Infection and Drug Resistance

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ORIGINAL RESEARCH

Effects of Embryo Microbial Contamination on ART and Neonatal Outcomes

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Purpose: This study investigated the incidence and microbial etiology of embryo contamination in assisted reproductive technology (ART), and its influence on embryo development, pregnancy and neonatal outcomes.

Methods: A retrospective analysis was conducted on embryo contamination at the Reproductive Centre of the Third Affiliated Hospital of Guangzhou Medical University, between 2018 and 2021.

Results: In the period from 2018 to 2021, the average incidence of embryo contamination was 0.12%. Bacterial growth was observed in 39 cases, with a preponderance of *Escherichia coli* (20, 51.28%), *Streptococcus agalactiae* (7, 17.95%). The fertilization rate of contaminated embryos was 18.18% (*Klebsiella pneumoniae*) to 94.79% (*S. agalactiae*), the cleavage rate was 9.09% (*Enterobacter cloacae*) to 98.90% (*S. agalactiae*), and the available embryo rate of Day 3 was 0 (*Klebsiella pneumoniae, Enterobacter cloacae*) to 63.33% (*S. agalactiae*). Blastocyst formation rate was 3.23% (*Proteus mirabilis*) to 64.29% (*Streptococcus mitis*). *E. coli* contamination occurred mostly on Day 1, and *S. agalactiae* on Days 3 and 5. After rinsing and rescuing treatment, six healthy male babies were born.

Conclusion: *E. coli* and *S. agalactiae* were the most common bacterial embryo contaminants. Most microbial contamination can significantly decrease the fertilization rate. Embryo transfer after rinsing and continuing culture had no negative effect on neonatal outcomes, but there was an increased risk of early abortion due to *E. coli* contamination.

Keywords: *in vitro* fertilization, microbial contamination, remedial treatment, pregnancy outcome, neonatal outcomes

Introduction

Assisted reproductive technology (ART) has rapidly developed as an effective treatment for infertility. An in vitro fertilization-embryo transfer (IVF-ET) culture system should be a sterile closed system, nevertheless microbial contamination of the embryo culture medium may occur.[1] Human gametes and their accompanying fluids carry several microorganisms. Biological fluids such as semen,[2] ovarian follicular fluid,[3] fallopian tube washings,[4,5] peritoneal fluid, and endometrial aspirates[6] are possible gateways to microbiological infection in the in vitro fertilization (IVF) system, placing embryos at risk of contamination.[7] Embryo contamination may also arise from the IVF culture system and environment, including the internal environment of the IVF culture room, laminar flow system, various instruments and equipment, (incubator, culture liquid, culture dish, glass and, plastic tubes), and embryologists' operational processes,[7] even though these are strictly monitored.

Received: 10 July 2024
Accepted: 19 September 2024
Published: 23 September 2024

Embryo contamination can result in a higher risk of cycle cancellation and a lower percentage of available embryos. When transferred, embryos can carry microorganisms to the microenvironment of the uterus, alter the local microbiota and compromise implantation and survival during pregnancy.[8,9] This, in turn, may increase the cost to patients and cause psychological damage. Most ART laboratories use culture media containing gentamicin (0.01 g/L) to minimize the risk of microbial growth. Nevertheless, oocytes and embryos are occasionally contaminated by microorganisms in culture dishes. The contamination rate of embryos during IVF is approximately 0.35%–0.69%.[1,10] Cottell et al[1] reported six instances of microbial contamination of the embryo culture media after 1691 oocyte collections, while Ben-Chetrit et al[10] reported five plates with yeast colonization in 729 cycles (0.69%). Adding this frequency to the number of IVF cycles performed each year in Europe[11] shows that many hundreds of IVF culture dish contamination incidents take place in Europe alone each year.

Given its serious consequences, medical workers should attach more importance to embryo contamination. There has been no systematic analysis of the microbial species, embryonic development, pregnancy outcomes, and neonatal outcomes of embryo contamination, thus a better understanding of embryo contamination in IVF culture systems is of value. In this study, 39 cycles of embryonic contamination over a period of four years were retrospectively examined. The characteristics of contamination and its effects on pregnancy and neonatal outcomes were explored. This retrospective study serves as an overview for embryologists on how to manage and avoid microbial contamination in the daily operations of ART laboratories.

Methods

Participants

This is a retrospective study conducted at the Centre for Reproductive Medicine, The Third Affiliated Hospital of Guangzhou Medical University, between January 1, 2018 to December 31, 2021. This study was approved by the ethics committee of the Third Affiliated Hospital of Guangzhou Medical University on the 2th of April 2022[Ethic no. (2022) 026]. Data was acquired from electronic medical records and pseudonymised after collection. Written informed consent was obtained from all the participants.

Inclusion criteria: All IVF cycles, including embryo contamination and non-contamination. Exclusion criteria: ICSI cycles, cycles when oocyte retrieval was cancelled, cycles when no oocytes were retrieved. Finally, this study included 39 (0.12%) cases with culture dishes contaminated with microorganisms in 31307 cycles. The time of infection of microbial contamination was recorded for only 22 of the 39 cases and was used to identify the effect on outcomes.

We collected baseline data for these cycles (female age, BMI, type of infertility, years of infertility, infertility factors), laboratory outcomes (number of oocytes retrieved, fertilization rate, cleavage rate, available embryo rate, blastocyst formation rate), pregnancy outcomes (implantation rate, HCG positive rate, clinical pregnancy rate, ectopic pregnancy rate, abortion rate, live birth), and neonatal outcomes (height, weight, fetal gender).

ART Procedures

Controlled ovarian stimulation, transvaginal oocyte retrieval, gradient centrifugation of semen, fertilisation, embryo transfer, freezing, thawing and luteal support were carried out in strict accordance with the Centre's standardized operating protocols. Tailored ovulation induction regimens are employed based on the unique circumstances of individual patients. These protocols are selected based on factors such as the patient's age, body mass index (BMI), antral follicle count (AFC), and anti-Mullerian hormone (AMH) levels. Egg retrieval is performed via the vaginal route, approximately 35–36 hours after the injection of human chorionic gonadotropin (hCG). Routine in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) is performed based on semen parameters and previous fertilization outcomes. During the luteal phase, vaginal microprogesterone (400 mg/day) is administered from the day of ovarian puncture.

Good-quality cleavage-stage embryos were defined as those with 7–9 symmetrical blastomeres without obvious fragmentation.[12] Good-quality blastocysts were defined as those having reached at least grade 3 expansion and grade A or B for the inner-cell mass and trophectoderm parameters. Poor-quality cleavage-stage embryos were defined as those with blastomere numbers greater than four, fragmentation < 30%, and positive size differences. Poor-quality blastocysts

were defined as those having reached at least grade 3 expansion but having grade C inner-cell mass or trophectoderm parameters.[13]

Culture Conditions

For conventional IVF, cumulus-oocyte complexes were inseminated with progressively motile spermatozoa in fertilization culture medium (G-IVF PLUS, Vitrolife, Gothenburg, Sweden). Fertilization was checked approximately 16 h after insemination and was determined by the presence of two pronuclei. Embryos were placed in the incubator (K-MINC-1000, Cook Medical, Bloomington, IN, USA) and cultured at 6% CO_2, 5% O_2, and 37°C. G-1 PLUS and G-2 PLUS (Vitrolife, Gothenburg, Sweden) was used for culturing cleavage-stage and blastocyst-stage embryos, respectively.[14] On day 1, the granulosa cells were stripped with sterile denuding pipette (RI, 7–72-2145/5, UK), then the zygotes were transferred to G-1 PLUS medium for a single-drop culture. Using a sterile denuding pipette (RI, 7–72-2145/5, UK) to transfer embryos to G-2 PLUS medium for blastocyst single-drop culture on day 3.

The majority of embryo microbial contamination occurred before the freeze. All medium containing 0.01mg/mL gentamicin. The culture solution was made into microdroplets and covered with sterile mineral oil. All reagents are used within the validity period. And all the consumables are disposable. All laboratory procedures are performed by experienced embryologists in strict accordance with the SOP.

IVF laboratory is a constant temperature, constant humidity clean space, with a strict laminar flow system. The gamete/embryo operation area is class 100 (particles/m^3 of air), and the embryo culture room is class 1000 (particles/m^3 of air). The interior decoration materials are selected without VOC volatilization. Monitor laminar flow pressure gauge readings, temperature and humidity every day, periodically change laminar flow and incubator filter membranes, and wipe the benchtop with sterile wipes that are non-toxic to embryos before and after operation. Perform bacterial culture on countertops and air regularly.

Follow-Up

The primary clinical outcomes encompassed the HCG positivity rate, clinical pregnancy rate, early abortion rate, late abortion rate, ectopic pregnancy rate, and live birth rate. Furthermore, the secondary outcomes were gender distribution, average height and weight.

Clinical outcome: The level of human chorionic gonadotropin (β-hCG) was measured by blood sampling 14 days after embryo transfer, and β-hCG≥10 IU/L was defined as positive. After transplantation, vaginal B-ultrasonography was performed, and the pregnancy was diagnosed as clinical pregnancy if the pregnancy sac and the original cardiac tube beat. Early abortion before 12 weeks of gestation; Late abortion at 13 to 27 weeks of gestation.

Neonatal outcome: Neonatal gender, height and weight were tracked.

Remedial Treatment

At days 1, 2, 3, and 5 of embryo culture, we removed the culture dishes and observed embryo development and contamination under a microscope. As the presence of suspicious microorganisms in the culture solution is found, positive remedial treatment was performed immediately. The method is formed by making appropriate adjustments on the basis of practice of many embryologists in China and internationally[15]: First, the surviving embryos were sucked out of the contaminated droplets and placed in a four-well dish containing pre-balanced tubal fluid supplemented with 0.15 g/L gentamicin at 37 °C in 5% CO_2 four times in sequence, then rinsed with human tubal fluid containing 10% serum, and finally transferred those non-degenerated embryos to a new culture dish for single-drop culture. On the third or fifth day, if there are no visible bacteria after rinsing under the microscope, the embryo can be frozen or transferred according to a strategy developed by the doctor.

Microbial Culture

After the contaminated embryos were transferred, a 1 mL sterile syringe was used to aspirate the culture liquid from the contaminated culture drops, and inoculated onto blood and Sabouraud agar plates for microbial culture. The plates were incubated overnight in a 36 °C, 5% CO_2 incubator and observed the next day for growth of bacteria. In a biosafety

cabinet, suspicious colonies were selected and seeded on a Macro 96-well target plate with sterile toothpicks. After natural drying, 1 μL formic acid solution was added to the surface of the target plate (provided with the instrument), after which 1 μL matrix solution was added. After drying, the Bruker Microflex MS system was used to collect and identify the colonies.[16] Each strain was similarly tested thrice.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 22.0. Continuous variables were expressed as mean ± standard deviation ($\bar{x} \pm SD$), and between-group comparisons were made using one-way analysis of variance. For categorical variables, data were presented as frequency and percentage n (%), and between-group comparisons were made using chi-square test. The significance level for hypothesis testing was set as two-sided, with $P<0.05$ indicating statistical significance.

Results

In the period from 2018 to 2021, the incidence of embryo contamination was 0.05%–0.25% (Table 1). Baseline demographics and cycle characteristics of the study cohort are shown in supplementary material (Table S1). No significant differences were observed in age, BMI, type of infertility, infertility duration, and stimulation protocols between groups. Bacterial growth was observed in 39 cases with a preponderance of *Escherichia coli* (20, 51.28%), *Streptococcus agalactiae* (7, 17.95%), and *Proteus mirabilis* (4, 10.26%) (Figure 1). Figure 2A shows a degraded unfertilized oocyte and Figure 2C shows a degraded cleavage embryo that were contaminated with *E. coli*. Figure 2B shows a morphologically normal cleavage-embryo and Figure 2D shows a morphologically normal blastocyst that was contaminated with *S. agalactiae*.

Embryo contamination is highly likely to lead to egg degeneration, low fertilization rate, the potential for embryo developmental damage, resulting in fewer available embryos and poor IVF outcomes. *E. coli, Enterococcus faecalis* and *Klebsiella pneumoniae* contamination significantly reduced the fertilization rate and available embryo rate. *S. agalactiae* and *P. mirabilis* had no effect on the fertilization, cleavage and available embryo rates, but the high-quality blastocyst formation rate decreased significantly. When *Streptococcus mitis* contamination occurred, it negatively affected the high-quality cleavage embryo rate (Table 2).

The embryos were incubated for at least 1 day, after rinsing and rescuing treatment. Before transfer, we collected the medium again and perform microbial culture again. Only embryos that are microorganisms -free can be used for transfer and freezing. To explore whether fresh or frozen embryo transfer is better after contamination, the clinical and neonatal outcomes were analyzed separately by fresh embryo transfer and frozen embryo transfer (Tables 3–5). There were 20 cases of *E. coli* contamination, but only five patients had available embryos that could be transferred. Two patients underwent fresh ET, three patients underwent frozen ET, and two of them underwent twice frozen ET. In all seven cases of *Streptococcus agalactiae* contamination, available embryos were present. Among them, four patients underwent fresh ET, four patients underwent frozen ET including one after the failure of fresh ET, and one patient underwent twice frozen ET.

Three cases of early abortion occurred in embryos contaminated with *E. coli* (Tables 3–5). Embryos contaminated with *S. agalactiae* resulted in a late abortion at 39 weeks of gestation (Tables 3–4). Six healthy foetuses (two from fresh

Table 1 Incidence of Embryo Contamination

Year	Number of Cycles	Number of Embryo Contamination Cycles	Embryo Contamination Rate (%)
2018	8278	4	0.05
2019	9003	9	0.10
2020	6699	17	0.25
2021	7327	9	0.12
Total	31307	39	0.12

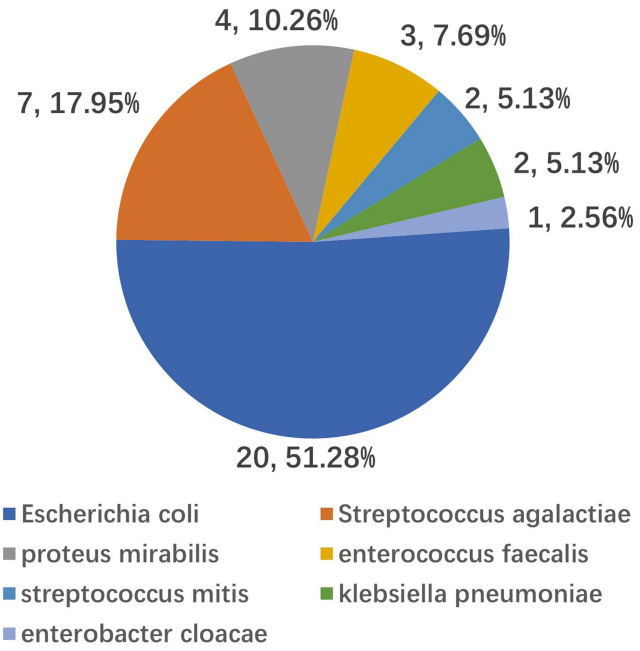

Figure 1 Microbial species and proportion.

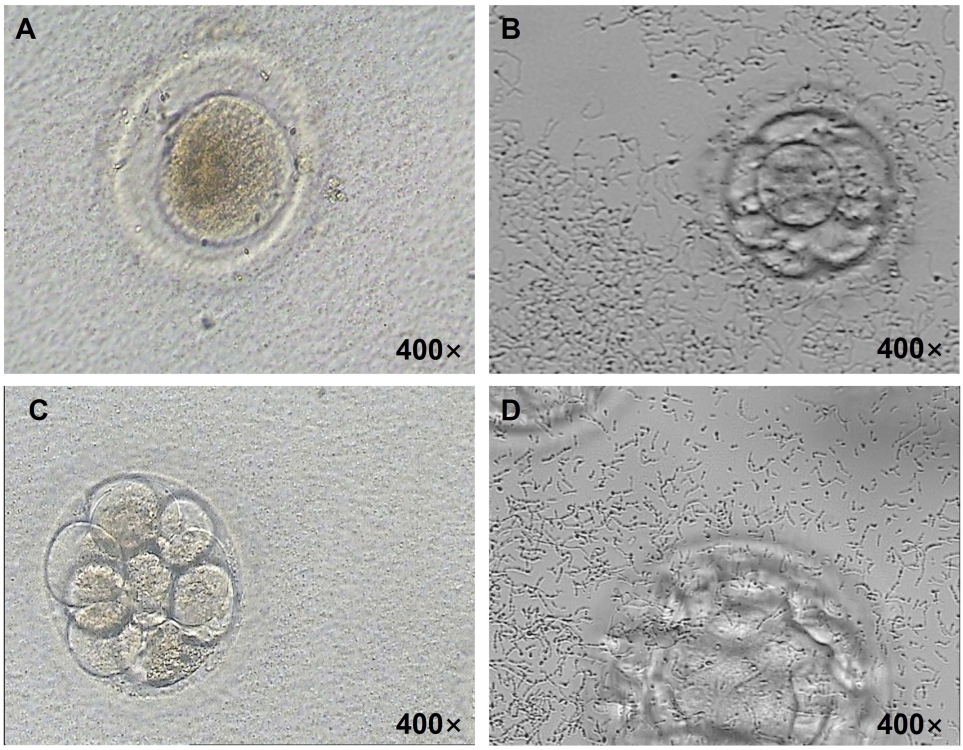

Figure 2 Oocyte and embryos contaminated with microorganisms. Oocyte (**A**) and embryo (**C**) contaminated with *E. coli* and embryos contaminated with *S. agalactiae* (**B** and **D**). 400×: under a microscope at 400 magnification.

ET and four from frozen ET) were born alive, all of whom were male (Tables 3–5). Only 22 of the 39 cases recorded the time of microbial contamination. The following investigation was done to further investigate whether contamination infection time influences the result. (Table 6) The result shows that *E. coli* was more likely to be detected on Day 1, and the embryo fertilization rate was significantly lower (44.34%). Eight cases were found on Day 2, the bacterial distribution

Table 2 Fresh IVF Cycles Outcomes of Patients Stratified by the Microbial Species

	Escherichia coli	*Streptococcus agalactiae*	*Proteus mirabilis*	*Enterococcus faecalis*	*Streptococcus mitis*	*Klebsiella pneumoniae*	*Enterobacter cloacae*	*Uncontaminated Embryos from IVF Cycles in the Same Period*
No. of cycles	20	7	4	3	2	2	1	31268
No. of oocytes retrieved	262	96	59	58	37	22	14	487212
No. of fertilized oocyte	167	91	46	24	31	4	11	376810
Fertilization rate (%)	63.74*	94.79	77.97	41.38*	83.78	18.18*	78.57	77.34
No. of cleavage embryo	83	90	45	24	29	1	1	351450
Cleavage rate (%)	49.70*	98.90	97.83	100.00	93.55	25.00*	9.09*	93.27
No. of embryos available	26	57	24	9	15	0	0	181032
Available embryo rate (%)	31.33*	63.33	53.33	37.50*	51.72	0.00*	0.00*	51.51
High quality cleavage-stage embryo rate (%)	5.73* (9/157)	28.92 (24/83)	28.21 (11/39)	25.00 (5/20)	3.70* (1/27)	0* (0/4)	0* (0/10)	25.33 (9372/37,001)
Blastocyst formation rate (%)	43.33 (13/30)	25.76* (17/66)	3.23* (1/31)	35.00* (7/20)	64.29 (9/14)	NA	NA	43.45 (110,355/253,981)
High quality blastocysts formation rate (%)	40.00 (9/30)	15.15* (10/66)	0* (0/31)	35.00 (7/20)	50.00 (7/14)	NA	NA	32.18 (81,733/253,981)

Note: *: There was a negative statistical significance compared with uncontaminated embryos from IVF cycles in the same period.
Abbreviations: NA, Not Available; IVF, In vitro fertilization.

Table 3 Pregnancy and Neonatal Outcomes Resulting of Fresh ET Stratified by the Microbial Species

	Escherichia coli	*Streptococcus agalactiae*	*Proteus mirabilis*	*Streptococcus mitis*
No. of cycles	2	4	2	1
Number of embryos transferred	3	7	2	2
Implantation rate (%)	33.33(1/3)	28.57(2/7)	50.00 (1/2)	0.00(0/2)
Positive HCG rate (%)	50.00(1/2)	50.00(2/4)	50.00(1/2)	0.00(0/1)
Clinical Pregnancy rate (%)	50.00(1/2)	50.00(2/4)	50.00(1/2)	0.00(0/1)
Early abortion, n	1(Day 5)	0	0	NA
Live birth, n	0	1(Day 5)	1(Day 5)	NA
Late abortion, n	0	1(Day 3)	0	NA
Fetal gender	NA	male	male	NA
Birth height(cm)	NA	50	51	NA
Birth weight(kg)	NA	3400	3100	NA

Abbreviations: NA, Not Available; ET, Embryo transfer; HCG, Human Chorionic Gonadotropin.

Table 4 Pregnancy and Neonatal Outcomes Resulting of First Frozen ET Stratified by the Microbial Species

	Escherichia coli	*Streptococcus agalactiae*	*Enterococcus faecalis*	*Streptococcus mitis*
No. of cycles	3	4	1	1
Number of embryos transferred	5	4	2	1
Implantation rate (%)	20.00(1/5)	50.00(2/4)	0.00(0/2)	100.00(1/1)
Positive HCG rate (%)	33.33(1/3)	50.00(2/4)	0.00(0/1)	100.00(1/1)
Clinical Pregnancy rate (%)	33.33(1/3)	50.00(2/4)	0.00(0/1)	100.00(1/1)
Early abortion, n	1(Day 5)	0	NA	0
Live birth, n	0	2(Day 5)	NA	0
Still birth, n	0	0	NA	1(Day 5)
Fetal gender	NA	male	NA	NA
Average birth height(cm)	NA	50	NA	NA
Average birth weight(kg)	NA	3000	NA	NA

Abbreviations: NA, Not Available; ET, Embryo transfer; HCG, Human Chorionic Gonadotropin.

Table 5 Pregnancy and Neonatal Outcomes Resulting of Second Frozen ET Stratified by the Microbial Species

	Escherichia coli	*Streptococcus agalactiae*	*Enterococcus faecalis*
No. of cycles	2	1	1
Number of embryos transferred	4	1	2
Implantation rate (%)	25.00(1/4)	100.00(1/1)	50.00(1/2)
Positive HCG rate (%)	50.00(1/2)	100.00(1/1)	100.00(1/1)
Clinical Pregnancy rate (%)	50.00(1/2)	100.00(1/1)	100.00(1/1)
Early abortion, n	1(Day 3)	0	0
Live birth, n	0	1(Day 5)	1(Day 5)
Fetal gender	NA	male	male
Birth height(cm)	NA	47	50
Birth weight(kg)	NA	3000	3650

Abbreviations: NA, Not Available; ET, Embryo transfer; HCG, Human Chorionic Gonadotropin.

Table 6 Microbial Species and ART Outcomes Stratified by the Infection Time

	Day 1	Day 2	Day 3	Day 5	*Uncontaminated Embryos from IVF Cycles in the Same Period*
Microbial species					
Escherichia coli	5	2	2	0	
Streptococcus agalactiae	0	1	4	1	
Proteus mirabilis	0	2	0	0	
Enterococcus faecalis	0	0	0	0	
Streptococcus mitis	0	1	1	0	
Klebsiella pneumoniae	1	1	0	0	
Enterobacter cloacae	0	1	0	0	
No. of oocytes retrieved	106	127	82	21	487212
No. of fertilized oocyte	47	97	77	21	376810
Fertilization rate (%)	44.34*	76.38	93.90	100.00	77.34
No. of cleavage embryo	43	54	72	21	351450
Cleavage rate (%)	91.49	55.67*	93.51	100.00	93.27
No. of embryos available	22	26	42	16	181032
Available embryo rate (%)	51.16	48.15	58.33	76.19	51.51
Blastocyst formation rate (%)	44.83 (13/29) *	0.00* (0/26) *	41.18 (21/51) *	12.50* (2/16) *	43.45 (110,355/253,981)
Early abortion, n	2	0	0	0	
Live birth, n	0	0	2	1	
Late abortion, n	0	1	0	0	
Still birth, n	0	0	1	0	

Note: *: There was a negative statistical significance compared with uncontaminated embryos from IVF cycles in the same period.
Abbreviation: ART, Assisted reproductive technology.

was relatively scattered, fertilization rate (76.378%) was not affected, but cleavage (55.67%) and blastocyst formation rates (0%) were greatly reduced, and one was a case of late abortion after fresh ET. On Day 3, 7 cases of contamination were found, mainly *S. agalactiae*. Embryos were not affected by this bacterium in the first three days of development, and the rate of fertilization, cleavage and availability of embryos were normal, but the proportion of blastocysts that developed by Day 5 was significantly reduced. Only one case of *S. agalactiae* contamination was discovered on Day 5 of in vitro culture. Due to the relatively late infection time, this microbe only affected the blastocyst formation rate, and had no adverse effects on other ART outcomes. All neonates were of normal height and weight, and no congenital malformations were found.

Discussion

Embryo contamination during IVF is uncommon, with an incidence of approximately 0.35%–0.69%.[17] In the period from 2018 to 2021, the incidence of embryo contamination was 0.05%, 0.10%, 0.25%, and 0.12%, respectively. According to previous reports, embryo contamination has a great impact on the outcome of IVF; it is highly likely to lead to egg degeneration, low fertilization rate, the potential for embryo developmental damage, resulting in embryo death, reduction in pregnancy and implantation rates, ultimately leading to IVF failure.[18] The data in this study are similar to those conclusions. Of the 39 cases of embryo contamination, 22 were degenerated and discarded, and only 17 had a chance of ET or cryopreservation.

It is currently considered that bacteria in semen may be the main source of contamination,[19,20] with *E. coli* and fungi being the most common contaminants.[21,22] The risk of contamination is greatly increased without urination or hand washing before semen extraction.[23] In accord with this, we detected the same species of microbes in remaining original semen, semen upstream after gradient centrifugation, and the contaminated culture media, which proving that the bacteria may have originated from semen samples. In addition, vaginal symbiotic bacteria can contaminate follicular fluid[3] which is theoretically sterile. The symbiotic microflora of the vagina cannot be completely eliminated by regular vaginal

scrubbing.[24] When the needle passes through the vaginal wall and enters the ovary, it may translocate bacteria from the vagina to the follicular fluid. Haemolytic *Streptococcus, Staphylococcus epidermidis, Mycoplasma hominis, Diphtheroids* and *Lactobacillus* can be cultured from follicular fluid.[24] Finally, embryo contamination may arise from the IVF culture system and environment,[7] including the internal environment of the IVF culture room, laminar flow system, various instruments and equipment, (incubator, culture liquid, culture dish, glass and, plastic tubes), and operators. Cottell et al[25] detected a very small proportion of *S. epidermidis* and *Streptococcus vivalis* in semen upstream (no such bacteria were present in the original semen), suggesting that the bacteria may have originated from laboratory culture and operational processes. Therefore, personnel factor as a potential source of contamination, it's important of non- touch techniques in all IVF laboratories.

These results showed that approximately half of the embryo contamination was caused by *E. coli*, followed by *S. agalactiae. E. coli* was more likely to be found on Day 1. Compared with normal IVF fertilization, *E. coli* contamination increased rate of degradation, reduced the fertilization and cleavage rates to some extent, but after rinsing, it had little effect on the blastocyst formation rate. *E. coli* contamination did not affect the implantation and pregnancy rates; however, it significantly increased the risk of early miscarriages (Tables 3–5). Our results showed that when embryos contaminated with *E. coli* were transferred, early abortion occurred in all patients who obtained clinical pregnancy. We suspect that due to the porous structure of the embryo's zona pellucida, washing alone is not effective in eliminating bacteria, as some still remain in the zona pellucida. Embryos can carry microorganisms to the micro-environment of the uterus, increase the possible risks of uterine and pelvic inflammation, alter the local microbiota and compromise implantation and survival during pregnancy, which ultimately leads to an increased incidence of early miscarriage. However, not all embryos contaminated with bacteria lead to increased miscarriage rates, which may be related to the different ability of bacteria to adhere to the zona pellucida.

Tables 3–5 shows the pregnancy and neonatal outcome of fresh or frozen embryo transfer. According to the findings (Table 3), among the 4 clinical pregnancies, 3 were transferred a blastocyst and 1 was transferred a cleavage embryo. Both two live births were from blastocysts. These findings imply that it is preferable to culturing embryos to blastocysts for ET after rinsing. No live birth can be achieved through the FET cycle (Table 4 and Table 5) by transferring an *E. coli*-contaminated embryo, whether it is a cleaved embryo or a blastocyst. These findings imply that, even if there are no visible bacteria after rinsing, extra caution should be utilized if embryos contaminated with *E. coli* are used for ET.

Consistent with the study by Prabha et al,[26] *E. coli* may impair the fertilization ability of sperm. *S. agalactiae* is not easily found, occurs mostly on Day and Day 5, but has no adverse effects on laboratory and clinical outcomes. The cumulative pregnancy rate was 71.43%. The essential difference between the two bacteria is that *E. coli* is a gram-negative bacillus while *S. agalactiae* is a gram-positive coccus. *E. coli* is known to reduce human sperm motility.[27] Additionally, Mulla et al[28] demonstrated that *E. coli* could also affect the acrosome reaction, which is another important sperm function, thereby reducing its fertilization ability. Endotoxins produced by gram-negative bacteria have a great impact on both fertilization and embryo development in IVF-ET.[29–31] All of the aforementioned factors may be responsible for *E. coli* negatively affecting the outcome of IVF. *S. agalactiae* which is gram-positive does not produce the damaging endotoxin. In addition, there are literature reports that *S. agalactiae* can secrete high levels of extracellular hyaluronidase[32] which can enhance blastocyst formation and reduce inner-cell mass apoptosis.[33] Finally, the results in Table 6 suggest that *E. coli* was easily found on Day 1, whereas *S. agalactiae* was more commonly found on Day 3. We think it might be because contamination was all along and not observed until the number of bacteria multiplied enough to be seen even. This may also explain why *S. agalactiae* has no effect on the embryo fertilization rate.

If embryo contamination is found, the embryo can be repeatedly decontaminated with antibiotics, such as penicillin, streptomycin, and gentamicin sulphate culture medium, followed by washing with pure culture medium.[34] In the next IVF cycle, intracytoplasmic sperm injection (ICSI) or appropriate shortening of fertilization time can be considered to reduce contact between the ovum and contaminated sperm.[35,36] We also found that all embryo contamination occurred during IVF cycles. Whenever the ICSI procedure was applied, no infections were observed, which implies a significantly reduced risk of colonisation in the culture dishes by microorganisms in ICSI culture dishes. This may be due to the isolation of single motile spermatozoa from the PVP solution during the ICSI procedure. Although the sperm samples for both IVF and ICSI were prepared by discontinuous gradient centrifugation, which removes most of the microorganisms

in the final sperm sample, the selection of a single sperm for injection may also reduce the risk of contamination. However, the reduced risk of infections during the ICSI procedure could also be due to the removal of cumulus and corona cells, the use of hyaluronidase or the extensive washing steps during the denudation procedure.[7]

Contaminated surviving embryos were remedied by repeated rapid rinses and transferred with informed consent from the patients. Ten became pregnant, six of whom had live births, two early abortions, one late abortion, and one stillbirth. All six live births and one stillbirth at 39 weeks were male, probably because male embryos may be more resistant to contamination and survive. Regardless of when embryo contamination occurs, there is a chance of pregnancy, thus embryos are not easily discarded.

It is feasible to use this method for the remedial treatment of IVF embryo contamination. However, a study[37] has demonstrated that washing alone is not effective in eliminating bacteria, as some still remain in the zona pellucida. In a recent case report by Li et al, a new method was proposed to rescue contaminated embryos, involving the removal of the contaminated zona pellucida using Tyrode acid. It has been observed that the porous structure of the zona pellucida makes it difficult to completely eliminate microorganisms. Therefore, removing zona pellucida using Tyrode acid is a promising method to solve the contamination problems. It is worth noting that the ZP plays vital roles during oogenesis, fertilization, and preimplantation development, lacking of zona pellucida may result in the collapse of the three-dimensional blastomere structure at cleavage stage.[38] There are published case reports that describe the successful use of laser to rescue contaminated embryos. However, since the embryo is a sphere, it is difficult to remove the complete zona pellucida with laser, so we think this method is difficult to implement.

However, measures should be taken to prevent contamination at various ART stages. If bacteria-contaminated embryos are rinsed and used for transfer, informed consent must be obtained from the patients, and patients must be fully informed of possible risks that embryos may carry microorganisms (as some still remain in the zona pellucida) to the microenvironment of the uterus, alter the local microbiota and compromise implantation and survival during pregnancy, and embryonic epigenetic changes. According to these results, the transfer of contaminated embryos had no effect on the height and weight of new-born, but its effect in the long-term needs a further prospective study with more samples. The different embryonic microbial species associated with different IVF cultures and clinical outcomes in patients undergoing IVF-ET might have profound implications for understanding microbial sources and improving the management of IVF culture systems.

Finally, to avoid the occurrence of embryo contamination, the contamination of semen sources can be reduced by strengthening education and guidance using a written prompt in addition to a verbal explanation which emphasises strict hand washing before sperm extraction; recommends drinking more water and urinating before sperm extraction; disinfecting the sperm collection room regularly; and not touching the inner wall of the semen collection cup when extracting sperm.

Strict vaginal irrigation before operation and the correct use of the negative pressure aspirator during operation can effectively prevent contamination of the follicular fluid source.

Contamination from the culture system can be avoided using strict aseptic procedures, proper storage of culture media, and the use of disposable tools.

This study summarized the types of embryo contamination and the outcome of ART for the first time, and proposed feasible and effective rescue methods. We found that E. coli and S. agalactiae were the most common bacterial embryo contaminants. Regardless of the etiology and time of infection, pregnancy is possible after effective remedial treatment. The limitation is that the sample size was small and came from a single center.

Data Sharing Statement

All the data that support the findings of this study have been presented in the manuscript.

Ethics Approval and Consent to Participate

This study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of the Third Affiliated Hospital of Guangzhou Medical University [Ethic no. (2022) 026]. Each patient has signed informed consent on obtaining and analyzing their clinical data prior to the initiation of IVF/ICSI-ET treatment. The datasets

generated and/or analysed during the current study are available from the corresponding author upon reasonable request. Please send your request for original data to the e-mail address of Professor Lei Li at lileigo@foxmail.com. No data other than those contained in the manuscript will be shared.

Consent for Publication

The author confirms that the study described has not been published before, that its publication has been approved by all co-authors and that its publication has been approved (tacitly or explicitly) by the responsible authorities at the institution where the study was carried out.

Acknowledgments

The study was performed under the auspices of the in vitro fertilization (IVF) unit of the Third Affiliated Hospital of Guangzhou Medical University.

Authors' Contributions

All authors made a significant contribution to the work reported. YLM and LL designed the study. LT, HYH, CYS and YQS made a contribution to acquisition of data. YMC was mainly responsible for the analysis and interpretation. YLM and YXH wrote the manuscript. LL and NNT revised the manuscript. All authors reviewed and agreed on all versions of the article. All authors have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This study was supported by the Funding by Science and Technology Projects in Guangzhou (2023A04J0579 to YLM), the Guangzhou Medical University Student Innovation Ability Enhancement plan (02-408-2203-2060 to YLM), Plan on enhancing scientific research in GMU Plan on enhancing scientific research in GMU (2024SRP114 to YLM).

Disclosure

All authors have declared no conflicts of interest in this work.

References

1. Cottell E, McMorrow J, Lennon B, Fawsy M, Cafferkey M, Harrison RF. Microbial contamination in an in vitro fertilization-embryo transfer system. *Fertil Sterility*. 1996;66(5):776–780.
2. Domes T, Lo KC, Grober ED, Mullen JBM, Mazzulli T, Jarvi K. The incidence and effect of bacteriospermia and elevated seminal leukocytes on semen parameters. *Fertil Sterility*. 2012;97(5):1050–1055.
3. Pelzer ES, Allan JA, Waterhouse MA, Ross T, Beagley KW, Knox CL. Microorganisms within human follicular fluid: effects on IVF. *PLoS One*. 2013;8(3):e59062
4. Pelzer ES, Allan JA, Cunningham K, et al. Microbial colonization of follicular fluid: alterations in cytokine expression and adverse assisted reproduction technology outcomes. *Hum Reprod*. 2011;26(7):1799–1812.
5. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Sterility*. 1985;44(4):493–498.
6. Kyono K, Hashimoto T, Kikuchi S, Nagai Y, Sakuraba Y. A pilot study and case reports on endometrial microbiota and pregnancy outcome: an analysis using 16S rRNA gene sequencing among IVF patients, and trial therapeutic intervention for dysbiotic endometrium. *Reprod Med Biol*. 2019;18(1):72–82.
7. Kastrop PM, de Graaf-Miltenburg LA, Gutknecht DR, Weima SM. Microbial contamination of embryo cultures in an ART laboratory: sources and management. *Hum Reprod*. 2007;22(8):2243–2248.
8. Borges ED, Berteli TS, Reis TF, Silva AS, Vireque AA. Microbial contamination in assisted reproductive technology: source, prevalence, and cost. *J Assist Reprod Genet*. 2020;37(1):53–61.
9. Castaneda CD, McDaniel CD, Abdelhamed H, Karsi A, Kiess AS. Evaluating bacterial colonization of a developing broiler embryo after in ovo injection with a bioluminescent bacteria. *Poult Sci*. 2019;98(7):2997–3006.
10. Ben-Chetrit A, Shen O, Haran E, Brooks B, Geva-Eldar T, Margalioth EJ. Transfer of embryos from yeast-colonized dishes. *Fertil Sterility*. 1996;66(2):335–337.
11. Nygren KG, Andersen AN. Assisted reproductive technology in Europe, 1999. Results generated from European registers by ESHRE. *Hum Reprod*. 2002;17(12):3260–3274.
12. Majumdar G, Majumdar A, Verma IC, Upadhyaya KC. Relationship between morphology, euploidy and implantation potential of cleavage and blastocyst stage embryos. *J Human Reprod Sci*. 2017;10(1):49–57.

13. He Y, Chen S, Liu J, Kang X, Liu H. Effect of blastocyst morphology and developmental speed on transfer strategy for grade ”C” blastocyst in vitrified-warmed cycles. *Jovarian Res*. 2021;14(1):51.
14. Mao Y, Tang N, Luo Y, Yin P, Li L. Effects of vitrified cryopreservation duration on IVF and neonatal outcomes. *Jovarian Res*. 2022;15(1):101.
15. Vaduva CC, Cilica RM, Nastase A, Carp-Veliscu A, Doroftei B. A successful case of assisted reproduction after contamination of the culture medium with Escherichia coli. *Eur Rev Med Pharmacol Sci*. 2022;26(16):5932–5938.
16. Wang J, Wang H, Cai K, et al. Evaluation of three sample preparation methods for the identification of clinical strains by using two MALDI-TOF MS systems. *J Mass Spectrom*. 2021;56(2):e4696.
17. Awonuga A, Nabi A, Govindbhai J, Birch H, Stewart B. Contamination of embryo transfer catheter and treatment outcome in in vitro fertilization. *J Assist Reprod Genet*. 1998;15(4):198–201.
18. Moreno I, Simon C. Deciphering the effect of reproductive tract microbiota on human reproduction. *Reprod Med Biol*. 2019;18(1):40–50.
19. Forman R, Guillett-Rosso F, Fari A, Volante M, Frydman R, Testart J. Importance of semen preparation in avoidance of reduced in vitro fertilization results attributable to bacteria. *Fertil Sterility*. 1987;47(3):527–530.
20. Krissi H, Orvieto R, Ashkenazi J, et al. Effect of contaminated preprocessed semen on fertilization rate and embryo quality in assisted reproductive techniques. *Gynecolog Endocrinol*. 2004;18(2):63–67.
21. Kim IH, Son DS, Lee HJ, et al. Bacteria in semen used for IVF affect embryo viability but can be removed by stripping cumulus cells by vortexing. *Theriogenology*. 1998;50(2):293–300.
22. Wolff H, Panhans A, Stolz W, Meurer M. Adherence of Escherichia coli to sperm: a mannose mediated phenomenon leading to agglutination of sperm and E. coli. *Fertil Sterility*. 1993;60(1):154–158.
23. Hewitt J, Cohen J, Fehilly CB, et al. Seminal bacterial pathogens and in vitro fertilization. *J in vitro Fert Embryo Transf*. 1985;2(2):105–107.
24. Bjurstrom L. Aerobic bacteria occurring in the vagina of bitches with reproductive disorders. *Acta Vet Scand*. 1993;34(1):29–34.
25. Cottell A, Denyer SP, Hanlon GW, Ochs D, Maillard JY. Triclosan-tolerant bacteria: changes in susceptibility to antibiotics. *J Hosp Infect*. 2009;72 (1):71–76.
26. Prabha V, Sandhu R, Kaur S, et al. Mechanism of sperm immobilization by Escherichia coli. *Adv Urol*. 2010;2010:240268.
27. Diemer T, Weidner W, Michelmann HW, Schiefer HG, Rovan E, Mayer F. Influence of Escherichia coli on motility parameters of human spermatozoa in vitro. *Int J Androl*. 1996;19(5):271–277.
28. el-Mulla KF, Kohn FM, Dandal M, et al. In vitro effect of Escherichia coli on human sperm acrosome reaction. *Arch Androl*. 1996;37(2):73–78.
29. Fishel S, Jackson P, Webster J, Faratian B. Endotoxins in culture medium for human in vitro fertilization. *Fertil Sterility*. 1988;49(1):108–111.
30. Nagata Y, Shirakawa K. Setting standards for the levels of endotoxin in the embryo culture media of human in vitro fertilization and embryo transfer. *Fertil Sterility*. 1996;65(3):614–619.
31. Kamiyama S, Teruya Y, Nohara M, Kanazawa K. Impact of detection of bacterial endotoxin in menstrual effluent on the pregnancy rate in in vitro fertilization and embryo transfer. *Fertil Sterility*. 2004;82(4):788–792.
32. Parida R, Samanta L. In silico analysis of candidate proteins sharing homology with Streptococcus agalactiae proteins and their role in male infertility. *System Biol Reprod Med*. 2017;63(1):15–28.
33. Marei WFA, Raheem KA, Salavati M, Tremaine T, Khalid M, Fouladi-Nashta AA. Hyaluronan and hyaluronidase, which is better for embryo development? *Theriogenology*. 2016;86(4):940–948.
34. Selman H, Mariani M, Barnocchi N, et al. Examination of bacterial contamination at the time of embryo transfer, and its impact on the IVF/ pregnancy outcome. *J Assist Reprod Genet*. 2007;24(9):395–399.
35. Seidman DS, Madjar I, Levron J, Levran D, Mashiach S, Dor J. Testicular sperm aspiration and intracytoplasmic sperm injection for persistent infection of the ejaculate. *Fertil Sterility*. 1999;71(3):564–566.
36. Park KS, Song HB, Chun SS. Late fertilization of unfertilized human oocytes in in vitro fertilization and intracytoplasmic sperm injection cycles: conventional insemination versus ICSI. *J Assist Reprod Genet*. 2000;17(8):419–424.
37. Shu YM, Prokai D, Berga S, Taylor R, Johnston-MacAnanny E. Transfer of IVF-contaminated blastocysts with removal of the zona pellucida resulted in live births. *J Assist Reprod Gen*. 2016;33(10):1385–1388.
38. Fan WH, Homma M, Xu RL, et al. The use of a two-step removal protocol and optimized culture conditions improve development and quality of zona free mouse embryos. *Biochem Biophys Res Commun*. 2021;577:116–123.