Nitric oxide synthase dysfunctionality in the umbilical cord vascular system during twin birth correlates with maturity and birth weight of the neonates

Ph.D. Thesis

Payal Chakraborty

Supervisor

Dr. habil. Edit Hermesz

Associate Professor

University of Szeged, Faculty of Science and Informatics,

Doctoral School of Biology

Szeged

2021

Introduction

Pregnancy has an excess demand for oxygen supply which can easily disrupt the redox homeostasis balance of the both maternal and fetal physiological state. In connection to that, there are increasing evidences of enhanced oxidative insults condition like preeclampsia, ectopic pregnancy, and placental abruption. Moreover, in cases of multiple/twin pregnancy, there is always an additional stress with greater risks of spontaneous miscarriage, preterm delivery, gestational hypertensive disorders, intrauterine growth restriction (IUGR) and perinatal death related to postnatal or neonatal illnesses. Since the last 30 years, by virtue of modern *in-vitro* fertilization therapies the proportion of twin/ multiple pregnancies have markedly increased and showed an elevation in the fetal, neonatal and perinatal mortality rate of 3-6 times in twins and 5-15 times in other multiple pregnancies in comparison to singletons. The mechanism in twinning depends on zygosity; twins can either develop from one zygote (monozygotic, MZ) or from two separate zygotes (dizygotic, DZ). MZ twins make up approximately 30% of twin pregnancies. In general, ~ 16% of twin gestations have discordance *i.e*. difference in the weights of siblings greater than 15-25%. As per the American College of Obstetricians and Gynaecologists discordant growth gets associated with anomalies like IUGR, preterm birth, infection of one of the fetus, stillbirth, umbilical arterial pH <7.10, admission to neonatal intensive care unit, respiratory distress, and death within 1 week of birth.

Fetal homeostatic condition and the intrauterine environment critically depends on the efficiency of the feto-placental circulation. The umbilical cord (UC) vascular system is the sole pathway of oxygen and nutrient transport to the fetus through the placenta. From the placenta, the oxygenated blood and nutrition are transported by the vein to the fetus and arteries carry the deoxygenated blood back to the placenta. In this scenario, it is clear that the UC vein primarily and directly gets exposed to the toxic materials/free radicals unfiltered by or generated in the placenta. Any alteration of it may serve as fingerprints of the damage affecting the *in utero* development. Major complications in fetal development can be directly or indirectly connected to the umbilical cord disorders causing intrauterine hypoxia and/or impaired blood flow to the developing fetus.

The major part of the umbilical cord lacks innervations and hence the vascular tone mainly depends on the level of nitric oxide (NO), i.e. mainly synthesized by endothelial nitric oxide synthase (NOS3). The NO, being a potent vasoactive agent, causes vasodilation and increases the perfusion rate of oxygen and nutrient supply. Any kind of impaired response and loss in the bioactivity of endothelium-derived NO within the umbilical cord vascular system causes intrauterine hypoxia, increased feto-placental vascular resistance and retardation in the fetal growth. NOS3 activation gets regulated by its substrate L-arginine concentration, availability of cofactors, rate of electron transfers, subcellular localization, post-translational modifications and diverse interacting proteins. The process of NOS3 coupling/dimerization is one of the most crucial step towards its activation; low concentration of L-arginine substrate causes uncoupling of NOS3 and thereby becomes dysfunctional. As NOS3 is in direct competition with the Arginase for their common substrate L-arginine, an increased presence of Arginase indirectly influences the NOS3 activation process. Among the post translational modification steps, phosphorylation of NOS3 seems to be important for its activation. In the presence of serine/threonine kinase Akt, NOS3 gets catalytically active by the phosphorylation at Ser1177, whereas Thr495 residue of NOS3 acts as the negative regulatory site, and its phosphorylation is associated with a decreased electron influx.

Under severe oxidative stress conditions, influenced by any intrinsic or extrinsic pathophysiological factors, there are obvious production of reactive oxygen species (ROS)/ strong oxidants; like superoxide anion, hydrogen peroxide and hydroxyl radical. The superoxide anion, in excess, undergoes spontaneous reactions and scavenge the bioavailable NO in the vascular system by forming deleterious pro-oxidant peroxynitrite entity. The major etiological factor for development of the endothelial dysfunction (ED) is the reduced production/bioavailability of NO, resulting in an impaired endothelial growth and vasodilation.

UC endothelium comes in direct contact with all the blood components and especially with the predominant circulating red blood cells (RBCs). Mainly RBCs were ascribed as carriers for

transmitting oxygen and NO into the vascular bed. Emergence of a recent, ground breaking publication presented the evidence for a NO synthase protein within the RBCs (RBC-NOS3). Supposedly, there exists an intimate crosstalk between the vascular endothelium and circulating RBCs. Thereby, ED can be sensed by circulating RBCs which might further increase the production of NO by the RBC-NOS3 activation pathway.

Aims

The presence of intrauterine hypoxia causes excessive production of ROS and leads to fetal oxidative stress, which variedly contributes to the occurrence of pathological conditions in post-natal or adult life. From the clinical purview, comparison of singleton versus twin neonates always experience an enhanced intrauterine hypoxia with advanced cellular stress conditions, that might directly or indirectly gets associated with several RBC and umbilical cord disorders. Establishment of an efficient cross-talk between the vascular endothelium and circulating RBCs is necessary to improve the rate of blood flow in the cord vessels by vasodilation. The developmental status of neonates is mainly determined by a number of multifactorial and complex processes, where the involvement of the NOS3 signalling pathway and its subsequent activation steps becomes pivotal. The NOS3 pathway significantly controls the level of bioavailable NO and in this way can modulate the supply of oxygen and nutrition to the developing fetus.

In our work, taking into account the highly hypoxic intrauterine circumstances due to twin pregnancy, we have pursued the molecular and functional consequences and its harmful effects on the vascular cord endothelial layer and the circulating RBCs of the developing fetuses. We searched for major alterations in the regulatory parameters which play crucial roles in the NOS3 signalling pathway to maintain the bioavailable NO level. Further we tried to shed light on the activation of any rescue/or compensatory mechanisms to combat higher demand of bioavailable NO in both the umbilical cord vessels and fetal circulating RBCs. In detail following the NOS signalling pathway our study targeted to identify an early marker for cardiovascular disorders and/or other relevant comorbidities.

In connection to this, we studied in detail to find answers for the following questions: -

- How alterations in the NOS3 expression and its post translational modifications along with the ROS-inducible NOS3 competitor of Arginase1 expression affects the functionality of the UC endothelial layer and circulating fetal RBCs?
- Is there any evidence of the circulating fetal RBCs that can show a compensatory or alternative mechanism to increase the bioavailable NO level in the vessels?
- What is the level of free radicals' /oxidants' formation in the RBCs derived from the twins and the singletons?
- What are the consequences/extent of macromolecular damages occurred due to higher level of pro-oxidants both in the UC vessels and circulating RBCs?
- What are the significant changes in the distribution pattern of different phenotypic variants in the RBC populations derived from singletons and twin population?
- Is there any connection between the birth weight of the fetus and the expression/activation of NOS3 system in the UC vessels and in the circulating RBC population?

Materials and methods

1. Collection and processing of Human samples originated from pre mature and mature single and twin pregnancies; umbilical cords and cord blood samples.
2. Eosin stained blood smear image processing and data analysis by an intelligent analysis software platform like the Advanced Cell Classifier
3. Immunolabelling and image processing of the umbilical cord sections and blood samples
4. Immunohistochemistry on the isolated RBCs and analysis by florescence activated cell sorting (FACS)
5. Measurement of hydrogen peroxide production in the RBCs populations
6. Determination of the peroxynitrite level in the RBCs populations
7. Assay for viability of RBCs

Results and Conclusion

In our work, we followed significant changes involved in the activation process of NOS3 that might affect the redox status and functionality of both the UC vessel's endothelial layer and RBCs- with a direct correlation on the birth weight and maturity level of the new born twins. Our study selected the parameters that influences the dimerization step of the NOS3 enzyme, in presence of the enzyme Arginase1. Secondly, an upregulation of inducible NOS (NOS2) enzyme might be an alternative approach towards a compensatory/rescue mechanism. Hence we collected and examined the umbilical cord and blood samples from both mature and preterm twins and neonates born from singleton pregnancies with different percentile values.

Our main findings can be summarized as follows:

(1) During twin pregnancy, the activation of NOS3 pathway in the umbilical cord vessels depends on the maturity level of the developing fetus - mature: > 37 weeks and preterm: 33-35 weeks' birth – that follows a vascular-specific regulation:

(a) Expression and phosphorylation of NOS3 at position Ser1177 in the umbilical vein endothelial cells of premature twin siblings is comparable to age matched neonates, regardless of the neonatal birthweight.

(b) Contrastingly, in matured twins, NOS3 expression and its activation by phosphorylation at Ser1177 residue is significantly lower than the level measured to their age matched singletons irrespective of their birthweight.

(c) In the umbilical cord arteries, with exception to the mature and premature normal-birthweight neonates, there is no significant changes detected in the NOS3 expression/activation when compared to their age matched singletons.

(2) Except the mature twins with normal birthweight, in all the other examined population showed, that the RBC-NOS3-NO pathway under hypoxic conditions may significantly contribute to the improvement of vascular tone and thus able to maintain the fetal blood flow due to an elevation in the NOS3 expression and/or activation.

(3) Apart from the preterm neonates having gestational age of 95th percentile, most likely the levels of bioavailable NOS3-NO and RBC-NOS3-NO are unsatisfactory in all the other investigated groups, and thus an alternative NO-producing pathway NOS2, is highly induced to improve the fetal blood flow.

(4) The expression level of Arginase1 in the RBCs of twin neonates is increased, which is partly due to the excessive presence of superoxide anions in the vascular system.

(5) In case of twin pregnancies, the level of pro-oxidant peroxynitrite anion in fetal RBCs is increased, which can also be associated with the higher availability of the superoxide anions.

(6) Increased macromolecular damages was observed in connection to the elevated levels of the peroxynitrite anion, which was detected and determined by measuring the accumulation of 4-hydroxynonenal (4-HNE) level. Irrespective of maturity, arteries and circulating RBCs were primarily affected. It is also shown that at the matured stages of twin pregnancy, the 4-HNE levels gets significantly increased in the arteries.

(7) Irrespective of the maturity level in the twin neonates, there occurs an increased frequency of morphological variants in the fetal RBCs. These phenotypic differences may be a useful indicator for any disturbances in the functional and/or physicochemical properties of the RBCs.

Funding

This work was supported by the European Union and the Hungarian Government in the framework of the GINOP-2.3.2-15-2016-00040 project.

List of Publications

Number of scientific publications: **6**

Total impact factor: **31.695**

MTMT identification number: **10053026**

1. **Payal Chakraborty**, Krisztina N. Dugmonits, Hajnalka Orvos, Edit Hermesz. Mature Twin Neonates Exhibit Oxidative Stress via Nitric Oxide Synthase Dysfunctionality: A Prognostic Stress Marker in the Red Blood Cells and Umbilical Cord Vessels. Antioxidants (2020); 9(9):845. doi: 10.3390/antiox9090845. **IF: 6.312**

2**. Payal Chakraborty**, Ali Khamit, Edit Hermesz. Fetal oxygen supply can be improved by an effective cross-talk between fetal red blood cells and the vascular endothelial layer. Biochim Biophys Acta Mol Basis Dis (2021); 1867(11), 166243. doi: 10.1016/j.bbadis.2021.166243. **IF: 5.187**

3. Balogh Gábor*, **Chakraborty Payal***, Dugmonits Krisztina*, Péter Mária*, Végh Attila G., Vígh László, Hermesz Edit. Sustained maternal smoking-associated changes in the physico-chemical properties of fetal RBC membranes might serve as early markers for vascular comorbidities. Biochim Biophys Acta Mol Cell Biol Lipids (2020); 1865(4):158615. doi: 10.1016/j.bbalip.2020.158615. **IF: 4.698**

4. **Chakraborty P***, Dugmonits KN*, Végh AG, Hollandi R, Horváth P, Maléth J, Hegyi P, Németh G, Hermesz E. Failure in the compensatory mechanism in red blood cells due to sustained smoking during pregnancy. Chem Biol Interact (2019); 313:108821. doi: 10.1016/j.cbi.2019.108821. **IF: 5.192**
*** Co-first authorship**

5. Dugmonits KN, **Chakraborty P**, Hollandi R, Zahorán S, Pankotai-Bodó G, Horváth P, Orvos H, Hermesz E. Maternal Smoking Highly Affects the Function, Membrane Integrity, and Rheological Properties in Fetal Red Blood Cells. Oxid Med Cell Longev. (2019); 1509798. doi: 10.1155/2019/1509798. **IF: 6.543**

6. Nikolett Bódi, Diána Mezei, **Payal Chakraborty**, Zita Szalai, Bence Pál Barta, János Balázs, Zsolt Rázga, Edit Hermesz, Mária Bagyánszki. Correlation between the region-specific thickening of ganglionic basement membrane and regionally decreased matrix metalloproteinase 9 expression in myenteric ganglia and its environment in type 1 diabetes. World Journal of Diabetes. (2021); 12(5): 658–672. doi: 10.4239/wjd. v12.i5.658. **IF: 3.763**

Supervisor Declaration

As supervisor of the doctoral candidate and being the corresponding author of the publications listed below, I declare that the data set, used in the dissertation reflects the significant contribution and involvement of the candidate to the articles listed as 1 and 2. Furthermore, results reported in these publications were not previously used to acquire any type of degree.

Szeged, 09/02/ 2021.

_____

Dr. Edit Hermesz
Associate professor