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Pravastatin and-L-arginine combination improves umbilical artery blood flow and neonatal outcomes in dichorionic twin pregnancies through an nitric oxide-dependent vasorelaxant effect

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ABSTRACT

The increase in fetal and neonatal morbidity and mortality associated with twin pregnancies correlates with an increased risk of preterm delivery, low birth weight, and intrauterine growth restriction (IUGR). Although the pathogenesis of IUGR is unclear and thus management remains a major challenge, feto-placental blood vessels are compromised, and altered umbilical blood flow is observed. In this pilot observational study we investigated the effects of pravastatin plus L-arginine on umbilical artery (umb art) blood flow. Between 2013 and 2016, five women received daily doses L-arginine and pravastatin when an umb art pulsatility index above limits for gestational age was observed and concerns about selective growth restrictions arose. All patients showed selective absent or reversed end-diastolic umbilical artery Doppler flow (AREDV) associated with increased perinatal mortality. Pravastatin (PRAV) plus L-arginine (L-Arg) treatment diminished umb art resistance significantly and allowed pregnancy to continue. No signs of acidosis or hypoxia, normal cardiotocography tracing, normal fetal movement and fetal weight gain were observed in the twins that showed abnormal umb art Dopplers. All neonates were born around 33 weeks (median 33 weeks, IQR [31.4–33.0]), thus diminishing substantially the chances for any prematurity-associated adverse neonatal outcomes. The infants now show normal growth and development. In in vitro studies, pravastatin induced relaxation of aortic rings. Murine studies identified were performed to investigate the mechanism behind PRAV + L-Arg beneficial effects. A nitric oxide (NO)-dependent synergistic vasorelaxant effect of PRAV + L-Arg was demonstrated using aortic rings. Increased levels of placental NO and increased synthesis of eNOS in placental endothelial cells were observed in mice treated with PRAV + L-Arg compared to untreated mice and mice treated with PRAV- or L-Arg alone.

This study suggests that PRAV plus L-Arg might be a good therapeutic option to improve blood flow in umbilical arteries prolonging pregnancy and improving pregnancy outcomes in twins. A RCT should be organized to confirm these results.

1. Introduction

The incidence of twin pregnancy is increasing, mainly due to delayed childbirth and advanced maternal age at conception and the extensive use of assisted reproduction techniques [1]. Twin pregnancies are associated with a greater risk of preterm delivery, low birth weight, intrauterine growth restriction (IUGR) and intrauterine fetal death (IUFD). A fivefold increase in the rate of stillbirths is observed in dichorionic twins (DC) compared with singleton pregnancies. Preterm birth occurs in up to 60% of twin pregnancies, contributing to the increased risk of neonatal mortality [2]. The recent global drive to prevent stillbirth has highlighted multiple pregnancy as a major risk factor [2].

IUGR, strongly associated with fetal demise, is commonly observed in twins, with an incidence of 25–35%, and can affect one or both fetuses. In the IUGR surviving fetuses, a positive correlation between low birth weight, abnormal neurodevelopment and increased risk of adult cardiovascular disease have been documented [3].

Although the pathogenesis of IUGR is unclear and thus management remains a major challenge, feto-placental blood vessels are

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compromised, resulting in altered umbilical artery (Umb art) blood flow in the severest cases.

Severe reduction in Umb art blood flow as reflected by absent or reverse end diastolic velocity (A/REDV) during pregnancy is highly associated with fetal morbility and mortality and expeditious delivery should be contemplated to prevent IUFD.

L-arginine (L-Arg), a precursor for synthesis of vasodilator nitric oxide (NO), has been used to treat IUGR in humans and sheep [4, 5]. While some studies have associated L-arginine supplementation to improved placental blood flow and increased birth weight compared to standard care, other studies including a well-designed double blinded trial of oral arginine versus placebo failed to show a benefit in fetal growth [6]. Efforts to develop effective treatments to improve umbilical arteries blood flow and prevent IUGR and IUFD would be of important clinical significance.

In a recent study, pravastatin treatment improved uterine arteries blood flow and pregnancy outcomes in women with obstetric antiphospholipid syndrome and placental insufficiency-related pregnancy complications: preeclampsia and IUGR [7]. Among the many pleiotropic effects attributed to statins, increased nitric oxide (NO) synthesis, vascular dilatation and anti-inflammatory and antithrombotic effects are described [8]. Thus, there is a strong basis for using pravastatin to improve umbilical arteries (Umb Art) blood flow, ameliorate IUGR and protect twin pregnancies. Pravastatin showed not to be teratogenic in mice and women [9–11]. Five women with twin pregnancies that presented umb art pulsatility index above limits for gestational age and thus at risk of fetal growth restriction and death were treated with L-arginine and pravastatin. We hypothesized that this combined therapy might have a synergistic modulatory effect on nitric oxide synthesis leading to Umb art vasodilation, blood flow improvement and prevention of growth restriction and IUFD in dichorionic twin pregnancies. In vitro studies, using aortic rings and isolated placental endothelial cells from mice were performed to test our hypothesis.

2. Methods

2.1. Human studies

From 2013 to 2016, five women (median age 37, IQR [33.5–38.5]) with dichorionic twin pregnancies and selective abnormal Umb art Dopplers were treated with L-arginine and pravastatin to prevent IUGR and IUFD. None of them had previous live births. Four of them conceived after in vitro fertilization. Normal fetal development and normal placental haemodynamics were observed until the end of the second trimester (median: 23 weeks, IQR [21.3–23.7]), when selective Umb art pulsatility index (PI) above limits for gestational age was detected. At this time, all women were treated with daily oral doses of pravastatin (PRAV, 40 mg) and L-arginine (L-Arg, 1.5 g) until the end of the pregnancy. Patients were scanned every week to monitor changes in vascular resistance in Umb Art and fetal well-being and growth. Uterine arteries Doppler ultrasound parameters were normal throughout pregnancy in all patients. Approval for investigational drug use was obtained from the Narodni Front University Hospital Ethical Review Committee at the University of Belgrade Medical School, Serbia and written informed consent was obtained from all pregnant patients. All research was performed in accordance with relevant guidelines and regulations.

2.1.1. Ultrasonography of pregnancies

Doppler examinations were performed by two examiners (AJ and ZJ) using RM6C matrix 4D convex probe (Voluson E10, GE Healthcare) and V4-8 4D convex probe (Medison V20 Prestige, Korea) with the high-pass filter at 60 Hz. Spectral Doppler analysis of flow velocity waveforms in fetal blood vessels (middle cerebral artery, umbilical artery) was performed after obtaining a minimum of 10 heart rate cycles without fetal movement and fetal breathing. When AEDV was detected in umbilical artery, measurements were repeated 3 times on different free loops of umbilical cord.

The following variables were recorded: i) Umbilical artery pulsatility index (Umb Art PI) obtained from a free loop of the umbilical cord; ii) middle cerebral artery (MCA) pulsatility index (PI) measured in the straight portion of the artery avoiding head compression by the transducer and iii) the cerebroplacental ratio (CPR) was calculated as the ratio of the MCA PI divided by the Umb Art PI.

2.2. Animal studies

All animal studies were performed in accordance with the 1986 UK Home Office Animal Procedures Act. Approval was provided by the local ethics committee, Animal Welfare & Ethics Board (AWERB).

FWB & St Thomas' Hospital at King's College London. C57BL/6 mice were maintained at 20–22 °C, with standard rodent chow available ad libitum and under 12:12 h light dark schedule. Non pregnant and day 15 pregnant C57BL/6 females were used in this study. After mating, the presence of a vaginal plug was considered day 0 of pregnancy. Animals were killed by cervical dislocation and the thoracic aortas were removed and placed into ice cold physiological salt solution for further analysis of vascular reactivity using wire myography. A group of pregnant (15 dpc) females were treated with pravastatin (PRAV (0.6 mg/mouse, intraperitoneal Sigma Aldrich), L-Arginine (L-Arg, 0.5 mg/mouse) or PRAV + L-Arg 4 h prior to the isolation of the aortas and placentas for endothelial cell isolation and nitric oxide production measurements. These doses are equivalent to the doses of 40 mg of PRAV and 1.5 g L-Arg used in the human studies.

2.2.1. Wire myography

To examine the effects of L-Arg and PRAV on vascular reactivity, aortic rings and wire myography were used [2]. The aorta was dissected, cleaned of surrounding fat, cut into approximately 2.5 mm rings and mounted into a chamber unit in a multi-wire myograph system (610 M, Danish Nyo Technology, Denmark). Vessel tension data was recorded and stored on a computer using Myodaq 2.02 analysis software (Danish Nyo Technology, Denmark). Vessels were then left to equilibrate for 30 min in physiological salt solution (PSS) and then subject to constriction with 125 mM potassium - substituted PSS (KPSS) as previously described [12]. Following incubations with KPSS, vessels were constricted with 50 µL of 10^{-4}mol/L phenylephrine (Sigma Aldrich, UK) to induce a 80% maximum contraction in arteries. After vasoconstriction with phenylephrine, incubations with PRAV (10 ng/mL, Sigma Aldrich) and L-Arg (350 µM, Sigma Aldrich), alone or in combination (L-Arg added 30 min before pravastatin), were performed to assess vasorelaxant effects. Pravastatin concentration was calculated based on the studies performed using the ex vivo technique of dual perfusion of placental lobule [13]. The perfused placenta studies showed that 18 ± 4% of the perfusion concentration (50 ng/mL equivalent to an in vivo dose of 40 mg [13]) was transferred to the fetal circuit at the end of a 4-hour perfusion [13]. L-Arg concentration was calculated based on the dose used in humans and a volume of distribution of 24 L [14].

Incubation for 30 min with nitric oxide (NO) synthase inhibitor NG-nitro-L-arginine methyl ester (100 µL 10^{-2} M L-NAME; Sigma Aldrich, UK) prior to the addition of pravastatin and L-Arg was performed to evaluate the role of NO in the effects these drugs on the vascular tone. Aortic rings with intact endothelium from pregnant mice (untreated and treated with PRAV, L-Arg and PRAV + L-Arg) were incubated with acetyl choline (Ach, 10^{-6}M, Sigma Aldrich) after preconstriction with phenylephrine to evaluate NO-dependent vasorelaxation.

After the experiments, contractile ability of the blood vessels was tested. Vessels which failed to contract to KPSS or phenylephrine were not included in the study.