Association between Bacterial Contamination of the Uterine Cervix at Embryo Transfer and Success Rate in In Vitro Fertilization/Intracytoplasmic Sperm Injection

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In the last two decades, in-vitro fertilization and embryo transfer (IVF-ET) have been successful in the alleviation of long-standing infertility due to female or male factors. It is well documented that the results of IVF in the presence of male factor are not as good as those in patients with normal semen parameters. Intracytoplasmic sperm injection (ICSI) is now one of the most successful and viable techniques in assisted fertilization. It leads to an increase in the fertilization rates but without corresponding increase in implantation rates. Therefore, efforts were directed to create a perfect relationship between good quality embryos and receptive endometrium. The aim of this study was to evaluate the effect of Chlamydia trachomatis infection and microbial flora of the cervix at the time of embryo transfer on fertilization and implantation in women undergoing ICSI procedure. From June 2003 to November 2003, thirty participants with a male cause of infertility were enrolled in the study in Ain Shams University Maternity hospital. All were recruited from the outpatient clinic of Assisted Reproduction unit. After embryo transfer (ET), cervical scrapings were taken and tested for Chlamydia trachomatis DNA by PCR technique. Also the distal tip of the embryo transfer catheter was cut off and tested for bacterial growth by conventional culture techniques. Pregnancy tests were done 2 weeks later, and participants were accordingly divided into pregnant and non-pregnant groups. Chlamydia was detected in 30% of the non-pregnant group versus 10% of the pregnant one but with no statistical significance. Non significant difference was found between both groups regarding cervical bacterial growth. E. coli had border line significance in the non pregnant group. In conclusion, the present study supports the hypothesis that microbial flora of the cervix detected during ET have no role in the implantation process, and does not affect pregnancy rates in women undergoing ICSI procedure for infertility. However further larger scale studies are recommended to assess the possible role of Chlamydia trachomatis and E. coli.

VF-ET procedures involve needle puncture of the vagina and placement of embryos through the cervix, thus contamination is possible from vaginal-cervical microorganisms particularly because vaginal antiseptics usually are not used during egg retrieval or embryo transfer to avoid injury of the egg or embryos (Yaron et al., 1994).

The minimal inflammation in response to microorganisms that enter the endometrium from the cervix during embryo transfer provides another mechanism that could damage the developing embryo and prevent pregnancy (Egbase et al., 1999). Regarding the mechanisms by which micro-organisms may be responsible for damaging the developing embryo. First, it is conceivable that intense concentrations of microorganisms on the cervix may be associated with subclinical chronic endometritis and therefore poor uterine receptivity. Second, the ET procedure may inoculate cervical microorganisms into the uterine cavity, potentially altering the biochemical or ultrastructural characteristics of the endometrium required for satisfactory embryo implantation and early development. Third, the possible direct contamination of embryos during transcervical embryo transfer may cripple their ability to implant (Tabibzadeh and Babakina, 1995). In fact a 50% reduction in pregnancy rate has been
reported in subjects with bacteria compared with those without bacteria isolated from the embryo transfer catheter tip (Fanchin et al., 1998).

Previous studies reported the role of Chlamydia trachomatis as a major cause of tubal occlusion. There is also increasing evidence that Chlamydia trachomatis infection may result in a number of adverse pregnancy outcomes, including early and late abortion, intrauterine infection of the fetus, still birth, prematurity, premature rupture of membranes, and postpartum endometritis (Mardh., 2002).

The aim of this study was to determine whether presence of cervical microorganisms at embryo transfer had an effect on the success of IVF.

**Subjects, Material and Methods**

The study is a prospective follow-up study, and was carried out in Ain Shams University Maternity Hospital in the Assisted Reproduction Unit, during the period from June 2003 to November 2003.

**Subjects:**

The study included 30 consecutive patients after a given consent, from those attending the unit with the diagnosis of primary infertility who were assigned for treatment with intra-cytoplasmic sperm injection (ICSI). The indication for ICSI was sperm abnormalities (male factor). The inclusion criteria included women age ≤ 35 years, whose uteri were morphologically normal as confirmed by HSG, U/S, and hysteroscopy to limit additional factors that may affect the results of the study. Moreover, none of the women included in this study had clinical evidence of vaginitis or cervicitis.

**Study Maneuver:**

A precise clinical history was taken from all the women under study. An abdominal and a pelvic examination were also performed. Initial transvaginal ultrasound (TVS) was done to exclude the presence of any uterine or ovarian abnormalities. Estimation of serum FSH, LH, and prolactin levels was done to all participants. Down regulation and induction of ovulation were initiated for every subject according to the chosen protocol, whether short or long, depending on her condition and the regulations of the unit. Follicular monitoring and estimation of endometrial thickness were performed using TVS day after day beginning 4 days after initiation of gonadotrophins.

Human chorionic gonadotrophins (hCG) was administered in a dose of 10,000 units deep intramuscularly when four or more follicles achieved a diameter of 18mm or more to induce final maturation of the oocyte. Oocyte retrieval by transvaginal-guided aspiration of follicles was done under general anesthesia 34-36 hours later using a suction catheter. The standard procedures of transvaginal follicle aspiration under vaginal ultrasound guidance were followed according to Rabe et al., 1997. Then, the oocytes were placed in culture medium and intracytoplasmic sperm injection was performed. The injected oocytes were incubated at 37°C. Fertilization was diagnosed by the presence of two pronuclei in the injected oocyte (Yao and Schust, 2002).

Embryo quality was assessed according to the presence of anuclear fragments. Embryos with less than 10% anuclear fragments, with 10-20% fragments and with more than 30% are referred to as grade 1, grade 2, and grade 3 respectively. Embryo transfer (ET) was done 48-72 hours after oocyte retrieval using a cook ET catheter. A maximum of four embryos were transferred at a time.

After embryo transfer, Cyclogest 400mg vaginal tablet was given daily till results of the pregnancy test were available, in addition to the administration of natural progesterone capsules, in a dose of one capsule 3 times daily. Patients were scheduled for β-hCG two weeks after oocyte retrieval. A titre of 72 SIU/ml was considered chemical pregnancy. TVS was done two weeks later to confirm fetal life.

**Specimen Collection and Bacteriological Methods:**

Chlamydia trachomatis:

Cervical scrapping was done to each patient, immersed and transported into phosphate buffered saline for detection of Chlamydia trachomatis by polymerase chain reaction (PCR). This involved three steps, namely DNA extraction, amplification, and detection of specific DNA product.
DNA extraction:
It was done according to Bailey et al., (1994). Tubes were centrifuged at 12,000 rpm to pellet cellular material. After removal of the supernatant, the pellet was resuspended in 40 ul of lysis buffer, incubated at 60°C for one hour in a dry bath to provide the optimum temperature for proteinase K (Amersham pharmacia biotech, Austria), then at 100°C for 10 minutes to inactivate proteinase K. The resulting crude DNA preparations were stored at -20°C until used. For positive control preparation, deep agar culture of E. coli, transfected with TA recombinant plasmid copy MOMP of Chlamydia trachomatis and ampicillin resistance gene, was kindly supplied by DR Linda Bobo, Ph.D., professor, Johns Hopkins University, School of Medicine, Division of Infectious Diseases.

DNA amplification:
A PCR mix was prepared, as shown in the following table, containing the DNA polymerase and its buffer, 2 primers, the dNTPs mix, and distilled water. The water volume was calculated to give the PCR mix a final volume of 50 ml.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Original Concentration</th>
<th>Final Concentration</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA polymerase buffer</td>
<td>10 x</td>
<td>1 x</td>
<td>5</td>
</tr>
<tr>
<td>Sense primer</td>
<td>5 Pmol</td>
<td>20 Pmol</td>
<td>4</td>
</tr>
<tr>
<td>Anti sense primer</td>
<td>5 Pmol</td>
<td>20 Pmol</td>
<td>4</td>
</tr>
<tr>
<td>dNTPs mix (Advanced Biotech UK)</td>
<td>2.5 mM</td>
<td>0.2 mM</td>
<td>4</td>
</tr>
<tr>
<td>DNA polymerase (Advanced Biotech UK)</td>
<td>5 U/ul</td>
<td>1 U</td>
<td>0.2</td>
</tr>
<tr>
<td>DEPC treated water</td>
<td>12.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNS</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primer Sequence (Research Genetics, USA): The sequence of the primers was chosen from the common (conserved) sequence of the MOMP of Chlamydia, sense (154-176, 5’-3’): ACC ACT TGG TGT GAC GCT ATC AG, antisense (1187-1166): CGG AAT TGT GCA TTT ACG TGA G.

The mix was then distributed in the PCR tubes; 30 µl of mix per tube. Then, 20µl of each sample and positive control were added to the PCR mix instead of the sample.

The thermal cycler (Biometra UnoII, Germany) was programmed first to heat the lid to 10°C then 95°C for 5 minutes (hard denaturation), followed by 35 cycles each consisted of: denaturation at 52°C for one minute, and extension at 72°C for 2 minutes. Prolonged extension at 72°C for 5 minutes was programmed to be followed by cooling to 4°C and holding at this temperature until the tubes were taken out of the machine to be examined by agarose gel electrophoresis.

Detection of specific DNA product:
This was done according to Harris et al., (1996) by agarose gel electrophoresis, utilizing DNA molecular size marker: 1X174/Hae III, 250µg/ml (Advanced biotechnology, UK).

The sample was considered positive when there was a band at the same level of the positive control band (which was 1034 bp) (Figure 1).

Detection of cervical bacteria:
The embryo transfer catheter distal tip (2 cm) was cut aseptically and directly inoculated on blood agar,
chocolate agar, and MacConkey’s agar plates. The plates were incubated aerobically and anaerobically in 5% CO₂ at 37°C. Bacteria was isolated for identification by standard laboratory procedures according to Collee et al., (1996).

**Statistical analysis:**
Data entry was done using SPSS 11.0 statistical software packages. Data analysis was done using the same program, in addition to Epi-Info 6.04 computer software package. Quantitative continuous variables were compared using Student t-test for the difference between two means. Qualitative variables were compared using Chi-square test. Whenever the expected values in one or more of the cells were less than 5, Fisher exact test was used instead. In larger tables with small numbers, the statistical tests results were not valid. Statistical significance was considered at p-value <0.05.

**Results**
From June 2003 to November 2003, thirty female patients with primary infertility due to male factor were included in this study at Ain Shams University Maternity Hospital and were divided into: group I (pregnant), who had positive pregnancy test two weeks after embryo transfer, and group II (non pregnant), who had negative pregnancy test.

Comparison of the socio-demographic characteristics between the two groups, revealed non significant differences; group I included 10 patients with mean age 31.3 ± 2.9 and mean duration of marriage 5.9 ± 1.6, and group II included 20 patients with mean age 30.6 ± 8.6 and mean duration of marriage 5.7 ± 6.2. Non significant differences were found between the husbands in the two groups regarding their age, smoking and medical history.

As regards the female and male diagnosis, the two groups were matching, all women were normal, except one in group I who had ovulatory dysfunction. For males, the most common diagnosis in the two groups was oligospermia with or without asthenospermia.

Regarding the results of ultrasound (US) and hormonal levels, table (1) shows that the non pregnant women had slightly higher rates of US abnormalities in the ovaries. Their mean levels of FSH and prolactin were slightly higher, and their LH mean level slightly lower, compared to the group of pregnant women. Nonetheless, no differences of statistical significance could be noticed.

**Table 1: Comparison of ultrasound findings, and hormonal levels among women according to pregnancy status.**

<table>
<thead>
<tr>
<th>US abnormalities in:</th>
<th>Group I (n= 10)</th>
<th>Group II (n= 20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>- endometrium</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>- right ovary</td>
<td>1</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>- left ovary</td>
<td>2</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>FSH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- range</td>
<td>1.6 -9.1</td>
<td>1.2 -9.8</td>
<td></td>
</tr>
<tr>
<td>- mean ± SD</td>
<td>5.6 ± 2.1</td>
<td>5.8 ± 2.6</td>
<td>0.80</td>
</tr>
<tr>
<td>LH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- range</td>
<td>2.8-15.4</td>
<td>2.5-10.8</td>
<td></td>
</tr>
<tr>
<td>- mean ± SD</td>
<td>6.0 ±3.7</td>
<td>5.2 ± 2.1</td>
<td>0.48</td>
</tr>
<tr>
<td>Prolactin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- range</td>
<td>7.8 –37.6</td>
<td>8.5 – 64.0</td>
<td></td>
</tr>
<tr>
<td>- mean ± SD</td>
<td>20.6 ± 9.6</td>
<td>22.3±11.7</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Group I: pregnant, Group II: non-pregnant, p> 0.05 NS
The embryo transfer data were also quite similar in the two groups regarding the number of embryos transferred, the size of the uterine cavity, or the use of US guidance.

Concerning cervical bacterial growth, table (2) indicates that klebsiella species, anaerobic cocci, and E. coli were higher in group II. Yet, this didn’t reach significance.

Non significant increase was found regarding Chlamydia trachomatis DNA detection in the non pregnant group, as it was detected in the cervices in only one patient of those who became pregnant (10%), versus 6 patients (30%) in the non pregnant group. table (3).

Table 2: Comparison of bacterial growth among women according to pregnancy status

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No  %</td>
<td>No  %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klebsiella</td>
<td>1 10</td>
<td>3 15</td>
<td>1.00</td>
</tr>
<tr>
<td>Anaerobic cocci</td>
<td>0 0</td>
<td>2 10</td>
<td>0.54</td>
</tr>
<tr>
<td>E. coli</td>
<td>0 0</td>
<td>7 35</td>
<td>0.06</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>2 20</td>
<td>2 10</td>
<td>0.58</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>3 30</td>
<td>2 10</td>
<td>0.17</td>
</tr>
</tbody>
</table>

GroupI: pregnant, GroupII: non-pregnant
p> 0.05 NS

Table 3: Comparison of Chlamydial DNA detection according to pregnancy status

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No  %</td>
<td>No  %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlamydia trachomatis</td>
<td>1 10%</td>
<td>6 30</td>
<td>0.37</td>
</tr>
</tbody>
</table>

GroupI: pregnant, GroupII: non-pregnant
p> 0.005 NS

Comparison of various combinations of bacterial and/or Chlamydial growth did not show any significant difference. Sixty % of women in each of the two groups had some type of bacterial growth. Meanwhile, only one woman in each group had combined bacterial and Chlamydia growth.

Discussion

Embryo implantation is the main event that limits the success of IVF-ET. Genital infections, particularly caused by sexually transmitted microorganisms, rank among the leading causes of infertility.

The present work aimed to determine whether presence of cervical microorganisms at embryo transfer has an impact on the success of pregnancy in women undergoing ICSI procedure. Non significant difference was detected between the groups of pregnant and non pregnant women as regards age, duration of infertility, and serum FSH level measured on day 3 of the cycle. This was important in order to be able to compare pregnant versus non pregnant women as regards the presence of Chlamydia and other microorganisms, and to investigate the relation between the presence of these microorganisms and the success or failure rates of the process of IVF.

Similarly, there were no significant differences between the two groups, as regards the number of retrieved oocytes and the number of transferred embryos. This is again to ensure that both groups had equal chance of success, and of getting pregnant, as mentioned by Sharara et al., (1996).

The present study has investigated the presence of Chlamydia infection and the success of IVF. This was based on previous debates about its role as a major cause of tubal occlusion. There is also increasing evidence that Chlamydia trachomatis infection may result in a number of adverse pregnancy outcomes, including early and late abortion, intrauterine infection of the fetus, still birth, prematurity, premature rupture of membranes, and postpartum endometritis (Mardh., 2002).
Ectopic pregnancy is commonly associated with a previous tubal Chlamydia infection where immunological reactions seem to play a role. Genital Chlamydia infection has been associated with problems in insemination and attempts at IVF (Mardh., 2002). This is particularly important since these infections are asymptomatic in most individuals and can persist in the genital tract for long periods of time in a form resistant to immune destruction.

The present study detected non significant increase in the rate of Chlamydia detection among the non pregnant group (6/20-30%) compared to those who became pregnant (1/10-10%). This is in accordance with Witkins et al., (1994) who found that unsuspected Chlamydia trachomatis infection or reactivation of an immune response to its heat shock protein may induce an inflammatory reaction in the uterus that impairs embryo implantation and/or facilitates immune rejection after uterine transfer of in vitro fertilized embryos.

Again Witkins et al., (1995) have investigated the prevalence of Chlamydia trachomatis in the endocervices of 307 asymptomatic culture-negative women undergoing IVF. Chlamydia trachomatis was detected by PCR in 20 subjects (6.5%), and there were strong correlations between a positive finding and both failure of pregnancy and spontaneous abortion after embryo transfer. There were no relationships between PCR findings and maternal age, cause of infertility, number of oocytes retrieved or fertilized, or number of embryos transferred. They concluded that an asymptomatic Chlamydia trachomatis infection might be responsible for implantation failure or spontaneous abortion after IVF and embryo transfer.

In a more recent study, Cortinas et al., (2004) have investigated the relationship between the presence of anti- Chlamydia trachomatis antibodies in serum (IgG) and anti Chlamydia trachomatis heat shock protein in follicular fluid (IgA) of infertile women. A significant association was found between the presence of IgG in serum and IgA in follicular fluid. Although the authors have reached to the conclusion that Chlamydia trachomatis infection might be triggering an autoimmune process that could negatively affect the success of IVF, they have not reported the pregnancy rates achieved, and whether it was associated with the levels of these markers.

In contradiction with the foregoing studies, Lessing et al., (1991) have determined the prevalence of specific Chlamydia IgG and IgA antibodies in 86 subfertile women undergoing IVF. Pregnancy was later achieved by IVF in 13 of 32 seropositive and 19 of 32 seronegative women. The results have demonstrated that high levels of IgG and IgA are not correlated with the outcome of IVF-embryo transfer treatment.

De Barbeyrac et al., (2006) estimated the prevalence of Chlamydia trachomatis infection in subfertile couples and studied the relationship between markers of C. trachomatis infection and male infertility as well as pregnancy rates after in vitro fertilization (IVF). Couples consulting for infertility and IVF were screened for C. trachomatis by direct (PCR test) and serological methods. The presence of positive markers was not associated with altered semen characteristics. Couples with positive markers had a pregnancy rate of 23.1% (12 out of 52) compared with 20.2% (24 out of 119) among those with negative markers. They concluded that the presence of past or current C. trachomatis infection was associated with neither semen characteristics nor outcome of IVF in subfertile couples.
As regards cervical bacterial growth, the present work demonstrated that Klebsiella, anaerobic cocci, and *E. coli* were slightly higher in the non-pregnant group while lactobacilli were slightly lower among them. However, no difference of statistical significance could be noticed, although the difference in *E. coli* had a borderline significance.

These findings are in agreement with Gaudion et al., (1999) who reported that women who have bacterial growth achieved pregnancy rates with IVF treatment similar to those of women with no evidence of such infections.

On the same line, Liversedge et al., (1999) stated that although the prevalence of bacterial infection was much higher in infertile patients having IVF treatment, compared to others in antenatal and general gynecological populations, no significant effect upon fertilization and implantation rates was found.

The lack of such association between bacterial vaginal growth and success of IVF has been explained by Michelmann (1998); he indicated that the effect of bacteria and/or leukocytes on the outcome of IVF or ICSI is influenced by three factors, which have little in common with in vivo conditions. These are namely the process of ejaculate preparation (swim-up, Percoll) with antibiotic buffered media; the small amount of inseminated spermatozoa (100,000 per culture); and the short cultivation time. From the very beginning, these factors limit whatever the influence of bacteria and leukocytes on fertilization and embryonic development in vivo may be. Despite the contradictory results published so far, the influence of bacteria and/or leukocytes on the functional integrity of spermatozoa during the process of IVF or ICSI can be ignored.

Furthermore, during IVF or ICSI the spermatozoon does not act as a vector for the transportation of bacteria into the ooplasm.

On the other hand, these present study results of no difference between pregnant and non-pregnant women as regards cervical bacterial growth are incongruent with those of Egbase et al., (1996) who reported that the presence of microorganisms on the cervix, as detected on the catheter used for ET, was associated with poor IVF-ET outcome in 110 cycles.

Also, Fanchin et al., (1998) found that the presence of cervical microbial flora (particularly *E. coli*) at the time of ET, as detected through bacteriological assessment of ET catheter tip is associated with decreased IVF-ET success. This finding may be in partial agreement with the present study finding, where a borderline significant difference was detected between pregnant and non-pregnant women as regards *E. coli*.

In conclusion, the present study supports the hypothesis that microbial flora of the cervix detected during ET have no role in the implantation process, and does not affect pregnancy rates in women undergoing ICSI procedure for infertility. *Chlamydia trachomatis* and *E. coli* might have a role. However, further larger scale studies are recommended to assess their role.

References


PMID: 9678092


