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IFPA Meeting 2015 Workshop Report IV: Nanomedicine Applications and Exosome Biology, Xenobiotics and Endocrine Disruptors and Pregnancy, and Lipid Mediators and Placental Function

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Abstract

Workshops are an important part of the IFPA annual meeting, as they allow for discussion of specialized topics. At the IFPA meeting 2015 there were twelve themed workshops, three of which are summarized in this report. These workshops were related to various aspects of placental biology but collectively covered areas of pregnancy pathologies and placental metabolism: 1) nanomedicine applications and exosome biology; 2) xenobiotics and endocrine disruptors and pregnancy; 3) lipid mediators and placental function.

1. Nanomedicine applications and exosome biology

Chairs: Carlos Salomon and Jeffrey Keelan

Speakers: Carlos Salomon, Claudia Gohner, Jeffrey Keelan, Helen Jones, Linda Harris and Roger Smith.

1.1 Outline

During the past decade, there have been major advances in the field of extracellular vesicles (EV) research, especially in characterising the compostion and functions of EVs originating from endosomal compartments called exosomes. Exosomes are a specific subtype of secreted vesicles; they are small (~30-120 nm), very stable, and are released from a wide range of cells and tissues including the human placenta. This workshop discussed the nature and content of endogenous placenta-derived nanovesicles (i.e. exosomes) and their potential prognostic and diagnostic applications in pregnancy. In addition, the workshop explored recent advances in the design and application of biosynthetic nanocarriers for targeted drug delivery in pregnancy, with a focus on placental nanovesicle targeting and uptake.

1.2 Summary

Dr Carlos Salomon first discussed the hypothesis that placenta-derived exosomes are a potential early biomarker for complication of pregnancies. Dr Salomon presented data on the different isolation methods to enrich subpopulations of extracellular vesicles (EVs) including exosomes. The term EVs is non-specific classification that include all membrane-bound vesicles with different sizes and origins. Interestingly, the placenta releases exosomes into the maternal circulation from as early as 6 weeks of gestation. Release is modulated by the microenvironment milieu (*e.g.* oxygen tension and glucose concentration) and correlates with placental weight and perfusion. The concentration of placenta-derived exosomes in maternal plasma increases progressively during gestation. At early gestation (i.e. 11-14 weeks), women who develop GDM later in gestation presented with a higher total number of exosomes and placenta-derived exosomes compared with values observed in normal pregnancies at the same stage (i.e. 11-14 weeks).

Dr Claudia Göhner presented data on the release of syncytiotrophoblast microvesicles (STBMV) and exosomes from the human placenta. These extracellular vesicles may show an immune-modulatory function in normal pregnancy and are believed to be involved in the pathophysiology of preeclampsia, where they are released in increased number and may contribute to the inflammatory state apparent throughout the disease. Normal STBMV and exosomes have been shown to guide monocyte maturation from $CD14^{++}CD16^{-}$ classical monocytes to $CD14^{++}CD16^{+}$ intermediate monocytes and monocyte activation (increased CD11b expression). In contrast, preeclamptic STBMV lose their ability to lead monocyte maturation and activation, which indicates an impairment of the STBMV function and may be connected to the inflammatory reaction in preeclampsia.

As an introduction to the remaining talks, Professor Jeff Keelan discussed how the nanoparticulate delivery systems offer a potential avenue for delivering therapeutics to maternal, placental and fetal tissues for the treatment of a wide range

of diseases and conditions in pregnancy. The placenta constitutes a primary drug target and a drug barrier, as well as a potential target of any toxicity. Prof Keelan presented a number of options for delivering drugs in pregnancy using targeted nanoparticle-mediated delivery systems and discussed their advantages, barriers to further progress, and therapeutic applications. Finally, he presented data from his own studies exploring biodistribution and placental uptake of polymer-based particles using both ex-vivo and in-vivo models.

Dr Helen Jones discussed her studies focused on using nanoparticle-mediate gene therapy to enhance intrauterine growth in a rodent model. Dr. Jones pointed out that no treatment is available at the moment for fetal growth restriction (FGR) save early delivery. Dr Jones described the development of a self-polymerizing HPMA-DMAEMA co-polymers incubated with PLAC1-human IGF-1 plasmids at an N:P ratio of 5:1 form nanoparticles that achieved cell-specific gene expression and function 96 hours after treatment in single and syncytialized BeWo cells and a murine model of FGR. In pregnant mice, Texas red conjugated nanoparticles were turned over within 72 hours of systemic or placental delivery and no aberrant transgene expression seen in maternal or fetal organs. Birthweights in the FGR model were restored to control levels with nanoparticle treatment. These exciting studies constitute building blocks for development of potential treatments for FGR in humans.

Dr Linda Harris pointed out that enhancement of placental function has been shown to alleviate maternal symptoms and improve fetal growth in animal models of pregnancy complications. Candidate drugs have been identified that promote placental function; however, in many cases it is not appropriate to administer them systemically to pregnant women. Targeted delivery of therapeutics to the placenta offers two advantages: the ability to minimise detrimental side effects in maternal and fetal tissues, and to reduce the dose of therapeutic required to achieve the desired biological effect. Dr Harris's studies have led to the identification of novel placental homing peptides for placental targeting; this has enabled the creation of nanoscale therapeutics for targeted manipulation of placental function. The processes of peptide screening, nanoparticle synthesis and in vivo testing were discussed.

Professor Roger Smith presented data on targeted liposomes as a drug delivery system for the uterus. Pregnancy and child birth are dangerous stages of life for both the mother and fetus and complications may arise during the term of the pregnancy, or in relation to labor itself. In this regard, it may be necessary to administer therapeutic intervention to the uterine tissue; however, how can we administer therapeutic intervention without affecting other tissues and organs? Liposomes are artificial phospholipid nano-vesicles ranging in size from 50 – 1000 nm and the most common and well-investigated nanocarriers for targeted drug delivery. Professor Smith and colleagues have exploited the selective expression of the oxytocin receptor (OTR) in the uterus to construct liposomes targeting the myometrium using anti-OTR antibodies. In vivo, he showed that targeted liposomes successfully accumulate in tissues that express the OTR (uterus and mammary tissue). Interestingly, some brain regions also express OTR, however, the targeted liposomes were not localized to other major organs (*i.e.* heart, brain, kidney and lung) and were not detected in the neonate. The targeted liposomes were capable of delivering active drug to human

myometrial strips, providing proof of concept that selective drug delivery could be accomplished using this strategy.

1.3 Conclusions and perspective

EV subtypes from the syncytiotrophoblast (microvesicles and exosomes) are formed in different processes, and may have different immunologic functions. To meaningfully ascribe biological functions and/or diagnostic and therapeutic utility to "extracellular vesicles", and in particular exosomes, greater specificity and vesicle characterization is required. Exosomes are a specific population of EVs, requiring standardisation of isolation methods to enrich a specific population. The functionality of syncytial EVs differs between normal pregnancy and preeclampsia (PE). For example, monocytes alter their phenotype after stimulation with PE-derived microvesicles or those from uncomplicated pregnancies. Finally, it should be recognised that the net biological effect of all microvesicles and exosomes derived from the placenta on maternal physiology needs to be studied and understood, as well as the individual contributions of all the various sub-types, in order to understand their significance with respect to dialog between the placenta and mother in normal and complicated pregnancies.

Placental targeting to increase selectivity and minimise risks of maternal side-effects or fetotoxicity is showing great promise. Finding a targeting strategy that is selective, efficient and allows placental uptake is key, but challenging. Estimating fetal uptake, placental transport and placental accumulation is also key with highly sensitive quantitative measures required. Adeno-viral mediated placental gene therapy could lead to over-expression of human growth factors in mouse placenta, alteration of placental vascularity and placental nutrient transport and improve fetal growth in a mouse model of Fetal Growth Restriction. Manipulation of intrinsic signalling pathways within the placenta may represent an alternative treatment strategy. Identification of placental homing peptides is critical to nanotherapeutics for targeted manipulation of placental growth. Interestingly, targeted delivery of IGF-II (Harris et al) increased placental weight but did not increase fetal weight in wild type mice. Manipulation of intrinsic placental growth signalling with nanoscale therapeutics enhancing placental growth signalling might reduce the risks associated with systemic drug administration in pregnancy. We are able to use liposomes to target the delivery of therapeutics to human uterine tissue in vitro. This targeting system is extremely flexible; encapsulated different classes of chemical compounds either block or promote contractions.

The attraction and benefits of targeted nanoparticle-mediated drug delivery in pregnancy are exciting and undeniable. From this workshop it is clear that significant progress has been made in achieving placental and uterine targeting and delivery of a variety of classes of drugs (DNA, small molecule drugs) for therapeutic purposes. Placental targeting using a homing peptide approach (Harris et al) is inexpensive, effective and appears to be highly selective, while the antibody-based approach (Smith et al) which is well-validated in other areas of medicine, has been successfully exploited for uterine delivery. The way has now been paved for others to begin exploring different nanoparticle construction and cargo configurations for the treatment of placental disorders in a variety of ex-vivo and in-vivo models. Different drug classes or combinations of drugs that target multiple aspects of the disease

process (e.g. receptor expression, intracellular signalling, transport and export) can be evaluated using the nanoparticle-based drug delivery strategies discussed in this workshop. Detailed biodistribution/pharmacokinetic studies and accurate and sensitive assessment of fetal exposure, drug accumulation and toxicity will be critical in allowing these approaches to move past the animal model stage into preclinical trials. Currently, only 0.1% of the trials listed at Clinicaltrials.gov are in nanoparticles, mainly due to the many challenges to transition nanomedical research into a commercial product. Of the multiples barriers are the FDA (Food and Drug Administration) approval process that can delay the initiation of the trial for over 2 years, patient enrolment, comparison with conventional medicine, investment as well as safety concerns. Interestingly, establishment of regulatory guidelines from the FDA, which are specific for nanomedicine and nanotechnology products, has been proposed. Finally, the possibility needs to be explored that exosomes and other vesicles that are shed from the syncytiotrophoblast may act as decoy receptors for placenta-targeted nanoparticle drug delivery systems. This needs to be carried out in order to assess the potential impact of this phenomenon on nanoparticle drug dose, clearance and efficacy in the therapeutic context.

2. Xenobiotics and endocrine disruptors and pregnancy

Chairs: Padma Murthi Murray Mitchell

Speakers: Murray Mitchell, Jeffery Keelan, Cathy Vaillancourt, Richard Saffery, Vicki Clifton and Padma Murthi.

2.1 Outline

Endocrine disruptors are exogenous substances that alter endocrine function and consequently causes adverse health effects in an intact organism, its progeny, or subpopulations. They may do so by interfering with the production, release, transport, metabolism, binding, action, or elimination of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental processes. Pregnant women are exposed to various potential endocrine disrupting chemicals through diet, medication use, occupational or environmental activities and other lifestyle factors. Epidemiological studies have associated altered pregnancy and fetal outcomes with exposure to contaminants such as heavy metals, polychlorinated biphenyls, dioxins and pesticides. The main focus of our workshop was to discuss how endocrine disruption leads to change in endocrine-regulated physiology in utero and contributes to adverse pregnancy and neonatal outcomes.

2.2 Summary

Professor Murray Mitchell reviewed the use of a method of ex vivo perfusion of the human placenta to assess transplacental transfer of substances, with emphasis on xenobiotics and endocrine disruptors. The use of this approach to determine the transfer of endocrine disruptors across the human placenta was presented. In particular, Professor Mitchell presented data on the transfer of Bisphenol A, 4-nonylphenol and genistein. Comparisons with their structural similarity to estradiol were attempted. Finally, information from data on the placental transfer of gangliosides and transthyretin was discussed.

Professor Jeffery Keelan discussed that bisphenol A (BPA) is a ubiquitous endocrine disrupting chemical that exerts a wide range of cellular effects, through multiple mechanisms. Professor Keelan presented data on human exposure to BPA during pregnancy and the implications for fetal development and the incidence of pregnancy complications. Professor Keelan also presented his own data on BPA levels in the amniotic cavity of pregnancy and the need for critical interpretation of these data in order to arrive at justifiable conclusions around exposure and risk.

Dr. Cathy Vaillancourt presented data on depression, which occurs in up to 25% of pregnant women, a third of which undergoes antidepressant treatment, mainly with selective serotonin-reuptake inhibitors (SSRIs). The placenta serves as an early source of the serotonin, which is critical in embryonic and fetal developmental processes. Serotonin can also induce aromatase (CYP19) in placental cell lines. However, the effects of SSRIs on the serotonin and estrogen systems placenta have never been studied. Using both a unique co-culture of BeWo (human trophoblast-like) and H295R (human fetal-like adrenocortical) cells, a model of feto-placental steroidogenesis and a rat model, it was demonstrated that SSRIs i) induced alterations in serotonin systems in the placenta and fetal heart and ii) disrupts placental serotonin transport and estrogen biosynthesis.

Professor Vicki Clifton discussed several placental mechanisms that function in a sex specific manner and could be potentially be affected in a sex specific manner by endocrine disruption. More recently, this team has found several different isoforms of the glucocorticoid receptor in the human placenta which varies in relation to fetal sex and could result in alterations in the placental response to phathlates. The exact mechanisms mediated by the steroid receptors that result in detrimental responses to endocrine disruption in the placenta are yet to be defined.

Dr. Richard Saffery discussed the molecular mechanisms underpinning the link between in utero exposure to plastic product chemicals (PPC) and adverse offspring outcomes in humans, a linkage which remains largely unexplored. The advent of longitudinal birth cohorts, commencing in pregnancy, offers unparalleled opportunities to address this issue and builds the level of causal evidence. The Barwon Infant Study (BIS) is a longitudinal, population-derived study of 1074 infants with antenatal recruitment. A total of nineteen plastic product chemicals are being measured in 1000 maternal urine samples collected in pregnancy. Cognitive, language and motor development have been assessed in infants at two years of age with the extensively validated Bayley-III scale. Planned genome-wide studies in placenta and blood at birth and age one will provide unparalleled insights into the link between PPC exposure in utero, early life epigenetic profile, and childhood health outcomes.

Dr. Padma Murthi presented some preliminary studies on the effect of bisphenol A (BPA) on human placental growth control gene expression using choriocarcinoma derived trophoblast cell line BeWo as an in vitro model system. Cultured cells were exposed to environmentally relevant concentrations of BPA (0.1-2 μg/ml) for up to 24h, after which levels of polarity genes Scribble, Disc Large Homolog1 (DLG1) and Lethal Giant Larvae (LGL1 and LGL2) mRNA, protein and activity were determined by qRT-PCR and Western immunoblotting. BPA dramatically decreased levels of Scribble and LGL protein and mRNA in a time- and concentration-dependent manner (< 2-fold). The functional role Scribble and LGL was determined using sequence

specific siRNAs. Both Scribble and LGL selectively inhibited trophoblast cell adhesion to matrices including laminin and fibronectin.

2.3 Conclusions

Evidence suggests that BPA can disrupt estrogen-sensitive developmental processes, although several studies indicate that environmental exposure at typical levels does not have effects on reproduction or development. In this workshop findings suggesting that disturbances in serotonin signalling may be responsible for the pregnancy complications associated with SSRIs treatment was discussed. Interestingly, sex difference in cortisol-regulated pathways of the placenta has been observed. Preliminary data link elevated maternal phthalates with poorer neurodevelopmental outcomes at age two, and to measureable differences in the cord blood epigenetic profile. BPA severely disrupts human placental polarity gene expression in vitro, which suggests that exposure to BPA may contribute to altered placental function and consequent pregnancy complications.

3. Lipid Mediators and Placental Function

Chairs: Denise Hemmings and Christiane Albrecht

Speakers: Christiane Albrecht, Isabella Caniggia, Denise Hemmings, Alicia Jawerbaum, Ed Johnstone, Rohan Lewis, Theresa Powell, Christian Wadsack.

3.1 Outline

Despite progress over the past few years in understanding the role of lipids in placental function and implications of lipid dysfunction in poor pregnancy outcomes, many questions remain. The format of this workshop was highly interactive, providing the attendees an opportunity to join one of two small group discussions (two topics per group) led by experts in the field. The workshop began with Dr. Albrecht and Dr. Hemmings highlighting outstanding questions in each topic area that were raised by the facilitators.

3.2 Summary

There is a lack of understanding as to how much signalling occurs by the high levels of fatty acids and lipids in maternal blood to regulate placental function and how much lipid is utilized by the placenta itself compared to the amount transported to the fetus. What is the gatekeeper that determines how much and what lipid species, for example, goes to the fetus for brain development versus how much is converted to cholesterol for use by the placenta? Is the storage pool versus the amount transferred determined by compartmentalization? New data suggests fatty acid signalling occurs through Toll-like receptor (TLR) or G protein-coupled receptors (GPR) receptors on the trophoblast that impacts function, development and growth of the placenta. Additional data is needed to understand whether lipid levels reflect the mother's diet or the release later in pregnancy of certain fatty acids and lipids stored in maternal adipose tissue in response to signals from the placenta. Can the syncytiotrophoblast determine what lipids are needed by the fetus and only transport those and utilize less critical fatty acids for oxidation? It is well established that lipids are required for fetal

brain development and fat deposition in the fetus with brown fat essential for thermoregulation of the newborn. However, no other primates are born with fat deposits. The possibility was raised during the IFPA meeting (by Professor Roger Smith) that fat is stored in the fetus for utilization in brain development after birth in the event that the lipids from lactation are not optimal.

Dr. Christian Wadsack discussed possible maternal to fetal lipid uptake routes through the placenta, e.g. the selective uptake of cholesteryl-ester derived from maternal derived high-density lipoprotein (HDL) or uptake of the complete HDL that carries lipids like sphingosine 1-phosphate (S1P). Alternatively, S1P can signal through S1P receptors on the trophoblast to generate responses. Dr. Wadsack and Dr. Lewis are using stable isotope labelled fatty acids to study uptake, metabolism and transfer in perfused placentas as a basis for computational modelling. It is unknown whether the uptake of fatty acids are concentration dependent but from the total amount of fatty acids associated with albumin and infused in a placental perfusion system, 5-12% appear to cross to the fetal side suggesting large amounts of lipid are taken up and stored or utilized by the placenta itself. There is evidence both for and against preferential transport of docosahexaenoic acid (DHA) by the placenta. Dr. Wadsack's work suggests that the more unsaturated fatty acids remain in the placenta longer. Dr. Powell and Professor Roger Smith indicated that DHA transfer was slower in placentas from pregnant women with high BMIs.

Dr. Theresa Powell raised the question of whether high triglycerides are toxic to the placenta leading to inflammation in light of some unpublished work showing that some obese women have high triglycerides but small rather than the expected large babies. The group then discussed the presence, function and regulation of lipid droplets in trophoblasts, particularly in light of work presented by Professor Yoel Sadovsky showing that sequestration of lipids by the trophoblasts can protect against lipotoxicity. PPAR gamma may act as a master regulator where the placenta packages excess lipids into intracellular storage droplets. Excess accumulation of lipids in droplets is typically detrimental and human placentas have been observed to contain lipid droplets. Dr. Powell suggested that lipid droplets are only observed under abnormal conditions such as diabetes and that DHA supplementation can alter these levels. Although Dr. Alicia Jawerbaum did not specifically investigate lipid droplets in a diabetic rat model, she found that PUFA-enriched diets increased placental lipid content but reduced lipids in fetal circulation and fetal liver. Sequestration in the placenta is a potential mechanism. It will be important to determine the role of accumulated lipids in the lipid droplets.

Both Dr. Powell and Dr. Jawerbaum raised the concept of transgenerational programming of lipid dysfunction in obesity and maternal high fat diets. The role of PPARs and the potential benefits of PUFA intake during pregnancy were discussed. Lipid droplets were observed in an obese mouse model where fat was deposited in inappropriate organs and the offspring were born with fatty liver disease. Placental desaturase activities are decreased in diabetes, but were not normalized if the obese mothers were put on high PUFA diets. Further work is needed to understand the putative benefits of these diets in preventing programming effects.

Discussions of models to study lipid transport centred on the positives and negatives of using cell lines such as SGHPL4 and Swan 71 (Dr. Ed Johnstone) or

BeWo (Dr. Isabella Caniggia) compared to polarized primary cytotrophoblasts (Dr. Christiane Albrecht), placental explants (Dr. Kent Thornburg and Dr. Isabella Caniggia) or whole lobule perfusion models (Dr. Albrecht, Dr. Lewis and Dr. Wadsack). The use of fluorescent dyes, which are transported and metabolized, to examine lipid droplets compared to the use of stable isotopes was discussed. The validity of very rapid transport of "BODIPY" lipid probes observed using the perfusion system was discussed with a suggestion that the lipophilic nature of the label itself could explain the rapid transfer. The panel discussed the secondary placental lobes of the non-human primates and the normal physiology of bilobed placentas and how these affect lipid transport. The question of whether lipid transport across species was the same arose with no conclusive answer. A high fat diet in macaques leads to a higher stillbirth rate that could indicate a negative impact on transporter uptake and activity. Dr. Caniggia discussed autophagy to raise the question of improving models to examine lipid metabolism and transport by using mass spectrometry to identify and localize lipids that could then be related to protein receptors and/or effectors. Lipid signalling and implantation appears to be an area of research that is understudied. Ceramide polarizes cells in general and could therefore impact lipid transport across the placenta (Dr. Caniggia); however, little appears to be known about the transport of circulating ceramide.

3.3 Conclusions

The group discussions focused on the importance of lipid metabolism for placental function, the tools we have for assessment and the impact of pregnancy complications on both placental usage and transport of lipids to the fetus, e.g. during obesity and diabetes. Identification of outstanding research questions on the role of lipids in placental function and fetal outcomes was a valuable outcome of this workshop. It is hoped that researchers in this field and those who are new will begin to focus on these important questions. We have ignored the lipid status of the mother for too long – it is time to make this a priority.

Conflict of interest statement

None of the authors has any conflict of interest to declare.