REPRODUCTIVE PHYSIOLOGY AND DISEASE

Protective placental inflammatory and oxidative stress responses are attenuated in the context of twin pregnancy and chorioamnionitis in assisted reproduction

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Abstract

Purpose Assisted reproduction technologies (ART) are associated with increased risks of pregnancy complications and obstetric interventions. Here, we aimed to determine if ART affects placental inflammation and oxidative stress as a mechanism for unfavorable pregnancy outcomes.

Methods The levels of six cytokines (IFN-γ, IL-1β, IL-6, IL-8, IL-10, TNFα) were measured using multiplex ELISA. The activity of four antioxidant enzymes (glutathione S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase, superoxide dismutase) and levels of two antioxidants (GSH, vitamin E) were measured using commercial/in-house assays. Markers were compared between ART and unassisted pregnancies, and then groups were stratified using ICD9/10 codes to determine differences in specific clinical contexts.

Results In unassisted twin pregnancies, there was a trend of decreased cytokine levels (IL-1β, IL-6, IL-8, TNFα, $p < 0.05$), but cytokines in ART twins were the same or higher. Additionally, GST and GPx activities were lower in unassisted twins, and vitamin E levels were higher in ART twins ($p < 0.05$). In pregnancies complicated by chorioamnionitis, there was a trend of increased cytokine levels in unassisted pregnancies (IL-1β, IL-6, and IL-8, $p < 0.05$). No increase was observed in ART, and IFN-γ and TNFα were decreased ($p < 0.05$). Placental GST and GPx activities were higher in unassisted pregnancies with chorioamnionitis compared to ART ($p < 0.05$).

Conclusion Attenuation of protective placental inflammatory and oxidative stress responses may play a role in the underlying pathogenesis of negative birth outcomes in ART, expanding our understanding of adverse pregnancy outcomes when ART is used to conceive.

Keywords Pregnancy · Inflammation · Infection · Oxidative stress · Assisted reproduction

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Introduction

The developed world has experienced a major decline in fertility in the last few decades [1]. Although the reasons for falling fertility and birth rates are not completely understood, each year, more people access assisted reproduction technologies (ART) to conceive [2, 3]. While the procedures involved in ART are considered safe, there is evidence of increased risks for a number of maternal, obstetric, and neonatal complications, including induced labor, emergency cesarean section, premature labor, and small-for-gestational-age infants [4–8]. There is also a higher incidence of twin pregnancy in ART, which independently increases these complications [9]. Even so, the increased risk of obstetric complications remains after correction for multiple births [9, 10]. While the number of multiple births has been greatly

decreased by a general move to single embryo transfer methodologies [11], ART pregnancies still account for close to 20% of multiple births in the USA, even though the prevalence in the total population is only 1–2% [12, 13].

Specific mechanisms underlying the higher rates of negative outcomes in ART remain unknown, but studies have pointed to placental dysfunction playing a critical role [7, 14, 15]. Higher placental weights, higher rates of placenta previa, and premature rupture of the membranes have been observed when ART is used to conceive in humans [5, 7, 8]. These conditions are also associated with placental inflammation and oxidative stress [16–18]. Pregnancy has been characterized as both an inflammatory and oxidative state, but these processes must be precisely controlled and balanced for obstetric success [19–21]. In this context, the placenta performs a number of important synthesis, transfer, and immunologic functions throughout pregnancy, including maintaining a physiological balance between pro- and anti-inflammatory signals [19]. Additionally, placental pro- and anti-inflammatory profiles shift depending on the stage of pregnancy [19, 22]. Within this paradigm, an antioxidant defense network is in place in the placenta that controls the production of reactive oxygen species (ROS) [22, 23]. Previous investigators have shown that increased mitochondrial activities in the placenta generate high levels of ROS, which can cause placental dysfunction leading to preeclampsia and gestational diabetes mellitus [24–26]. Glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), and superoxide dismutase (SOD) are key enzymes in the antioxidant defense network that are present in the trophoblast to protect against the action of ROS. Similar to inflammatory signaling, the involvement of oxidative stress in the development of preeclampsia and gestational diabetes highlights the importance of the development of these processes being completed in a spatially and time-regulated manner within the placenta [23, 27, 28].

Previous studies from our laboratory, using a mouse model of assisted reproduction, have found ART is associated with dysregulated inflammation and oxidative stress [29]. Specifically, ART placentas showed greater levels of apoptosis and degraded nucleotides, as well as increased IL-6 levels, suggesting placental inflammation and cellular stress. Placentas from mouse pregnancies achieved by ART also had lower activity of antioxidant enzymes SOD, GST, GPx, GR, thioredoxin reductase, and xanthine oxidase. Notably, ART pregnancies fertilized using intracytoplasmic sperm injection (ICSI) exhibited more severe effects than regular in vitro fertilization (cavitation) techniques with respect to a decline in RNA purity and decreased placental activity of antioxidant enzymes. A systematic review of ART practices found ICSI was used in approximately 70% of cases, the justification of which has been debated [30–32]. Precisely how ART affects inflammatory and oxidative stress

responses is not completely understood; however, differences between ART and natural conception exist that could account for these differences. The period around conception is associated with widespread epigenetic changes, which are known to be influenced by ART [33, 34]. Another proposed mechanism for pregnancy complications in ART is the absence of the corpus luteum which can affect the placental vasculature [35]. This may also affect trophoblast function in early pregnancy during implantation and placentation.

Here, we aimed to use human tissues to determine whether differences in inflammation and oxidative stress, observed in our previous murine studies, also occur between placentas from ART and unassisted pregnancies. We performed a retrospective cohort study of placentas from ART and unassisted pregnancies, with matched gestational age, maternal age, and ethnicity. We hypothesized that negative maternal, obstetric, and neonatal outcomes observed in ART pregnancies may be mediated by dysfunctional inflammatory and antioxidant responses within the placenta. The long-term objective is to improve the safety and success of ART, from conception to childhood development.

Materials and Methods

Reagents

Multiplex ELISA kits were purchased from Meso Scale Diagnostics (Rockville, MD, USA); assay kits for GR, GPx, SOD, and GSH were purchased from Cayman Chemical Company (Ann Arbor, MI, USA); solvents were obtained from VWR International (Mississauga, ON, Canada), and all other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Human placenta sample collection and processing

The human placentas used in this study ($n = 126$) were collected immediately after birth, with informed maternal consent for inclusion into the Hawaii Biorepository and for use in future investigations. This study was approved by the Research Ethics Board (Clinical) at the University of British Columbia (H14-00,092). Placentas from unassisted pregnancies ($n = 56$) and ART pregnancies ($n = 56$) were baseline matched for gestational age ($+/-6$ days), maternal age ($+/-5$ years), ethnicity, and whether they were singleton or twin pregnancy. Cohort demographics are presented in Table 1. Significant differences in the cohort at baseline were that ART pregnancies were significantly more likely to be delivered by cesarean section ($p = 0.001$) and were significantly more likely to be complicated by gestational diabetes ($p < 0.0001$) and significantly more likely to have high blood pressure ($p = 0.002$).

Table 1 Demographics of the cohort used in this study

	Unassisted pregnancy (n=56)	Assisted reproduction (n=56)	P value
Maternal age (years)	35.57±4.80	36.60±4.34	0.24
Gestational age (days)	262.18±22.89	259.86±22.47	0.60
Maternal BMI	24.43±5.70	24.20±5.51	0.83
Delivery method			
Caesarian	45% (n=26)	70% (n=40)	**0.01**
Vaginal	54% (n=30)	30% (n=16)	
Membrane ruptured			
Yes	36% (n=20)	30% (n=16)	0.39
No	64% (n=36)	70% (n=40)	
Preterm labor	32% (n=18)	36% (n=20)	0.69
Pregnancy weight gain (kg)	13.3±7.3	12.6±4.8	0.56
Birth weight (g)	2850.5±743.2	2711.9±781.7	0.39
Singleton			
Yes	82% (n=46)	82% (n=46)	1
Twin (dichorionic)	18% (n=10)	18% (n=10)	
Ethnicity			
Asian	66.7%	67.2%	0.96
White	21.2%	27.4%	0.64
Native Hawaiian/Pacific Islander	7.1%	2.3%	0.56
Hispanic	3.6%	3.1%	0.97
Gestational diabetes	0	21.4% (n=12)	**<0.001**
Blood pressure >140/90 mm Hg	0	16.1% (n=9)	**0.002**
Cigarettes	Unknown	Unknown	1
Alcohol	n=2	n=2	1

Stratified groups	UP/no chorio	UP/chorio	ART/no chorio	ART/chorio	UP/singleton	UP/twin	ART/singleton	ART/twin
Maternal age (years)	36.2±4.3	32.0±6.1	36.6±4.3	37.1±4.1	35.9±4.7	33.9±5.5	36.7±4.5	36.1±3.7
Gestational age (days)	266.9±13.8	233.9±42.1	259.5±23.4	260.2±29.7	266.2±21.7	243.8±20.0	263.5±24.4	244.5±18.9

Bolded p values are significant

ART/chorio, assisted reproduction with chorioamnionitis; *ART/no chorio*, assisted reproduction, no chorioamnionitis; *ART/singleton*, assisted reproduction, singleton; *ART/twin*, assisted reproduction, twin pregnancy; *UP/chorio*, unassisted pregnancy, with chorioamnionitis; *UP/no chorio*, unassisted pregnancy, no chorioamnionitis; *UP/singleton*, unassisted pregnancy, singleton; *UP/twin*, unassisted pregnancy, twin pregnancy

The villous placenta was collected by blunt dissection, and the decidua was stripped off manually; membranes and the chorionic plate were not included. Villous samples were washed, snap-frozen in liquid nitrogen, and then stored at $-80℃$ at the Hawaii Biorepository. Upon request, pieces of the villous placenta (0.2–0.5 g) were cut frozen and shipped to the University of British Columbia on dry ice under executed material transfer agreement M17-00,402. Placental pieces were mechanically processed into lysates in Tris–HCl buffer, aliquoted, and stored frozen at $-80℃$ until use, as previously described [36]. A portion of the lysate made was further processed to S9 fraction by centrifugation at $10,000 \times g$ for 20 min at $4℃$. The resulting supernatant was removed, aliquoted, and stored at $-80℃$ until use. The protein content of both lysate and S9 fraction was determined using the BCA assay, with bovine serum albumin as a protein standard [37].

Detection of cytokines using ELISA

Commercial multiplex ELISA kits were purchased from Meso Scale Diagnostics (Rockville, MD, USA) and performed according to the manufacturer's instructions. Antibodies for the V-PLEX Cytokine Panel 1 Human Kit were purchased to simultaneously detect the cytokines IFN-γ, IL-1β, IL-6, IL-8, IL-10, and TNFα.

Spiking studies were performed to validate the assay and determine the specificity of the ELISA for the detection of analytes in the placental lysate. Positive control placentas were spiked with analytes and included in the screening, blinded to the bench scientists. Samples were unblinded once all samples were screened.

Biochemical assays for antioxidant enzyme activity

Total GST activity was measured using an in-house assay with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate [38, 39]. Briefly, 1 μL of 50 mM CDNB, placenta S9 2 mg/ml total protein in well, and 79 μL of 0.1 M KPO4 buffer (pH 6.5) were added to a 96 well plate. Following a 2-min incubation at $37℃$, 10 μL of 10 mM glutathione is added to initiate the reaction. Absorbance is measured every 10 s for 5 min at 340 nm. Specific activity is calculated using $e = 9.6$ mM/cm.

Determination of GR activity was performed using a commercially available kit from Cayman Chemical (Catalogue #703,202, Ann Arbor, MI, USA). Placenta S9 samples were assayed in triplicate at a standardized protein concentration of 2 mg/mL. The assay was performed according to the manufacturer's instructions, and absorbance was read at 340 nm every 20 s for 5 min. The activity of GR was determined by calculating the change in absorbance over time and calculated using $\varepsilon = 0.00622$ μM^{-1}/cm for NADPH.

The activity of GPx was measured in placenta S9 using a commercially available kit from Cayman Chemical (Catalogue #703,102, Ann Arbor, MI, USA). Samples were standardized to 1 mg/mL total protein, and the assay was performed as per the manufacturer's instructions, using cumene hydroperoxide as the substrate. Absorbance was read at 340 nm every 20 s for 5 min. The mean values were plotted, and the change in absorbance over time was converted to GPx activity using $\varepsilon = 0.00622$ μM^{-1}/cm for NADPH.

The activity of SOD was measured in placenta S9 using a commercially available kit from Cayman Chemical (Catalogue #706,002, Ann Arbor, MI, USA). Placenta S9 samples were diluted to 0.05 mg/mL to fall into the standard curve range. The assay was performed as per the manufacturer's instructions. For all enzymatic assays, results were accepted if %CV was < 15%, and a positive control sample of pooled placenta S9 was assessed on each plate.

Biochemical assays for glutathione and vitamin E

Levels of vitamin E were measured in placenta S9 samples using a plate-based method based on the method by Tütem et al. [40] using DL-α-tocopherol as a standard. A standard curve was generated over the range of 0–500 μM. Briefly, 100 μL of 10 mM CuCl$_2$·2H$_2$O, 250 μL of 3 mM neocuproine (prepared in absolute ethanol), 300 μL of absolute ethanol, 100 μL of 1 M ammonium acetate, and 50 μL of S9 sample at 2 mg/mL total protein were added to borosilicate glass tubes. Tubes were incubated at room temperature for 30 min, and 100 μL of the product was transferred to a clear 96 well plate in triplicate. Absorbance was measured at 450 nm, and vitamin E levels were determined by comparison to the standard curve.

Glutathione levels in placenta S9 samples were measured using a commercial kit from Cayman Chemical Company (Catalogue #703,002). This kit quantifies GSH using an optimized enzymatic recycling method which utilizes glutathione reductase, meaning GSH and its oxidized form GSSG are both measured (representing total glutathione). The assay was performed according to the manufacturer's instructions, using 50 μL of placenta S9 at 1 mg/mL total protein.

Demographic and statistical analyses

The cohort reported in this retrospective analysis contains five major ethnicities: East Asian, White, Native Hawaiian, Pacific Islander, and Hispanic. Ethnicity was reported to the grandparent's generation, generating partial ethnicities that were statistically compared by determining the percentages of ethnicity for each individual and calculating the mean and standard deviation for each ethnicity.

We first evaluated if there were any differences in measured outcomes between unassisted and ART placentas. A *t*-test with Welch's correction for unequal variance was used to test for differences in cytokine levels, antioxidant enzyme activity, and antioxidant levels between unassisted and ART placentas. Correlations between cytokines, antioxidant enzyme activity, and antioxidant levels were evaluated using Pearson correlation to determine the degree of association between two variables. A biologically significant association was defined as Pearson's $r > 0.2$. Next, unassisted and ART groups were stratified based on 35 ICD9/10 clinical chart codes encompassing various maternal, obstetric, and neonatal outcomes which were collected with the samples for inclusion into the Hawaii Reproductive Biobank. Case groups with a minimum of 4 samples were included in the analysis. Measured outcomes were assessed for normality and, if normality assumptions were met, analyzed using two-way ANOVA with appropriate post-hoc analysis. Statistical analyses were performed using R Studio (Boston, MA) and visualized using Graphpad Prism (San Diego, CA).

Results

Correlations between cytokines, antioxidant enzymes, and antioxidant levels with continuous clinical variables

No significant correlations were found between cytokines, antioxidant enzymes, or antioxidant levels with maternal age (years), gestational age (days), maternal weight gain in pregnancy (kg), maternal BMI (kg/m^2), baby weight (g), baby length (cm), or baby head circumference (cm) using linear regression analysis (*data not shown*). Biologically significant associations were pre-defined as Pearson's $r > 0.2$.

Differences between cytokines, antioxidant enzymes, and antioxidant levels with discrete variables

No significant differences were found between cytokines, antioxidant enzymes, or antioxidant levels between ART and unassisted placental samples unstratified for outcome using a *t*-test with Welch's correction for unequal variance (*data not shown*). For discrete clinical variables, data were further stratified by pregnancy outcome using ICD9/10 clinical chart codes. There were no significant differences observed for the following discrete variables: augmentation, gestational diabetes, induction, IUGR, spontaneous membrane rupture, preterm birth, or small-for-gestational-age infants (*data not shown*). Interestingly, we did not observe differences in the levels of cytokines between delivery method (vaginal vs. cesarian, *data not shown*). However,

two important associations were observed, alteration of placental inflammatory and oxidative stress responses in the context of twin pregnancy and pregnancies complicated with chorioamnionitis.

Cytokines, antioxidant enzymes, and antioxidants in twin pregnancy

Twin pregnancies were associated with attenuated villous inflammatory signaling in unassisted pregnancies (Fig. 1). The cytokines IL-1β, IL-6, and TNFα were significantly decreased in unassisted twin pregnancies compared to unassisted singleton pregnancies (Fig. 1B, C, F, $p < 0.05$). The decrease in IL-8 also approached significance (Fig. 1D, $p = 0.06$). No attenuation of any of the six cytokines was observed in ART, which showed a trend toward increasing levels in twin compared to singleton pregnancy placentas (Fig. 1). Levels of TNFα in ART twin pregnancies were significantly higher than unassisted twin pregnancies (Fig. 1F).

With respect to the antioxidant defense system, we observed a decrease in antioxidant enzyme activity in unassisted twin pregnancies. These placentas had significantly decreased levels of GST activity compared to both unassisted and ART singletons (Fig. 2A). A similar effect was observed in GPx activity, where unassisted twin pregnancies had lower activities compared to unassisted singletons (Fig. 2B). Finally, levels of antioxidant vitamin E were higher in ART twins compared to unassisted twins (Fig. 2C).

Cytokines, antioxidant enzymes, and antioxidants in chorioamnionitis

ART attenuates villous placental cytokine signaling in chorioamnionitis, a potentially problematic response (Fig. 3). Across all six cytokines measured, a trend of increased cytokine levels occurred in chorioamnionitis of placentas from unassisted pregnancies, and levels of IL-1β, IL-6, and IL-8 significantly increased (Fig. 3, $p < 0.05$). In ART placentas, INF-γ was significantly decreased in placentas with chorioamnionitis compared to ART placentas without chorioamnionitis (Fig. 3A, $p < 0.05$). Additionally, there was a trend of decreasing TNF-α in ART placentas with chorioamnionitis compared to ART placentas without chorioamnionitis (Fig. 3F, $p = 0.06$).

Moreover, the activities of antioxidant enzymes GST and GPx were affected differently in unassisted and ART pregnancies complicated with chorioamnionitis (Fig. 4). GST activity was significantly lower in ART pregnancies with chorioamnionitis compared to ART pregnancies without chorioamnionitis; this did not differ without ART (Fig. 4A). Furthermore, unassisted pregnancies with chorioamnionitis had significantly higher placental GST activities compared to ART pregnancies with chorioamnionitis (Fig. 4A). The
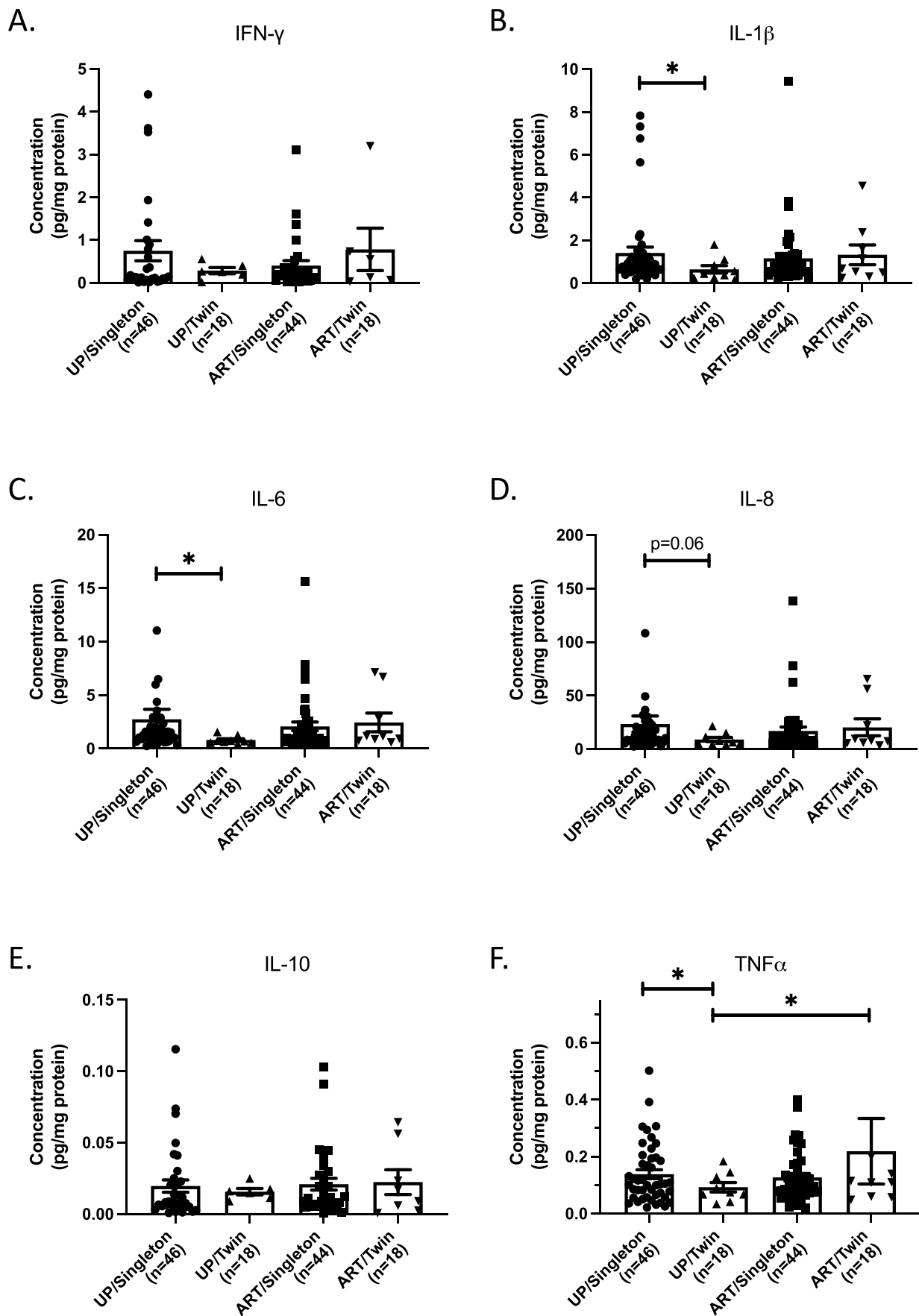
A.

IFN-γ

B.

IL-1β

C.

IL-6

D.

IL-8

E.

IL-10

F.

TNFα

activity of GPx was significantly higher in unassisted pregnancies with chorioamnionitis compared to uncomplicated pregnancies and ART pregnancies with chorioamnionitis (Fig. 4B, $p < 0.05$). Placental levels of the antioxidants GSH and vitamin E were not different between unassisted and ART pregnancies with chorioamnionitis (*data not shown*).

Discussion

In this study, we have identified that protective villous placental inflammatory signaling and oxidative stress responses are attenuated in ART pregnancies complicated by multiple pregnancies or chorioamnionitis. In unassisted twin pregnancies, levels of cytokines and specific antioxidant enzymes were similar or lower compared with singleton placentas, meaning these responses appear to be dampened in unassisted twin pregnancies. Lower cytokine levels could indicate an adaptive response to protect the fetuses from the maternal immune system, preventing the immune system from identifying the baby as "not self" and activating, thereby maintaining pregnancy and preventing preterm birth [41]. Mechanistic support for this assertion comes from studies demonstrating that the levels of IL-10, and the balance of IL-10 to uterine natural killer (uNK) cells is vital in controlling inflammatory responses and maintaining pregnancy [41–43]. In contrast, cytokine levels in ART placentas were similar or increased in twin pregnancies over levels observed in ART singletons. This opposite response to the natural physiological order could be contributing to the significant increases in preterm labor and first-trimester spontaneous fetal loss observed in ART [42, 44]. The processes of inflammation and oxidative stress are intricately linked. Free radicals can induce cytokine release, which can in turn alter the expression and activity of GST and other antioxidant enzymes [45–47]. Therefore, it is not surprising that we observed altered activities of GST and GPx when cytokine levels were decreased. Traditionally in ART, the number of multiple births was very high due to the transfer of multiple embryos to increase the success rate per cycle. However, due to the risks associated with multiple births, many physicians have moved to single embryo transfer as standard practice [11]. Risks associated with multiple-order pregnancy include preterm birth, which increases the risk of perinatal mortality or disability [48] and risks of preeclampsia, embolism, and heart failure in the mother [49–51]. Despite this, many individuals opt for double embryo transfer to increase the odds of successfully achieving pregnancy [52]. As such, with the increasing use of ART, it is important to understand the complications occurring in twin pregnancy to provide better outcomes to both mothers and neonates. If ART twin pregnancy exhibits dysregulated inflammation and antioxidant defense, physicians and pregnant people should be aware of the risks. However, it is important to note that the twin pregnancies included in this study are dichorionic, and the effects observed here may not be applicable to other types of twins such as monochorionic/diamniotic or monochorionic/monoamniotic twins.

The other major finding of this work is that inflammatory and oxidative stress responses were altered when pregnancies were complicated by chorioamnionitis. Chorioamnionitis complicates less than 5% of all births in the USA, but in preterm birth, the rate is 40–70% [53]. Several risk factors have been identified for chorioamnionitis including immune-compromised individuals, meconium passage in utero, bacterial vaginosis, and African American ethnicity [54–57]. Notably, the prevalence of chorioamnionitis in the
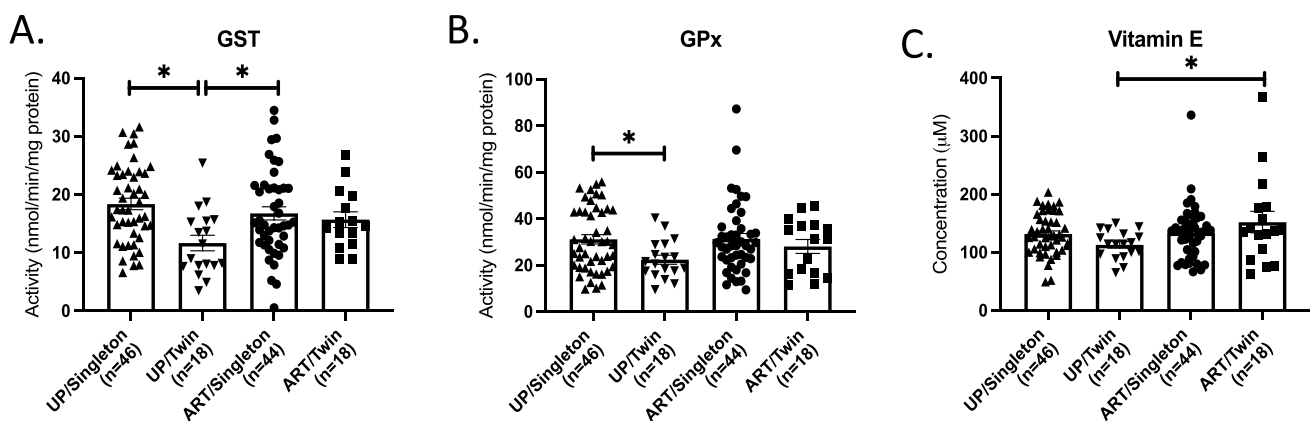

Fig. 2 Activities of glutathione S-transferase (GST) and glutathione peroxidase (GPx) and levels of vitamin E in placentas of unassisted pregnancies (UP) and assisted reproduction technology pregnancies (ART), singletons and twins. Tukey's box plots. *$P < 0.05$
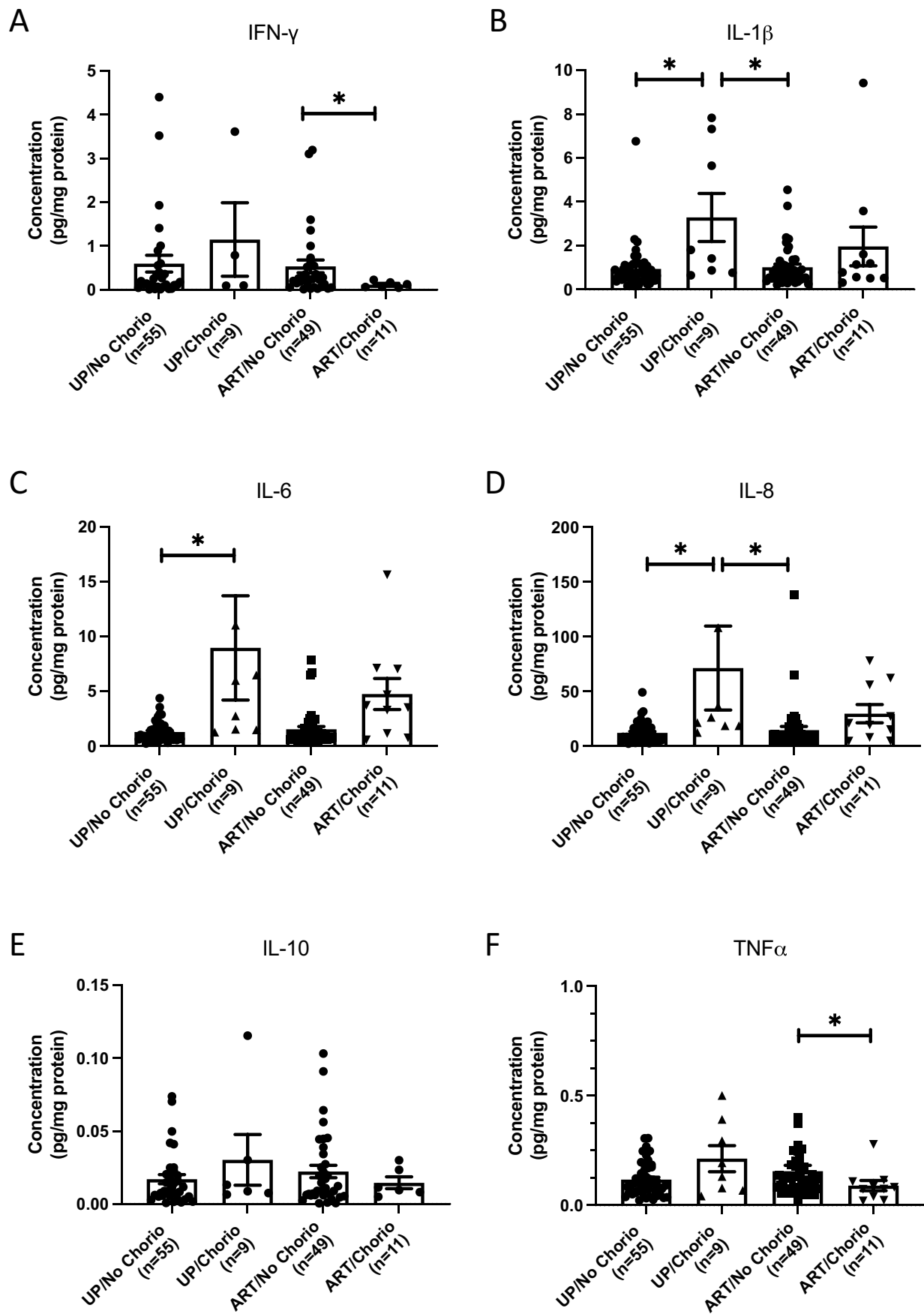
population has not been shown to be increased in ART pregnancies compared to unassisted pregnancies. Chorioamnionitis can be a sterile infection, but the intra-amniotic infection usually occurs as a result of ascending infection from the genital tract and leads to the activation of the immune system with subsequent release of both pro- and anti-inflammatory cytokines in the maternal and fetal compartments [58, 59]. The inflammatory response mimics the natural progression of labor, leading to prostaglandin release, fetal ACTH release, and increased estrogen synthesis that can cause transcription and translation of contraction-associated proteins [56]. For this reason, most pregnancies complicated by chorioamnionitis result in preterm birth. In our cohort, 4/10 ART pregnancies with chorioamnionitis were complicated by preterm birth (40%), and 5/8 unassisted pregnancies with chorioamnionitis were complicated by preterm birth (63%). Preterm birth in response to infection is feto-protective, where cytokines produced as part of the inflammatory cascade activate the immune system and initiate parturition to "remove" the fetus/neonate from the noxious stimuli [56, 59]. Similar to our findings, 4-hydroxy-2-nonenal, a marker of oxidative stress, is increased in placentas with chorioamnionitis, and these authors also demonstrated that activation of oxidative stress pathways is critical in combating

intrauterine infection [60, 61]. Therefore, our finding of lower activities of GST and GPx in ART placentas with chorioamnionitis implies that more oxidative stress is occurring and could be a mechanism for negative pregnancy outcomes in ART, including consequences for fetal growth, development, and well-being.

Inter-individual variation is an important consideration with respect to the findings of this study. In both chorioamnionitis and multiple births, there were trends of increasing or decreasing cytokine levels which approached statistical significance and seemed to be biologically relevant. The number of unassisted and ART placentas to be included in this study were powered a priori from previous studies in our laboratory using mouse models of ART. However, the mice used in the prior studies were not outbred and were housed in facilities with the same diet and environment, meaning that inter-subject variability in mice is much lower than in human populations where maternal age, gestational age, ethnicity, environment, and presence of underlying fertility problems vary widely. To partially mitigate human variability as a confounding factor, and to preserve power, our unassisted and ART groups were baseline matched for maternal age, gestational age, and ethnicity. This worked well for primary analysis, but precise matching was lost in the secondary stratification of all groups, including for chorioamnionitis and singleton/twin, where we observed significant effects. This has two potential outcomes: (1) loss of power in the secondary analysis (although the significant results tend to indicate a strong signal-to-noise ratio and
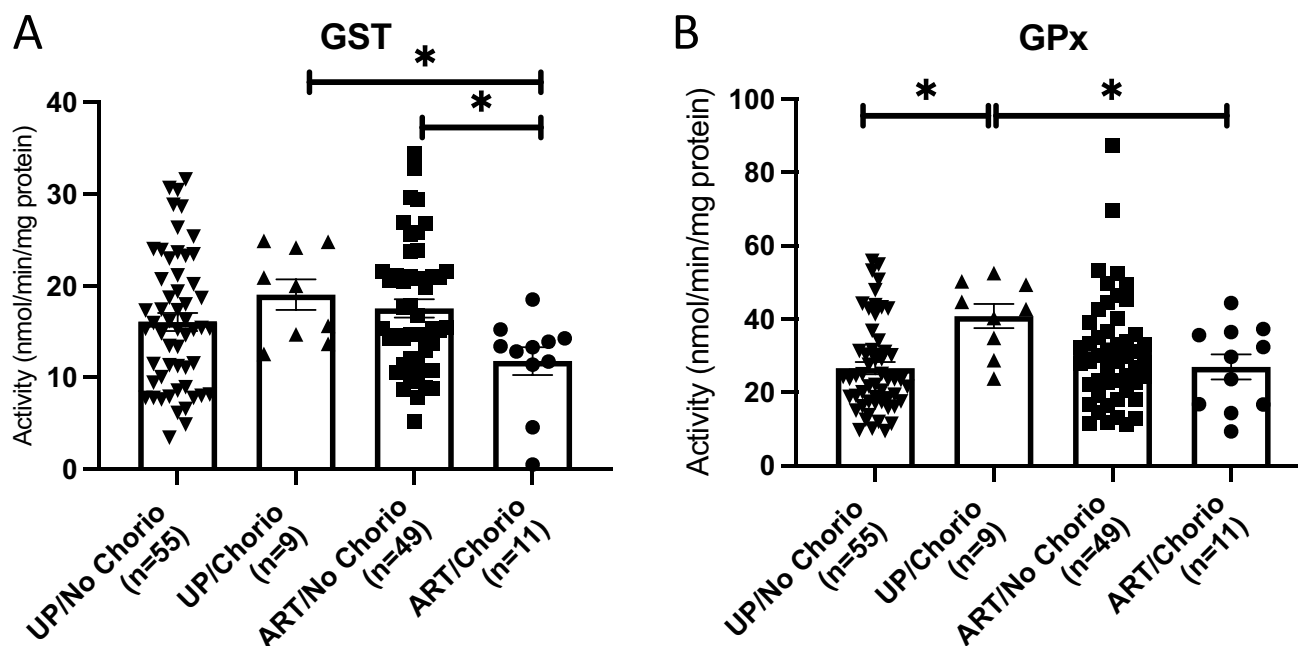

Fig. 4 Activities of glutathione S-transferase (GST) and glutathione peroxidase (GPx) in placentas of unassisted pregnancies (UP) and assisted reproduction technology pregnancies (ART), with and without chorioamnionitis. Tukey's box plots. *$P < 0.05$

high biological significance) and (2) confounding of variables once the secondary stratification occurs (e.g., all older patients could randomly end up in one group). An important difference between ART and unassisted pregnancies is the number of vaginal births *vs.* the number of cesarian sections. Cesarian sections are known to be more common in ART, and this is reflected in our cohort, where birth by caesarian section was significantly more common in the ART group (Table 1). However, there were no significant differences in the levels of any cytokines between the delivery methods in either ART or unassisted pregnancies. Similarly, the ART cohort had significantly higher rates of gestational diabetes and high blood pressure in pregnancy, but there were no significant associations between gestational diabetes or high blood pressure and cytokine levels. Certain limitations also exist with respect to the cohort described here. The clinical charts accompanying the samples lacked information on the technical type of ART used (IVF or ICSI). Additionally, it is unknown if the ART pregnancies were from fresh transfers, frozen transfers, or natural cycle, which may affect pregnancy outcome. Another limitation of the cohort is that we did not have information regarding the underlying cause of infertility in the ART group. The cause of infertility has been proposed to be involved in the increased risk of pregnancy complications observed in ART and may impact inflammatory and oxidative stress responses within the placenta. However, in the current landscape of ART outcomes, it is unclear how underlying infertility is related to the increased risks of pregnancy complications observed.

In summary, this is the first report in humans that inflammatory signaling and antioxidant defense in the placenta is attenuated in the contexts of chorioamnionitis and multiple births when ART is the method of conception. Assisted reproduction is known to affect trophoblast function with respect to the expression of growth factors, vascular development, invasion, and proliferation [62]. Dysregulation of these processes within the developing placenta may have a lasting effect on inflammatory and oxidative stress responses throughout pregnancy. Due to these changes, the effect accounting for adverse pregnancy outcomes likely extends beyond inflammatory and antioxidant effects. Additionally, the mechanistic basis of altered inflammatory signaling may have been partially revealed by a pilot study from our laboratory investigating differences in the expression of suppressors of cytokine signaling (SOCS) 1, 2, and 3 in ART [63]. That study indicated significantly lower SOCS3 occurred in placental syncytiotrophoblasts in ART as compared to villous placentas from unassisted pregnancies. Moreover, positive correlations between SOCS1/IL-10, SOCS2/IFN-γ, and SOCS3/IFN-γ in syncytiotrophoblasts of unassisted pregnancy were lost in ART. Hence, the alterations in SOCS expression observed in the pilot study may provide a mechanistic basis for the attenuated responses observed here. We

also build upon prior animal work in which we found the altered activity of antioxidant enzymes when ART is used to conceive [29]. However, here we find ART alters antioxidant enzyme activity in specific clinical situations, but not as compared to all pregnancies achieved with ART. Further studies investigating both up- and downstream cytokine regulatory mechanisms such as SOCS or Jak/STAT that function to dampen the immune response in pregnancy may elucidate the bases for the associations observed here with pregnancy outcome and attenuated cytokine signaling. Additionally, the investigation of levels of other pro-inflammatory cytokines may provide more insight into the balance between pro- and anti-inflammatory signaling. In the long term, these studies can provide an improved understanding of the risks faced in ART pregnancy and eventually provide targets for therapeutic intervention and improve pregnancy outcomes.

Data Availability Data are available upon request.

Code availability N/A.

Declarations

Ethics approval This study was performed in line with the principles in the Declaration of Helsinki. Approval was granted by the Ethics Committee of The University of British Columbia (H14-00092).

Consent to participate The human samples used in this project were collected at birth, with informed consent from patients for inclusion of their tissues into the Hawaii Biorepository, including consent for future investigation after anonymization and de-identification.

Consent for publication Consent for inclusion of tissues into the Hawaii Biorepository included consent for future investigations and publications.

Conflict of interest The authors declare no competing interests.

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