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# Fetal asphyctic preconditioning in rats results in a preserved placental inflammatory phenotype at birth

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ABSTRACT

*Introduction:* Perinatal asphyxia (PA) is a major cause of neonatal mortality and morbidity. Research has shown that in rats fetal asphyxia (FA) can provoke neuroprotection against a subsequent more severe perinatal asphyctic insult. This is called fetal asphyctic preconditioning (PC). Our objective was to investigate alterations in the placental inflammatory phenotype associated with PC.
*Methods:* FA was induced in the rat at embryonic day 17 by reversibly clamping the uterine circulation and PA was induced at birth by submersion of the uterine horns in a saline bath for 19 min. The effect of PC was studied by inducing FA at E17, followed by PA at E21. Placental TNF-α, IL-1β, IL-6 and IL-10 mRNA and protein levels were measured by qPCR and ELISA.
*Results:* IL-1β mRNA increased in the labouring FA group, but IL-1β protein decreased after both FA and PA. In the PC group, IL-1β mRNA and protein levels were similar to controls. IL-6 protein increased 6 h after FA, however decreased 24 h after FA. IL-6 mRNA was higher in the labouring PA group. IL-10 protein decreased 24 h after FA. At birth, IL-10 mRNA increased in the PA group; however, IL-10 protein decreased in both the PA and the FA group. In the PC group, IL-10 mRNA and protein were similar to control levels.
*Discussion:* Depleted protein concentrations of IL-10 and IL-1β after one single asphyctic insult were reversed after fetal asphyctic PC. In addition, PC placentas showed less up-regulation of IL-6 mRNA compared to the PA ones. This modulated placental inflammatory phenotype might contribute to the improved neonatal outcome showed after fetal asphyctic PC.


## 1. Introduction

Pre- and perinatal asphyxia are caused by fetal lack of oxygen (hypoxia) and reduced blood flow (ischemia). It is a major cause of neonatal mortality and morbidity [1,2], leading to infant neuronal injury and impaired neurodevelopment [3–5]. To date, there is no effective therapy, except for post-asphyctic hypothermia in term infants [6–8]. Nevertheless, this intervention is only effective in moderate cases and therefore there is an urgent need to develop new therapeutic alternatives.

Interestingly, we have previously reported that in rats sub-lethal fetal asphyxia (FA) can induce neuroprotection against a subsequent more severe perinatal asphyctic insult. In this unique experimental rat model of acute global feto-placental asphyxia, FA was induced by clamping the uterine arteries for 30 min at embryonic day 17 (E17). Perinatal asphyxia (PA) was induced at E21 (P0) by submersing the uterine horns in saline bath for 19 min [10]. The FA insult acted as a preconditioning (PC) stimulus that subsequently improved animal outcome after a PA insult. PC resulted in

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decreased perinatal mortality rates, short-term protection against vascular alterations, decreased apoptosis and attenuated astrogliosis in the striatum, prefrontal cortex and hippocampus and long-term preserved motor and cognition functions [9–11]. Moreover, recent studies performed by our research group show changes in the brain following fetal asphyctic PC (genomic reprogramming in the fetus and altered transcription in the neonate) [12,13]. A better understanding of the precise mechanisms underlying the protective endogenous phenomenon could offer specific targets for the development of new neuroprotective therapies.

We have shown that changes in pro- and anti-inflammatory cytokines in the developing rat brain and cerebellum may play a role in the induction of neuroprotection at birth [14,15], although no chronic changes could be detected [16]. The acute adapted inflammatory response following asphyctic PC was also seen in the liver [17]. Knowing that the placenta is a critical organ for fetal wellbeing and that it is also subjected to asphyxia, we sought to analyze if the placenta plays a role in fetal asphyctic PC by modulating the inflammatory process. A proper balance between pro- and anti-inflammatory placental cytokines is commonly considered critical for a successful pregnancy [18].

Although there is no current literature describing the production of placental cytokines in a model of acute pre- and perinatal asphyxia, both acute and chronic hypoxia are known to influence cytokine levels in non-gravid [19,20] and gravid conditions [21,22]. It has been reported that cultured human placental tissue secretes pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β) and interleukin 6 (IL-6) [21]. Despite the fact that IL-6 is produced by a healthy placenta, this cytokine is generally viewed as detrimental to pregnancy [18]. It is also known that increased levels of TNF-α and IL-1β can contribute to impaired placental angiogenesis and endothelial dysfunction observed in preeclampsia [21,23]. In contrast, the anti-inflammatory and immunoregulatory cytokine interleukin 10 (IL-10) is believed to be beneficial [18]. The placental bed presents a high IL-10 secretory pattern [24,25].

To gain more insight regarding the placental inflammatory reaction ensuing pre- and perinatal asphyxia and the potential protective role of the placenta during asphyctic PC, we chose to investigate both placental mRNA and protein levels of TNF-α, IL-1β, IL-6 and IL-10 in prenatal and labouring rat placentas. To the best of our knowledge, this is the first report attempting to determine these levels in a clinically relevant rat model of global feto-placental asphyxia. Most studies have used models of focal brain asphyxia, which are not representative of a global insult and do not include the placental compartment. Moreover, the rat model is one of the most appropriate placental animal models since rodents and humans both exhibit haemochorial placentation. This model provides us the unique opportunity to elucidate involved placental inflammatory mechanisms and explore their contribution to PC.

We hypothesized a lower inflammatory response in the placentas of PC fetuses, potentially reflected by an increased placental production of the anti-inflammatory cytokine IL-10 and/or a reduced production of the pro-inflammatory cytokines, analogous to the down-regulation of the pro-inflammatory cytokines occurring in the preconditioned central nervous system [14,15].

## 2. Methods

### 2.1. Animals and placental tissue processing

All animal experiments were approved by the Animal Ethics Board of Maastricht University according to Dutch governmental regulations. Timed-pregnant Sprague–Dawley rats (Charles River, France) were housed individually under standard laboratory conditions in which food and water were given ad libitum. Pregnant rats (n = 22) were randomly assigned to an experimental group and euthanized afterwards by decapitation [10]. Placental tissues were collected by hysterectomy at several time points. Placentas were snap-frozen in liquid nitrogen after clamping and cutting the umbilical cords and assessing placental weight. Control and FA placentas were collected immediately after hysterectomy, PA placentas were collected following the PA procedure. All placenta samples were then preserved at −80 °C until further analysis.

### 2.2. Study groups

An overview of study groups is given in Table 1. Nine study groups were included providing a total of 44 placental samples.

The prenatal placentas (FA and Control) were collected by hysterectomy at three different time points (E17, E18 and E21 before onset of labour). The non-labour control group at E21 was included to determine the effect of birth on placental cytokine levels.

The labouring placentas (FA, Control, PA, and PC) were collected at term birth (E21). To assure full-term pregnancy, hysterectomy was only performed after the vaginal delivery of the first-born pup. The effect of preconditioning (PC) was studied by inducing FA at E17, followed by PA at E21. Control placentas were collected by hysterectomy from untreated mothers.

### 2.3. Fetal asphyxia (FA) procedure

#### 2.3.1. FA was induced at embryonic day 17 (E17)

Pregnant rats were anesthetized with isoflurane (1.5–2.0%). The uterine horns were exposed by performing a midline laparotomy. Both uterine and ovarian arteries were clamped with removable clamps. After 30 min reperfusion was permitted by detaching the clamps, the uterine horns were placed back intra-abdominally and the abdominal cavity was closed. (Fig. 1A and B) [10]. The FA procedure did not induce fetal death.

### 2.4. Perinatal asphyxia (PA) procedure

After the first pup was born per vaginam at E21 (Fig. 1A) pregnant rats were euthanized by decapitation and immediately hysterectomized (E21/P0). In order to induce severe PA the uterine horns containing the fetuses and placentas were detached and submersed in a saline bath (0.9% NaCl, 37 °C) for 19 min (Fig. 1B). Afterwards, the umbilical cords were ligated and cut to separate the placentas from the pups.

### 2.5. Assessment of cytokine mRNA levels

Total RNA was prepared from 50 mg of placental tissue using Trizol Reagent (Invitrogen, Breda, Netherlands) according to manufacturer's instructions. mRNA quality and quantity were measured using a NanoDrop spectrophotometer (ND-1000 V3.3.0, Thermo Fisher Scientific Inc, USA). Reverse transcription was achieved using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc, USA), also according to manufacturer's guidelines. cDNA was amplified in duplicate using real time PCR in a Lightcycler® 480 instrument (SYBR Green 1 Master mix, Roche Applied Science, Mannheim, Germany). A comparative cycle of threshold fluorescence (Ct) method was used. Values are reported relative to the geomean mRNA expression of two housekeeping genes. Primer sequences are reported in Table 2. Quantification cycle values were extracted with the Lightcycler 480 software (Conversion LC and Linge PCR).