Effects of extracellular vesicles on placentation and pregnancy disorders

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Abstract

In humans, pregnancy maintenance depends on normal placental formation following trophoblast invasion into the endometrium and vascular remodeling. In the early stages of pregnancy, immune tolerance, inflammatory response and adaptation to hypoxia need to be precisely regulated in the placental microenvironment. Various types of cells, such as trophoblasts, endothelial cells, immune cells, mesenchymal stem cells (MSCs) and adipocytes, induce normal placental development via intercellular interactions through soluble factors. Extracellular vesicles (EVs) are used to diagnose various diseases because their constituents vary depending on the type of cell of origin and pathological characteristics. EV-derived microRNAs (miRNAs) and proteins in the placenta regulate inflammatory responses and the invasion of trophoblasts through intercellular delivery in the placental microenvironment. If the placenta does not adapt to the changed environment during early pregnancy, pregnancy disorders such as pre-eclampsia, preterm birth and gestational diabetes mellitus can occur. Thus, the important roles of EVs during pregnancy and development is fast emerging. This review describes the physiological role of EVs during placentation and their composition in the human placenta. It also suggests the possibility of finding EV markers that can diagnose pregnancy disorders. Furthermore, it describes the properties of EVs that affect pregnancy in livestock.


Introduction

In humans, a successful pregnancy depends on the normal implantation and development of the placenta, which is a temporary organ responsible for fetal growth and development during the pregnancy period. In the fetomaternal interface during early pregnancy, an inner layer called the cytotrophoblast (CT) is attached to the basement membrane and an outer layer called the syncytiotrophoblast (ST) originates from the fusion of CT. ST is in direct contact with maternal blood and is responsible for the exchange of gas, nutrients and waste products between the mother and fetus. Several evidences show that the release of apoptotic debris from the ST causes a systemic inflammatory response. The villous trophoblasts (VTs) avoid immune system attacks by forming an immune privilege at the fetomaternal interface (Sargent et al. 2003). The extravillous trophoblasts (EVTs) penetrate the endometrium and the underlying myometrium to induce intimate interactions between the placenta and uterine wall. Another group of EVT enter the uterine spiral arteries and induce remodeling of the blood vessels. During the first trimester, EVT invasion is controlled by cytokines and chemokines secreted by the decidual and immune cells. In the early stages of fetal implantation, the oxygen tension is ~3% in the uterus and 8~12% in the decidua and myometrium (Jauniaux et al. 2003). In the fetomaternal interface, oxygen tension regulates EVT invasion and vascular remodeling in the myometrium.

Extracellular vesicles (EVs) are found in most body fluids, including blood, urine, saliva and breast milk and contain numerous types of RNAs, lipids and proteins. miRNA expression in trophoblast cells is reportedly altered in hypoxic and inflammatory placental environments. Not only are miRNAs abundant in EVs implicated in the pathological changes in diseases, but they can also stably exist in the circulatory system, which makes them potential biomarkers for diagnosing pregnancy disorders. Recent studies have shown that maternal obesity also contributes to the formation of pro-inflammatory environments and endothelial cell dysfunction in the placenta, which is related to the amount of maternal circulating EVs (Elfeky et al. 2017). Since EVs contain the metabolic products of cells, they make it possible to predict the physiological and pathological conditions of the cell of origin. Numerous trophoblast cell lines, such as JEG-3 choriocarcinoma and HTR8/SVneo EVT cell lines, have been used to elucidate the physiological effects of EVs. Furthermore,
the placental explant culture model or perfusion methods have also helped to analyze the endothelial dysfunction mechanism using placenta-derived EVs (Gupta et al. 2008).

This review describes the effect of placenta-derived EVs on placental immune response regulation and trophoblast cell characteristics in humans. The contribution of EVs to pregnancy disorders such as pre-eclampsia (PE), preterm birth (PB) and gestational diabetes mellitus (GDM) is also analyzed. Finally, the role of EVs in interactions between the conceptus and endometrium in livestock is described.

Identification of placenta-derived EVs

The characterization of EVs is not yet fully established. The components of the recovered EVs can vary depending on the extraction methods (Lotvall et al. 2014). In addition, extracellular RNA can be delivered through non-EV carriers, which further makes research on EVs challenging (Vickers et al. 2011). The exosome is the most widely studied EV, but a method to accurately distinguish between exosomes has not been established. Numerous proteomic analyses of EVs have been conducted, but specific protein markers for each type of EV remain elusive.

Many studies have identified the composition of placental EVs derived from maternal circulation. Furthermore, the effect of EVs on placentation has been elucidated via in vitro studies by analyzing the changes in expression of miRNAs and proteins in EVs depending on the types and invasiveness of the trophoblast cells (Tong et al. 2016). The chromosome 19 miRNA cluster (C19MC), including miR-520c, is a highly expressed miRNA in placenta-derived EVs that changes its expression under hypoxic conditions and is likely to function in placental–maternal signaling (Donker et al. 2012).

Flow cytometry and nanoparticle tracking analysis (NTA) have not only enabled the detection of EVs in maternal circulation, but have also helped identify differences in the concentration and size of EVs between women with pregnancy disease and normal healthy women. Additionally, quantitative analysis of CD63, a typical EV marker, and placental alkaline phosphatase (ALPP), a placenta-specific marker, revealed that the concentration of placental EVs increased with the progress of pregnancy (Sarker et al. 2014). By injecting EVs labeled with fluorescent dyes into a rodent model, it was possible to image the delivery pattern of the EVs within the fetomaternal interface and other organs such as the lungs, liver and kidneys (Sheller-Miller et al. 2016, Tong et al. 2017). Engineered materials that can help analyze placenta-derived EVs more directly and sensitively have also been developed recently (Boriachek et al. 2019).

Physiological functions of EVs in the placenta

The functional activity of isolated EVs has been the focus of recent in vitro studies. However, investigation of physiological changes following delivery of EVs has been hindered by some experimental limitations (Lotvall et al. 2014). It is not easy to secure a treatment group capable of acting as a negative control in which EVs are depleted. It is also difficult to confirm the function of each EV subpopulation because various extraction methods are used. Since most of the studies that report the functional role of exosomes in the placenta have included these limitations, we have used the more general term ‘EVs’ rather than ‘exosomes’.

Pregnancy is a condition characterized by mild immunosuppression and induced inflammation (Redman & Sargent 2007). The activation of T cells along with the expression of inflammatory cytokines during pregnancy has been associated with the onset of pregnancy disorders such as PE and intrauterine growth retardation (IUGR). Moreover, during early pregnancy, the placenta is hypoxic, which increases endothelial cell proliferation and ultimately increases the surface area of the blood vessels, maximizing oxygen and nutrient transfer. By determining the number of ALPP-positive EVs, it is possible to investigate the effects of placental EVs in comparison to those of the total EV population in maternal blood (Table 1).

Trophoblasts express a number of immunoregulatory proteins that regulate maternal immune cell function (Petroff et al. 2005). EVs exhibit either immunostimulatory or immunosuppressive properties depending on their origin and composition. For instance, placental Fas ligand (FASLG) is released from the ST via EVs at the fetomaternal interface in order to promote an immune privilege status (Stenqvist et al. 2013). EVs carrying FASLG and CD274 inhibit T-cell activation signals such as those from Janus kinase 3 (JAK3) and have immunomodulatory effects (Sabapatha et al. 2006). In addition to FASLG, TNF superfamily member 10 (TNFSF10) is also released from the ST via EVs, leading to apoptosis of T cells and/or peripheral blood mononuclear cells (PBMCs) (Stenqvist et al. 2013). Furthermore, EVs bearing soluble MHC class I chain-related molecules (MIC) and UL16-binding proteins (ULBP) inhibit the killer cell lectin like receptor K1 (KLRK1)-dependent cytotoxic responses of PBMCs and induce fetal immune escape during early pregnancy (Mincheva-Nilsson et al. 2006, Hedlund et al. 2009). Moreover, endogenous retrovirus group W member 1, envelope (ERVW-1) transfers to PBMCs through EVs and inhibits the response of lipopolysaccharide (LPS)/phytohemagglutinin (PHA)-induced cytokines. C19MC in the EVs of trophoblast cells also induces viral resistance mediated by autophagy in nonplacental recipient cells (Delorme-Axford et al. 2013). Notably, miR-517a-3p is abundantly contained in EVs derived from trophoblast cells, is delivered to T cells and NK
cells and is known to target the PRKG1 gene in recipient cells (Kambe et al. 2014).

Excessive pro-inflammatory effects in the placenta are associated with the onset of pregnancy disorders, which are characterized by systemic inflammation such as PE and PB. During pregnancy, macrophages are abundant in the maternal decidua and they regulate the inflammatory response at the fetomaternal interface by secreting various cytokines and chemokines. Fibronectin is transferred to macrophages via EVs derived from the trophoblast cells to promote the production of interleukin-1β (IL1B) (Atay et al. 2011b). In addition, EVs derived from trophoblasts increase the migration of monocytes, thus generating an inflammatory environment through the production of IL1B, IL6, SERPINE1, colony stimulating factor 2 (CSF2) (Atay et al. 2011a). The expression of miR-146a-3p in trophoblasts is increased in cell lysates and EVs via antiphospholipid antibodies, which promotes IL8 secretion through Toll-like receptor 8 (TLR8) activation (Gysler et al. 2016). On the contrary, miR-548c-5p, which is highly expressed in EVs derived from PE patients, inhibits the proliferation of macrophages and the expression of inflammatory cytokines such as IL12 and TNF (Wang et al. 2019). Maternal plasma EVs were also shown to cause inflammatory responses and PB in pregnant mice, which clarified the role of paracrine signaling in the progression of pregnancy inflammatory diseases (Sheller-Miller et al. 2019).

During the first trimester of pregnancy, placenta-derived EVs also induce vasculogenesis and angiogenesis through an oxygen-sensing mechanism under hypoxic conditions. Proteomic analysis suggests that trophoblast EVs induce the activation of matrix metalloproteinases (MMPs) and mitogen-activated protein kinase (MAPK) signaling pathways. miR-520c-3p of EVs promotes the invasiveness of EVTs, which occurs by targeting CD44 in EVTs (Takahashi et al. 2017). Other C19MC miRNAs as well as miR-520c-3p are also speculated to regulate EVT migration. In addition, ST-derived EVs containing endothelial nitric oxide synthase (eNOS) facilitate the prediction of low nitric oxide (NO) biological activity in PE patients (Motta-Mejia et al. 2017). miR-155, which is highly expressed in the plasma and placenta of PE patients, is delivered to endothelial cells via EVs and inhibits the expression of eNOS (Shen et al. 2018). Thus, many miRNAs and proteins in placental EVs regulate the placental immune response and invasiveness of trophoblast cells. However, more research is needed regarding the genetic and epigenetic networks involved in the development of pregnancy disorders (Fig. 1).

The microenvironment around placental trophoblast cells has important physiological functions with respect to placental development. In the placental microenvironment, soluble factors secreted by various types of cells such as endometrial cells, mesenchymal stem cells (MSCs), adipocytes and macrophages play various roles during intercellular interactions in the fetomaternal interface. Several tracking experiments have shown that miR-30d and let-7a secreted from endometrial cells can be transferred to the trophoblast through EVs (Vilella et al. 2015, Niu et al. 2017). Moreover, endometrial EVs are internalized by trophoblast cells, which mediate focal adhesion kinase (FAK) signaling, thereby increasing their ability to attach to the endometrium (Greening et al. 2016). MSCs, which can be easily isolated in the placenta, contribute to the formation of the vascular system in the placenta by producing numerous pro-angiogenic factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and fibroblast growth factor2 (FGF2) (Kong et al. 2013). EVs derived from placental MSCs (pMSCs) promote migration and tube formation via placental microvascular endothelial cells in order to adapt to low oxygen tension. Moreover, pMSCs increase VEGF and miR-126 levels in the EVs and promote angiogenic processes in response to NO stimulation (Du et al. 2017).
The placenta also induces the secretion of pro-inflammatory cytokines by internalizing macrophage-derived EVs. Adipose EVs regulate glucose and glycogen metabolism in the placenta explant culture, which is associated with the onset of GDM. The endothelial cell EVs released due to high glucose levels associated with GDM may induce the disruption of the fetoplacental vascular system. The onset of GDM via endothelial cell-derived EVs has been reported to activate ROS production and the MAPK1 and AKT signaling proteins (Saez et al. 2018). Thus, pregnancy disorders such as GDM can be caused by interactions of various types of cells, including trophoblast cells, in the placental microenvironment (Fig. 2).

**Diagnosis of pregnancy disease using EVs**

The ratio of placenta-specific EVs to total circulating EVs was reported to be positively correlated with fetal growth. Several studies have suggested that placental miRNAs may be involved in fetal growth restriction. Cohort studies show that miRNAs in the serum EVs of pregnant women in their second trimester can also help predict fetal growth (Rodosthenous et al. 2017). In addition to predicting fetal growth, much evidence suggests that placental EVs can be used to diagnose pregnancy disorders.

**Pre-eclampsia**

PE, characterized by hypertension and proteinuria after 20 weeks of gestation, adversely affects both the mother and fetus, exhibiting symptoms such as kidney and liver dysfunction and prematurity. PE is caused by abnormal placental development and is difficult to diagnose early due to insufficient understanding of its molecular pathogenesis mechanism. In a previous study, concentrations of placenta-specific EVs were clinically correlated to distinguish between normal pregnancy, early onset of PE and late onset of PE (Pillay et al. 2016). The expression of circulating miRNAs was also reported to be altered in the body fluids of PE patients when compared to controls. Notably, miR-803-5p exhibited higher expression than apoptotic bodies or microvesicles in the plasma-derived EV-rich fractions of PE patients. Moreover, miR-136, -494 and -495 in the EVs secreted by umbilical cord mesenchymal stem cells (UCMSCs) were also highly expressed in PE patients.
when compared to healthy controls. The expression of miR-210 was found to be higher in PE placenta when compared to normal placenta; coincidentally, hypoxic conditions induce a high level of miR-210 release via EVs (Biro et al. 2019).

Proteins present in placental EVs can also be diagnostic markers due to their altered expression in pregnant women with PE when compared to that in normal pregnant women. The FLT1 binds to VEGF and interferes with vascular homeostasis. Levels of sFlt-1 in placenta-derived EVs are higher in patients with severe PE. Moreover, plasma-derived EVs of PE patients contain a large amount of FLT1 and endoglin (ENG), which cause vascular dysfunction when administered to pregnant mice (Chang et al. 2018). Proteomic analysis using umbilical cord blood-derived EVs revealed 29 instances of significantly different protein expression between PE patients and the control group (Jia et al. 2015). It is also likely that EVs isolated from urine can be used in the diagnosis of PE (Nielsen et al. 2017). Syncytin-2 expression, which was found to be reduced in the serum EVs of PE patients, is important for the cell uptake of EVs (Vargas et al. 2014). Galectin 13 (LGALS13) is one of the galectins that is specifically expressed in the placenta. The reduction of LGALS13 level in placental tissue and placenta-derived EVs is also associated with PE progression (Than et al. 2014).

**Preterm birth**

The precise cause of PB is still unclear; however, the action of biochemicals released by mature fetal organs is suspected to cause an inflammatory response that interferes with the maintenance of pregnancy. EVs derived from UCMSMCs in term or preterm infants have been shown to have different oxidative metabolism (Panfoli et al. 2016). This suggests that the different effects of EVs on aerobic respiration by gestational age may affect the onset of preterm delivery. EVs derived from the milk of women who underwent PB survived even after gastric and pancreatic digestion, and miRNAs in EVs displayed different expression patterns when compared to same from mothers who underwent term birth (TB) (Kahn et al. 2018). Recent studies have shown that there are 173 different miRNAs in EVs between PB and TB, and bioinformatic analysis suggests that these miRNAs target TGFBI and TP53 signaling (Menon et al. 2019). Various circulating microparticle proteins extracted from the plasma of 10- to 12-week pregnant women show correlation with PB. Moreover, evidence suggests that miRNA in the EVs of plasma can act as a biomarker to predict PB (Fallen et al. 2018). Complex genetic networks, including P3K/AKT and VEGF signaling, regulated by miRNAs in EVs are assumed to be involved in the onset of PB. Furthermore, cytoscape analysis of the UCMSMCs of PB and TB infants revealed a difference between inflammation and metabolic cluster between the two groups (Bruschi et al. 2018).

**Gestational diabetes mellitus**

GDM affects 9–15% of all pregnancies and is screened according to oral glucose tolerance tests at 24–28 weeks of gestation (Chu et al. 2007). An earlier diagnosis of GDM would allow for faster response to the damage accumulated by glucose intolerance. Hyperglycemia increases the release of EVs from primary trophoblast cells, which is further enhanced under hypoxic conditions (Rice et al. 2015). According to a cohort study, both normal and GDM pregnancies show a greater increase in the concentration of placenta-derived EVs as pregnancy progresses; however, a larger increase can be observed in GDM pregnancies (Salomon et al. 2016). The plasma EVs of pregnant women with GDM further promote the release of inflammatory cytokines from endothelial cells. Placental EVs from GDM pregnancy also show different miRNA expression patterns when compared to normal EVs and are associated with skeletal muscle insulin sensitivity. Notably, placental EVs in normal pregnant women increase glucose uptake in the diabetic skeletal muscle (Nair et al. 2018).

**The effect of EVs on livestock pregnancy**

In livestock, the endometrium secretes numerous soluble substances that are responsible for the growth and elongation of the conceptus, which is abundant in the EVs. Pregnancy loss occurs frequently during blastocyst hatching and conceptus implantation onto the endometrium; however, the factors responsible for intercellular interactions are still not completely understood. Even in livestock, placental EVs likely play an important role in maintaining pregnancy (Table 2). Several evidences suggest that the endometrium and placenta release microvesicles, including exosomes, to induce conceptus-endometrial interactions via the uterine luminal fluid (ULF). EVs isolated from the ULF of pregnant ewes show many differences in protein and miRNA profiles in EVs when compared to cyclic ULFs. Furthermore, EVs identified in the ULF of pregnant ewes contain endogenous beta retrovirus (enJSRV) RNA, which can be delivered to heterologous cells (Burns et al. 2014). Exosomes containing enJSRV protein also promote the proliferation and the secretion of interferon tau (IFNT) on ovine trophoderm cells (Ruiz-Gonzalez et al. 2015). Moreover, EVs labeled with PKH67 dye were observed in both the trophoderm and uterine epithelium (Burns et al. 2016). This suggests that the cell-to-cell interactions via EVs play an important role in maintaining normal pregnancy in sheep. Additionally, EVs isolated from the uterine flushings of pregnant ewes had proteins such as IFNT, macrophage-capping protein...
According to Boriachek et al. (2019), miR-150 is reduced in the umbilical cord of porcine pregnancies, and this reduction is associated with the development of placental dysfunction. Zhao et al. (2018) observed that EVs released from bovine uteri may play an important role in embryo development and pregnancy maintenance.

In porcine pregnancies, placentalization begins with the expression of adherent molecules in the trophectoderm and uterine luminal epithelium over days 15-20 during the gestation period. The expression of EV-related genes in the porcine uterus shows a specific difference depending on the cell type. The EVs derived from porcine trophectoderm cells induce the proliferation of the maternal endothelial cells and promote angiogenic processes (Bidarimath et al. 2017). The expression of porcine miR-150 is reduced in the umbilical cord blood-derived EVs of pigs undergoing IUGR (Luo et al. 2018). The increase in miR-150 in the EVs increases the proliferation and migration of endothelial cells, thereby exhibiting a pro-angiogenic effect.

When bovine EVs isolated from a luteal phase uterus were supplemented with bovine somatic cell nuclear transfer (SCNT) embryos, the blastocyst formation rate and inner cell mass/trophectoderm cell ratio were observed to increase (Qiao et al. 2018). This suggests that EVs released from bovine uteri may play an important role in embryo development. Treatment of EVs from the flushed fluids of a bovine uterus during pregnancy (at days 17, 20, and 22) leads to differences in the expression of apoptosis-related genes and adhesion molecules in endometrial epithelial cells depending on the cell type. The EVs derived from bovine uteri show differences in the expression of EV-related genes and adhesion molecules in endometrial epithelial cells depending on the cell type.

In conclusion, the placenta during early pregnancy is in an environment of mild inflammation and hypoxia, and normal proliferation and invasion of trophoblasts is necessary for the maintenance of pregnancy. Various miRNAs and proteins in placenta-derived EVs are known to play a role in pregnancy maintenance in the trophoblast and placental microenvironment. Therefore, understanding the physiological activity of EVs during early pregnancy will present a new approach to overcome abnormal placentation and pregnancy disorders.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement
G S and W L designed and directed the study. C Y, G S and W L wrote and prepared the manuscript. C Y designed the figures. All authors provided critical feedback and helped to shape the manuscript.

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