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A successful case of assisted reproduction after contamination of the culture medium with *Escherichia coli*

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Abstract. – INTRODUCTION: We report a case of successful pregnancy after *in vitro* fertilization culture (IVF) medium contaminated with *Escherichia coli* (*E. coli*).

CASE REPORT: To remove *E. coli*, oocytes were isolated in a PVP-ICSI medium and then in a culture medium supplemented with gentamicin. We selected two embryos that were vitrified and frozen. In the next natural cycle, we performed the embryo transfer of 2 embryos.

RESULTS: Only one pregnancy was uncomplicated until term and was completed by the live birth of a healthy baby. The child's subsequent development until the date of publication of this article is normal.

CONCLUSIONS: In cases of *E. coli* contamination of the IVF culture medium, specific laboratory methods allow the procedure to be continued, and a healthy baby can be obtained.

Key Words:
IVF, Embryo, Culture medium, Contamination, *E. coli.*

Introduction

Reproductive tract infections account for almost 15% of all male infertility cases. The most isolated bacterium in the ejaculate of infertile men is *E. coli*, which causes 60-85% of cases of chronic bacterial prostatitis leading to sperm damage[1]. Bacterial contamination of cultures used for *in vitro* fertilization (IVF) procedures has a low frequency[2] of 0.35-0.86%. In most cases, these bacteria are resistant to prophylactic therapy with antibiotics contained in culture media; of these, the most common bacterium is *Escherichia coli* (*E. coli*)[3]. Previous studies have shown that, in addition to bacteria, the endotoxins secreted by them cause fragmented embryos, leading to a decrease in the pregnancy rate[4,5]. In the event of bacterial contamination, the policy is not to transfer embryos[3-5].

In the present study, we present the case of a couple with primary infertility who obtained a pregnancy following the IVF procedure, although the semen and the embryonic culture were infected with *E. coli*. The subsequent evolution of the conception product was normal.

Case Report

A 30-year-old couple presented at the IVF clinic in 2018 with declared primary infertility for approximately 5 years. Informed consent was obtained from the patients for this case report.

The couple was firstly investigated clinically and para-clinically, and then followed by IVF, embryo freezing, and embryo transfer (ET) in another cycle. From a microbiological point of view, the content of embryonic culture media was followed by the evolution of the embryos and the product of conception.

The patient presented on day 2 of the cycle normal hormonal values, corresponding to age: FSH

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= 8.3 mUI/ml, LH = 6.2 mUI/ml, estradiol = 23 pg/ml, progesterone = 0.7 ng/ml, TSH=1.37 mUI/ml and AMH = 5.35 ng/ml. Blood tests, infectious markers, and genetic tests (karyotype and thrombophilia) were within the normal limits. Extensive cultures (microbial, chlamydia, mycoplasma, and fungi) of the cervix and vagina were negative. The gynecological clinical and ultrasound examinations, as well as endocrinological examination results, were within normal limits. The hysteroscopic examination showed a normal-looking uterine cavity, permeability of fallopian tubes, and presence of a small polypoid formation that we extracted and sent for pathological examination, but no pathological aspects were revealed.

The partner presented with normal hormonal tests: prolactin = 16 mUI/mL; testosterone = 2.59 ng/ml; FSH = 1.89 mUI/ml; TSH = 3.11 mUI/ml and LH = 1.51 mUI/ml. Usual blood tests and borderline infectious markers. The genetic analysis results were within normal limits. Clinical and ultrasound examinations were performed by a urologist, and the results were within normal limits. The initial spermogram showed severe male factor infertility with oligoastenoteratozoospermia: volume 2 ml, number 7 × 10^6/ml, total motility 21%; progressive motility 6%; morphologically normal 2%; rare leukocytes (according to the WHO laboratory manual for the examination of human semen).

E. coli infection was detected after sperm culture. According to the antibiogram, treatment with quinolone derivatives was administered for 10 days. The subsequent spermograms were sterile. In addition, a sterile uroculture was used. Next, the couple presents to the IVF clinic to undergo ovarian puncture and IVF.

Thus, in a sterile container, the couple's partner collected the semen sample by masturbation, fulfilling the collection conditions regarding abstinence, hygiene, consumption of energizing substances, and treatment. After harvesting, the semen container was transported to the andrology laboratory and incubated for 30 min at a constant temperature of 37°C, respected under sterile conditions. After liquefaction (30 min), the sample was mixed using a sterile pipette for homogenization, and the concentration and motility parameters were evaluated. Following the examination, the following results were obtained: a volume of 2 ml of semen with a sperm concentration of 0.09 x 10^6/ml, of which 0.06 x 10^6/ml sperm with progressive motility, without the presence of leukocytes in the sample before and post preparation. A major change in the spermogram parameters was observed on the day of the puncture compared to the previous spermogram, due to infection and antibiotic treatment. Therefore, the patient was diagnosed with cryptozoospermia.

The sample was processed using the density gradient method to perform intracytoplasmic sperm injection (ICSI). Thus, 1 ml of 90% gradient, lower layer, and 1 ml gradient of 45% upper layer were placed in two 15 ml tubes. The 90% gradient was formed by mixing 9 ml SpermGrad medium (Vitrolife) and 1 ml G-IVFplus medium (Vitrolife), and the 45% gradient was formed by mixing 4.5 ml SpermGrad medium with 5.5 ml G-IVFplus. Over a 2 ml gradient, 1 ml of the semen sample collected by the patient was added to each tube. The two tubes were then centrifuged for 20 minutes at 1,500 rpm. After centrifugation, the supernatant was removed, and the pellet (approximately 0.5 ml total) was diluted with 5 ml of G-IVFplus medium, a medium containing gentamicin as an antimicrobial agent, and centrifuged for 10 min at 2,000 rpm. 300 µl of sperm was obtained, incubated at 37°C, and used for the ICSI procedure.

The patient underwent ovarian stimulation with a long antagonist protocol. Since there is a risk of ovarian hyperstimulation in connection with tapering the rFSH dose, a GnRH antagonist was administered twice (0.25 mg/12 h) on the day before the hCG trigger[6]. 31 ovarian follicles were punctured.

Follicular fluid obtained by puncturing the ovarian follicles was subjected to microscopic examination using a stereomicroscope (Nikon SMZ). In 60 mm collecting vessels, 21 oocytes were identified and collected using G-MOPS medium (Vitrolife), pre-balanced at 37°C. After examination of the entire amount of follicular fluid, the oocytes were incubated for 2 hours in 4-well culture vessels containing 0.5 ml G-IVF and 0.4 ml Ovoil (Vitrolife) at 37°C in a Planner cell incubator with 6% CO_2, and 5% O_2 gas atmosphere.

Oocytes were decumulated in Hyase (Vitrolife), containing penicillin, at a dilution of 1:10 with G-Mops medium. Decumulated oocytes were evaluated at 20x objective magnification to determine the degree of maturity. Of the 21 oocytes, 17 were mature (MII) and 4 were in the germinal vesicle stage. The ICSI procedure was then performed using an inverted microscope Nikon Eclipse Ti-U and a Narshige micromanipulator system. 17 oocytes were used for injection.

The embryologist used the technique of intracytoplasmic morphologically selected sperm

injection (IMSI) for better sperm selection, as we described in a previous study[7].

For injection of oocytes, culture vessels with 50 µL droplets of G-MOPS medium were used, in which the oocytes were inserted and put next to two drops of 50 µL each of the prepared semen sample, covered with OVOIL. After injection, the 17 oocytes were incubated for 18 h in a culture vessel with G-IVFplus and OVOIL medium.

Results

After the first day of culture (18 h after injection), infection was detected in the culture during the fertilization evaluation (Figures 1 and 2). As shown in these figures, the degree of bacterial multiplication was impressive.

The culture conducted in the harvested environment later proved that it was *E. coli*. Sperm culture performed on the same day also showed the presence of *E. coli*. 14 oocytes (2 PB) from the 17 injected oocytes were fertilized. To remove the infection, fertilized oocytes were washed in PVP-ICSI medium (Vitrolife) by successive pipetting. After washing in PVP-ICSI, the oocytes were washed in G-TLplus medium (medium containing gentamicin as an antimicrobial agent), supplemented with gentamicin (100 IU/ml). The oocytes were then transferred to a new culture vessel with a pre-balanced G-TLplus medium, which was incubated at 37°C, 6% CO_2, and 5% O_2. The infection was no longer present on day 2 of culture.

On day 3 of the culture, 10 embryos were obtained, 2 with normal development and 8 with high degrees of fragmentation, as shown in Table I.

We decided to cryopreserve two embryos on day 3 with B10 and B12 scores, the remaining embryos were destroyed with the consent of the patients. The transfer was canceled because the patient was also suspected to have ovarian hyperstimulation. The two embryos were cryopreserved and stored in the cell bank by the rapid vitrification method using a Kitazato cryopreservation kit. A single straw containing two frozen embryos was inserted into the cell bank.

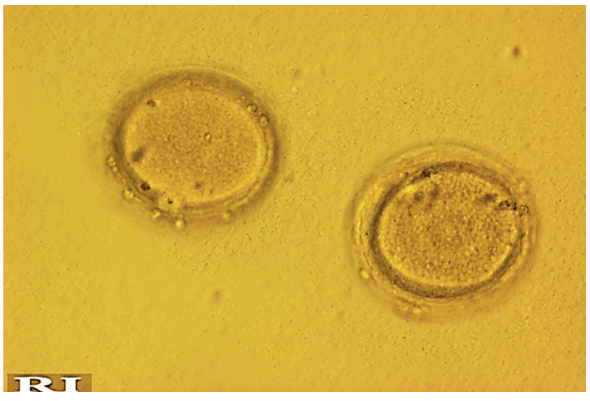

Figure 1. Oocytes in the contaminated culture at 18 h (×200).

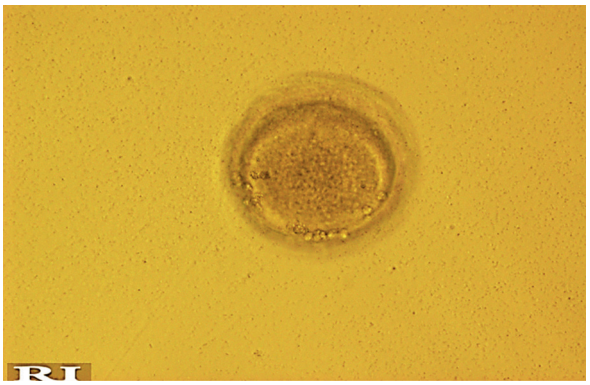

Figure 2. Oocyte in the contaminated culture at 18 h (×200).

The medium used for day 3 embryos culture was analyzed under sterile microbiological conditions and did not develop any bacteria on specific culture media.

The couple was then treated with a combination of antibiotics: cefoperazone-sulbactam and quinolone derivatives for another 10 days.

After 34 days from cryopreservation, ET of thawed embryos was performed in a natural cycle. After devitrification with Kitazato devitrification medium, two day 3 embryos were viable and were transferred (Figure 3).

At ET, we did not use pre-injection of the uterine cavity with the supernatant of the embryo

Table I. Fragmentation degree of day 3 embryos obtained.

Number of cells	Score	Degree of fragmentation
12	B	5%
10	B	0%
6	C	20%
7	C	20%
5	C	10%
2	C	40%
10	C	30%
8	C	50%
6	D	50%
5	D	70%

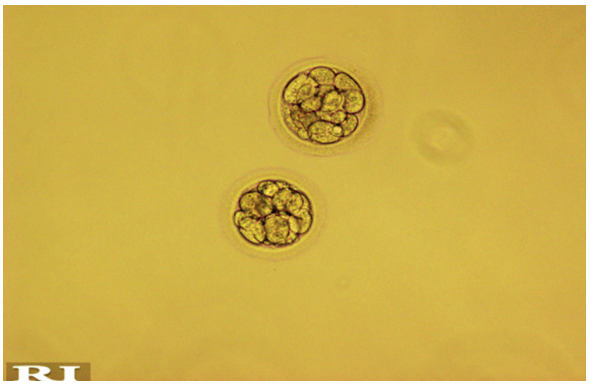

Figure 3. Day 3 embryos transferred after devitrification (×200).

culture because previous studies[8] have already shown that this has no advantages.

Only one intrauterine pregnancy was confirmed, which progressed normally to term. Blood tests, non-invasive prenatal genetic screening, and clinical and ultrasound examinations throughout pregnancy were all normal. At the end of 2019, the pregnancy was completed by cesarean section with the live birth of a perfectly healthy female child weighing 3,400 g and an Apgar score of 10. Furthermore, the child's evolution was subjected to a periodic medical examination. The subsequent evolution of the child, for 2.5 years until the date of publication of this article, was normal.

Discussion

In the case of IVF procedures, there are three sources of microbial contamination. The first is the semen used to fertilize oocytes but, in this case, most incriminated organisms are non-pathogenic, and after washing and swim-up procedures, the contamination is reduced[9] from 63% to 5%. The second source of contamination is transvaginal oocyte puncture. The third source is IVF and ET environment, instruments, culture dishes or media, incubators, or operatives.

Microbiological investigations of semen may reveal the probable infection. Bacteriospermia is common (35.3%), but the microorganisms found are rarely replicated and most likely originate from contamination[10]. Between 15% and 60% of infertile men are positive for at least one pathogen, and there is an important relationship between bacteriospermia and sperm damage[11].

Washing and preparing semen according to the concentration gradient method in a culture medium rich in antibiotics eliminated 95% of microorganisms. Presence of microorganisms in semen can affect the ability of sperm to fertilize and develop embryos[12].

A study of 132 infertile couples showed that 19 men and 11 women were positive for *E. coli*, which is quite common, especially in men with infertility[13]. *E. coli* has negative effects on the morphological parameters of the spermogram[13]. Other common microorganisms isolated from semen are also *E. faecalis*, *Staphylococcus aureus*, *gonococci*, *Candida sporules*, and *Klebsiella sporules*[14].

The direct effect of *E. coli* on sperm has been demonstrated in *in vitro* studies[15] performed by direct incubation of both cells. Authors have shown that *E. coli* comes in contact with sperm and causes a decrease in sperm motility, alters acrosomal function, and decreases vitality. Bacterial-sperm interactions have been widely studied, especially in human spermatozoa with *E. coli* as a model of contamination, concluding that the spermicidal effect is concentration-dependent[16]. Several adverse effects of bacterial contamination on sperm quality have been reported, such as sperm motility affected by bacterial adhesion and agglutination, induction of morphological changes, impaired sperm function, increased phosphatidylserine translocation, and activation of apoptosis[17].

In our case, it was shown that the microbial contamination came from the collected semen. Although the patient was previously treated and had a negative sperm culture, the existence of a hidden, chronic source of infection probably led to recolonization of the semen. Even if this colonization was initially minimal, impressive multiplication was observed, which was due to the favorable conditions of the incubator and cell culture environment.

A study[18] published in Human Reproduction by microbiological examination emphasizes that most of the contaminants were *E. coli* (58.9%): 73.7% of cases of contamination with a single germ, of which 80% were with *E. coli*; 73.2% of cases were resistant to penicillin and streptomycin, and most cases of *E. coli* infection were detected on the first day of culture. Regarding the influence of E. coli on the quality of sperm, they showed a significant change in their number, motility, and morphology[10,18,19]. *E. faecalis* and *Escherichia coli* are often the main microorganisms isolated from semen, with the most negative influence on sperm motility and morphology[20-23].

Studies[24] dealing with cognitive, emotional, behavioral, relational, and social aspects of IVF

treatment have shown that both men and women need psychological support. In this context, culture contamination in IVF procedures has a huge impact on the would-be parents and is enormously costly for both parents and the laboratory concerned.

Literature has aimed at avoiding replacing embryos from contaminated culture dishes. This is because of the fear that these microorganisms carried by the embryos may be introduced into the uterus and cause infections during the embryo transfer procedure. In addition, embryo development is deficient in these germs[3]. Bacterial endotoxins secreted into the environment by these microbes have a negative impact on embryonic development[5]. In patients with genitourinary tract infections, almost all spermatozoa showed an abnormal meiotic process which led to a significant increase in aneuploidy and sperm necrosis[25]. When bacteria die or decompose endotoxin will release into the surrounding medium, even from the living bacteria, which is highly toxic to tissue and cells. This endotoxin could affect the development of embryos, as well as decrease the clinical pregnancy rate and living birth rate[5]. Fragmentation and bleeding of embryos have negative effects on the pregnancy rate[4,5]. There are extremely rare reports[3,18] in the literature that one method that can be used to reduce the risk of microbial contamination is ICSI. The isolation of a single motile sperm from the solution during ICSI may explain the low risk of contamination. In our case, however, we found considerable contamination of the culture medium after the ICSI procedure.

We were put in a position to stop this culture, but we continued because we saw that after successive washing procedures in the media, the germs from the culture disappeared. It is likely that isolating fertilized oocytes in a solution of PVP and then the subsequent washing in the gentamicin-containing G-IVFplus medium in which a surplus of gentamicin was added contributed to the disappearance of the microbial load. The evolution of the two embryos continued until day 3 of cultivation when they were frozen by vitrification.

After a month we performed devitrification and ET of the two embryos. This process of vitrification-freezing and devitrification may have contributed to the disappearance of the microbial load. In addition, rapid freezing on day 3 decreased the duration of embryo exposure to culture conditions. The fertilization rate did not seem to have been influenced by *E. coli* infection. In the present case, 17 oocytes were fertilized with 14 of them (82%), but the percentage of viable embryos was low (approximately 20%). 60% of the embryos obtained had degrees of fragmentation between 20-50%, and 20% of these degrees of fragmentation were over 50%.

We must point out that this case of microbial contamination of the embryo culture medium can also occur when there is no time to examine and treat the couple, as is the case in cases where immediate cancer treatment must be given. IVF is an option for surgical treatment to preserve fertility. GnRH antagonists are most used to preserve fertility through a vitrification system for oocyte retrieval, as in our case[26]. Current advances in diagnostic and prognostic methods make it possible to select patients who can receive fertility-preserving treatment with increasing oncological safety[27,28].

Conclusions

This study presents a successful case of conception following an IVF procedure in which bacterial contamination of the culture medium with *E. coli* occurred. Laboratory methods, such as isolation in the environment of PVP-ICSI, successive washing in the environment with gentamicin, vitrification, freezing and devitrification used for handling and culturing the gametes and embryos, and the short incubation period up to the third day, contributed to the success of the procedure. The intrapartum and postpartum development of the mother and the fertilized product was completely normal. We believe that this study encourages us to continue IVF treatment, even in case of contamination of the culture medium with *E. coli*. Further studies are needed to accept this behavior in general.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Informed Consent
All patients were well informed and written informed consent was obtained.

Authors' Contribution
C.-C.V., A.N. and R.-M.C. (conceptualization, data curation, investigation, formal analysis, methodology, and writing of the original draft); C.-C.V., R.-M.C. and A.N. (conceptualization, methodology, writing–review and editing); C.-C.V., A.V. and B.D. (supervision, validation, project administration).

Data Availability Statement
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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