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

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Running title: Undetectable SARS-CoV-2 RNA in oocytes

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Abstract

A central concern for the safe provision of ART during the current coronavirus disease 2019 (CODIV-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.

Keywords: 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 (CODIV-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

SARSCoV-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, Glover, and Zernicka-Goetz 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, CTSL1* 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 23/06/2020 (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. Further, 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, Netherlands) from the second day of the menstrual cycle. When ovarian follicles reached a size of 14 mm diameter on average, a GnRH antagonist (Orgalutran 0.25 mg, Merck Sharp & Dohme, Haarlem, 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 > 18mm diameter were detected by ultrasound. Oocyte retrieval was performed 36 hours 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 A 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 $2x10^5$ 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, CTSL1* 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; $2x10^5$ 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.

Further, 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.

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 *TMRPSS2* 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 (less than 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. Further, 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 statement

Fully anonymized data are available upon request.

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Authors' roles

MB involved in study design, data collection, data analysis, statistical analysis and manuscript preparation; J.J Guillén involved in data collection and manuscript revision; N. Martin-Palomino involved in data collection and manuscript revision; A. Rodriguez involved in expert knowledge and manuscript revision; R. Vassena involved in study design, implementation and supervision, and manuscript preparation.

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Conflict of interest

MB has nothing to declare, JJG has nothing to declare, NMP has nothing to declare, AR has nothing to declare, R. has nothing to declare.

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Table I 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 SARS-CoV-2 N1 R	GACCCCAAAATCAGCGAAAT TCTGGTTACTGCCAGTTGAATCTG	99.9	CDC (2020)
SARS-CoV-2 Gene N (Pair 2)	**SARS-N2**	txid2697049	SARS-CoV-2 N2 F SARS-CoV-2 N2 R	TTACAAACATTGGCCGCA GCGCGACATTCCGAAGAA	105.1	CDC (2020)
RNAse P	**RNAse P**	NM_006413	RP F RP R	AGATTTGGACCTGCGAGCG GAGCGGCTGTCTCCACAAGT	117.3	CDC (2020)
Angiotensin I converting enzyme 2	***ACE2***	NM_001371415	ACE2 F ACE2 R	AAACATACTGTGACCCCGCAT CCAAGCCTCAGCATATTGAACA	81.0	Ma et al. (2020)
Transmembrane serine protease 2	***TMPRSS2***	NM_005656	TMPRSS2 F TMPRSS2 R	ACTCTGGAAGTTCATGGGCAG TGAAGTTTGGTCCGTAGAGGC	85.3	Ma et al. (2020)
Basigin	***BSG***	NM_001728	BSG F BSG R	CTGCAAGTCAGAGTCCGTGC CTACACATTGAGAACCTGAAC	86.0	This paper
Cathepsin L	***CTSL***	NM_001912	CTSL F CTSL R	CTGGTGGTTGGCTACGGATT CTCCGGTCTTTGGCCATCTT	96.4	Ruan et al. (2014)
Actin beta	***ACTB***	NM_001101	ACTB F ACTB R	GGACTTCGAGCAAGAGATGG AGCACTGTGTTGGCGTACAG	102.6	Barragan et al. (2017)
DNA (cytosine-5-)-	***DNMT1***	NM_001130823	DNMT1 F	TGGACGACCCTGACCTCAAAT	75.8	Barragan et al. (2017)

methyltransferase 1			DNMT1 R	GCTTACAGTACACACTGAAGCA		
Ubiquitin C	***UBC***	NM_021009	UBC F UBC R	ATTTGGGTCGCGGTTCTTG TGCCTTGACATTCTCGATGGT	81.7	Barragan et al. (2017)
Bacteriophage MS2	**MS2**	txid12022	MS2 F MS2 R	CTCTGAGAGCGGCTCTATTGGT GTTCCCTACAACGAGCCTAAATTC	NA	Bal et al. (2018)