

Undetectable viral RNA in oocytes from SARS-CoV-2 positive women

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ABSTRACT: A central concern for the safe provision of ART during the current coronavirus disease 2019 (COVID-19) pandemic is the possibility of vertical transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection through gametes and preimplantation embryos. Unfortunately, data on SARS-CoV-2 viral presence in oocytes of infected individuals are not available to date. We describe the case of two women who underwent controlled ovarian stimulation and tested positive to SARS-CoV-2 infection by PCR on the day of oocyte collection. The viral RNA for gene N was undetectable in all the oocytes analyzed from the two women.

Key words: coronavirus / COVID-19 / oocyte / SARS-CoV-2 / IVF / ICSI / ovarian stimulation

Introduction

In the months since December 2019, when the new coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in our species, it has become apparent that the virus can affect several tissues and organs. A central concern for the safe provision of ART treatments during the current coronavirus disease 2019 (COVID-19) pandemic is the possibility of vertical transmission of SARS-CoV-2 infection through gametes and preimplantation embryos. Despite the large body of literature gathered so far, the effects of SARS-CoV-2 infection on reproductive function are still mostly unknown. Specifically, it is not clear whether the virus can infect human gametes, and whether the use of oocytes from women harboring the virus can result in an infection of the developing embryo. The ability of SARS-CoV-2 to affect a tissue is determined by its capacity to infect cells and replicate, which requires expression of the SARS-CoV-2 receptors angiotensin-converting enzyme 2 (ACE2) and Basigin (BSG), and the proteases transmembrane protease serine 2 (TMPRSS2) and cathepsin L (CTSL) (Hoffmann *et al.*, 2020; Wang *et al.*, 2020). The mRNAs of these genes are expressed in most of the human female reproductive tract, whole ovary (Hikmet *et al.*, 2020) including cumulus cells (Stanley *et al.*, 2020), endometrium (Henarejos-Castillo *et al.*, 2020) and during the early developmental stages of the human embryo (Weatherbee *et al.*, 2020). Importantly, protein expression in these tissues has not been confirmed.

A further limiting factor of all these analyses is their reliance on samples from healthy women, as no published data are available from women with a confirmed SARS-CoV-2 infection.

We report here on the detection of SARS-CoV-2 viral RNA, and gene expression of ACE2, TMPRSS2, CTSL and BSG, in mature oocytes from two women who underwent controlled ovarian stimulation and oocyte retrieval while positive to PCR for the SARS-CoV-2 virus.

Case report

Ethical approval

This study was approved by the Research Ethics Committee of Clinica EUGIN on 23 June 2020 (reference: CEUGIN-2020-09-COROVA). The two women were given oral and written information about the study, ample time to consider their participation, and consented in writing. Furthermore, both women were informed and gave written consent for their case to be published.

Stimulation and oocyte collection

The women in this study (A and B) contacted the clinic in late February 2020, wishing to donate their oocytes. While A did not report any respiratory symptoms in the preceding weeks, B did report symptoms compatible with a mild cold in early February. At the time

of A and B screening for donation, regulations in Spain mandated that only individuals with active symptoms compatible with COVID-19 should be screened for SARS-CoV-2 infection, therefore, they were not tested by PCR at that time. Following clinical, genetic, psychological and family history screening, both women were accepted as donors. Their ovarian stimulation was carried out with recombinant FSH (Gonal-f 1050, Merck Europe B.V. Amsterdam, the Netherlands) from the second day of the menstrual cycle. When ovarian follicles reached a size of 14mm diameter on average, a GnRH antagonist (Orgalutran 0.25 mg, Merck Sharp & Dohme, Haarlem, the Netherlands) was added. Ovulation was triggered with 0.3 mg of a GnRH agonist (Decapeptyl 0.1 mg, IPSEN PHARMA, Hospitalet de Llobregat, Spain) when three or more follicles >18 mm diameter were detected by ultrasound. Oocyte retrieval was performed 36 h later (both on the 25th of March). Oocytes were denuded by exposure to 80 IU/ml hyaluronidase (Hyase-10x, Vitrolife, Sweden) in G-MOPS medium (Vitrolife, Sweden), followed by gentle pipetting. Once denuded, oocytes were scored for polar body presence and mature (MII) oocytes were vitrified using an open method following standard procedures (Cryotop[®], Kitazato[®], BioPharma Co., Ltd; Japan).

SARS-CoV-2 test of oocytes donors

Controlled ovarian stimulation of donors screened in February was carried out in March (starting with the following menstrual cycle). At this time, given the expected high rate of undetected SARS-CoV-2 infection among the general population, and the great uncertainty on the possibility of vertical transmission of the virus, the clinic had decided to systematically perform a diagnostic PCR (VIASURE SARS-CoV-2 Real-Time PCR Detection Kit, CerTest, Zaragoza, Spain; TaqMan[™] 2019nCoV Assay Kit v1, ThermoFisher Scientific, Waltham, MA, USA) in all oocyte donors on the day of oocyte retrieval, and to vitrify all MII oocytes collected while waiting for the PCR results and storing them for clinical procedures later on. In the second half of March, 24 donors underwent oocyte retrieval and PCR, and two (8.3%) were positive (A and B; Cq < 37). All donors were given oral and written information about the SARS-CoV-2 PCR test, and all of them consented to the test. Nasopharyngeal swabs were taken by the anesthesiologist during the anesthesia for oocyte retrieval. Samples were sent to a certified independent diagnostic laboratory, and PCR results were obtained 2 days later.

Whole transcriptome amplification

Six oocytes from A and 10 from B were warmed according to standard procedures (Cryotop[®], Kitazato[®], 150 BioPharma Co., Ltd; Japan), and individually processed for whole transcriptome amplification (WTA) using the REPLI-g WTA Single-Cell Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions for amplification of total RNA from single cells by using a mixture of random and oligo dT primers. Briefly, individual oocytes were placed in a PCR tube containing 7 µl RNase-free-PBS and snap-frozen in liquid nitrogen. A positive control for viral RNA amplification was run to control for the recovery of potential viral particles and to assess potential amplification inhibition during the WTA protocol (Bal et al., 2018). One immature oocyte from a COVID-19 negative woman was included as positive control for viral RNA recovery: in this case, the oocyte was placed in 7 µl RNase-free-PBS containing 2×10^5 copies of MS2 bacteriophage

(MS2Φ; 10165948001; Roche-Merck; Madrid, Spain). After thawing on ice, 4 µl lysis buffer provided in the kit was added to each tube and all oocytes were processed simultaneously.

Quantitative PCR detection of SARS-CoV-2 and related genes

WTA samples were diluted 1:100 following manufacturer's instructions and 2 µl were used for quantitative PCR (qPCR) in technical triplicates. A standard curve for the SARS-CoV-2 gene N was performed by serial dilutions of a 2019-nCoV_N_Positive Control (10006625; IDT; Coralville, IA 52241; USA) on the single-oocyte WTA control sample. Transcripts for SARS-CoV-2 N gene, MS2Φ, and human *ACE2*, *TMPRSS2*, *CTSL* and *CD147* genes were quantified by SYBRgreen fluorescence (Bio-Rad, Hercules, CA, USA) using a CFX Real-Time PCR system (Bio-Rad). Baseline correction, threshold setting and relative expression were performed using the automatic calculation of the CFX Manager Software (Bio-Rad). The software includes algorithms to analyze gene expression results with multiple reference genes (Vandesompele et al., 2002). Actin B (*ACTB*), ubiquitin C (*UBC*) and DNA methyltransferase-1 (*DNMT1*) were used as normalizers (Table I).

Results

The REPLI-g WTA single-cell kit can isolate, reverse transcribe and amplify viral particles present in a single oocyte; 2×10^5 copies of single strain RNA of MS2 bacteriophage were added to a single oocyte at the lysis step. After amplification, specific MS2 RNA was detected (mean Cq = 17.42; SD = 0.12). Additionally, the standard curve for the SARS-CoV-2 N gene determined that the limit of detection (LOD) in our set-up is Cq < 35, which corresponds to 100 copies per well (R = 0.997; from 10^6 copies (mean Cq = 21.1, SD = 0.15) to 10^2 copies (mean Cq = 34.3, SD = 0.61)). The RNA for the SARS-CoV-2 gene N was undetectable (Cq > 38) in the six oocytes from A and 10 from B that were analyzed.

Furthermore, we analyzed the expression of genes involved in controlling SARS-CoV-2 infection, to understand whether oocytes could get infected, regardless of current undetectable viral RNA.

We detected levels of *ACE2* in 2/6 oocytes from A and 3/10 from B (LOD Cq < 36; range (25.51–33.85)). Additionally, *TMPRSS2* was not detected in any of the oocytes (LOD Cq < 38). The putative receptor *BSG* (6/6 for woman A and 9/10 for woman B) (LOD Cq < 38; range (28.58–33.74)) and the protease protein *CTSL* (6/6 for woman A and 10/10 for woman B) (LOD Cq < 38; range (24.31–32.33)) were both expressed at similar levels in 50% oocytes for woman A and 60% for woman B.

Discussion

To our knowledge, this is the first report on the detection of the viral RNA of SARS-CoV-2 in oocytes from women who were positive by PCR. We found that the viral RNA was undetectable in all 16 oocytes tested from two asymptomatic positive women.

Table 1 Primer sequences for real-time PCR.

| Gene name | Gene symbol | NCBI accession no. | Primer name | Primer sequence (5'→3') | Efficiency (%) | Study |
|--|-------------|--------------------|-----------------|---------------------------|----------------|------------------------|
| Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Gene N (Pair 1) | SARS-N1 | txid2697049 | SARS-CoV-2 N1 F | GACCCCAAAATCAGCGAAAT | 99.9 | CDC (2020) |
| | | | SARS-CoV-2 N1 R | TCTGGTTACTGCCAGTTGAATCTG | | |
| SARS-CoV-2 Gene N (Pair 2) | SARS-N2 | txid2697049 | SARS-CoV-2 N2 F | TTACAAACATTTGGCCGCA | 105.1 | CDC (2020) |
| | | | SARS-CoV-2 N2 R | GCGCGACATTTCCGAAAGAA | | |
| RNAse P | RNAse P | NM_006413 | RP F | AGATTTGGACCTGCGAGCG | 117.3 | CDC (2020) |
| | | | RP R | GAGCGGCTGTCTCCACAAGT | | |
| Angiotensin I converting enzyme 2 | ACE2 | NM_001371415 | ACE2 F | AAACATACTGTGACCCCGCAT | 81.0 | Ma et al. (2020) |
| | | | ACE2 R | CCAAGCCTCAGCATATTGAACA | | |
| Transmembrane serine protease 2 | TMPRSS2 | NM_005656 | TMPRSS2 F | ACTCTGGAAGTTTCATGGGCAG | 85.3 | Ma et al. (2020) |
| | | | TMPRSS2 R | TGAAGTTTGGTCCGTAGAGGC | | |
| Basigin | BSG | NM_001728 | BSG F | CTGCAAGTCAGAGTCCGTGC | 86.0 | This paper |
| | | | BSG R | CTACACATTGAGAACCTGAAC | | |
| Cathepsin L | CTSL | NM_001912 | CTSL F | CTGGTGGTTGGCTACGGATT | 96.4 | Ruan et al. (2014) |
| | | | CTSL R | CTCCGGTCTTTGGCCATCTT | | |
| Actin beta | ACTB | NM_001101 | ACTB F | GGACTTCGAGCAAGAGATGG | 102.6 | Barragan et al. (2017) |
| | | | ACTB R | AGCACTGTGTGGCGGTACAG | | |
| DNA (cytosine-5-)-methyltransferase I | DNMT1 | NM_001130823 | DNMT1 F | TGGACGACCCTGACCTCAAAT | 75.8 | Barragan et al. (2017) |
| | | | DNMT1 R | GCTTACAGTACACACTGAAGCA | | |
| Ubiquitin C | UBC | NM_021009 | UBC F | ATTTGGTCCGGGTTCTTG | 81.7 | Barragan et al. (2017) |
| | | | UBC R | TGCCTTGACATTCTCGATGGT | | |
| Bacteriophage MS2 | MS2 | txid12022 | MS2 F | CTCTGAGAGGGCTCTATTGGT | NA | Bal et al. (2018) |
| | | | MS2 R | GTTCCCTACAAACGAGCCTAAATTC | | |

Regardless of the detection of viral RNA in oocytes, one wonders about the possibility of the virus infecting them, perhaps when present in higher concentration in the reproductive organs. Therefore, we have analyzed the expression of two functionally related pairs of genes: *ACE2* and *TMPRSS2* on the one hand, and *CTSL* and *BSG* on the other. *ACE2* acts as receptor for SARS viruses by interacting with the S1 domain of their S protein, while *TMPRSS2* facilitates the virus entry by cleaving and activating viral envelope glycoproteins. Viruses such as human coronavirus 229E (HCoV-229E), Middle East respiratory virus coronavirus (MERS-CoV), SARS-CoV and SARS-CoV-2 do use these proteins for cell entry (Hoffmann *et al.*, 2020). *BSG* is a transmembrane glycoprotein that has been identified as a putative receptor for virus infection (Wang *et al.*, 2020), while *CTSL* could cleave the S1 subunit of the SARS-CoV-2 spike protein in the absence of functional *TMPRSS2* (Hoffmann *et al.*, 2020).

It was previously suggested that expression of *ACE2* and *TMPRSS2* was likely in human oocytes, based on their mRNA expression in a non-human primate oocytes (up to antral follicular stages), and in human cumulus cells (Stanley *et al.*, 2020). We found variable expression (<30% of the oocytes) of *ACE2*, and undetectable expression for *TMPRSS2* in our cohort. These results extend the observation that neither *ACE2* mRNA nor protein were consistently detected in human ovaries (Hikmet *et al.*, 2020). However, as we report expression of *BSG* and *CTSL*, we cannot exclude the possibility of multiple avenues through which SARS-CoV-2 may infect human oocytes. Furthermore, we have tested RNA presence, while a more definitive assessment would need to include protein presence in the oocytes.

Although all oocytes were visually inspected under the microscope at warming for absence of cumulus cells, we cannot fully discard the possibility of contamination of RNA from cumulus cells in our samples, given the physiological continuum between cumulus cells and oocytes during oocyte maturation. Prospective analysis of follicular fluid and cumulus cells in infected women would help clarify this point.

The two women included in this study were asymptomatic. Although it is not possible to determine whether symptomatic women may harbor viral particles in their oocytes, the most likely clinical situation for the provision of ART treatments is with asymptomatic patients. Our report suggests that vertical transmission in these women may not occur through their oocytes during treatment, and that handling of this material in the clinical embryology laboratory may not constitute a hazard for healthcare professionals; nevertheless, more extensive reports are needed to confirm our findings.

Data availability

Fully anonymized data are available upon request.

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Authors' roles

M.B. involved in study design, data collection, data analysis, statistical analysis and manuscript preparation; J.J.G. involved in data collection

and manuscript revision; N.M.-P. involved in data collection and manuscript revision; A.R. involved in expert knowledge and manuscript revision; R.V. involved in study design, implementation and supervision, and manuscript preparation.

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Conflict of interest

The authors have nothing to declare.

References

- Bal A, Pichon M, Picard C, Casalegno JS, Valette M, Schuffenecker I, Billard L, Vallet S, Vilchez G, Cheynet V *et al.* Quality control implementation for universal characterization of DNA and RNA viruses in clinical respiratory samples using single metagenomic next-generation sequencing workflow. *BMC Infect Dis* 2018; **18**:537.
- Barragan M, Pons J, Ferrer-Vaquer A, Cornet-Bartolome D, Schweitzer A, Hubbard J, Auer H, Rodolosse A, Vassena R. The transcriptome of human oocytes is related to age and ovarian reserve. *Mol Hum Reprod* 2017; **23**:535–548.
- CDC. CDC 2019–Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel. 2020. <https://www.fda.gov/media/134922/download> (1 August 2020, date last accessed).
- Henarejos-Castillo I, Sebastian-Leon P, Devesa-Peiro A, Pellicer A, Diaz-Gimeno P. SARS-CoV-2 infection risk assessment in the endometrium: viral infection-related gene expression across the menstrual cycle. *Fertil Steril* 2020; **114**:223–232.
- Hikmet F, Mear L, Edvinsson A, Micke P, Uhlen M, Lindskog C. The protein expression profile of *ACE2* in human tissues. *Mol Syst Biol* 2020; **16**:e9610.
- Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A *et al.* SARS-CoV-2 cell entry depends on *ACE2* and *TMPRSS2* and is blocked by a clinically proven protease inhibitor. *Cell* 2020; **181**:271–280.e8.
- Ma D, Chen CB, Jhanji V, Xu C, Yuan XL, Liang JJ, Huang Y, Cen LP, Ng TK. Expression of SARS-CoV-2 receptor *ACE2* and *TMPRSS2* in human primary conjunctival and pterygium cell lines and in mouse cornea. *Eye (Lond)* 2020; **34**:1212–1219.
- Ruan J, Zheng H, Fu W, Zhao P, Su N, Luo R. Increased expression of cathepsin L: a novel independent prognostic marker of worse outcome in hepatocellular carcinoma patients. *PLoS One* 2014; **9**:e112136.
- Stanley KE, Thomas E, Leaver M, Wells D. Coronavirus disease-19 and fertility: viral host entry protein expression in male and female reproductive tissues. *Fertil Steril* 2020; **114**:33–43.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; **3**:RESEARCH0034.

- Wang K, Chen W, Zhou YS, Lian JQ, Zhang Z, Du P, Gong L, Zhang Y, Cui HY, Geng JJ et al. SARS-CoV-2 invades host cells via a novel route: CD147-spike protein. *bioRxiv*. 2020. doi: 10.1101/2020.03.14.988345.
- Weatherbee BAT, Glover DM, Zernicka-Goetz M. Expression of SARS-CoV-2 receptor ACE2 and the protease TMPRSS2 suggests susceptibility of the human embryo in the first trimester. *Open Biol* 2020; **10**:200162.