonstrated for the first time the presence of ROS, LPO and TAC in human HSF. There was a trend towards a higher blastocyst development rate where ROS was detected in the HSF, compared with HSF devoid of ROS. However, we speculate that these low concentrations of ROS were beneath the threshold for being deleterious to the embryos and may represent normal ROS generation by a functional endosalpingeal damage. Secretory products from healthy tubal mucosa have embryotrophic effects (Liu et al., 1995; Morgan et al., 1995; Vlad et al., 1996). Thus, the detection of ROS at low concentrations may be a marker of normal tubal secretary function.

Similarly, we have documented that follicular fluid ROS concentrations were positively correlated with IVF pregnancy rates. This may also reflect healthier follicles with more metabolically active granulosa cells (Attaran et al., 2000). However, the source of ROS in HSF may also be from the immune cells associated with chronic salpingitis, as polymorphonuclear leukocytes are a very well known source of ROS (Kubo et al., 1987). The presence of detectable yet non-significant concentrations of LPO in all of our HSF samples indicates that an oxidative stress reaction was going on during the acute phase of the disease.

We also noted a concentration-dependent embryotoxic effect of HSF but only at high concentrations, 50 and 75%. This may represent a dilutional effect of required media nutrients and not the result of a true embryo toxin. Several investigators did demonstrate HSF-induced embryotoxicity even at very low concentrations, i.e. <10% (Mukherjee et al., 1996; Rawe et al., 1997; Sachdev et al., 1997). Few investigators noted an embryo toxic effect only at high concentrations, i.e. 50–100%, similar to our findings (Murray et al., 1997; Koong et al., 1998; Spandorfer et al., 1999). On the other hand, some studies suggested that hydrosalpingeal fluid had no direct embryotoxic effects (Granot et al., 1998; Strandell et al., 1998).

The exact biochemical nature of HSF is unknown and no controls are available, hence it is difficult to compare the results with reference values. In our earlier work we measured ROS, TAC and LPO concentrations in follicular fluid or peritoneal fluid from healthy controls as well as in women with endometriosis (Wang et al., 1997; Attaran et al., 2000). In the follicular fluid, the log (ROS + 1) values were 1.01 ± 0.14 in the pregnant cycles compared with 0.69 ± 0.08 in the non-pregnant cycles (Attaran et al., 2000). In the same study, we reported TAC concentrations of 819.22 ± 54.23 Trolox equivalent in the pregnant cycles compared with 722.15 ± 48.76 Trolox equivalent in the non-pregnant ones. The median and interquartile range of ROS in the peritoneal fluid of patients undergoing tubal ligation, patients with idiopathic infertility and endometriosis patients were 99.6 (73.0, 169.0),
230.5 (105.6, 515.1) and 115.8 (25.4, 194.2)×10^4 respectively (Wang et al., 1997b).

We believe that follicular fluid and peritoneal fluid from normal patients are not ideal controls for hydrosalpingeal fluid. The ideal control would be the normal tubal secretions. However, due to very small volumes and technical difficulties, we were not able to use this physiological control. We are currently analysing various growth factors, cytokines and prostaglandins in HSF. The dynamic nature and micromovements of fluid within normal Fallopian tubes pose obvious difficulties in obtaining fluid to provide normative data regarding the concentrations of growth factors, cytokines, prostaglandins, ROS and LPO, which would make comparisons to HSF more relevant.

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References

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