

Detection of SARS-CoV-2 in follicular fluid, cumulus cells, spermatozoa, and seminal plasma of COVID-19 patients undergoing IVF using nested RT-PCR: a prospective, observational study

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Abstract

Purpose

What is the risk of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) contamination in reproductive specimens from patients infected during controlled ovarian stimulation?

Methods

This prospective, observational, single-center study enrolled 354 cycles between December 2022 and May 2023, in which at least one partner was infected with SARS-CoV-2. Nested RT-PCR was performed to detect viral RNA in reproductive specimens. Embryo quality, pregnancy outcomes, perinatal outcomes, and 1-year infant growth were compared between groups stratified according to viral detection status in reproductive specimens.

Results

Among women with positive oropharyngeal swabs, 12.7% tested positive for SARS-CoV-2 in follicular fluid/cumulus cells, whereas no virus was detected in women with negative swabs. The positivity rate in spermatozoa/seminal plasma was 8.3% in men with positive swabs and 4.3% in those with negative swabs. Viral positivity was detected in immature oocytes (5.9%) and arrested embryos (3.1%) from couples positive in follicular fluid/cumulus cells. A positivity rate of 1.1% was observed in arrested embryos from couples with positive spermatozoa/seminal plasma. Viral presence in reproductive specimens had no significant adverse effects on oocyte maturation, normal fertilization, embryo quality, clinical pregnancy, miscarriage, live birth, neonatal outcomes or 1-year infant growth.

Conclusions

Continuing IVF treatment for asymptomatic or mildly symptomatic COVID-19 patients during ovarian stimulation is relatively safe. However, the low but non-negligible risk of viral vertical transmission should be discussed with couples.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) first emerged in 2019 and rapidly evolved into a global public health emergency. Although the World Health Organization declared the end of the global emergency on May 5, 2023, coronavirus disease 2019 (COVID-19) continues to spread worldwide [1]. While the respiratory system is the primary target of SARS-CoV-2, growing evidence indicates that the virus can infect multiple organs [2, 3].

SARS-CoV-2 employs angiotensin-converting enzyme 2 (ACE2) receptor and transmembrane serine protease 2 (TMPRSS2) for viral attachment to and entry into host cells. Co-expression of ACE2 and TMPRSS2 has been documented in granulosa cells, cumulus cells (CC), Sertoli cells, germ cells, and preimplantation embryos [4–7], implying that the reproductive system may be susceptible to viral infection. SARS-CoV-2 RNA has been detected in semen during the acute phase of infection [8]. However, most studies have failed to detect viral RNA in ovarian follicles, including follicular fluid (FF), CC, and oocytes [9–11]. Only one study reported the detection of virus in a patient's immature oocytes [12]. Nearly all prior studies used quantitative reverse transcription polymerase chain reaction (RT–qPCR) for virus detection in samples. Currently regarded as the gold-standard assay for SARS-CoV-2 diagnosis [13, 14], RT–qPCR offers high sensitivity, typically detecting 200 – 1000 copies/mL. Nevertheless, this sensitivity may be inadequate to identify extremely low or trace viral loads. Thus, for reproductive samples from asymptomatic or mildly symptomatic patients, a more sensitive assay such as nested RT-PCR may be required to detect minimal viral nucleic acid.

Since December 2022, China has implemented nationwide relaxation of COVID-19 pandemic control policies. Consequently, in vitro fertilization (IVF) centers have encountered an increasing number of infected patients. However, it remains unclear whether trace virus exists in reproductive tissues. This study used nested RT-PCR to examine viral RNA in FF, CC, semen, immature oocytes, and arrested embryos from couples testing positive for SARS-CoV-2 during controlled ovarian stimulation (COS). We further evaluated whether viral presence in these specimens affected embryonic development, pregnancy and perinatal outcomes, as well as growth parameters of infants within 1 year after birth.

Materials and methods

Study design and population

Between December 19, 2022, and May 25, 2023, all couples underwent nasopharyngeal swab testing for SARS-CoV-2 within two days before oocyte retrieval. Couples with COVID-19 symptoms or known high-risk exposure during COS also received screening. If one partner tested positive but was asymptomatic or mildly symptomatic, the couple received counseling regarding the potential impact of infection on IVF outcomes and the unknown risk of vertical transmission. If couples chose to continue treatment, they could select: (1) Oocyte insemination with fresh embryo transfer; (2) Oocyte insemination with total embryo cryopreservation; (3) Oocyte vitrification. Couples were invited to donate FF, CC, immature oocytes, semen, and arrested embryos for viral detection. Semen samples were exempted from testing if using autologous or donor sperm cryopreserved before policy relaxation. For couples undergoing oocyte cryopreservation, semen samples were not collected during oocyte retrieval. Before oocyte thawing, semen samples were tested, and thawing was performed only if results were negative.

This prospective, observational, single-center study enrolled all couples with at least one partner infected with COVID-19. Patients were excluded if they: (1) Declined sample donation; (2) Underwent a second oocyte retrieval during the study; (3) Had no oocytes retrieved; (4) Had no mature oocytes; (5) Had

samples with failed viral detection. This study was approved by the Ethics Committee of the Reproductive & Genetic Hospital of CITIC-Xiangya (LL-SC-2023-014).

IVF procedures

Ovarian stimulation was performed according to a previously published protocol [15]. When two-thirds of follicles reached 18 mm, 5,000–10,000 IU hCG (Pregnyl, Merck, Darmstadt, Germany) was administered. Oocyte retrieval was performed 34–36 hours later under transvaginal ultrasound guidance. Retrieved oocytes were either vitrified (Kitazato vitrification kit, Cryotop open straws) or inseminated by IVF or intracytoplasmic sperm injection (ICSI) based on clinical indications. Normal fertilization was confirmed by two pronuclei (2PN) 16–18 hours after insemination. Embryos were cultured in a tri-gas incubator (37°C, 6% CO₂, 5% O₂, 89% N₂) using sequential media (Vitrolife, Gothenburg, Sweden). On day 3, embryos were evaluated using Puissant's morphological criteria [16], and then transferred, cryopreserved, or cultured to the blastocyst stage based on clinical indications and patient willingness. Blastocysts were graded using Gardner and Lane criteria [17]. Those graded $\geq$ 4BC/4CB on day 5 were eligible for transfer, surplus blastocysts on days 5–7 were vitrified. For preimplantation genetic testing (PGT) cycles, a 25- μ m laser-assisted zona pellucida (ZP) opening was created on day 5 morning to facilitate trophectoderm herniation. Blastocyst biopsy was performed on days 5–7 for embryos reaching $\geq$ 4BC/4CB, as previously described [18].

Sample collection

Clear, blood-free FF was collected after oocyte retrieval, centrifuged at 1710 $\times$ g for 5 minutes, and 1 mL supernatant was transferred to an RNase-free tube. CC were collected in ICSI and oocyte vitrification cycles. Oocyte-cumulus complexes were denuded with hyaluronidase, and CC were transferred to RNase-free tube, centrifuged at 1710 $\times$ g for 5 minutes, and supernatant was removed. Semen samples were collected by masturbation or surgical sperm retrieval on the day of oocyte retrieval. For ejaculated semen, 0.5 mL was collected after liquefaction. For testicular or epididymal sperm, residual samples post-ICSI were saved. Semen was centrifuged at 3,000 $\times$ g for 15 minutes to separate spermatozoa and seminal plasma (SP). FF, CC, spermatozoa, and SP samples were stored at – 80°C. Immature oocytes were collected after ICSI or oocyte vitrification and cryopreserved in liquid nitrogen. For viral detection, thawed immature oocytes underwent ZP removal using Tyrode's acid and were transferred individually or in groups into RNase-free tubes. Arrested embryos were denuded of their ZP and tested immediately (individually or pooled) or cryopreserved for later analysis.

SARS-CoV-2 testing of oropharyngeal swabs

Total RNA was extracted from 200 μ L of oropharyngeal swab samples using a magnetic bead-based Viral RNA Extraction Kit (EasyDiagnosis, Wuhan, China) with an automatic nucleic acid extraction system (Tianlong, Suzhou, China). RNA was eluted in 60 μ L buffer and immediately tested by real-time RT-PCR

using a SARS-CoV-2 detection kit (Mingde, China). A 5 µL of template RNA was thoroughly mixed with the reaction premix to a final volume of 25 µL. Amplification was performed on a real-time fluorescent quantitative PCR instrument with the following program: reverse transcription at 50°C for 15 minutes; pre-denaturation at 95°C for 5 minutes; followed by 45 cycles of denaturation at 95°C for 3 seconds and annealing/extension at 60°C for 40 seconds (fluorescent signals were collected during this stage). Each detection batch included positive, negative, and internal standard controls. If the internal standard curve was valid and the fluorescent signals of SARS-CoV-2-specific targets (typically ORF1ab and N genes) reached the preset threshold (Ct value ≤ 40), the sample was deemed positive. If no amplification curve was observed for the targets (Ct value > 40 or undetectable Ct value), the sample was classified as negative.

SARS-CoV-2 testing of reproductive samples

For FF and SP, RNA was extracted from 200 µL specimen using a Viral RNA Extraction Kit (EasyDiagnosis, Wuhan, China) with an automatic nucleic acid extraction system (Tianlong, Suzhou, China). The extracted nucleic acids were eluted into a single EP tube with 60 µL of elution buffer. Standardized nucleic acid extraction was combined with 3M sodium acetate treatment, concentrating 60 µL of RNA to a final volume of 10 µL. For CC, spermatozoa, immature oocytes, and arrested embryos, RNA was extracted using the TRIzol method. The extracted nucleic acids were eluted into a single EP tube with 10 µL of elution buffer and stored at – 80°C.

Nested RT-PCR assay was performed in a 25 µL reaction volume using PCR solution (EasyDiagnosis). The reaction mixture comprised the following components: 5 µL of RNA template, 0.1 µL of LNA-outer ORF1ab forward primer 2 (10 µM), 0.1 µL of LNA-outer ORF1ab reverse primer 1 (10 µM), 0.1 µL of LNA-outer N forward primer 2 (10 µM), 0.1 µL of LNA-outer N reverse primer 1 (10 µM), 0.1 µL of TaKaRa Ex Taq® Hot Start Version, and 19.5 µL of PCR mixture. Amplification was performed on a PCR instrument (Tianlong, Suzhou, China) with the following program: 50°C for 15 minutes, 95°C for 30 seconds, 10 cycles of 95°C for 5 seconds, 64°C for 20 seconds, and 72°C for 40 seconds; 40 cycles of 95°C for 5 seconds, 60°C for 20 seconds, and 72°C for 40 seconds; with simultaneous fluorescent signal collection. The FAM channel was used for N gene detection, the HEX channel for ORF1ab gene detection, and the ROX channel for reference gene detection. Each run included positive and negative controls. Samples were considered positive if the Ct values of both target genes (ORF1ab and N) were ≤ 28, and negative if both Ct values were > 29. If only one target gene had a Ct value ≤ 28 while the other exceeded 28 (i.e., Ct values falling within the gray zone of 28–29, an ambiguous result), retesting of the relevant gene was required. If the repeat test confirmed positivity for only one target gene, the sample was interpreted as single-gene positive; otherwise, it was classified as negative.

Outcome measures

For IVF laboratory indicators, oocyte maturation rate was determined by dividing the number of metaphase II (MII) oocytes by the total number of oocytes retrieved in ICSI or oocyte vitrification cycles.

Normal fertilization rate was calculated by dividing the number of 2PN zygotes by the number of MII oocytes in ICSI cases or by the total number of oocytes in IVF cases. Cleavage rate was calculated by dividing the number of 2PN cleavage embryos on day 3 by the number of 2PN zygotes. Day 3 high-quality embryo rate was calculated by dividing the number of day 3 2PN high-quality embryos (defined as having ≥ 6 evenly sized blastomeres, $< 20\%$ fragmentation) by the number of 2PN cleavage embryos. Blastocyst formation rate was determined by dividing the number of blastocysts by the number of day 3 embryos available for blastocyst culture. High-quality blastocyst formation rate was calculated by dividing the number of high-quality blastocysts (defined as having at least grade 4 expansion and grade A or B for both the inner cell mass and trophectoderm) by the number of day 3 embryos available for blastocyst culture.

Pregnancy and neonatal outcomes were defined according to international guidelines [19]. Monozygotic twin pregnancies were characterized as one sac. Clinical pregnancy rate was calculated by dividing the number of clinical pregnancies by the number of embryo transfer cycles. Miscarriage was defined as a nonviable intrauterine pregnancy occurring before 28 weeks of gestation. Miscarriage rate was calculated by dividing the number of miscarriages by the number of clinical pregnancies. Live birth rate was calculated by dividing the number of live birth cycles by the number of embryo transfer cycles. Furthermore, we tracked the developmental milestones of children, including the onset of babbling, crawling, tooth eruption, as well as their height and weight at 1 year of age.

Statistical analysis

Continuous variables were expressed as the mean $\pm$ standard deviation (SD), and categorical variables were presented as frequencies (percentages). The normality of continuous variables was assessed using the Kolmogorov–Smirnov test. Between-group comparisons were performed using the two-sample t-test, Kruskal–Wallis test, or Pearson χ^2 test, as appropriate for variable type. Multivariable linear regression was used to adjust for female age when comparing laboratory parameters. Multivariate logistic regression was applied to control for confounding factors in the analysis of pregnancy outcomes. For perinatal outcomes and 1-year infant growth parameters, multivariable logistic regression or multivariable linear regression was used to adjust for female age. All statistical analyses were performed using R software (version 4.5.1). A *P*-value < 0.05 was considered statistically significant.

Results

During the study period, 399 cycles had at least one partner infected during COS. After excluding cycles that did not meet the inclusion criteria, 354 cycles were included. Among 244 women with positive oropharyngeal swab results, 31 (12.7%) tested positive in FF/CC; 110 women with negative swabs had no viral detection in FF/CC. Among 173 men with positive swabs, 133 underwent spermatozoa/SP testing, yielding a positivity rate of 8.3%. Among 181 men with negative swabs, 117 underwent spermatozoa/SP testing, with a positivity rate of 4.3% (Table 1). There were no cycles where both FF/CC and spermatozoa/SP tested positive.

A total of 17 immature oocytes and 64 arrested embryos were contributed by 18 couples with FF/CC positivity, yielding positivity rates of 5.9% and 3.1%, respectively. Ninety arrested embryos were obtained from 16 couples with spermatozoa/SP positivity, with a positivity rate of 1.1%. In 39 couples negative for viral detection in both FF/CC and spermatozoa/SP, 31 immature oocytes and 169 arrested embryos were collected, and no virus was detected in any of these specimens (Table 2 and Supplementary Table 1).

Baseline characteristics were compared among the FF/CC-positive group, the spermatozoa/SP-positive group, and the group with no positive samples (Table 3). No significant intergroup differences were observed in age, BMI, infertility duration, infertility type, cycle rank, ovarian stimulation protocol, treatment regimen, or sperm source. Of the 31 cycles positive for FF/CC, 15 were fresh oocyte insemination cycles and 16 were oocyte vitrification cycles. Oocyte thawing was performed in 6 of these 16 vitrification cycles; one cycle was excluded from subsequent analysis due to the combination of thawed and fresh oocytes. As semen viral testing was not performed in vitrification cycles, all 16 spermatozoa/SP-positive cycles were from fresh oocyte insemination cycles. Of the 307 cycles negative for both FF/CC and spermatozoa/SP, 236 were fresh oocyte insemination cycles and 71 were oocyte vitrification cycles. Subsequent oocyte thawing was performed in 57 of these 71 vitrification cycles, with thawed and fresh oocytes combined in 15 cycles (Fig. 1).

Laboratory outcomes are presented in Table 4. In both fresh oocyte insemination and oocyte thawing cycles, no significant differences were observed in oocyte maturation rate, normal fertilization rate, cleavage rate, day 3 high-quality embryo rate, blastocyst formation rate, or high-quality blastocyst formation rate among the FF/CC-positive group, the spermatozoa/SP-positive group, and the group with no positive samples. Clinical pregnancy, miscarriage, and live birth rates were similar among groups (Table 5). In the FF/CC-positive group, one patient with viral detection in immature oocytes achieved a healthy live birth. Among two patients with viral identification in arrested embryos, one delivered a healthy live birth while the other did not conceive. In the spermatozoa/SP-positive group, one patient with viral detection in arrested embryos failed to conceive.

With respect to neonatal outcomes, including gestational age, sex ratio, birth weight, and rates of preterm birth, low birth weight, and birth defects, no significant differences were observed among groups in both fresh oocyte insemination and oocyte thawing cycles. The onset age of babbling, crawling, and tooth eruption, as well as weight and height at 1 year of age, were comparable among groups (Supplementary Table S2).

Discussion

Previous studies on the detection of SARS-CoV-2 infection in reproductive samples have predominantly employed the RT-PCR method. This study utilizes a more sensitive nested RT-PCR approach, enabling the detection of trace amounts of viral RNA in the samples. Corona *et al.* conducted a summary of studies that explored the presence of SARS-CoV-2 mRNA in semen, revealing that the overall positivity rate was 8% [8]. In our study, the viral detection rate in the semen of male SARS-CoV-2 infected patients

was 8.3%. Our detection rate of the virus in semen is consistent with that of previous studies, suggesting a relatively high viral load in the semen of infected individuals, which exceeds the lower limit of detection (LOD) of the RT-PCR assay employed in prior researches. To date, viral RNA has remained undetectable in FF, granulosa cells, and CC. For the first time, we identified the presence of viral RNA in FF and CC from infected women. The virus detection rate in FF exceeds 10%, while that in CC is around 4%. These findings suggest a low viral load in ovarian samples, which falls below the LOD of the RT-PCR assay. The primary route of viral invasion into gonadal tissues is hematogenous dissemination. The testis exhibits a higher degree of vascularization and greater blood flow compared with the ovary [20]. Moreover, the expression level of ACE2, which is critical for SARS-CoV-2 entry, is significantly higher in the testis than in the ovary [21]. Therefore, viral abundance in testicular tissues from infected males should be higher than that in ovarian tissues from infected females. Differences in viral abundance within gonadal tissues between male and female infected individuals may underlie the divergent impacts of viral infection on testicular and ovarian function. SARS-CoV-2 infection can adversely affect spermatogenesis and testosterone levels, whereas most studies have not reported a significant impact of COVID-19 on ovarian reserve and ovarian function [22, 23].

In the present study, viral RNA was not detected in FF or CC from women with negative oropharyngeal swabs during COS. A viral detection rate of 4.3% was observed in semen samples from men with negative swabs, suggesting that these men had been infected with COVID-19 prior to the COS cycles. Previous studies have found that viral detection in semen is rare among males who have recovered from COVID-19 [24]. To date, the virus has been found in the semen of only one recovered patient [25]. Using a relatively sensitive detection method, we identified viral presence in the semen of five males during the non-acute infection phase, suggesting that a small proportion of males may still harbor low levels of virus in semen after recovery from infection. However, due to the lack of data on the participants' prior infection status and timing of infection, we were unable to provide further information regarding the risk of viral vertical transmission in males after recovery.

ACE2 and TMPRSS2 are co-expressed in human oocytes and preimplantation embryos [5]. Few studies have investigated the presence of SARS-CoV-2 in oocytes and embryos. In one study, no viral RNA was detected in 16 oocytes obtained from two asymptomatic SARS-CoV-2-positive women [9]. Another study identified viral RNA in immature oocytes from one out of 16 COVID-19-positive individuals [12]. An in vitro investigation demonstrated that infectious SARS-CoV-2 is capable of infecting human preimplantation embryos [26]. However, one study analyzed discarded blastocysts from three COVID-19 patients, all of which tested negative for SARS-CoV-2 RNA [12]. In the present study, no virus was detected in immature oocytes or arrested embryos from couples with negative test results in both FF/CC and semen. In couples with positive viral detection in either FF/CC or semen, the virus was identified in arrested embryos for the first time; however, the positive detection rate is low. These findings suggest that for asymptomatic or mildly symptomatic COVID-19 patients undergoing IVF treatment, the risk of vertical viral transmission via gametes is negligible when FF/CC and semen are virus-negative, and remains low even if these samples are positive. This conclusion warrants further validation through additional studies.

Although the probability of direct SARS-CoV-2 infection in oocytes and embryos remains low, virus in FF/CC and semen may increase oxidative stress and inflammation in the gamete microenvironment, potentially impairing embryonic development [27, 28]. Numerous studies have investigated the impacts of SARS-CoV-2 infection on embryo quality. Existing evidence demonstrates no significant discrepancies in IVF outcomes between couples with prior COVID-19 recovery and uninfected control populations [29, 30]. Nevertheless, inconsistent findings have been reported regarding couples infected by SARS-CoV-2 during COS. Several studies have showed that infection in either male or female partner throughout ovarian stimulation does not significantly compromise IVF outcomes [31, 32]. In contrast, other investigations have indicated that infection in both partners is negatively correlated with embryo quality [33, 34]. We found no adverse effect of viral positivity in FF/CC on embryo quality, regardless of whether patients received fresh oocyte insemination or oocyte vitrification protocols. Similarly, the presence of viral in seminal specimens also showed no obvious correlation with compromised embryo quality. Our data support that SARS-CoV-2 does not appear to damage gametes either via direct infection of gametes or disruption of their local microenvironment. However, it cannot be ruled out that the virus may affect gamete development by interfering with systemic physiological homeostasis, such as hormonal secretion and immune regulation [35, 36].

One study indicated that COVID-19 infection in either female or male partners during COS does not affect the clinical pregnancy rate, early miscarriage rate, or live birth rate [34]. In contrast, another study reported that COVID-19 infection in females prior to oocyte retrieval significantly reduced the clinical pregnancy rate and live birth rate [37]. None of these studies performed virological detection of SARS-CoV-2 in reproductive specimens. This study is the first to focus on the association between viral presence in FF/CC or semen and clinical outcomes, showing no impairment of pregnancy or live birth outcomes. Our data support the relative safety of continuing IVF cycles for patients infected during COS. However, since virus was detected in embryos from patients with positive viral testing in FF/CC or semen, the risk of vertical transmission should be discussed with couples. A more prudent strategy is to perform embryo biopsy and proceed with transfer only when the biopsied cells test negative.

The strengths of this study include the prospective consecutive enrollment of patients with COVID-19 infection, systematic sample collection, and the adoption of viral detection methods with higher sensitivity than those utilized in previous studies. We followed perinatal outcomes and monitored infant growth through 1 year of age. Several limitations of the present study should be acknowledged. First, semen samples were not tested in patients undergoing oocyte vitrification or using previously cryopreserved sperm, which may bias the true prevalence of viral detection in semen. Second, although the overall sample size included in this study was relatively large, the positive rate for SARS-CoV-2 was low. Consequently, the sample size of the positive groups was small for comparisons of clinical outcomes; notably, the number of positive cases was further reduced in neonatal analyses, thereby limiting the statistical power. Third, only patients infected during COS who were asymptomatic or had mild COVID-19 manifestations were enrolled. Therefore, the current findings cannot be directly generalized to individuals with resolved SARS-CoV-2 infection or those experiencing moderate/severe symptoms. Fourth, given that the Omicron variant was the predominant circulating strain in China during

the study period [38], these results may not be extrapolated to other SARS-CoV-2 variants or populations of different ethnicities.

In summary, we report for the first time the detection of SARS-CoV-2 RNA in FF and CC from infected women. Low positivity rates in oocytes and embryos indicate a potential but low risk of vertical transmission. Viral detection in FF/CC and semen did not negatively affect embryo quality, perinatal outcomes, or 1-year infant growth. Further studies are warranted to evaluate long-term offspring safety.

Declarations

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Data Availability All data are available from the corresponding author upon reasonable request.

The authors declare no conflicts of interest.

Ethics approval and consent to participate This study was approved by the Ethics Committee of the Reproductive & Genetic Hospital of CITIC-Xiangya (LL-SC-2023-014). Written informed consent was obtained from all patients enrolled in the study.

Consent for publication All authors have consented to the publication of this manuscript.

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Tables

Tables are available in the Supplementary Files section.

Figures

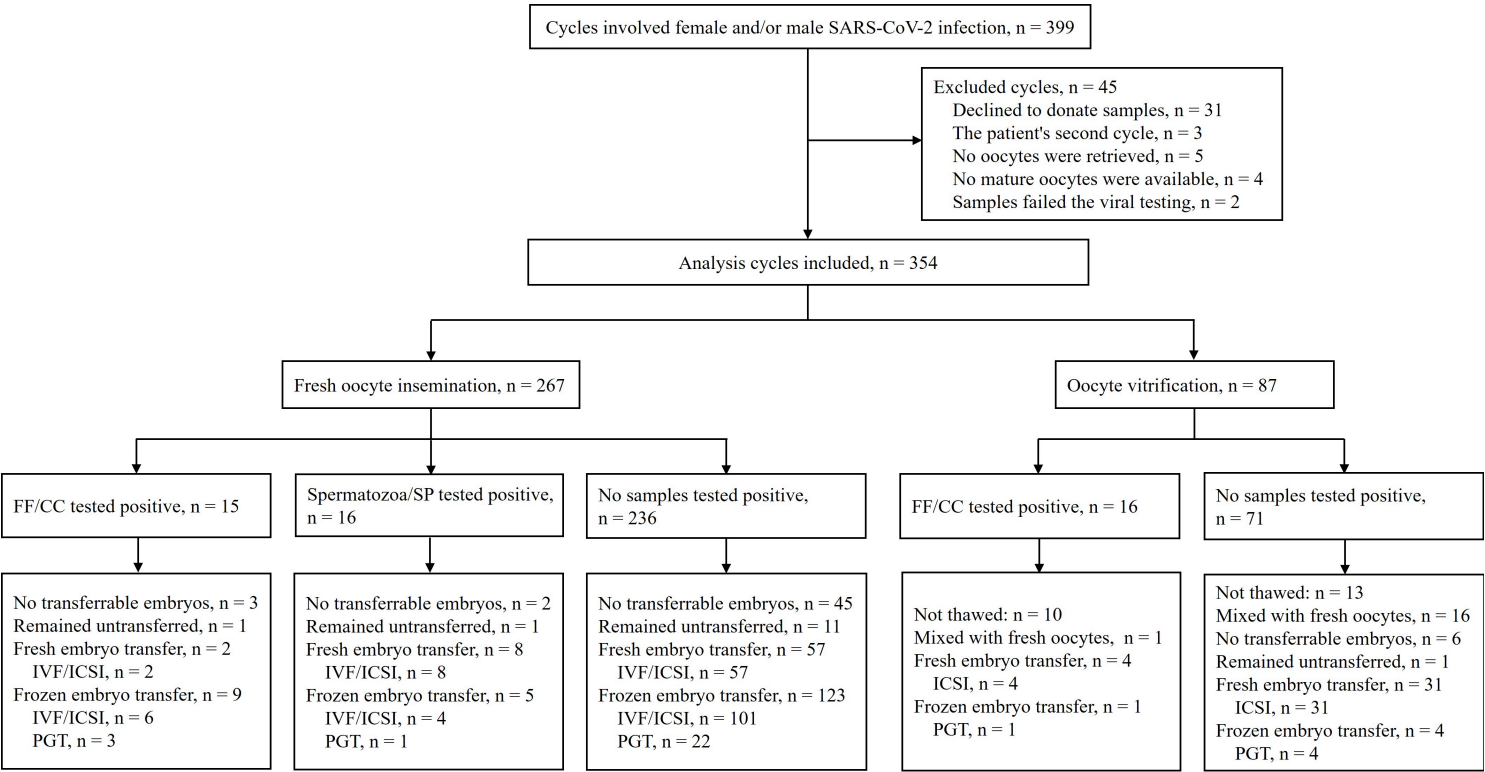


Figure 1

Study flowchart. FF, follicular fluid; CC, cumulus cells; SP, seminal plasma

Supplementary Files

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