MEDDOCS Open Access Publisher

ISSN 2641-6522

Annals of Obstetrics and Gynecology

Open Access | Research Article

Microbe Contamination During Embryo Cultures in an *In Vitro* Fertilization-Embryo Transfer System

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Received: Jun 16, 2020

Accepted: Jul 31, 2020

Published Online: Aug 05, 2020

Journal: Annals of Obstetrics and Gynecology

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Keywords: IVF-ET; Embryo; Bacteria; Contamination; Live birth.

Abstract

Microbe contamination during embryos culture occasionally occurs in an IVF-ET system. To understand the microbe contamination in embryo cultures in the IVF laboratory, the microbe contamination rate, the sources of microbes, the early development of embryos and the clinical outcomes of the contaminated IVF cycles were examined. The relevant data of microbe contamination in IVF/ICSI cycles from January 2010 to May 2020 were evaluated. A total of 65 microbe contaminated cases occurred in 27713 oocyte retrieving cycles (0.23%), and was observed only in IVF embryos but never in ICSI embryos. Thirty-seven contaminated cases occurred on day 2 with the highest ratio (56.9%). Semen was the most common source inducing microbe contamination. All of the microbe contaminations were proven to be induced by bacteria. In particular, *E. faecalis* in follicular fluid and *E. coli* in semen were the most common bacteria. Compared with the all contaminated cycles, the partial contaminated cycles had a lower rate of no available embryos (84.6% vs 23.1%) and a higher rate of blastocyst formation (0.0% vs 30.0%), and six cases of live birth were obtained in partial contaminated cycles while there were no live births in any of the all contaminated cycles.

Abbreviations: IVF-ET: In Vitro Fertilization-Embryo Transfer; IVF: In Vitro Fertilization; COCs: Cumulus Oocyte Complexes; HCG: Human Chorionic Gonadotropin; ICSI: Intracytoplasmic Sperm Injection; h-ICSI: IVF-ICSI Split Insemination; R-ICSI: Rescue ICSI; E. faecalis: Enterococcus faecalis; E. coli: Escherichia coli.

Cite this article: Wang W, Du F, Luo H, Li R, Zhu Y, et al. Microbe Contamination During Embryo Cultures in an *In Vitro* Fertilization-Embryo Transfer System. Ann Obstet Gynecol. 2021; 4(1): 1015.

Introduction

Most infertile couples receiving assisted reproductive treatment have to experience several months of clinical treatment before the oocyte retrieval operation and the laboratory embryo culture; therefore, it is important for patients to receive good quality embryos for transfer. Although the IVF laboratory enforces strict standards control within the laboratory environment, laminar flow systems, operating instruments, utensils and technical operations, the embryo culture system is still not a completely sterile system [1]. Bacteria, fungi, and viruses may exist in the IVF laboratory environment, also, it may exist in the patients' follicular fluid coming from oocyte retrieval operations and the semen coming from masturbation [2]. Even if an antibiotic was added into the culture medium, the microbes could still survive. When microbe contamination occurs, it may result in the degradation of gametes, a low fertilization rate, the blocking of the development potential of embryos, a reduction in the number of available embryos and may even cause result of finding no available embryos to use. Worse still, it may lead to contamination of the entire IVF culture system [3]. When transferring contaminated embryos to patients, microbes may be transmitted to patient's uterus, which would result in either decreased endometrial receptivity for embryo implantation or infections of the uterus [4].

Under normal conditions, the follicular fluid is sterile [5,6], but it is still possible to become one source that induces embryonic contamination. The positive rate of bacterial culture in the solution of a washing needle after oocyte extraction and the follicular fluid obtained by left and right ovary puncture was 27%-40% [7]. In male patients undergoing IVF-ET who have no infection symptoms, a variety of microbes, including bacteria, fungi and mycoplasma could be cultured from their semen; the positive rate of sperm microculture was 13%-97% [7-9]. Improper operations can also induce contaminations [10]. Some reports showed that in Europe alone, hundreds of IVF culture dishes are contaminated each year [11].

The literatures on embryonic microbe contamination in human IVF laboratories are limited. Furthermore, no studies have reported the occurrence of microbe contamination on different culture days and the adverse effects of microbe contamination on embryo development and clinical outcomes. The purpose of this study was to investigate microbe contamination during embryo cultures in the IVF laboratory as well as to make a systematic evaluation of microbe contamination in the IVF-ET system, and to provide knowledge that would enable in the improved management of the sterile micro-environment for embryo growth.

Materials and Methods

Patients

This retrospective study included all patients who received ovarian stimulation and fresh oocytes retrieving cycles from January 2010 to May 2020, at the Reproductive Medicine Center of Sun Yat-Sen Memorial Hospital. Cycles where no oocytes were retrieved or where oocytes were frozen, were also excluded from this study.

Semen preparation

The fresh ejaculated semen samples were collected directly following masturbation and stored in sterile plastic cups. Before sample collection, the men were asked to wash their hands with soap and water, and to pay attention to avoid the semen being contaminated. After the semen liquefaction, sperm concentration, motility and vitality were assessed according to WHO guidelines, then the semen were put into a tube containing gradient centrifugation (90 and 45%, SpermGrad; Vitrolife) below to centrifuge. For the normal concentrations of semen which prepared for IVF procedure, after the gradient centrifugation and separating out seminal plasma and other cells, the semen was washed twice with the medium of SPR (Vritrolife; Swiss) and GIVF (Vritrolife; Swiss) and then received swim-up for 30 minutes, regulating the concentration of sperm to 0.3-1.5× 10^6/mL, which was then placed in the incubator for reserve. For the low concentrations of semen which prepared for ICSI procedure, the semen was washed only once with the medium of GIVF, and then swim-up for 20 minutes and keep the upper semen for reserve.

Oocyte retrieval

All patients underwent oocyte retrieval under pain anesthesia. Before oocyte retrieval, all the clinical doctors, nurses, and laboratory technicians washed and disinfected their hands, and worn sterile hair covers, face masks, gloves and operating coats; patients were also asked to change into sterile operating coats and wear hair covers. In additional, all the surgical instruments were sterile. When the operation began, the patient's vagina was swabbed with sterile cotton wool soaked in a culture medium, and through the vagina each patient used a sterile oocyte-taking needle to puncture the left and right ovaries with 8-50ml of sterile saline to flush. In the course of the surgery, the follicular fluid was delivered to the laboratory technicians to pick up the exposed COCs under laminar air-flow conditions. The COCs would be quickly collected into the G-mops (Vritrolife, Sweden) medium, then washed twice with the GIVF medium and transferred into a new fertilization dish with the GIVF medium before being placed into incubators with 6% CO_2 at 37.

Fertilization and embryo culture

All operations on gametes or embryos were carried out under laminar air-flow conditions. After about forty hours of HCG (Profasi, Serono, Swiss)) injection, the prepared sperm was added into the oocytes for conventional in vitro fertilization, or the sperm was injected into the oocyte for ICSI or IVF-ICSI split insemination to prevent fertilization failure or low fertilization (h-ICSI). The R-ICSI procedure was taken in two situations, one was that there were no pronucleus observed in oocytes or only in very few oocytes after IVF the next morning, the other one was that there were no second polar body observed in oocyte or only in very few oocytes after IVF that afternoon after 6 hours of insemination. The oocyte retrieval day was called Day 0. On Day 1, the fertilization was checked by observing the pronucleus of zygote, then the zygote was transferred into cleavage dishes made by G1-Plus (Vritrolife, Sweden) for the following 3 days, after which the embryos were transmitted into blastula dishes made by G2-Plus (Vritrolife, Sweden) for the following 2 days. On days 2 through 6, the growth of the embryos was checked and graded under a microscope, and the available embryos were frozen or transferred into the uterus of patients.

Microbiologic examination and treatment of contaminated embryos

During the in vitro culture of gametes and embryos, we checked the fertilization of oocytes on day 1 and the development of the embryos on days 2 through 6. If the fertil-

ization dishes or the drops in the cleavage or blastula dishes were not clear or turned to a yellowish color, and suspected microbe contamination occurred when checked under microscope (sometimes with embryos degenerated or dead), then we made a preliminary judgment that the embryos were contaminated by microbes. When a microbe contamination accident occurred, the first thing we did was use the equilibrated medium to prepare new culture dishes, along with repeatedly washing the available embryos with a sterile culture medium to remove as many of microbes as possible, before transferring all the embryos into a new culture dish. The contaminated cases were divided into two groups: the all contaminated group and the partially contaminated group. The basis for grouping was whether all embryos were contaminated or not when examined under a microscope. If partial embryos were contaminated, we washed contaminated embryos and uncontaminated embryos separately, and kept embryos culturing in different dishes. After the washing treatment, the second thing we did was collect samples of the patient, including the semen and follicular fluid preserved on oocyte retrieval day, and the fertilization medium or contaminated drops in culture dish. Then the samples were sent to the Department of Clinical Laboratory of our hospital for microbe culture and identification, with the report coming out 3 days later. In this study, we collected 147 samples of which 65 were contamination cases.

Embryo transfer

Most embryos were transferred on days 3 and 5, while others were transferred on days 2, 4 and 6. If the embryos were partially contaminated, the normal embryos would be transferred while the contaminated embryos would be discarded. If the embryos were all contaminated, all would be discarded. In this study, eight cases transferred contaminated embryos after a thorough anti-inflammatory treatment, due to the patients' strong requests.

Clinical outcomes and statistical analysis

Live birth was defined as a live birth after 24 weeks of pregnancy. Pregnancy loss was considered as a biochemical pregnancy or early/late spontaneous abortion. All pertinent clinical data were collected retrospectively and evaluated at the end of the study period. All data analyses were conducted with SPSS software version 23.0 (IBM, New York, USA). Categorical data were described as a frequency and as percentages and were analyzed using a Chi-square test.

Results

In the 39 all contaminated cases, 33 cases resulted in no available embryos to use (84.6%) and the remaining cases did not result in pregnancy or live birth, even after 4 cases of fresh embryo transfer and 2 cases of frozen embryo transfer (Table 4). In the 26 partially contaminated cases, 6 cases had no available embryos (23.1%), even after 15 cases of fresh embryo transfer and 5 cases of frozen embryo transfer, a total of 6 cases of live birth and 3 cases of pregnancy loss were obtained, while the two cases in which were embryos contaminated before did not get pregnant. Moreover, when embryos were cultured to blastocyst, there was no blastocyst forming in any of the all contamination groups, while 3 cases in the partially contaminated group did form blastocyst (0.0% vs 30.0%).

Table 1: The incidence of microbe-contamination during in vitro culture and the distribution number of different fertilization treatment cycles.

Year	IVF			h-ICSI			ICSI			R-ICSI		
	T (N)	C(N)	%	T (N)	C(N)	%	T (N)	C(N)	%	T (N)	C(N)	%
2010	606	5	0.83	20	0	0.00	120	0	0.00	11	0	0.00
2011	1388	7	0.50	134	0	0.00	284	0	0.00	29	0	0.00
2012	1832	2	0.11	96	0	0.00	329	0	0.00	57	0	0.00
2013	1830	8	0.44	284	0	0.00	399	0	0.00	57	0	0.00
2014	2117	3	0.14	350	1	0.29	466	0	0.00	48	1	2.08
2015	2281	14	0.61	245	0	0.00	530	0	0.00	38	0	0.00
2016	2744	4	0.15	190	1	0.53	540	0	0.00	48	1	2.08
2017	2612	3	0.11	267	0	0.00	530	0	0.00	46	0	0.00
2018	2380	5	0.21	376	2	0.53	517	0	0.00	25	0	0.00
2019	2203	5	0.23	359	2	0.56	617	0	0.00	29	0	0.00
2020	494	1	0.20	63	0	0.00	118	0	0.00	4	0	0.00
Total	20487	57	0.28	2384	6	0. 25	4450	0	0.00	392	2	0.51

IVF: *In Vitro Fertilization*; h-ICSI: IVF-ICSI Split Insemination; ICSI: Intracytoplasmic Sperm Injection; R-ICSI: Rescue ICSI; T: Total Cycles; C: Contamination Cycles.

During a 11-year period there was a total of 27713 fresh oocytes retrieving cycles, including 20487 IVF, 2384 h-ICSI, 4450 ICSI and 392 R-ICSI of which 65 cases were contaminated by microbes (0.23%). The average age of the women was 34.1 (Table 1). The mean frequency of microbe contamination in IVF, h-ICSI and R-ICSI cycles was 0.28%, 0.25% and 0.51% respectively, while no contaminations occurred in ICSI cycles. In 2010, the contamination frequency in IVF cycles was 0.83%, which was the highest. Besides, the contamination only occur in IVF embryos but never in ICSI embryos.

Table 2: The number of samples examined and microbe-contaminated on different culture days.

	D1	D2	D3
Contaminated cases (n=65)	23 (35.4)	37 (56.9)	5 (7.7)
Samplesexamined(n=147)			
Follicular fluid (n=46)	16	26	4
Semen (n=48)	18	26	4
Culture medium (n=53)	19	30	4
Total (n=147)	53	82	12
Samples contaminated			
Follicular fluid (n=21)	6 (37.5)	13 (50.0)	2 (50.0)
Semen (n=41)	15 (83.3)	22 (84.6)	4 (100.0)
Culture medium (n=53)	19 (100.0)	30 (100.0)	4 (100.0)
Total (n=115)	40 (75.5)	65 (79.3)	10 (83.3)

D1, Day 1; D2, Day 2; D3, Day 3.

At last, 147 samples were examined, including 46 follicular fluid samples, 48 semen samples and 53 culture medium samples (Table 2). It is shown that the contaminated cases mainly detected on day 2 (56.9%), while there were only five contaminated cases on day 3 (7.7%). A total of 115 samples were detected to be contaminated (78.2%), and on these 3 days, the culture mediums were all contaminated (100%), while the semen contaminated rate was higher than follicular fluid every day (83.3%, 84.6%, 100% vs 37.5%, 50.0%, 50.0%, respectively). All in all, the samples we examined were mostly contaminated by microbes on day 3 (83.3%).

Table 3: Incidence of identification of microbes in different contaminated samples and on different culture days.

Identified microbes	Samples microbe-contamination identified			Day microbe-contamination identified		
	Follicular fluid (n=46)	Semen (n=48)	Culture medium (n=53)	D1 (n=53)	D2 (n=82)	D3 (n=12)
Escherichia coli (n=73)	6 (13.0)	30 (62.5)	37 (69.8)	32 (60.4)	41 (50.0)	-
Enterococcus faecalis (n=15)	7 (15.2)	4 (8.3)	4 (7.5)	2 (3.8)	6 (7.3)	7 (58.3)
Streptococcus agalactiae (n=5)	2 (4.3)	1 (2.1)	2 (3.8)	2 (3.8)	-	3 (25.0)
Klebsiella pneumoniae (n=4)	-	1 (2.1)	3 (5.7)	-	4 (4.9)	-
Streptococcus Pasteurella (n=2)	2 (4.3)	-	-	-	2 (2.4)	-
Flavobacterium (n=2)	-	1 (2.1)	1 (1.9)	-	2 (2.4)	-
Klebsiella aerogenes (n=2)	1(2.2)	-	1 (1.9)	-	2 (2.4)	-
Staphylococcus (n=2)	-	-	2 (3.8)	2 (3.8)	-	-
Proteus mirabilis (n=1)	-	-	1 (1.9)	-	1 (1.2)	-
Corynebacterium glucuronolyticum (n=1)		1 (2.1)			1 (1.2)	
Escherichia coli + *Enterococcus faecalis* (n=4)	2 (4.3)	-	2 (3.8)	1 (1.9)	3 (3.7)	-
Escherichia coli + *Enterococcus avium* (n=1)	1(2.2)	-	-	-	1 (1.2)	-
Escherichia coli + *Klebsiella pneumoniae* (n=1)	-	1 (2.1)	-	-	1 (1.2)	-
Escherichia coli +*Staphylococcus* (n=1)	-	1 (2.1)	-	1 (1.9)	-	-
Enterococcus faecalis +*Staphylococcus* (n=1)	-	1 (2.1)	-	-	1 (1.2)	-
Total (n=115)	21 (45.7)	41 (85.4)	53 (100.0)	40 (75.5)	65 (79.3)	10 (83.3)

"+" indicate in pair

After the examination of 147 samples, all of the microbe contaminations were proved to have been induced by bacteria, and there were 15 groups of bacteria consisting of 11 types of bacteria alone or in pairs (Table 3). Follicular fluid was typically contaminated by *E. faecalis* (15.2%), while semen and culture medium were most commonly contaminated by *E. coli* (62.5%, 69.8%). Additionally, there were several samples contaminated by two types of bacteria in each sample groups, of which *E. coli* was the most involved bacteria. In general, on days 1 and 2, the samples were most commonly contaminated by *E. coli* (60.4%, 50.0%), while it was *E. faecalis* (58.3%) that most commonly contaminated samples on day 3. At the same time, there were five samples contaminated by two types of bacteria on day 2, with an additional two on day 1 and none on day 3.

Table 4: The development and clinical outcomes of embryos in all contaminated cycles and partially contaminated cycles.

	All contaminated (n=39)	Partial contaminated (n=26)
No available embryos	33 (84.6)	6 (23.1)
Fresh embryo transfer		
ET case	4	15
Embryos contaminated before	4	1
Live birth	0 (0.0)	4 (26.7)
Pregnancy loss	0 (0.0)	2 (13.3)
Blastocyst culture		
Culture case	5	10
Blastula formation	0 (0.0)	3(30.0)
Embryos frozen	2	8
Frozen embryos transfer in new cycles		
FET case	2	5
Embryo contaminated before	2	1
Live birth	0 (0.0)	2 (40.0)
Pregnancy loss	0 (0.0)	1 (20.0)

ET: Fresh Embryo Transfer; FET: Frozen Embryo Transfer.

Discussion

In our study, the percentage of microbe contamination was 0.23% in total, with 27713 fresh oocytes retrieving cycles, which was lower than the 0.35% reported by Peter M and 0.69% reported by Ben-Chetrit [7,12]. In IVF cycles in 2010, the contamination ratio was as high as 0.83%, which may be due to the fact that the IVF laboratory had just started IVF-ET operations in which the environment, consumables, technicians and other quality control were not strict nor efficient enough.

In ICSI cycles, no contamination occurred, which was in accordance with the findings of Kastrop et. al. [2] in which all contaminations occurred in IVF embryos but never in ICSI embryos. On the one hand, it may be due to the sperm preparation; compared with the conventional IVF procedure, ICSI only needs to select one sperm for each oocyte to fertilize, which makes it easier to avoid contaminations caused by microbes from semen. On the other hand, the removal of cumulus granulosa cells, the use of hyaluronidase, and the repeated washing steps before the ICSI procedure may help to avoid contaminations from the oocytes, while the hyaluronidase treatment had been applied for virus decontamination for porcine embryos produced *in vitro* [13]. There were two contamination cases in R-ICSI cycles; in one case, the embryos of IVF were partially contaminated, while in the other case all embryos, including those from ICSI, were contaminated, but the bacteria were detected only in semen and culture medium which means it could be suggested that the ICSI part were contaminated earlier during the previous IVF fertilization. Also, two couples went through a recurrent embryonic contamination when taking several oocytes retrieval cycles with different fertilization procedures for each, and the recurrent contaminations only occurred in IVF embryos. Consequently, we suggested that applying ICSI procedure could effectively prevent embryonic contamination in culture dishes.

There were two contamination cases in donor sperm IVF cycles, in which the frozen semen came from the Provincial Human Sperm Bank. This serves as an important reminder that it is important to take a semen infection examination before accepting a sperm donation. For the donors who do not have to manage the pressure of giving birth, it is especially necessary to emphasize the importance of the aseptic operation. At the same time, laboratory technicians should pay great attention to the risk of microbe contamination when freezing, storing, transporting and thawing semen, for many studies have reported the potential hazard of microbes or disease transmission through cryopreserved and banked semen or embryos in liquid nitrogen [14-16].

In our study, 56.9% cases were contaminated on day 2, and we speculated that this high contamination rate may have been due to the fact that the number of microbes too small to be detected on day 1, while when cultured to day 2, the half-life of the antibiotics expired and the number of microbes reached the detection standard. Thus, the contaminations on day 3 may be more likely to reflect the unclear culture environment or improper operations. The samples of contamination cases were most contaminated on day 3 (83.3%), which may suggest that when the samples were placed in vitro for 3 days with the half-life of antibiotics expiring before then, the microbes had grown to the maximum amount to be detected, as compared to days 1 and 2.

Twenty-one follicular fluid samples were contaminated in total. As we know, normal follicular fluid is aseptic, but the *β-hemolytic streptococcus*, *Staphylococcus epidermidis*, *Mycoplasma hominis*, *diphtheria*, *Lactobacillus*, *Streptococcus aeruginosa*, *Gardnerella vaginalis* and other microbes can be separated and cultured from follicular fluid [7,17], which are almost symbiotic bacteria of the vagina and cannot be completely eliminated by routine vaginal scrubbing. Also, the follicular fluid itself had certain antibacterial abilities, but the antibacterial activity was selective to different bacteria, which suggested that not every type of bacteria could not have been successfully disinfected [17].

Compared with follicular fluid, semen was the most common source inducing contaminations on days 1-3. which corresponds to the study reported by Pomeroy et al. [18], who discovered that semen (32%) and improper sterile techniques (23%) were the major factors inducing contaminations. However, we did not research the operative technique in this study. In male patients in IVF cycles, it has been reported that the contamination of symbiotic bacteria in the urethra and perineal skin were the most common sources inducing semen-carrying bacteria [9,19,20] and that the infection of any part or gland of the male reproductive system may lead to bacteriozoospermia. When using the more sensitive PCR detection method, bacteria were detected in 65% of the men in IVF cycles [21]. It seems that reducing semen contamination may be the key step to prevent embryonic contamination occurrence. Kim et al. reported when using 4% chlorhexidine and 10% polyolypyrone iodine to clean the perineum, penis and hands before ejaculating, the positive rate of semen bacterial culture was significantly reduced [22]. Furthermore, it is suggested the patients should drink more water and empty urine before ejaculating, which can reduce the concentration of bacteria and help to reduce the occurrence of embryonic contamination.

At present, antibiotic therapy is widely used in patients. However, whether the preliminary use of antibiotics in patients

could ultimately ameliorate the microbe contamination in an IVF culture system or improve the clinical pregnancy outcome is still unclear. It has been reported that prophylactic antibiotics administered routinely to women at the time of oocyte retrieval have been associated with a reduction in positive microbiology cultures of embryo catheter tips in 78.4% of patients, and that the implantation and clinical pregnancy rates increased significantly (21.6% vs 9.3%, p<0.001; 41.3% vs 18.7%, p<0.01) [23]. Also, the administration of amoxycillin and clavulanic acid prior to embryo transfer reduced upper genital tract microbe contamination but did not alter clinical pregnancy rates, and the effect on live birth was unknown [24]. In male patients without infection symptoms, it has been suggested that there is no need to use prophylactic antibiotics for treatment, since this runs the risk of destroying the normal flora balance and leading to the selection of drug-resistant bacterial strains, which in turn could induce contaminations in an IVF culture system [25].

The addition of antibiotics to an IVF culture medium is an effective method to prevent microbe contamination, but for now, gentamicin has replaced penicillin and streptomycin due to its the longer half-life at 37 in the IVF laboratory. In the study by Cottell et al. [10], only 5% of specimens remained positive for bacterial contamination after washing the sperm with antibiotic containing media. However, antimicrobials in culture media probably provide little inhibition to the potentially large amount of bacteria, which could contaminate the embryo transfer catheter when traversing the cervix. Moreover, the report by Magli et al. found that antibiotic supplementation of media had an adverse effect on the growth rate of human preimplantation embryos, even in reduced concentrations [26]. Therefore, what type of antibiotics should be used in culture medium and culture dishes to make microbes more sensitive and to minimize the adverse effects on the survival of oocytes or embryos is also an unresolved problem.

In our study, all contaminations were induced by bacteria in 115 samples, and *E. coli* was the most common bacteria, which was in accordance with the study by Kastrop et al., who found that *E. coli* (58.9%) was the most common microbe [2]. However, Kimball et al. revealed that in 32 IVF laboratories, 49% of the time the microbes were identified as a bacterium but 51% of the time they were identified as fungi, and the most common species were *E. coli*, *C. albicans* and gram negative cocci [18]. Fungi is most likely incurred from follicular aspiration or an unclean incubator. In our study there was no fungi contamination. On one hand, this may be due to efficient clinical work, including the women's gynecological inflammation treatment and the disinfection and cleaning processes of the vaginas before oocyte retrieval operation, while on the other hand, the strict quality control of the micro-environment of embryo culture in the laboratory may have helped. Few studies have researched the influence of *E. faecalis* on gametes or embryos, while we found that in all four cases contaminated by *E. faecalis*, the embryos did not degrade or die; in fact, it seemed that *E. faecalis* did not inhibit the development of the embryos, but the contaminated embryos could continue to be cultured to blastula stage and form available blastocysts. However, when transferring two embryos contaminated by *E. faecalis* on day 3, the patient did not get pregnant. We hypothesized that the source of *E. faecalis* may be incurred during follicular aspiration. While it is a common bacterium in the vagina or anus, it belongs to facultative anaerobe like yeast, and it may have had a beneficial effect on the embryos by producing antibiotics and bacteriocins in synthesis and metabolism, either by decreasing the concentration of oxidative free radicals or decreasing oxygen tension in culture medium.

When embryonic contamination occurs, the mechanism of how bacteria affect fertilization and embryo development is, as of yet, not very clear. Many studies have suggested that bacteria have different influences on the structure and function of sperm. For example, some suggest that when the sperm is contaminated by *E. coli,* a lot of bacteria adhere to the sperm, which can be observed under an electron microscope, and results in damage of the sperm's ultrastructure and decrease sperm motility [28]. Also, it is worth nothing that, in this case, the acrosome reaction of spermatozoa was significantly inhibited, which would have damaged the sperm's fertilization ability [29]. Moretti et al. reported that almost all the spermatozoa showed abnormal meiotic process in patients with genitourinary tract infections, which led to a significant increase of sperm chromosome aneuploidy and a large proportion of necrosis [30]. In addition, when bacteria die or decompose, endotoxin, which is a macromolecular substance on the outer membrane surface of Gram-negative bacteria, will release into the surrounding medium, even from the living bacteria, which is highly toxic to tissues and cells. In humans it was reported that the endotoxin could affect the development of embryos as well as decrease the clinical pregnancy rate and live birth rate. It has been confirmed that the acceptable level of endotoxin in IVF culture medium is ≤ 2pg/ml while the optimum level should be less than 1pg/ml [31].

It is difficult for patients to get live birth after transferring contaminated embryos; therefore, it is suggested that for non-special cases, washing and continuing to culture contaminated embryos should not be recommended, nor the process of embryo transfer and freezing. However, the embryos that are uncontaminated in the contamination cycles can be used to transfer or freeze. In our study, the low pregnancy rate and the high pregnancy loss rate may be related to the sources of contaminations. Many studies have demonstrated that the microbes isolated from the fundus of the vagina, the cervix or the tip of the embryo transfer catheter may be related to decreased pregnancy rates [4,32]. We speculate that there are several possible mechanisms for decreased pregnancy rates and increased pregnancy loss rates: 1) The presence of high levels of bacteria in the cervix, which may be due to chronic endometritis and may result in a lowered endometrial receptivity. That is to say, even if the embryo is successfully planted in the uterus, it cannot grow there successfully. 2) The transfer procedure may carry bacteria from the cervix into the uterus, where it may alter the endometrium and hinder implantation and development. 3) The process of embryo transfer may contaminate the embryo with bacteria, resulting in a direct negative effect on the embryo.

Conclusion

In conclusion, our study demonstrated that the application of an ICSI procedure could effectively prevent microbe contamination. Bacterial contamination mainly detected on day 2, and semen is the most common source that induces embryonic contamination. Contaminations in semen are mainly caused by *E. coli*, while contaminations in follicular fluid are typically caused by *E. faecalis*. This study gives a comprehensive systematic evaluation of the occurrence of microbe contaminations in IVF-ET system, and the statistics contain the largest number of oocytes retrieving cycles and the longest number of years to date. Also, we are the first to report the clinical outcomes of the embryonic contamination cycles. Yet, further study is still necessary to bet-

ter understand the sources that induce microbe contaminated embryos, and more efficient methods are required to remove the microbes on these contaminated embryos so as better develop and manage a sterile micro-environment for successful embryo growth.

Declarations

Ethics approval and consent to participate

The Ethics Committee of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University approved and consent to participate this research.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Funding Sources

This work was supported by Natural Science Foundation of Guangdong Province of China (no. 2018A030313911), National Natural Science Foundation of China (no.81801431) and Chinese Medical Association clinical medical research special fund, Research and development of young physicians in reproductive medicine (no. 18010060735). The funding agencies had no input on the design of the study, collection, analysis and interpretation of the data, and writing of the manuscript.

Authors' Contributions

WJW conceived the project and led the writing. FJD designed studies, collected and analyzed data, and drafted the manuscript. HJL assisted with collecting relevant patients' data, and assisted with writing. RQL planned study, conducted field Surveys. YQZ and JZ assisted with samples senting for examine and collecting bacteriological test results. PY and QXZ provided valuable advice on the analysis of the results. All authors have read and approved this manuscript.

Acknowledgement

We thank all the colleagues in the IVF laboratory in Reproductive Medicine Centre of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University.

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