Intrauterine fetal demise in the third trimester of pregnancy associated with mild infection with the SARS-CoV-2 Delta variant without protection from vaccination

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Summary: We describe intrauterine fetal demise in an unvaccinated women with mild symptoms of SARS-CoV-2 Delta variant infection. Histology and elevated proinflammatory responses of the placenta suggest that fetal demise was associated with placental malperfusion due to Delta variant infection.

Abstract

SARS-CoV-2 has a higher infection rate in pregnant women than age-matched adults. With increased infectivity and transmissibility, the Delta variant is predominant worldwide. Here, we describe intrauterine fetal demise in an unvaccinated women with mild symptoms of SARS-CoV-2 Delta variant infection. Histology and elevated proinflammatory responses of the placenta suggest that fetal demise was associated with placental malperfusion due to Delta variant infection. This study suggests that the Delta variant can cause severe morbidity and mortality to fetuses. Vaccination should continve to be advocated and will likely continue to reduce SARS-CoV-2 infection risks for pregnant women and their fetuses.

Keywords: Delta variant, SARS-CoV-2, COVID-19, pregnancy, intrauterine fetal demise, vaccination, coronavirus, placenta, cytokine storm, acute inflammation

Background

The COVID-19 pandemic has been caused by the SARS-CoV-2 virus and has led to >266 million cases wordwide, of which ~5.26 million have been fatal as of 8 December, 2021. Pregnant women are particularly vulnerable to COVID-19 and have a 70% higher infection rate than age-matched adults [1]. Between January 22, 2020 and December 6, 2021, a total of 150,036 pregnant women were confirmed with COVID-19 infections in the United States (US) alone. A PCR positivity rate of 15.4% with SARS-CoV-2 was reported in women presenting in labor in New York City, regardless of symptoms [2]. Pregnant women with COVID-19 typically experience mild illness, and their births are often uncomplicated [3]. However, there have been reports of pregnancy complications like pre-eclampsia, preterm birth, intrauterine fetal demise (IUFD), and stillbirth, as well as serious maternal illness which required intensive care, and even maternal death [4]. The recent Morbidity and Mortality Weekly Report on November 19, 2021 indicated that a COVID-19 diagnosis during the delivery hospitalization was associated with an increased risk for stillbirth in the US, with a stronger association when the Delta variant was predominant [5].

SARS-CoV-2 has evolved rapidly and novel genetic variants continue to emerge and challenge disease prevention and control. The Delta variant has become the predominant variant worldwide, with a prevalence of 98.8% in the US as of Aug 24, 2021. The Delta variant has been reported to be more contagious and some individuals still have breakthrough infections despite vaccination [6, 7]. A large-scale cohort study showed patients with the Delta variant had more risk of hospital admission compared to patients with the Alpha variant, indicating that the Delta variant in unvaccinated populations may lead to higher healthcare burdens [8]. In this study, we describe IUFD associated with placental malperfusion due to cytokine storm during the third trimester of a pregnancy complicated by mild Delta infection.

Methods

Ethics approval and biosafety. This study has been approved by the University of Missouri institutional review board (IRB #2063342 and #2025449) and proper consent was obtained. All work

with live SARS-CoV-2 was performed in a BSL-3 laboratory in compliance with state and federal regulations and with the approval of the University of Missouri Institutional Biosafety Committee.

Placental Immunohistochemistry (IHC) antigen staining. Sections of placenta were deparaffinized, rehydrated, and underwent antigen retrieval and endogenous peroxidase activity quenching, then blocked with 10% normal goat serum before incubation with SARS-CoV-2 spike-specific mouse monoclonal antibody (Invitrogen, Carlsbad, CA). Sections were treated with a biotinylated goat anti-mouse IgG polyclonal secondary antibody, and ABC reagent (Vector lab, Burlingame, CA) was applied following the manufacturer's instructions. Sections were counterstained with hematoxylin, washed, dehydrated, and covered with coverslip.

Viruses. The prototype SARS-CoV-2 virus isolate, USA-WA1/2020 (WA1; NR-52281), was obtained through BEI Resources. MU8944NPS (Delta-142G) and MU8946NPS (Delta-142D) were isolated from two COVID-19 positive patients' nasopharyngeal swabs in Missouri.

RNA extraction. Supernatant from homogenized placental tissue were used for RNA extraction. Viral RNA was extracted using the RNeasy Kits with RNase-Free DNase set (QIAGEN, Germantown, MD) following the manufacturer's protocol.

Viral RNA quantification and sequencing. The quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using SARS-CoV-2 primer/probe sets (Integrated DNA Technology, Coralville, Iowa). The protocol was adapted from CDC's RT-PCR Panel for detection of 2019-novel coronavirus.

PrimeScript One-Step RT-PCR Kit (Mountain View, CA) and spike gene-specific primers were used for PCR. PCR products were sent for Sanger sequencing by the Genomics Technology Core at the University of Missouri-Columbia.

Cytokine and chemokine expression. Total RNA was transcribed to cDNA using SuperScript™ III Reverse Transcriptase with Oligo (dT)20. The qPCR amplification mixture contains: 7µl of water, 10µl of PowerUp™ SYBR® Green Master Mix, 1µl of each forward- and reverse- primers (10µM),

1µl of cDNA. The parameters of the qPCR were as follows: one cycle at 50°C for 2 minutes, one cycle at 95°C for 2 minutes, followed by 40 cycles at 95°C for 1 second and 60°C for 30 seconds. The relative mean fold changes for genes of interest are normalized with house keeping genes (Supplementary Table 1).

Micro-neutralization assay. Sera were heat- inactivated for 60 minutes at 56°C. The DMEM high glucose supplemented with 2% FBS and Antibiotic-Antimycotic was used as diluent. Two- fold serial dilutions of the serum, starting from 1:10, were mixed with an equal volume of virus with 100 $TCID_{50}$ of SARS- CoV- 2. The serum- virus mixture was incubated for 1 hour at 37°C. After incubation, Vero E6 cells was added into each well. The cytopathogenic effect was observed for neutralization titers after a 72-hour incubation at 37°C with 5% CO_2.

Results

A 25-year-old pregnant multigravida at 27+5 weeks gestation age, with diet-controlled gestational diabetes and a body mass index of 17, presented to the emergency department (ED) with mild COVID-19 symptoms, including 103ºF fever, scratchy throat, and cough. The patient was diagnosed with SARS-CoV-2 infection, with reassuring fetal monitoringin the ED. Three days after diagnosis, the patient presented with vaginal bleeding and decreased fetal movement, went into active labor, and delivered a demised male infant vaginally. The patient's postpartum course was complicated by disseminated intravascular coagulation, and she was discharged home after two days of hospitalization.

As per routine IUFD evaluation, patient was IgM negative but IgG positive against parvovirus B19 and cytomegalovirus. She was immune to rubella and negative for toxoplasma. IHC was negative in placenta for cytomegalovirus and herpes simplex virus. Her urine toxicology screen was negative for controlled substances. The fetus did not display any gross anomalies. Fetal chromosomal microarray confirmed a normal male with no clinically relevant copy number changes or loss of

heterozygosity. The patient declined an autopsy for further investigation. These results suggested most likely cause of IUFD did not include infections, chromosomal abnormalities, or harmful drugs.

Viral RNA from the placenta was COVID-19 positive.The virus was determined to be a Delta variant by sequencing, with the spike genetic mutation signature including T19R, R158G, L452R, T478K, D614G, P681R, and D950N, and deletions at positions 156 and 157 compared with the prototype pandemic Wuhan strain. Currently, two Delta variants (Delta-142G and Delta-142D), are co-circulating in the United States, and this patient was infected with Delta-142D, which has been predominant in Missouri since May 2021.

Pathological analysis of the placenta was performed. The umbilical cord was slightly twisted and torn near the insertion site. There was no sign of maternal vascular malperfusion, viral inclusions, erythroblastosis, villous edema, suspicious lesions on the placental disk, membrane inflammation, or umbilical cord phlebitis or arteritis. However, pathology confirmed intervillous inflammation composed of neutrophils and monocytes. The placenta showed villi with rims of fibrin, areas of degeneration, and loss of syncytiotrophoblastic layers. Syncytiotrophoblastic knots and stromal vascular karyorrhexis was seen (Figure 1A). This was observed in all examined sections, indicating diffuse involvement.

To understand the association of SARS-CoV-2 infection with IUFD, IHC and qPCR were performed to confirm antigen presentation and expression of inflammatory mediators in the placenta was measured. Extensive SARS-CoV-2 antigen was identified throughout the placental disc in villous cytotrophoblasts and throughout the cytoplasm of syncytiotrophoblasts (Figure 1B). Additionally, abundant SARS-CoV-2 RNA was detected. Thus, we interpret this case as a Delta variant inducing an acute inflammatory response. Gene expression level of proinflammatory markers associated with viral infection in the placenta were quantified. Compared with those markers in the placenta from a healthy woman, 9 of 13 dramatically increased from COVID-19 infection including tumor necrosis factor (TNF)-α with an increase of 199.60 [±29.02, standard deviation; p=0.0329]-fold, interferon gamma-induced protein 10 (IP-10) (80.38±27.21; p<0.0001), interferon (IFN)-α (54.97±2.33; p<0.0001),

interleukin (IL)-6 (19.38±8.25), IFN-γ (16.96±7.80), IL-8 (13.41±3.03; p=0.0016), monocyte chemoattractant protein-1 (CCL2) (11.86±0.24; p<0.0001), IL-10 (9.55±4.41), and granulocyte colony-stimulating factor (G-CSF) (4.34±1.93) (Figure 2). This response suggested that high levels of these proinflammatory markers stimulated by the Delta variant were associated with the trophoblastic necrosis and lesions crossing the placenta, which ultimately led to placental abruption.

Neutralizing antibody titers typically peak around 60 days after illness onset of mild COVID-19 infections [9]. Neutralizing antibody titers against both Delta variants from the patient's sera 2-months after disease onset were quantified. Results showed that the patient had homologous geometric mean titers of 320.00±0.00 against Delta-142D and heterologous geometric mean titers of 201.59±75.42 against Delta-142G. In comparison, the neutralizing titers against the early US variant (WA1) were still present but considerably less, with titers of 50.40±18.86. The high neutralizing antibody level of the patient indicated stimulation of the immune response against SARS-CoV-2, suggesting that the patient was not immunocomprised and demonstrated a proinflammatory response to the pathogen.

Discussion

The placenta is a physical and immunological barrier against fetal infection. Viral infection of the placenta triggers immune responses at the maternal-fetal interface. A cytokine storm at the placenta may trigger placental malperfusion and even abruption, and the intravasal coagulation at the utero-placental level can cause IUFD. The human placenta expresses receptors of SARS-CoV-2 and proteases that trigger viral fusion [10]. Pregnant women infected with SARS-CoV-2 during the first and third trimesters may have higher risks of inducing exaggerated responses causing a cytokine storm [11]. In the third trimester IUFD present in this study, our data suggests that the excessive infiltration of immune cells and cytokine storm at the placenta due to the Delta variant caused severe placental inflammation and damage, which likely resulted in placental abruption and the demise of the fetus. The limitation of our study is that we did not have access to control placental tissue from a

gestational age-matched pregnant individual with gestational diabetes, but no SARS-CoV-2 infection for comparison of the pathological and cytokine expression levels. Thus, our comparison was to a normal placenta. However, a previous study reported no significant differences in the expression of TNF-α, IL-6, IL-8, and several other genes of interest in the placentas of women with gestational diabetes mellitus relative to those of healthy pregnant women [12], whereas we found TNF-α and IL-8 were significantly elevated in our COVID-19 patient.

Studies showed that symptomatic pregnant women with COVID-19 are at increased risk for severe COVID-19 associated illness compared to non-pregnant women [1, 13]. During the third trimester, SARS-CoV-2-positive women were shown to have more maternal and fetal vascular malperfusion, villous agglutination, and subchorionic thrombi [14]. The presence of SARS-CoV-2 infection in placenta has also been reported in COVID-19 patients [3]. Among these cases of placental infections, one mother experienced a complicated second trimester pregnancy with preeclampsia and placental abruption, one had mild COVID-19 symptoms with a normal, healthy birth, and one experienced moderate symptoms while the neonate also contracted COVID-19 and suffered from neurological manifestations. However, IUFD associated with maternal SARS-CoV-2 infections have been generally rare, and all of these cases were associated with severe maternal complications [15-17]. The maternal characteristics and symptoms included overweight or obese individuals experiencing mild to moderate COVID-19 symptoms. Additionally, different extents of placental inflammation were reported, such as inflammation and fibrin deposition in and between the villi [15], chorionitis, infarction, and necrosis of the placental parenchyma [16] and mixed inflammatory infiltrates in the subchorial space [17].

The histopathological changes of placenta in patients with diabetes were varied and inconsistent. The most frequent placental changes were represented by the immaturity of chorionic villus characterized by a high villus density with increased villus lumenus and presence of syncytial nodules. Less frequent changes include the presence of stromal edema in the terminal villus, the presence of collagen fiber densifications in villus trunks >1mm, and diffuse calcifications in the villus

stroma. There is also evidence of minimal to no placental changes in some diabetic women compared to control groups [18].

This report adds to the literatures a case of IUFD in a pregnant woman caused by Delta variant. Evidence suggests that risk for stillbirth was significantly higher during the period of Delta predominance than during the pre-Delta period [5]. The rapid fetal demise indicates that close fetal monitoring of infected pregnant women, regardless of symptom severity, should be considered.

COVID-19 vaccines are widely accepted to be safe and stimulate robust antibody responses in pregnant women [19-23]. However, among pregnant women, there is only 24% vaccine coverage of individuals with at least 1 dose in the US as of November 27, 2021. The rise of the Delta variant coupled with high vaccine hesitancy in pregnant women raises public health concerns regarding the potential for greater disease severity and worse outcomes, as observed in the unvaccinated patient discussed in this case. Evidence shows that the COVID-19 vaccine has high levels of effectiveness against symptomatic disease with the Delta variant after full vaccination [6, 24].Thus, vaccination should continue to be strongly advocated and will likely continue to reduce SARS-CoV-2 infection risks for pregnant women and their fetuses.

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FIGURE LEGENDS

Figure 1. Placental histopathology. A) Hematoxylin and eosin staining of placenta sections from the patient and a healthy control. Control placenta is with intact syncytiotrophoblasts on the outer villous surface and no inflammation. The placenta of SARS-CoV-2 patient showed intervillous inflammation composed of neutrophils and monocytes (upper right); villi with rim of fibrin and areas of degenerating and loss of syncytiotrophoblastic layer (bottom left); and villi with syncytiotrophoblastic knots and stromal vascular karyorrhexis (bottom right). B) SARS-COV-2 spike protein-specific immunostaining of placenta sections from the patient and a healthy woman. The presence of SARS-CoV-2 antigens were identified in villous cytotrophoblast cells and cytoplasm of syncytiotrophoblast cells throughout the placenta (brown staining). The placenta from the healthy woman was collected before the emergence of the COVID-19 pandemic in the US.

Figure 2. Immunolgoical responses. Gene expression for 13 proinflmmatory markers associated with virus infection in the placeta was determined against the housekeeping gene, succinate dehydrogenase complex. Proinflmmatory markers include interleukin (IL) cytokines, granulocyte colony-stimulating factor (G-CSF), interferon (IFN) gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1/CCL2), tumor necrosis factor (TNF)- α, IFN- α and IFN-γ. The relative mean fold changes for gene of interest are determine by $2^{-\Delta Ct}$ (Ct is the cycle threshold) methods for qPCR data analysis. Here, ΔCt represents: [$Ct_{\text{gene of interest}}$ -$Ct_{\text{housekeeping gene}}$]. The mean fold changes ($2^{-\Delta Ct}$) and standard deviations of the patient and a normal placenta were represented. Two-way ANOVA was conducted between the two placentas. Differences were considered significant when $p \leq 0.05$.

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Figure 1

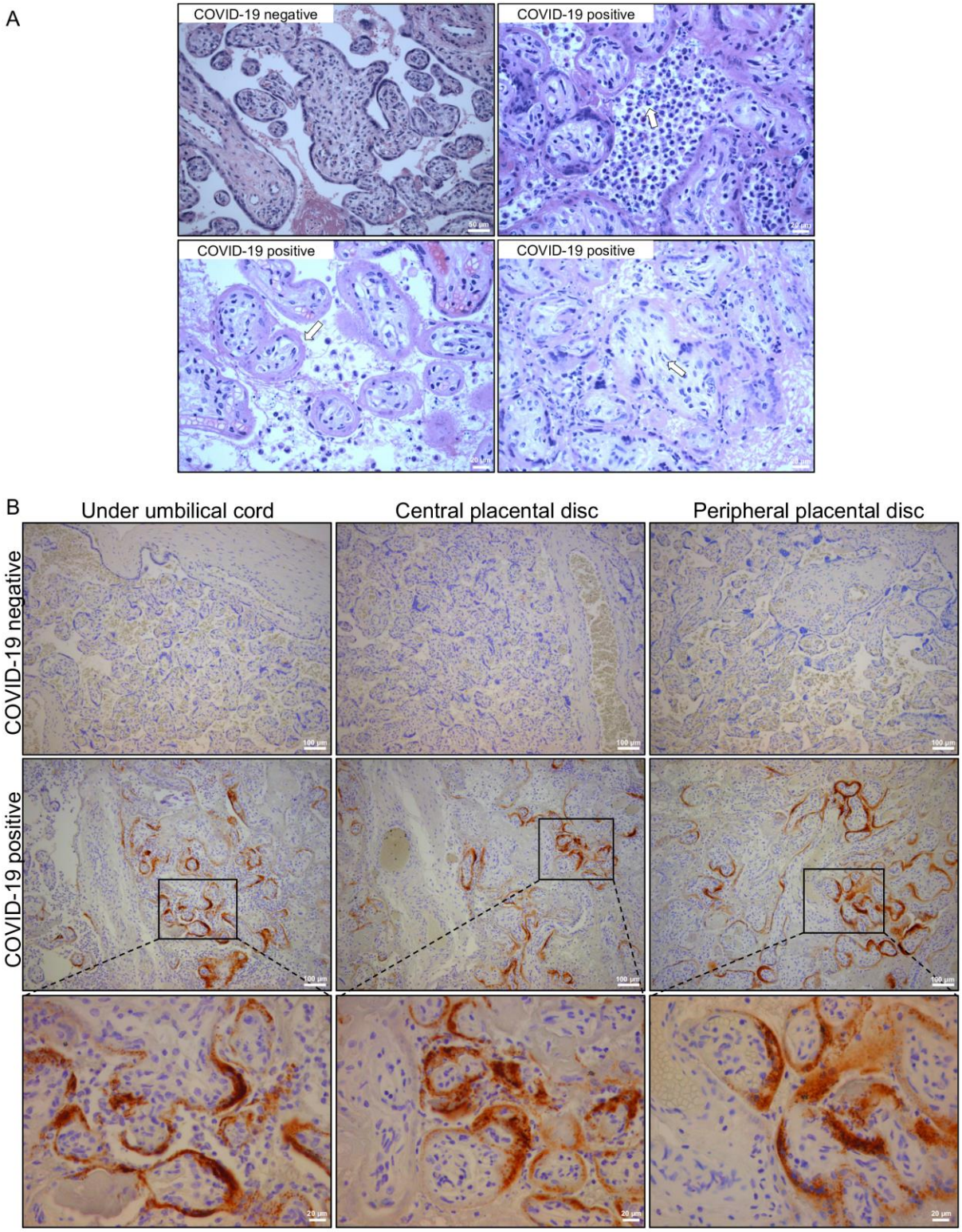
A
COVID-19 negative
COVID-19 positive
COVID-19 positive
COVID-19 positive
B
Under umbilical cord
Central placental disc
Peripheral placental disc
COVID-19 negative
COVID-19 positive

Figure 2