The Significance of Fetal Inflammation in the Pathogenesis of Perinatal Brain Injury

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Abstract
Clinical, epidemiological and experimental studies indicate that in-utero exposure to infection plays an important role in the pathogenesis of fetal or neonatal injury leading to cerebral palsy and chronic lung disease. Thus, after chorioamnionitis the incidence of immature neonates suffering from periventricular white matter damage and peri- or intraventricular hemorrhage is significantly increased. Recent clinical and experimental data support the hypothesis that a fetal inflammatory response links antenatal infection with brain white matter damage and subsequent motor handicap. A variety of studies support the view that cytokines released during intrauterine infection directly cause injury to the immature brain. In this review article we provide evidence that in-utero exposure to bacterial infection may severely alter fetal cardiovascular function, resulting in dysregulation of cerebral blood flow and subsequent hypoxic-ischemic brain injury.

Keywords: intrauterine infection, brain damage, cytokine, chorioamnionitis, newborn

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Introduction
Despite improvements in perinatal medicine, the prevalence of cerebral palsy has risen over the last two decades (1), and the etiology of cerebral palsy remains poorly understood. As demonstrated by a variety of recent clinical and epidemiological studies, inflammatory reactions not only aggravate secondary neuronal damage after cerebral ischemia, but may also affect the immature brain directly. Thus, after chorioamnionitis the incidence of periventricular leukomalacia and peri- or intraventricular hemorrhage in immature newborn infants is significantly increased (2-7). Recently published articles on this topic provide evidence that chorioamnionitis gives rise to a fetal inflammatory response, and that this inflammation contributes to neonatal brain injury and subsequent cerebral palsy (2,6,8,9). Reports of elevated cytokine levels in both neonatal blood (10,11) and amniotic fluid (12,13) in children with cerebral palsy support the notion that cerebral palsy is preceded by a perinatal inflammatory disease. However, the pathogenesis of infection related neuronal and oligodendroglial cell damage remains unclear. Various experimental studies provide evidence that impaired fetal cardiovascular control during endotoxemia, resulting in sustained hypotension and a loss of cerebral autoregulation, precedes hypoxic-ischemic brain injury. The current studies on direct cytotoxic effects of endotoxins and proinflammatory cytokines on cerebral tissue are summarized in the first part of this article. The second part reviews the experimental data available on endotoxemia-induced alterations of the fetal cardiovascular function and outline their significance in the pathogenesis of infection related perinatal brain injury.
Clinical Data and Definitions

Clinical chorioamnionitis is an infection of the uterus and its contents during pregnancy. Its diagnosis is based on the presence of fever (T > 38°C) plus two or more of the following conditions: maternal tachycardia, fetal tachycardia, uterine tenderness, foul smelling of the amniotic fluid, or maternal leukocytosis (14). The incidence varies between 10 and 20% (15).

Histologically, chorioamnionitis is defined by the presence of polymorphonuclear infiltrates in the placenta and its membranes. It affects 20% of term pregnancies and up to 60% of preterm pregnancies and is often an occult finding (16). Recently, Dasse and co-workers (17) reported a simultaneous diagnosis of histological chorioamnionitis in mothers with clinical chorioamnionitis in 80% of cases, while 20% had no histological evidence of infection. Furthermore, Grether and Nelson (5) reported that both clinical and histopathological evidence of placental infection were associated with an increased risk of unexplained cerebral palsy (CP) [OR 9.3, 95% CI 2.7-31 for clinical chorioamnionitis (CA); OR 8.9, 95% CI 1.9-40 for histological CA]. Recently, a meta-analysis confirmed the potential association between CA and CP, in both full term and preterm infants (18).

I. INFECTION RELATED PERINATAL BRAIN DAMAGE: AN OVERVIEW

A wealth of experimental studies has emerged describing the pathophysiological mechanisms implicated in perinatal brain injury in response to hypoxia-ischemia. These involve the acute breakdown of cerebral energy metabolism leading to the release of excitatory amino acids such as glutamate. Glutamate binds to postsynaptically located glutamate receptors that regulate calcium channels (19). The resulting calcium influx, so-called calcium overload, activates proteases, lipases and endonucleases which in turn destroy the cellular skeleton (20). A second wave of neuronal cell damage occurs during the reperfusion phase. This cell damage is thought to be caused by the posts ischemic release of oxygen radicals, synthesis of nitric oxide, inflammatory reactions and an imbalance between the excitatory and inhibitory neurotransmitter systems (for review: 21).

A. Direct neurotoxic effects of endotoxins and cytokines followed by brain white matter injury (27)

An increasing body of evidence shows that endotoxins and released proinflammatory cytokines may also damage the fetal brain directly, especially the periventricular white matter. Thus, animal studies provide evidence that administration of endotoxin induces increased cytokine expression in adult rat brains (22-24) and in fetal hippocampal tissue (25). A rise in the release of TNF-α, in particular, has been thought to be associated with brain injury (26). In addition, increased expression of IL-1β and TNF-α mRNA has been observed in the brain of fetal rats after intraperitoneal application of LPS to the dam. This was followed by white matter injury (27). Similar observations have been described in immature rabbits after uterine infection with bacteria (28) and in newborn kittens after intraperitoneal injection of LPS (29).

Since PVL, the common type of fetal brain injury associated with ascending intrauterine infection, occurs well before the onset of active myelination, it has been suggested that cytokines may damage the progenitors of oligodendrocytes directly (4,30). In fact, inhibitory effects of TNF-α on the proliferation of enriched oligodendrocyte progenitors and their subsequent differentiation into mature myelinating oligodendrocytes have been reported (31). Moreover, it has been shown that TNF-α could compromise the growth of oligodendrocytes and the expression of mRNA for myelin basic protein (MBP) in cultures of mixed glial cells from rodent brains and be cytotoxic at high concentrations (32-34). The combined application of TNF-α and IFN-γ severely reduced survival and inhibited differentiation of oligodendrocyte progenitors in a primary culture prepared from neonatal rats (35). It is obvious that loss or functional alteration of these oligodendrocyte progenitors may underlie the disrupted myelination that characterises PVL.

B. LPS sensitizes the fetal brain to subsequent hypoxic-ischemic insults

Recently, Eklind and co-workers (36) reported a sensitization of the 7-day-old neonatal rat brain to hypoxic-ischemic insults by intraperitoneal injection of LPS. Moreover, it has been demonstrated in neonatal rat pups that LPS applied intracisternally enhances susceptibility to subsequent hypoxic ischemic brain damage (37).

II. IMPAIRED FETAL CARDIOVASCULAR CONTROL DURING ENDOTOXEMIA

A. Fetal circulatory redistribution induced by endotoxin

In addition to direct cytotoxic effects of cytokines on cerebral tissue, impaired fetal cardiovascular control during endotoxemia/bacteremia resulting in a loss of cerebral autoregulation may contribute to fetal brain injury (Figure 1). Lipopolysaccharide (LPS), an endotoxin extracted from the cell wall of gram-negative bacteria, seems to have a major impact in the pathophysiology of infection related perinatal brain injury. The effects of systemically administered LPS on the fetal cardiovascular function have been studied in various animal models. In chronically instrumented premature fetal sheep (0.7 of gestation) i.v. injection of LPS (E. coli; O127:B8, Sigma-Aldrich; 53 ± 3 μg per kg fetal BW) severely decreased placental blood flow within 1 hour, while blood flow to the peripheral organs, e.g. to the carcass rose (38). During a short period of superimposed asphyxia in utero there was clear evidence of circulatory de-centralization, i.e. both placental blood flow and cerebral oxygen delivery were nearly arrested, while hyperperfusion of peripheral organs, i.e. liver, lungs, gastro-intestinal tract, and carcass occurred (Figure 2). Since the umbilical and placental vessels lack autonomic innervation (39), the regulation of umbilical and placental blood flow must depend on circulating or locally released vasoactive substances (for review: 40). In fetal sheep the placental microcirculation is remarkably inert to many
vasoconstrictors, including norepinephrine and angiotensin II, whereas the umbilical artery and vein are more responsive to vasoactive substances (41,42). The decrease in placental blood flow was accompanied by sustained hypotension, hypoxemia and mixed acidosis causing dysregulation of cerebral blood flow.

In contrast to the findings in immature fetal sheep, mature fetuses appear to be less sensitive to endotoxin administered systemically. It was demonstrated previously that i.v. LPS (E. coli; 50 μg/kg fetal BW) treatment compromises fetal cardiovascular control during and shortly after in-utero asphyxia (43). After injection of endotoxin there were increases in arterial blood pressure, as well as in concentrations of hemoglobin, glucose, lactate, catecholamines, vasopressin, and angiotensin II and a decrease in base excess and granulocyte counts, while both arterial oxygen saturation and pO2 remained unchanged. Blood flow to the brain, placenta, and carcass decreased, while that to the lungs, heart, pituitary, gastro-intestinal-tract, pancreas, and liver increased. During asphyxia, blood flow to the brain did not increase, thus circulatory centralization was impaired. However, in contrast to immature fetuses all these changes occurred transiently and recovered partially within 1h of recovery (43).

B. Chronic LPS exposure
Recently, the effects of low dose LPS-treatment on cardiovascular control were studied in 0.7 gestation fetal sheep (Figure 3). Intravenous application of LPS to the premature fetus (E. coli; O127:B8, Sigma-Aldrich; 100 ng/kg fetal BW) caused a substantial and longlasting decrease in umbilical blood flow resulting in sustained fetal hypoxemia without acidemia (44,45). Placental blood flow began to fall 1 h after LPS and was Lowest (-40%) at 4 h -5 h after LPS (Figure 3), while placental vascular resistance rose by 75% during this period. Thereafter, placental blood flow slowly
returned to control values at 12 h - 16 h after LPS application. Both, fetal heart rate and mean arterial blood pressure increased at 4 - 5 h after LPS infusion and remained elevated for the following 12 h. Histopathological examination revealed an increase in the periventricular white matter cell count, accompanied by intense and uniform nuclear staining and chromatin condensation with karyorrhexis in response to systemically administered LPS (Figure 4) (44). Electron micrographs showed characteristic chromatin condensation and segregation, extracellular apoptotic bodies, and cell fragments phagocytosed in macrophages in the periventricular white matter.

Dalitz and co-workers studied the effects of sub-lethal doses of LPS on both cerebral and placental blood flow in the chronically prepared preterm ovine fetus (46). Endotoxemia impaired fetal oxygenation transiently and caused acidemia by reducing umbilico-placental blood flow. In the presence of hypoxemia, a prolonged decrease of oxygen delivery to the brain was observed of approximately 40%. Thus, mechanisms that normally increase fetal cerebral blood flow in the presence of hypoxemia are apparently impaired following systemic LPS exposure. Furthermore, they investigated the effects of systemically applied endotoxin on the perinatal brain in the preterm ovine fetus. In response to LPS injection the fetuses suffered from hypoxemia, acidemia, and hypotension for several hours (47). Interestingly, with increasing numbers of LPS injections the changes in hemodynamic variables became less and less pronounced, while white matter injury was present 10-11 days after the initial LPS injection. Injury was most prominent in the cerebral white matter and ranged from diffuse subcortical damage to focal periventricular leukomalacia, typical of white matter injury in preterm fetuses. Most recently, Mallard and co-workers compared neuropathological findings in response to intrauterine exposure to asphyxia or endotoxemia in mid-gestation fetal sheep (48). Both experimental paradigms resulted in white matter damage and inflammation. However, while LPS treatment resulted in selective white matter injury and regional infiltrates of inflammatory cells, injury following severe asphyxia involved cortical gray matter and was associated with microglia activation.

III. PATHOGENESIS OF IMPAIRED FETAL CARDIOVASCULAR CONTROL DURING ENDOTOXEMIA

A hallmark of endotoxemia and sepsis is the heterogeneous pattern of vasoconstriction and vasodilation in different organs, culminating in a fall in total peripheral vascular resistance concomitant with regional maldistribution of blood flow. These changes in the distribution of cardiac output are most likely caused by vasoactive substances, i.e. nitric oxide (NO), prostacyclin (PGI2), angiotensin converting enzyme (ACE) activity, endothelin and adrenomedullin, released from the endothelium under experimental septic conditions. In this context we will discuss the role of two important vasoactive agents, NO and endothelin, in the endotoxin-mediated alterations of fetal cardiovascular control.
P<0.01 in sustained fetal hypoxemia without acidemia (44,45). *P<0.05, tantial and longlasting decrease in umbilical blood flow resulting li; O127:B8, Sigma-Aldrich; 100 ng/kg fetal BW) caused a subs-
ated (n=8) premature fetal sheep. Intravenous endotoxin (E. co-
Figure 3. erences in mean arterial pressure between control and L-
sistance during hypoxia. Since there were no significant dif-
cardia and lowered carotid blood flow. This was accompanied
of the NOS inhibitor L-NAME resulted in bradycar-
dual changes in hypoxic cerebral vasodilation (51). In fetal she-
auer brain from neuronal injury by increasing cerebral blood
flow during hypoxia. Furthermore, NO is an important vaso-
dilator in the cerebral circulation, and developmental changes
itric oxide synthase (NOS) may contribute to developmen-
Flow distribution were further investigated during hypoxia in
R. Umbilical blood flow in control (n=6) and endotoxin tre-
ver, most of these studies were performed in adult animals,

ted by pretreatment with exogenous glucocorticosteroids.
endogenous glucocorticoids, develop a more severe form of
circulatory shock in response to LPS, which can be preven-
ted by pretreatment with exogenous glucocorticosteroids.
Smolich (56) investigated the effects of NO on fetal blood
flow redistribution between body and placenta as well as oxy-
gen extraction and oxygen consumption. In term fetal sheep
OS inhibition redistributes systemic blood flow towards the
placenta and increases fetal body oxygen extraction. The lat-
ner initially increased whole-body oxygen consumption and
interestingly, maintained it near baseline levels after a fall in
placental perfusion. The effects of NOS inhibition on blood
flow distribution were further investigated during hypoxia in
the chronically prepared preterm ovine fetus (57). Under nor-
moxic conditions, L-NAME infusion decreased blood flow to
the fetal body and to the placenta by more than 60%. During
a short period of superimposed acute hypoxia, L-NAME did
not change the redistribution of cardiac output towards the
central organs. However, the control of fetal heart rate and
blood pressure was altered in L-NAME treated animals. Af-
ter hypoxia L-NAME delayed the recovery of cardiac output
and blunted the increase in blood flow to the brain and heart.
Thus, NO plays an essential role in fetal cardiovascular con-
rol during normoxia and acute hypoxia.

A. The pivotal role of nitric oxide in fetal cardiovascular control during normoxia and hypoxia
Nitric oxide is known to be a potent mediator in the regulati-
on of resting tone in cerebral, renal, mesenteric and hindquad-
vascular beds and hence in blood pressure homeostasis in
adults (49). It has also been suggested that NO contributes to
vascular control during hypoxia in adults and fetal she-
ep (49-52). There is also evidence that NO might, in part, me-
diate the fetal circulatory centralization that occurs during par-
tial cord occlusion (53). This cardiovascular response to redu-
ced oxygen supply is a crucial mechanism that protects the fe-
tal brain from neuronal injury by increasing cerebral blood
flow during hypoxia. Furthermore, NO is an important vaso-
dilator in the cerebral circulation, and developmental changes
in nitric oxide synthase (NOS) may contribute to developmen-
tal changes in hypoxic cerebral vasodilatation (51). In fetal she-
ep, endothelial NOS is already present in blood vessels by 0.4
gestation (54). Moreover, cortical NOS catalytic activity in-
creases threefold between 0.6 and 0.9 gestation (55).

Green and co-workers (50) demonstrated the central role of
NO in maintaining fetal cardiovascular function during both
normoxia and hypoxia. In term fetal sheep intravenous appli-
cation of the NOS inhibitor L-NAME resulted in bradycar-
dia and lowered carotid blood flow. This was accompanied
by transient hypertension and an increase of both carotid and
femoral vascular resistance. During intra-uterine exposure to
hypoxia the magnitude of the subsequent chemoreflex brady-
cardia was reduced after L-NAME treatment and the well-
known rebound tachycardia during recovery was absent with
NOS inhibition (50). Moreover, NOS inhibition blunted the
rise in carotid blood flow and the fall in carotid vascular re-
sistance during hypoxia. Since there were no significant dif-
fences in mean arterial pressure between control and L-
NAME infused groups during hypoxia, it is thought that NO
mediates vasodilatation of the carotid vascular bed during
hypoxia in the fetus.

B. Nitric oxide as a mediator of fetal (circulatory) cardiovascular shock
During endotoxemia, an enhanced formation of NO con-
tributes to the acute and delayed therapy-resistant fall in blood
pressure and the vascular hyporeactivity that develops to en-
dogenous and exogenous catecholamines in adults. For in-
stance, in anaesthetized adult rats, LPS administration triggers
the release of NO, resulting in a fall in blood pressure and re-
duced responsiveness to intravenously applied vasocon-
strictor agents (58,59). Enhanced formation of NO following
the activation of the constitutive isoform of NO-synthase
(eNOS) present in endothelial cells, mediates the immediate
release of NO in response to LPS. Longer periods of endoto-
xemia are associated with the formation of cytokine-inducib-
le NO-synthase (iNOS) in many cells and organs including
the vessel wall. The enhanced generation of NO due to cyto-
kine- or endotoxin-related induction of iNOS contributes to
inappropriate vasodilation, delayed hyporeactivity to adre-
nergic agonists and the peripheral vascular failure associated
with endotoxic shock (59-61).

Interestingly, glucocorticoids, which are potent inhibitors of
the induction of NOS as well as of cyclo-oxygenase-2
(COX-2), protect against cardiovascular failure in septic and
hemorrhagic shock (62). Adrenalectomized animals, lacking
endogenous glucocorticoids, develop a more severe form of
circulatory shock in response to LPS, which can be preven-
ted by pretreatment with exogenous glucocorticosteroids.
Attenuation of the induction of NOS by endogenous glucoc-
corticosteroids accounts for endotoxin tolerance (62). Howe-
ever, most of these studies were performed in adult animals,
and their significance for the fetal circulation has not yet be-
en established.
C. The role of endothelin-1 during endotoxemia

In addition to the relaxing factors prostacyclin and NO, the vascular endothelium synthesizes the 21-amino acid peptide, endothelin-1 (ET-1). ET-1 is produced from a prepropeptide, which is proteolytically cleaved to big ET-1 (63) (Figure 5). Big ET-1 is subsequently converted to ET-1 by the metalloprotease endothelin-converting enzyme-1 (ECE-1) (64). The conversion of big ET-1 to ET-1 is essential for its biological activity, because the pressor action of big ET-1 is almost completely abolished by inhibition of ECE (65). ET-1 is the most active pressor substance yet discovered, with a potency some ten times that of angiotensin II (66).

ET-1 is released in response to several stimuli, including hypoxia, endotoxemia, increased pressure, and shear stress (for review: 67). In response to these stimuli, ET-1 causes both acute responses, such as vasodilation and vasoconstriction, as well as chronic changes, such as smooth muscle proliferation. Interestingly, the vascular responses to ET-1 vary during development in fetal and neonatal sheep and these effects are vascular-bed specific. Thus, ET-1 induced vasoconstriction increases with gestational age in femoral, middle cerebral, and renal arteries but not in adrenal arteries (68). These hemodynamic effects are mediated through two receptor subtypes: $\text{ET}_A$ and $\text{ET}_B$. $\text{ET}_A$ receptors and a small subpopulation of $\text{ET}_B$ receptors of vascular smooth muscle cells mediate vasoconstriction. A larger subpopulation of $\text{ET}_B$ receptors are located on vascular endothelial cells and are responsible for the vasodilating effects, mediated by NO-production (69).

Among the pathophysiological conditions known to involve the endothelin system, sepsis produces the highest plasma levels of ET-1 (70). The possible involvement of the endothel-
lin system in human septic shock is further supported by a correlation between endothelin plasma levels and morbidity and mortality in septic patients (71; for review: 67,72). Bacterial endotoxin and septic conditions increase ET-1 concentrations in fetal umbilical arterial plasma more than fivefold in both pigs and humans, reaching levels close to threshold vasocostriction (73). Other pro-inflammatory agents such as IL-1, TNF-α, and TGF-β, which are released during endotoxemia, also increase the production of ET-1 from endothelial cells. In fetal sheep ET-1 causes both constriction of the fetoplacental microcirculation and a decrease of fetal oxygen consumption (74). Prolonged alterations of placental gas exchange are known to induce fetal cardiovascular dysfunction with subsequent arterial hypotension and cerebral hypoperfusion. It is therefore quite conceivable that ET-1 induced effects on the placental vascular bed could finally result in hypoxic-ischemic fetal brain damage.

Another mechanism by which endothelin may affect fetal cardiovascular control during endotoxemia could be through its pronounced effects on pulmonary circulation. The pathophysiology includes a characteristic biphasic increase in mean pulmonary artery pressure and the pulmonary vascular resistance index and is thought to involve different mediators, including cytokines, which lead to increased expression of adhesion molecules, leukocyte activation and endothelial damage resulting in endothelial edema, vascular obliteration and vasoconstriction (for review: 75). The involvement of both the cyclo-oxygenase pathway in the early phase, and the endothelin system in the late phase of endotoxin-induced pulmonary hypertension has been shown, and unselective as well as selective endothelin ET_A receptor antagonism can counteract the late changes under experimental conditions (76,77). In contrast, intrauterine exposure to systemically applied endotoxin decreased pulmonary vascular resistance.

Figure 5. Brief schematic overview of the endothelin system. ET-1 is produced from a prepropeptide, which is proteolytically cleaved to big ET-1. Big ET-1 is subsequently converted to ET-1 by the metalloprotease endothelin-converting enzyme-1 (ECE-1), which is essential for its biological activity. ET-1 is released in response to several stimuli, including hypoxia, endotoxemia, cytokines, increased pressure, and shear stress. In response to these stimuli, ET-1 has vasodilating and vasoconstricting effects, which are mediated through two receptor subtypes: ET_A and ET_B. ET_A receptors and a small subpopulation of ET_B receptors of vascular smooth muscle cells mediate vasoconstriction. A larger subpopulation of ET_B receptors is located on vascular endothelial cells and is responsible for the vasodilating effects, mediated by nitric oxide-production.

aa = amino acid; cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; DAG = diacylglycerol; EC = endothelial cell; ECE = endothelin converting enzyme; IP_3 = inositol triphosphate; PKC = protein kinase C; VSMC = vascular smooth muscle cell.
and increased lung perfusion in fetal sheep (38). Although ET-1 produces systemic vasoconstriction, its effects on the pulmonary circulation vary with age and vascular tone (78-80). In the lung of the fetal lamb the ET\(_B\) receptor is highly expressed. ET-1 activates the ET\(_B\) receptor, which stimulates eNOS activity and NO production, mediating vascular smooth muscle relaxation and pulmonary vasodilation (81). In fact, pulmonary vasodilation has been described after exposure to NO, a substance, which is produced in large amounts after LPS application. These observations warrant further experiments investigating the effects of LPS on pulmonary vascular control.

**Conclusion**

It is obvious from recent clinical and epidemiological studies, that in-utero exposure to bacterial infection increases the incidence of periventricular leukomalacia and peri- or intraventricular hemorrhage in immature newborn infants. Recent data on this topic provide evidence that chorioamnionitis gives rise to a fetal inflammatory response and that this inflammation contributes to neonatal brain injury and subsequent cerebral palsy. Endotoxemia and systemic inflammation induce rapid and profound changes in endothelial function.

From the experimental data available, it is evident that intrauterine exposure to infection severely alters fetal cardiovascular control contributing to hypoxic-ischemic brain injury, especially in the periventricular white matter. During endotoxemia, enhanced NO formation contributes to inappropriate vasodilation, delayed hyporeactivity to adrenergic agonists and the peripheral vascular failure associated with endotoxic shock. In addition to relaxing factors, the vascular endothelium releases ET-1 in response to endotoxemia and hypoxia. Among the pathophysiological conditions known to involve the endothelin system, endotoxemia produces the highest plasma levels of ET-1. In fetal sheep ET-1 causes both constriction of the fetoplacental microcirculation and an inappropriate increase in pulmonary blood flow causing prolonged hypoxemia and decreased oxygen delivery to the brain. Thus, the impaired fetal cardiovascular control during endotoxemia resulting in dysregulation of placental, pulmonary and cerebral blood flow may have a significant role in the pathogenesis of perinatal brain white matter injury.

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