Transfer of IVF-contaminated blastocysts with removal of the zona pellucida resulted in live births

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Abstract

Purpose Bacterial contamination may cause loss or damage to cultured oocytes or embryos, resulting in cancelation or delaying of a fresh embryo transfer. While live births have been reported following the transfer of embryos contaminated with yeast, very little information is available on how to handle embryos with bacterial contamination. We report two cases of successful pregnancy in patients with bacterial contamination of embryo culture dishes.

Methods We retrospectively reviewed 878 oocyte retrievals performed between January 2011 and December 2014. Bacterial contamination was recorded in two split IVF/ICSI cases, where contamination occurred in embryo culture drops containing embryos from conventional insemination but not from ICSI on day 3.

Results To minimize the adverse effects of bacterial contamination on transfer outcomes, we removed the zona pellucida of contaminated frozen blastocysts and successfully obtained clinical pregnancies following transfer of zona-free blastocysts that were previously contaminated during IVF culture.

Conclusions Removal of the zona pellucida is an appropriate approach to handle blastocysts contaminated with bacteria during in vitro culture.

Keywords Bacterial contamination · In vitro fertilization · Zona pellucida · Blastocysts

Capsule Removal of the zona pellucida maintained embryo viability of blastocysts contaminated with bacteria during in vitro culture and resulted in successful pregnancy.

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Introduction

Bacterial contamination may cause loss or damage to cultured oocytes or embryos, resulting in cancelation or delaying of a fresh embryo transfer. By reviewing 13,977 IVF cycles over a period of 8 years, Kastrop et al. [1] reported 95 infections of IVF culture dishes (0.68 %). Considering the large number of IVF cycles performed, thousands of IVF microbial contaminations occur every year. Live births following transfer of embryos contaminated with yeast were reported [2]; however, there is very little information available on how to handle embryos derived from contaminated culture dishes with bacterial contamination.

This is a retrospective chart review of 878 oocyte retrievals performed between January 2011 and December 2014. Bacterial contamination on day 3 of culture was recorded in two split IVF/ICSI cases (0.22 %), where bacterial contamination occurred in embryo culture medium containing embryos from conventional insemination but not from ICSI. To minimize the adverse effects of bacterial contamination on transfer outcomes, we removed the zona pellucida of contaminated frozen blastocysts and successfully obtained clinical pregnancies following transfer of zona-free frozen blastocysts that were previously contaminated during IVF culture.

Case 1

A 37-year-old primary infertility patient presented with dysmenorrhea. She had tried to conceive for 6 years. Her husband's semen analysis was normal (sperm concentration, $50 \times 10^6/\text{ml}$; motility, 71 %; normal morphology, 10 %). Controlled ovarian stimulation was performed using a long GnRH-agonist protocol, where the patient started on 10 units/day of Lupron (Lupron Depot; TAP

Pharmaceuticals, North Chicago, IL) on day 21 of the previous cycle and decreased Lupron dose to 5 units/day when stimulation started on day 4. Stimulation with 150 IU/day of Gonal F (Merck-Serono, Switzerland) and two ampules of Menopur (Ferring, Parsippany, NJ, USA) was continued until the day of hCG trigger. Transvaginal oocyte retrieval was performed 36 h after hCG administration, with a total of 22 oocytes being retrieved. On the day of oocyte retrieval, sperm analysis showed normal semen parameters (sperm concentration, $60 \times 10^6/\text{ml}$; motility, 63 %).

Considering her history of years' primary infertility, split IVF/ICSI was suggested. Of the 11 oocytes assigned for conventional insemination, 8 were fertilized normally on day 1. Among the 11 oocytes for ICSI, 8 were mature and injected, with 5 oocytes being fertilized normally. Oocytes and embryos were cultured in 50 μl microdrops of Global medium (IVF Online, Montreal, Canada) supplemented with 10 % serum protein substitute (SPS; Cooper Surgical, Trumbull, CT, USA) and 10 $\mu\text{g}/\text{ml}$ of gentamicin until the morning of day 3. Bacterial contamination was noted in microdrops containing conventional insemination embryos (Fig. 1a). No contamination was observed in microdrops containing ICSI embryos. All eight embryos from contaminated IVF drops were at the 6–10 cell stage without significant fragmentation. After being thoroughly rinsed several times in Quinn's Modified HTF (Cooper Surgical, Trumbull, CT, USA) with 10 % SPS, eight contaminated IVF embryos were refreshed with a clean culture dish and kept until day 4, when they were re-examined for bacterial contamination. While one of the drops containing five IVF embryos was clear without bacteria, the other drop with three IVF embryos still showed light bacterial contamination on day 4. To prevent further bacterial growth, all IVF embryos were rinsed again in fresh culture medium and cultured until day 5, when a fresh embryo transfer was scheduled. A total of seven good-quality blastocysts were obtained on day 5, among which three were from ICSI, one from the later clean IVF drop, and three from IVF drop with light bacterial contamination on day 4 and day 5.

As per the patient's request, the best two blastocysts were chosen for transfer based on day 5 embryo morphology, with

one being from ICSI and the other from the later clean IVF drop. The remaining good-quality blastocysts on day 5 and day 6 were frozen and thawed using the Menezo two-step slow freezing and two-step rapid thawing protocols as previously described [3]. No pregnancy was achieved from her fresh embryo transfer. In a subsequent frozen embryo transfer, two blastocysts obtained from IVF drop showing light bacterial contamination on day 4 and day 5 were thawed and transferred, resulting in a biochemical pregnancy. After one fresh and one frozen transfer, two frozen blastocysts from ICSI and one from the lightly contaminated drop remained in storage.

Considering that an intact zona pellucida is not necessary for a day 5/6 blastocyst and to avoid the further growth of bacteria attached to the zona pellucida, the zona pellucida of the contaminated IVF blastocyst was removed with laser pulse lengths of 0.500 ms (ZILOS-tk; Hamilton Thorne Biosciences, Beverly, MA, USA) after warming. The zona-free frozen IVF blastocyst (Fig. 1b) was then transferred with two ICSI blastocysts in her second frozen transfer cycle. A singleton was confirmed by a vaginal ultrasound examination 4 weeks after embryo transfer, and a live birth was achieved with a normal baby weighing 2608 g at 37.5 weeks' gestation.

Case 2

Case 2 was a 27-year-old primary infertility patient with stage III endometriosis. She had three failed intrauterine insemination cycles before proceeding to IVF. GnRH-antagonist protocol was performed administering 225 IU/day of Gonal F (Merck-Serono, Switzerland) and one ampule of Menopur (Ferring, Parsippany, NJ, USA) from day 3. Ganirelix acetate (Ganirelix, Merck-Serono, Switzerland; 0.25 mg/day) was then given from the seventh day of stimulation (day 9 of the cycle). The FSH dose was adjusted to 187 IU/day from day 7 of stimulation (day 9 of the cycle) for 2 days and then 112 IU/day on day 10 of stimulation, when 10,000 units of hCG administration were performed. Oocyte retrieval was performed 36 h after hCG injection, with a total of 33 oocytes

Fig. 1 **a** Bacterial contamination in IVF embryo culture dishes; **b** zona-free contaminated IVF blastocyst. Bars = 50 μm

being obtained. Due to ovarian hyperstimulation, her fresh transfer was canceled.

Among the 17 oocytes assigned for ICSI, 15 were matured and injected, with 14 being fertilized normally. Sixteen oocytes were inseminated by conventional IVF, of which 12 were fertilized normally. Fertilized oocytes were cultured in fresh Global medium with 10 % SPS until embryonic evaluation on day 3, when bacilli-like structures were observed in all three microdrops containing IVF embryos. No sign of contamination was observed in the ICSI drops. No significant difference in embryonic development on day 3 was observed between the IVF and ICSI embryos. The twelve contaminated IVF embryos were rinsed thoroughly on both day 3 and day 4, with seven blastocysts being cryopreserved on day 5/6. In the ICSI group, 8 of 14 embryos formed good-quality blastocysts and were cryopreserved. In the subsequent two frozen cycles, four ICSI blastocysts were thawed and transferred, resulting in one negative pregnancy and one biochemical pregnancy. Per patient's request, the remaining 11 blastocysts (4 from ICSI, 7 from IVF) were thawed for preimplantation genetic screening (PGS). The zona pellucida was removed from the contaminated IVF blastocysts immediately after warming. Frozen-thawed blastocysts were then cultured in 50 μ l microdrops of Global medium supplemented with 10 % SPS. Once the blastocyst cavity was fully re-expanded, laser dissection of three to five trophectoderm cells was performed. After trophectoderm biopsy, shrunk blastocysts were vitrified using Cryotop as described by Kuwayama et al. [4]. All 11 blastocysts survived warming and were biopsied, with trophectoderm cells being sent to Genesis Genetics (Plymouth, MI) for aneuploidy screening using next-generation sequencing technology. Six blastocysts were identified as euploid, of which four were from the contaminated IVF embryos and two were from non-contaminated ICSI embryos.

One month later, the patient came back for a frozen embryo transfer using euploid embryos diagnosed by PGS. Embryos were selected for transfer based on their morphology after trophectoderm biopsy. Two zona-free euploid blastocysts originating from the IVF group were chosen for transfer in their third frozen transfer cycle. To warm vitrified blastocysts, the polypropylene strips of Cryotops were directly immersed into 0.5 ml pre-warmed 1.0 mol/l sucrose thawing solution (TS, Vit Kit-Thaw, Irvine Scientific, Santa Ana, CA) maintained on a warm stage at 37 for 1 min. Embryos were then transferred into 50 μ l 0.5 mol/l sucrose dilution solution (DS, Vit Kit-Thaw, Irvine Scientific, Santa Ana, CA) for 3 min, after which the warmed blastocysts were kept in washing solution (WS, Vit Kit-Thaw, Irvine Scientific, Santa Ana, CA) for 5 min before being moved to culture in Global medium with 10 % SPS. Positive hCG was obtained 8 days after embryo transfer, and a twin pregnancy was confirmed by ultrasound observation of fetal heart beat

4 weeks after the positive hCG. Caesarean section at 38.5 weeks resulted in the delivery of healthy twins weighing 3345 g (male) and 3091 g (female).

Discussion

In our practice, meticulous care is paid in gamete and embryo handling, but contamination still has occurred in 0.22 % of cases. Review of previously published reports indicated an incidence rate of IVF culture contamination ranged from 0.35 % [5] to 0.69 % [6]. IVF culture contamination had been attributed to poor laboratory techniques. A recent review by Pomeroy [7] supports that microbes from the non-sterile IVF culture system and non-sterile oocyte and semen collection process are the most common sources of contamination. In both cases, contamination was observed only in microdrops containing embryos from conventional insemination but not ICSI, which is consistent with Kastrop et al. [1], where bacterial contamination was observed in 95 IVF cycles (0.68 %) while no bacterial contamination was observed in 2926 ICSI cycles. Two important sources of contamination during embryo culture are semen and follicular fluids. Cumulus cells surrounding the oocytes were removed prior to ICSI, and there was no direct oocyte seminal fluid contact so that microorganisms from either follicular fluid or semen could be reduced or avoided [7]. ICSI procedure reduces risk of colonization of the culture dishes by microorganisms and should be offered for those patients with a previous infection of the IVF culture dishes. Identification of the contaminating microorganisms showed that infections in IVF culture dishes were mainly caused by bacterial strains insensitive to the antibiotics contained in culture media or due to yeast colonization by *Candida* species which frequently reside in the vagina [1].

Complete penetration and direct infection of embryonic cells is unlikely for bacteria; however, poisonous substances produced by bacteria (exotoxins and endotoxins) are suspected to cause embryo fragmentation and low pregnancy rates in human IVF [8, 9]. To minimize the adverse effects of bacterial contamination on embryonic development, cultured embryos should be removed from microdrops once contamination is identified. In most published reports, embryonic development was poor if embryo culture dishes were contaminated with bacteria [1]. Of the 20 embryos contaminated from the two cases of our report, 11 (55 %) developed to good-quality blastocysts that were either transferable or freezable. The blastocyst formation rate was similar to those from the ICSI group (11/19, 59 %). Bacterial contamination was not detected until day 3 of embryonic development in both cases for this report. The short duration of bacterial exposure and washing off attached bacteria and cellular debris from zona pellucida on both day 3 and day 4 might help explain the

decreased adverse effects of bacterial contamination on embryonic development.

At the cleavage stage, retention of the zona pellucida is both for the prevention of loss of blastomeres from the embryos and also to maintain the appropriate cell arrangement [10]. Once the embryo reaches the blastocyst stage, zona pellucida is no longer essential for continued normal embryonic development in vitro [11]. In fact, transfer of zona-free blastocysts has resulted in pregnancies and live births [12]. In a prospective, randomized study performed by Urman et al. [13], zona-free blastocyst transfer increases the success in patients with poor-quality blastocysts. Similarly, Jelinkova et al. [14] showed that chemical removal of the zona pellucida with acidic Tyrode's solution from day 5 blastocysts improved blastocyst implantation rate, especially of embryos with delayed development. Different from the above studies where chemical methods were used, laser-assisted zona removal was performed on collapsed frozen-thawed blastocysts in this study, which is safer, faster, and more precise.

While contaminated day 3 embryos were rinsed several times in clean culture drops, bacterial growth was still observed in the embryo culture drops on day 4, suggesting that there is remaining bacteria attached to zona pellucida. Simply rinsing contaminated embryos could not either completely remove the attached bacteria or avoid further contamination during embryo culture and transfer. Considering that the removal of zona pellucida would not compromise embryonic development potential while minimizing the occurrence of further contamination, we performed zona removal on contaminated blastocysts. No further bacterial growth was observed in the embryo culture medium of zona-free blastocysts that were previously contaminated in either case 1 or case 2.

There is no consensus among IVF clinics on how to handle embryos contaminated during IVF culture. Embryos cultured in the presence of yeast were usually not significantly affected and were recommended for transfer. As reported by Klein et al. in a recent study [2], three live births were achieved following the transfer of embryos from 21 yeast-contaminated cycles. However, considering the potential risk of introducing microorganisms into the uterus during embryo transfer and causing infections, some programs avoid replacing any embryos descended from contaminated dishes. No relevant policy was available at the timing of IVF culture contamination in our program. Considering that embryonic development was not significantly affected by contamination in case 1, one contaminated blastocyst from the clear IVF drop on day 4 and one blastocyst from the ICSI drop were selected for transfer based on embryo morphology on day 5 in her fresh cycle. After her failed fresh cycle and a subsequent biochemical pregnancy following transfer of two frozen IVF blastocysts from contaminated culture drop, we removed the zona pellucida of the remaining IVF blastocyst and had it transferred with two ICSI blastocysts. For this reason, it is still

unclear whether the resulting pregnancy was actually from conventional insemination or ICSI embryos. Different from case 1, both transferred blastocysts in case 2 were zona-free blastocysts that were originally from conventional insemination and underwent laser-assisted zona removal, demonstrating that successful pregnancy resulted from zona-free blastocysts when the contaminated zona pellucida was removed.

Removal of the zona pellucida minimizes further bacterial contamination in culture dishes or the uterus and is an appropriate approach to handle contaminated blastocysts. Our results demonstrate that embryo viability of contaminated blastocysts could be maintained if proper handling is provided.

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