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Impact of hydrosalpinx fluid on early human embryos

Hongchu Bao, Qinglan Qu, Xin Huang, Meimei Wang, Xinrong Wang, and Cuifang Hao

Department of Reproductive Medicine, Yantai Yuhuangding Hospital Affiliated to Qingdao University, Yantai, China

ABSTRACT

This study aimed to investigate the impact of hydrosalpinx fluid (HF) on early human embryonic development. A total of 33 patients who underwent laparoscopic surgery for hydrosalpinx were selected, and the HF specimens obtained from these patients were subjected to bacterial culture, Chlamydia antigen detection, biochemical analysis, and cytokine detection. Meanwhile, human embryos derived from three pronuclei (3PN) were cultured in various HF concentrations. There was no significant difference in the chemical components and physical characteristics between colorless and colored HF specimens, apart from the glucose concentration which was significantly higher in colorless HF. K⁺ and HCO₃⁻ were significantly increased (P < 0.05 and P < 0.01), and Ca²⁺, Mg²⁺, and glucose were significantly decreased (P < 0.05, P = 0.006, and P = 0.007) in the two HF specimens, compared to blastocyst culture medium (G-2 medium); no phosphates were detected in the HF specimens. Compared to colorless HF, the concentrations of tumor necrosis factor α (TNF-α) and interleukin 2 (IL-2) in the colored HF specimens were significantly increased (P < 0.05). There were no differences in the Chlamydia antigen-positive rate between the HF groups (62.5% vs. 70.6%), and no bacterial growth occurred in the HF specimens. There were no significant differences in the development of the 3PN embryos between the two HF groups (P > 0.05). High-concentration HF (75%) significantly affected the rates of blastulation, blastocyst hatching, and high-quality blastocyst formation (P < 0.05). HF is related to chlamydial infection. Embryonic development may be significantly affected only in high-concentration HF, possibly due to the deficiency of essential elements required for embryonic development. TNF-α and IL-2 concentrations were found to vary between the clear and colored HF specimens; however, TNF-α and IL-2 in HF do not appear to exert adverse effects on embryonic development.

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KEYWORDS

Blastocyst; cytokines; embryo; hydrosalpinx fluid

Introduction

Hydrosalpinx fluid (HF) formation may occur in 10–30% of patients who undergo in vitro fertilization-embryo transfer (IVF-ET) [Zhang et al. 2015]. Although hydrosalpinx negatively affects IVF-ET treatment outcomes [Zhang et al. 2015; Tsiami et al. 2016], the underlying mechanisms have not been elucidated [Polat et al. 2014; D’Arpe et al. 2015]. Furthermore, there is no consensus on the adverse effects of HF on embryos [Spandorfer et al. 1999; Lu et al. 2013]. Most researchers have shown via human and animal embryo experiments that HF adversely affects embryonic development as it contains microorganisms, tissue debris, lymphocytes, and embryotoxic substances, thereby reducing embryo implantation and pregnancy rates and increasing the abortion rate [Jastrow et al. 2002; Loutradis et al. 2005]. These adverse effects can be attributed to abnormalities in the HF components, such as ion concentration, pH, and cytokine concentration [Chen et al. 2002]. However, in clinical practice, colorless and clear HF are found in most cases (colorless HF), and some HF that appear brownish or reddish in color (colored HF) are also observed. It is unclear whether there are different reasons for the generation of colored and colorless HF and if their components differ. Furthermore, it not known if they exert the same negative effects on embryonic development. This study examined the biochemical components, microorganisms, and cytokine concentrations of colorless and colored HF; 3PN embryos were cultured in order to investigate the effects of HF on embryos and determine the possible underlying mechanisms.

Results

HF analysis

As seen in Table 1, glucose levels were higher in the colorless HF group than in the colored HF group (p < 0.01), but there were no differences in other chemical components and physical characteristics, such as pH and osmotic pressure (p > 0.05). The
Table 1. Comparison of chemical components between the two hydrosalpinx fluid (HF) groups and the blastocyst culture medium.

<table>
<thead>
<tr>
<th>Index</th>
<th>Colored HF (n=16)</th>
<th>Colorless HF (n=17)</th>
<th>Blastocyst culture medium (n=9)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.5±0.08**</td>
<td>7.5±0.09**</td>
<td>7.4±0.08</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Osmotic pressure (mOsm/kg)</td>
<td>265.4±10.25</td>
<td>260.3±5.72</td>
<td>271.0±16.24</td>
<td>NS</td>
</tr>
<tr>
<td>K⁺ (mmol/L)</td>
<td>3.75±0.09**</td>
<td>3.64±0.82**</td>
<td>5.80±0.72</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Na⁺ (mmol/L)</td>
<td>142.3±8.24</td>
<td>135.09±8.22</td>
<td>144.3±7.34</td>
<td>NS</td>
</tr>
<tr>
<td>Cl⁻ (mmol/L)</td>
<td>118.6±9.05</td>
<td>115.2±7.86</td>
<td>122.7±6.25</td>
<td>NS</td>
</tr>
<tr>
<td>CO₂⁻ (mmol/L)</td>
<td>26.5±1.94**</td>
<td>29.6±10.21**</td>
<td>19.9±1.24</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>9.1±0.72</td>
<td>9.1±0.72</td>
<td>9.1±0.72</td>
<td>NS</td>
</tr>
</tbody>
</table>

** Comparison between the HF specimens and blastocyst culture medium revealed significantly increased pH and HCO₃⁻ (p < 0.05 and p < 0.01, respectively) and significantly reduced K⁺, Ca²⁺, Mg²⁺, and glucose levels (p < 0.01, p < 0.05, p = 0.006, and p = 0.007, respectively) in the HF specimens. No phosphates were detected in the HF specimens; however, the phosphate level in the blastocyst culture medium was 0.26 ± 0.03 mmol/L. Additionally, no proteins were detected in the HF specimens and the blastocyst culture medium (G-2 medium); however, considering that appropriate protein supplements will be added to the control culture during blastocyst culture, it is apparent that the HF specimens were protein deficient.

Detection of cytokines and microbiology in the HF specimens

Enzyme-linked immunosorbent assay (ELISA) revealed significantly increased TNF-α and IL-2 expression levels in the colored HF group (from 32.7 ± 4.52 pg/mL to 29.9 ± 4.55 pg/mL) (Table 2). Meanwhile the HF specimens were subjected to routine bacterial culture and Chlamydia antigen detection. The Chlamydia antigen-positive rate was 62.5% in the colored HF group and 70.6% in the colorless HF group, and there were no significant differences between the two groups. Routine bacterial culture of the HF specimens showed no bacterial growth (Table 2).

Table 2. Comparison of cytokines and microbiological tests between the two HF groups.

<table>
<thead>
<tr>
<th>Index</th>
<th>Colored HF</th>
<th>Colorless HF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/mL)</td>
<td>32.7±4.52</td>
<td>23.40±4.09</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>IL-2 (pg/mL)</td>
<td>29.9±4.55</td>
<td>20.35±4.24</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Chlamydia antigen-positive rate (%)</td>
<td>62.5 (10/16)</td>
<td>70.6 (12/17)</td>
<td>NS</td>
</tr>
<tr>
<td>Bacterial culture-positive (%)</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
</tbody>
</table>

Blastocyst culture of 3PN embryos

Varying concentrations of HF were used to culture 3PN embryonic blastocysts in order to observe blastocyst formation in both the colorless and colored HF specimens. No significant differences in the rate of blastulation (17.6% and 17.2%), blastocyst hatching (48.9% and 43.9%), and high-quality blastocyst formation (64.4% and 58.5%) were observed between the colored and colorless HF group (Table 3). Further, low-concentration HF (<50%) did not affect the rates of blastulation, blastocyst hatching, and high-quality blastocyst formation; however, high-concentration HF (75%) significantly affected these rates (Table 3).

Blastocyst formation conditions were observed after culturing with 3PN embryos using different concentrations of tubal effusion, including different colored oviduct effusion. A total of 239 embryos were cultured in colored oviduct effusion, and a total of 255 embryos were cultured in colorless oviduct effusion. The blastocyst formation rate, hatching rate, and high-quality blastocyst formation rate of 3PN embryos in colored oviduct effusion and colorless oviduct effusion were 17.6% and 17.2%, 48.9% and 43.9%, and 64.4% and 58.5%, respectively (Table 3); there was no significant difference. Meanwhile, by summarizing culturing results, including colored oviduct effusion and colorless oviduct effusion, it was found that the comparison of the blastocyst formation rate, hatching rate, and high-quality blastocyst formation rate between the low-concentration and high-concentration HF was significant.

Table 3. In vitro impacts of hydrosalpinx fluid (HF) with different concentrations and colors on embryos.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of D3 embryos</th>
<th>Blastulation rate (%)</th>
<th>Blastocyst hatching rate (%)</th>
<th>High-quality blastocyst rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% HF</td>
<td>166</td>
<td>19.9 (33/166)*</td>
<td>51.5 (17/33)*</td>
<td>72.7 (24/33)*</td>
</tr>
<tr>
<td>25% HF</td>
<td>165</td>
<td>19.4 (32/165)</td>
<td>53.1 (17/32)</td>
<td>75.0 (24/32)</td>
</tr>
<tr>
<td>50% HF</td>
<td>166</td>
<td>20.5 (34/166)</td>
<td>52.9 (18/34)</td>
<td>73.5 (25/34)*</td>
</tr>
<tr>
<td>75% HF</td>
<td>163</td>
<td>12.3 (20/163)</td>
<td>25.0 (5/20)</td>
<td>20.0 (4/20)</td>
</tr>
<tr>
<td>Colorless HF</td>
<td>255</td>
<td>17.6 (45/255)</td>
<td>48.9 (22/45)</td>
<td>64.4 (29/45)</td>
</tr>
<tr>
<td>Colored HF</td>
<td>239</td>
<td>17.2 (41/239)</td>
<td>43.9 (18/41)</td>
<td>58.5 (24/41)</td>
</tr>
</tbody>
</table>

*p < 0.05, compared with 75% HF.
oviduct effusion (<50%) and oviduct effusion-free culture medium showed no significant difference, but during the culture in high-concentration oviduct effusion (75%). Blastocyst formation rate, blastocyst hatched rate, and high quality blastocyst rate were significantly affected and showed significant differences (Table 3).

Discussion

Biochemical composition of HF and related biological components

The presence of a variety of nutrients and bioactive factors in the external environment, such as carbohydrates, proteins, lipids, electrolytes, vitamins, and a complex cytokine network, is required for the fertilization of oocytes and embryonic development. Normal oviductal fluid contains nutrients required for early embryonic development, and contains higher levels of K\(^+\) and HCO\(_3\)- than does the plasma during the same period; further, oviductal fluid nutrients differ from those in the plasma and periodically change during the menstrual cycle [Leese et al. 2001]. In most cases, HF is clear, but the color of some HF ranges from brown to red. Some researchers sampled HF from IVF-ET patients on the oocyte aspiration day in order to identify microbes and found that 75% of the sampled HF was bacterial culture-positive and included alpha-hemolytic streptococci, *Bacteroides* spp., *Lactobacillus* spp., *Streptococcus milleri*, etc. However, these bacteria are part of the normal flora in the lower genital tract; therefore, the presence of these organisms due to vaginal contamination of the needle cannot be ruled out, and it is possible that infection would not lead to embryonic toxicity [Ng et al. 2000]. In the present study, we used laparoscopy to aseptically collect HF specimens from 33 patients, thus avoiding the possibility of contamination when the sample is collected via the vagina. Routine bacterial culture showed no bacterial growth, suggesting that the HF may have been due to pelvic inflammatory disease. However, the *Chlamydia* antigen assay of the HF specimens revealed a relatively higher *Chlamydia* antigen-positive rate of >60%. Further, there were no significant differences in the *Chlamydia* antigen-positive rates between the colorless and colored HF specimens, suggesting that *Chlamydia* infection may play an important role during HF formation, and is not strongly associated with the color of HF. However, repeated *Chlamydia* infections may lead to progressive damage and adhesions, occlusion, and hydrosalpinx [Chen et al. 2014; Yang et al. 2014]. Further studies are required to determine the effectiveness of macrolide antibiotics against *Chlamydia* in patients with HF [Hurst et al. 2001].

Under normal physiological conditions, human female oviduct epithelial tissue expresses cystic fibrosis transmembrane conductance regulator (CFTR); the main functions of CFTR are to regulate fluid secretion, dilute intrauterine fluid, and facilitate sperm migration. Studies have confirmed that HF induces an upregulation of CFTR in oviduct epithelial cells [Ajonuma et al. 2005]. Sequelae of pelvic inflammatory disease, such as *Chlamydia trachomatis* infection, can promote the formation of HF as well as simultaneously increasing the amount of fluid via the increase in CFTR levels. Several researchers have reported their findings on the chemical composition and biological activities of HF, but the results vary. Ng et al. [2000] and Granot et al. [1998] reported that the level of total protein and albumin in HF is lower than that in normal serum. With regard to the other biochemical components, Ng reported that Na\(^+\), K\(^+\), Ca\(^{2+}\), and glucose levels in human HF were similar to those in normal serum, with a pH ranging from 7.5 to 8.1, but the osmotic pressure differed. Granot found similar urea and K\(^+\) concentrations in normal serum and HF, but the Na\(^+\), Ca\(^{2+}\), and phosphate levels were lower in HF than in normal serum; the pH ranged from 8.6 to 8.7 and the osmotic pressure ranged from 268 to 280 mOsmol/L, similar to the values of normal serum. Because it is not possible to detect the chemical components and properties of HF under normal physiological conditions, we chose embryo culture medium for this study. The findings showed that there were no differences in the electrolyte, protein concentrations, pH, osmotic pressure, and chemical components between the colored and colorless HF specimens, but the glucose concentration in the colored HF specimens was significantly reduced as compared to that in the colorless HF. The reason for the reduced glucose levels needs to be determined. We also found that the pH and HCO\(_3\)- concentration was significantly increased and the K\(^+\), glucose, protein, Ca\(^{2+}\), Ma\(^{2+}\), and inorganic phosphate concentrations were significantly reduced in the HF specimens as compared to the embryo culture medium. Therefore, it can be assumed that these abnormalities in HF can affect embryonic development when the embryo comes in contact with HF.

HF may also contain certain cytokines that are toxic to embryos or affect endometrial receptivity; additionally, HF may lack certain cytokines that facilitate embryonic development or implantation. Strandell et al. [2004] found that the concentrations of IL-8, IL-12, IL-1α, TNF-α, transforming growth factor β2 (TGF-β2), granulocyte colony-stimulating factor, and
leukemia inhibitory factor in HF are increased to various degrees, suggesting upregulation of these cytokines by endothelial tissue due to HF. However, the concentrations of these cytokines differ largely in different HF specimens, and their impact on embryos and endometrial receptivity needs to be further studied. We grouped the HF specimens according to their color and found that TNF-α and IL-2 levels were significantly increased in the colored HF specimens. This is the first report of differences in the levels of inflammatory cytokines in HF specimens of different colors, and this finding suggests that there is an underlying reason for the formation of different colored HF. However, the specific mechanisms underlying the formation of colored HF and its effect on embryotoxicity and endometrial receptivity need to be investigated further.

**Effect of HF on early human embryos**

Under physiological conditions, early human blastocysts appear on the 4th or 5th day after fertilization, late blastocysts appear on the 5th or 6th day, and the full hatching of blastocysts occurs on the 6th or 7th day. Previous studies have shown that the levels of biochemical components and bioactive factors are abnormally increased or reduced in HF; during the process of IVF-ET, embryos implanted within the uterus may come in contact with HF that reflexes to the fallopian tubes, and it is unclear whether this ‘HF environment’ surrounding the embryos adversely affects development. Several researchers have investigated this issue using animal embryos; however, their findings vary considerably. Experiments conducted using murine embryos have shown that HF can affect embryonic development. Earlier studies found negative correlations between mouse blastocyst development and HF concentration when the HF concentration was 5–20% [Granot et al. 1998]. Mukherjee et al. [1996] collected HF samples from postmenopausal women and cultured mouse embryos in various concentrations of HF; they observed embryonic toxicity in all samples irrespective of the HF concentration. Spandorfer et al. [1999] believed that HF can only produce significant embryonic toxicity at high concentrations and the embryonic toxicity can be alleviated by the endometrium. Other researchers believe that HF has only slight or no toxic effect on embryonic development [Koong et al. 1998; Saito et al. 2000]. The differences in the findings reported can be attributed to differences in the mouse embryo strains used and their sensitivity to human HF, different HF collection periods, or variations in the inclusion criteria for HF patients.

Therefore, conclusions related to the effects of HF on mouse embryonic development may not necessarily be applicable to humans, and research on human embryos would be optimal. The toxic effects of HF on human embryos are rarely reported, and the conclusions derived from studies on in vitro mouse embryonic development differ [Granot et al. 1998; Strandell et al. 1998]. In the present study, we cultured human 3PN embryos in various concentrations of HF (0%, 25%, 50%, and 75%) to determine if the effect of HF on the blastocyst development rate differs with concentration, and studied the rates of blastulation, blastocyst hatching, and high-quality blastocyst formation. The results revealed that although the concentrations of TNF-α and IL-2 differ in the clear and colored HF samples, the rates of blastulation, blastocyst hatching, and high-quality blastocyst formation of 3PN embryos do not differ between the colored and colorless specimens, suggesting that the two inflammatory cytokines TNF-α and IL-2 do not exert adverse effects on the embryonic development of 3PN embryos. The findings from the blastocyst culture process in 0%, 25%, 50%, and 75% HF revealed that the 0–50% concentrations had no impact on the rates of blastulation, blastocyst hatching, and high-quality blastocyst formation, but the 75% concentration significantly reduced these rates. Further, the morphologies of the blastocysts were poor, the trophectoderm and inner cell mass were dispersed, and the number of the cells was smaller, suggesting that high-concentration HF exerts adverse effects on embryonic development. Since early embryos are very sensitive to pH, osmotic pressure, electrolytes, energy substance, and a variety of growth factors, changes to any of these components or physical properties may affect embryonic development. The glucose and protein concentrations in HF were found to be decreased, but pH and HCO_3^- were increased; meanwhile, Ca^{2+} and phosphates were decreased, and K+ levels were relatively low, which may be one of the reasons why high-concentration HF affects embryogenesis and reduces the quality of blastocysts. We found that, except for glucose levels, the biochemical compositions of the colored and colorless HF specimens were substantially identical, and no significant microbial growth was observed.

There were significant differences in the concentrations of the bioactive components TNF-α and IL-2, but these bioactive factors were not found to be associated with embryonic toxicity. Therefore, the negative impacts of TNF-α and IL-2 on IVF-ET outcomes should be determined in further studies focused on endometrial receptivity.
In conclusion, higher concentrations of HF may affect human blastocyst development, and pretreatments for hydrosalpinx, such as salpingectomy, salpingostomy, and proximal tubal obstruction, should be recommended before IVF-ET to improve outcomes.

Materials and methods

Subjects

A total of 33 patients who were treated for HF at the Department of Reproductive Medicine, Yantai Yuhuangding Hospital Affiliated to Qingdao University, from December 2009 to June 2011 were included in this study. Of these, 16 patients had colored HF and 17 had colorless HF. This study was conducted in accordance with the declaration of Helsinki and was conducted with approval from the Ethics Committee of Qingdao University. Written informed consent was obtained from all participants.

Inclusion criteria were: willingness to undergo laparoscopic surgery to treat HF; age between 25 and 35 y, normal menstrual cycle of 25–35 days, autologous period changes within ±3 days, weight between 45 and 70 kg, and body mass index of 18–25 kg/m². Exclusion criteria were: abnormal endocrine function, such as polycystic ovary syndrome or hyperprolactinemia, and acute reproductive tract or pelvic infection.

HF collection method

After the presence of HF was confirmed laparoscopically, a sterile needle was used to pierce the fallopian tube lumen through a region where the wall was relatively thin. The HF specimen was then aspirated, divided into three parts, and used immediately for routine bacterial culture and Chlamydia antigen detection, biochemical component detection (after appropriate processing), and analysis of TNF-α and IL-2 levels (centrifuged at 1,000 rpm for 15 min and then stored at -70°C until analysis). Culture of the 3PN embryos was performed simultaneously.

Biochemical detection of HF

After centrifugation at 300 rpm for 30 min to remove cellular components, 2 mL of the HF specimen was immediately used for biochemical component analysis (concentrations of electrolytes, proteins, glucose, etc.) performed using a Beckman LX20 (Beckman Coulter, USA) automatic biochemical analyzer.

Cytokine detection

The frozen HF specimens were first slowly restored to room temperature (18–25°C), and then 1 mL of HF was used for the detection of TNF-α and IL-2. The expression of TNF-α and IL-2 was measured by ELISA, according to manufacturer’s instructions (Wuhan Boster Biotechnology, Wuhan, China). The remaining HF was used to determine the effect of HF on the development of early human 3PN embryos.

Microbiological assay of HF

For Chlamydia antigen detection the HF specimens were subjected to the Rapid Chlamydia immunoassay (Shanghai Chemtronbio Co. Ltd.) using standard methods and manufacturer’s instructions immediately after the samples were obtained. For the bacteriological assay, routine bacterial culture was performed within 2 h of sampling.

Effects of HF on the development of 3PN embryos

Oocytes sampled from patients who underwent conventional IVF-ET at our center from January 1, 2009, to December 31, 2010, were observed 16 h after fertilization, and 3PN zygotes, once formed, were cultured (in different HF concentrations) until the third day of fertilization. The embryos that developed into 6–10 cells were vitrified routinely for later use. The G-2 medium (Vitrolife, Sweden) supplemented with 5% human serum albumin solution (Vitrolife) was prepared as the control culture medium. Culture medium containing various concentrations of HF (0%, 25%, 50%, and 75%) was prepared as the test medium. All solutions were placed in four-well culture dishes (Falcon, USA), covered with mineral oil, and balanced overnight at 37°C, 5% CO₂, and 80% relative humidity. This study followed the ethical guidelines for research on human gametes or embryos and was approved by the ethics committee of our hospital. All the patients provided signed informed consent for “voluntary donation of embryos for scientific research.” Every patient was assigned a number, and a certain number of blastocysts were cultured depending on the HF concentration. Specific methods and criteria [Strandell et al. 1998] were used. Embryonic development was observed at the same time each day. According to the formation and expansion of the blastocysts, the detailed conditions were recorded and scored: blastocysts with a D5 score of ≥3AA, 3AB, 3BA, or 3BB, or a D6-7 score of ≥4AA, 4AB, 4BA, or 4BB were determined to be high-quality blastocysts.
Statistical analysis

SPSS 11.0 software was used for statistical analysis. The t-test was used for intergroup comparison and analysis of variance was used for multi-group comparisons. The SNK-q test was used for paired comparisons between two groups. The count data were processed using the $\chi^2$ test in the $R \times C$ list data, and when the intergroup difference was statistically significant, the $R \times C$ table segmentation of the $\chi^2$ test was used for the comparison, with $p < 0.05$ considered as being statistically significant.

Declaration of interest

All authors have no conflict of interest regarding this article.

Notes on contributors

Conceived and designed the experiments: HB, CH; Performed the experiments: HB, QQ, XH; Analyzed the data: HB, MW; Contributed reagents/materials/analysis tools: HB, MW, CH, XW; Wrote the manuscript: HB, CH. All authors approved revisions and the final paper.

References


