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Chapter 2

Endothelial Nitric Oxide Synthase and Neurodevelopmental Disorders

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Abstract

Endothelial activity reflects the balance of endogenous factors regulating vasoconstriction and vasodilation. Among these factors, nitric oxide (NO) is the most important contributor to the acute regulation of vascular tone. Altered nitric oxide synthesis by the vascular endothelium plays several important roles in the pathogenesis of neonatal disease through its effects on vascular homeostasis. However, the role of NO in the pathogenesis of perinatal brain injury has not been fully investigated. The present chapter explores how NO synthesis is regulated under physiological and pathological conditions, the impact of acute and chronic hypoxia on NO synthase activity in the vascular endothelium, and the role of perinatal endothelial dysfunction in the pathogenesis of neurodevelopmental disorders later in life.

Keywords: endothelial dysfunction, eNOS, perinatal hypoxia, neuronal injury, neurodevelopmental disorders

1. Introduction

Endothelial function and the associated production of nitric oxide (NO) play a key role in the pathogenesis of diseases involving the disturbance of vascular homeostasis [1]. Enzymatic generation of NO in mammalian systems is accomplished by the oxidation of L-arginine to L-citrulline with the participation of NADPH as a cofactor. Thus, NO is produced by NO synthase isoforms including endothelial NO synthase (eNOS) and neuronal NO synthase (nNOS), with eNOS being the dominant isoform in the vasculature under physiological conditions [2]. eNOS, also known as nitric oxide synthase 3 (NOS3), participates in the regulation

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of vascular tone and has a wide range of actions that control cerebral blood flow and metabolism. In contrast, the main role of nNOS is the production of NO for retrograde signaling across neuronal synapses.

Altered NO production by the vascular endothelium contributes to the pathogenesis of neonatal disease and may influence developmental growth [3]. The actions of eNOS in endothelial dysfunction lead to vascular and metabolic disorders and are also implicated in hypoxic-ischemic brain injury [4]. Studies have shown that hypoxic brain injury is characterized by changes in vascular growth and endothelial dysfunction [5–8]. Despite the widespread confirmation a significant role for NO in physiological and pathological vascular homeostasis, the role of NO in the pathogenesis of perinatal brain injury has not been fully investigated. Specifically, the impact of chronic hypoxia on NO synthase isoenzymes in the neonatal brain is unknown. Therefore, the goal of this chapter is to present the results of recent investigations of the pathological role of eNOS in endothelial dysfunction in preterm infants with hypoxic-ischemic encephalopathy (HIE) and in early-age neurodevelopmental disorders.

2. Specification and function of vascular endothelium in fetoplacental circulation

The formation of the mammalian vasculature involves many interdependent processes, including the maturation of multiple cell types within tissue compartments, pulsatile blood flow, blood pressure, the activity of smooth muscle cells in vessel walls, and the transmigration of immune cells. Scientific investigations of endothelial remodeling have confirmed its relevance to vascular barrier function, inflammation, and vascular disease [9].

During embryonic development, the first endothelial cells are derived from the extraembryonic mesoderm and appear around embryonic day 7. The placental barrier to the maternal blood is gradually breached between 8 and 12 weeks of gestation owing to invasion of the uteroplacental spiral arteries of the placental bed by the extravillous trophoblast. Accordingly, placental oxygen tension rises and leads to a phase of branching angiogenesis that lasts 24 weeks [10]. The fetoplacental endothelium is continuous with the fetal circulation, such that its function and potential dysfunction have a profound impact on fetal development [11]. To this end, successful pregnancies are highly dependent on effective vasculogenesis.

The regulatory sites and mechanisms responsible for endothelial function in the uteroplacental and fetal circulation remain unclear; however, it is obvious that endothelial activity is regulated through the balanced production and action of local endogenous constricting and dilating factors. Among vasodilatory factors, NO appears to be a chief regulator of acute vascular tone. NO generated by NO synthase expressed in the uterine artery endothelium is a diffusible gas molecule that produces smooth muscle relaxation and therefore vasodilation in a cGMP-dependent manner [12]. In general, NO is essential for the formation of endothelial function. In pregnancy, NO promotes endovascular invasion by the cytotrophoblast; interstitial trophoblasts produce NO as they invade the maternal spiral arteries in the uterine

wall in order to maintain a low-resistance and high-caliber uteroplacental unit. If this process fails, endothelial dysfunction associated with increased vascular resistance and reduced fetoplacental blood flow results in placental ischemia, pregnancy complications, and restrictive effects on fetal growth [13, 14]. Moreover, placental hypoperfusion and ischemia lead to the release of antiangiogenic factors that cause oxidative stress and inflammation, further contributing to endothelial dysfunction.

eNOS is well established as a primary physiological source of NO. eNOS affects vascular tone, reduces uteroplacental resistance, regulates uterine and fetoplacental blood flow, and is involved in uterine quiescence prior to parturition in normal pregnancy. Several studies have confirmed that eNOS activity is increased in the uterine artery during pregnancy in several species. Yet, investigations of the role of NO modulators in normal and abnormal pregnancies have shown conflicting results. The concentration of NO in the fetoplacental system depends on many factors including L-arginine availability, the activity levels of NO synthase isoforms, the presence of endogenous NO synthase inhibitors, and species-dependent variation. While some studies have reported lower eNOS expression in preeclamptic syncytiotrophoblasts than in normal syncytiotrophoblasts [15, 16], a series of clinical studies revealed that increased NO concentration primarily caused altered fetoplacental circulation, endothelial dysfunction, and reduced flow-mediated vasodilatation in different pregnancy pathologies [17–19]. Moreover, these studies identified increased endothelial permeability and decreased eNOS expression in the peripheral vasculature under pathological conditions [17]. Norris et al. reported increased NO production in the uteroplacental and fetoplacental circulation during preeclampsia compared to normotensive pregnancies and reasoned that this increase was a compensatory mechanism to offset the pathological effects of preeclampsia [18]. In support of this hypothesis, uterine arteries of pregnant rats exposed to plasma from women with preeclampsia were found to have increased eNOS expression and decreased inducible nitric oxide synthase (iNOS) expression [19]. In contrast, Leiva et al. purported that the bioavailability of NO in the fetoplacental system is decreased in pregnancy pathologies such as preeclampsia, gestational diabetes mellitus, and maternal supraphysiological hypercholesterolemia [20]; the authors hypothesized that altered NO synthesis and bioavailability in these cases are owing to the transcriptional and posttranslational modulation of NO synthases during hypoxia and oxidative stress.

One controversial question regards the putative effect of eNOS depression on fetoplacental blood flow in acute and chronic pathological processes. This topic was investigated in a series of experimental animal models; systemic NO synthase inhibitor administration was found to decrease uteroplacental blood flow and increase peripheral vascular resistance in several species [21]. Rosenfeld and Roy argued that the uteroplacental vasculature is less sensitive to prolonged systemic NO synthase inhibition than the peripheral circulation, which might be explained by the activation of compensatory mechanisms such as those reported for NO synthase in ovine uterine artery smooth muscle [22].

Several studies have investigated eNOS gene polymorphisms and their effects in different pregnancy pathologies. Whereas chronic hypoxia selectively augments the pregnancy-associated upregulation of eNOS gene expression and endothelium-dependent relaxation of the uterine artery [23], women with eNOS gene mutations were found to be at risk for developing

preeclampsia in a study of Egyptian families [24]. However, other studies do not support a major role for eNOS gene variants in preeclampsia [25, 26]. Comparing the results of studies conducted worldwide, Ma et al. concluded that an eNOS gene polymorphism was related to pregnancy-induced hypertension risk in Asian populations but not in European and American populations [27].

In summary, altered functionality of the fetal endothelium likely contributes to the formation of extrauterine pathologies from the neonatal period onward. However, the mechanisms underlying fetoplacental vascular development and pathologies thereof remain incompletely defined, such that further studies are necessary to understand the exact role of eNOS in pregnancy pathologies and fetal growth problems.

3. The role of endothelial nitric oxide synthase activity in the pathophysiology of perinatal brain injury

Perinatal hypoxic-ischemic brain injury is a major cause of neonatal death and long-term disability. Approximately 15–25% of newborns with hypoxic-ischemic encephalopathy (HIE) die during the postnatal period, and surviving infants are at risk for the development of severe and permanent neuropsychological sequelae such as cerebral palsy, seizures, visual impairment, mental retardation, and learning and cognitive impairments [28–30]. Decreased cerebral perfusion, hypoxia, hypoglycemia, and severe anemia can cause critical energy shortages in newborn infants, and accordingly severe hypoxia/ischemia can also affect other tissues of the body [31]. Disorders affecting the peripheral organs are often caused by hemodynamic disturbances resulting from the centralization of the bloodstream and/or poor circulation to the internal organs [32].

In the early days of extrauterine life, the vascular endothelium is exposed to high concentrations of inflammatory stimuli and can become dysfunctional if exposed to a hypoxic environment [33]. Cerebral ischemia induces an inflammatory response in the brain parenchyma and systemic circulation [34, 35], resulting in the augmented secretion of proinflammatory cytokines and chemotactic molecules by the vascular endothelium in newborn infants with hypoxic-ischemic injury. Hence, cytokines are important upstream effector of brain injury after ischemia [36]. Vasoregulatory mechanisms play essential roles in brain injury and tissue reperfusion in critically ill children; endothelial dysfunction results in an imbalance between vasoconstriction and vasodilatation, which causes tissue reperfusion, cytotoxic edema, and brain injury [37]. A previous study determined that hypoxic inflammation was regulated via bioactive mediators synthesized by endothelium, whereas NO and the sources of its synthesis play a special role in the pathophysiology of leukocyte-endothelial interactions [38]. To this end, studies show that growth-retarded fetuses and infants with severe and long-lasting neuronal injuries exhibit decreased vascular growth and endothelial dysfunction [39].

Of note, brain injury after hypoxic-ischemic injury progresses over many days even after reperfusion has been achieved. For example, oxygenated blood flow is restored to ischemic brain areas after severe perinatal asphyxia; however, while reperfusion temporarily corrects

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energy failure, excitotoxicity, and the generation of reactive oxygen species during the ischemic period are together responsible for a significant degree of brain damage. Brain damage after hypoxia-ischemia includes the primary insult and secondary damage such as delayed neuronal death related to cerebral edema [40]. Primary perinatal insults resulting from hypoxia-ischemia are associated with the failure of ATPase-dependent ions channels, which can disrupt synaptic function and lead to the accumulation of extracellular glutamate [41]. The increased availability of reactive oxygen metabolites after reperfusion is also directly involved in augmented glutamate release after injury. Increase in extracellular glutamate concentrations and the activation of glutamate receptors lead to excitotoxicity [42], which involves increased intracellular flux of calcium through open NMDA receptor channels and the release of calcium from intracellular stores. Elevations in intracellular calcium activate lipases, proteases, and endonucleases that lead to cellular damage and death [43]. Moreover, the post-hypoxic reperfusion process results in oxidative stress; energy failure activates nNOS and increases NO production, increasing the likelihood of its reaction with superoxide anion to form the powerful oxidant peroxynitrite [44]. Together, cellular energy failure, acidosis, glutamate excitotoxicity, and oxidative stress lead to cytotoxic edema and neuronal death after hypoxia-ischemia injury [45]. Additionally, there is a continuum of necrosis and apoptosis after such injury: often, early (primary) cell death appears necrotic, whereas later (secondary) cell death appears apoptotic**.** Therefore, while severe insult results in cell necrosis, more moderate asphyxia can cause delayed neuronal death through apoptosis [46]. Secondary apoptosis involves multiple pathophysiological processes such as excitatory neurotransmission, altered growth factor production, and changes in protein synthesis [47].

NO serves diverse functions in the perinatal brain, including neuronal differentiation and survival and synaptic formation and plasticity [48]. NO also affects these processes in pathological contexts by (in part) mediating neuronal death and neurodegeneration [49]. Previous studies in growth-restricted infants demonstrated that elevated NO production was associated with decreased endogenous antioxidant activity, increased lipid peroxidation, and impaired neuronal function [50]. NO supplementation was also found to increase uteroplacental circulation and decrease biomarkers of neuronal injury in the cord blood of infants diagnosed with intrauterine growth retardation [51, 52]. Therefore, it is difficult to interpret the role of NO in the pathogenesis of perinatal neuronal injury: is the concentration of NO increased as a defensive mechanism or does it point to a more profound impairment? Clinical and experimental investigations describing the roles of different NO sources in the pathogenesis of brain injury have provided insight on this problem. In the prospective clinical trial conducted by the Azerbaijan Medical University Neonatology group (ACTRN12612000342819), NO, eNOS, and endotelin-1 were quantified in 240 preterm infants with high risk for perinatal HIE; the results indicated that while eNOS expression was reduced, NO concentrations were increased in accordance with the severity of HIE (**Figure 1**).

This result provided foundation evidence for nonendothelial sources of NO synthesis in tissue hypoperfusion and hypoxia. Thereafter, the balance of NO/eNOS and its effect on neuronal injury in preterm infants was investigated. An important finding was that infants with severe HIE had higher NO/eNOS ratios compared with mild/moderate HIE and control infants, suggesting a relationship between nonendothelial NO production and neuronal injury (**Figure 2**).

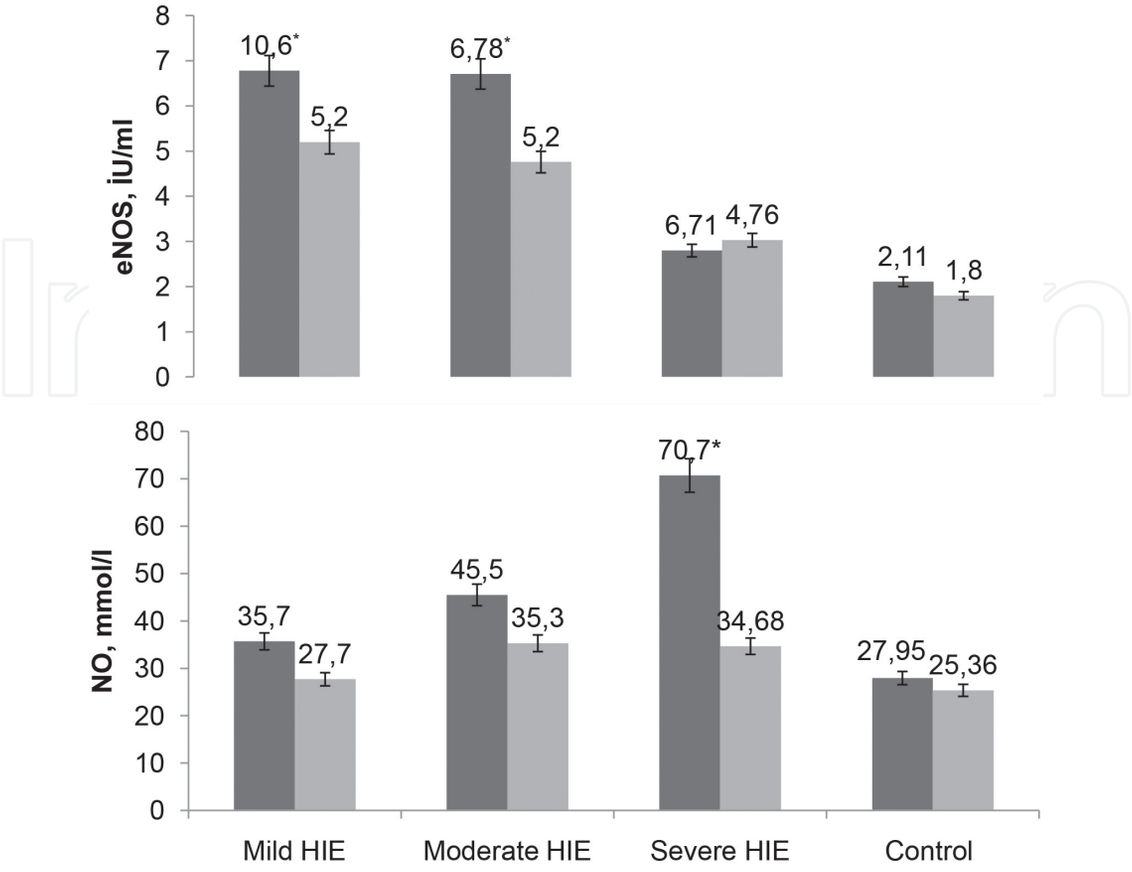


Figure 1. Mean total eNOS and NO values in preterm infants with HIE. Error bars indicate the standard error of the mean. Black bars show results from days 1 to 3 and grey bars show results from days 5 to 7. *$p < 0.05$ compared with the control group.

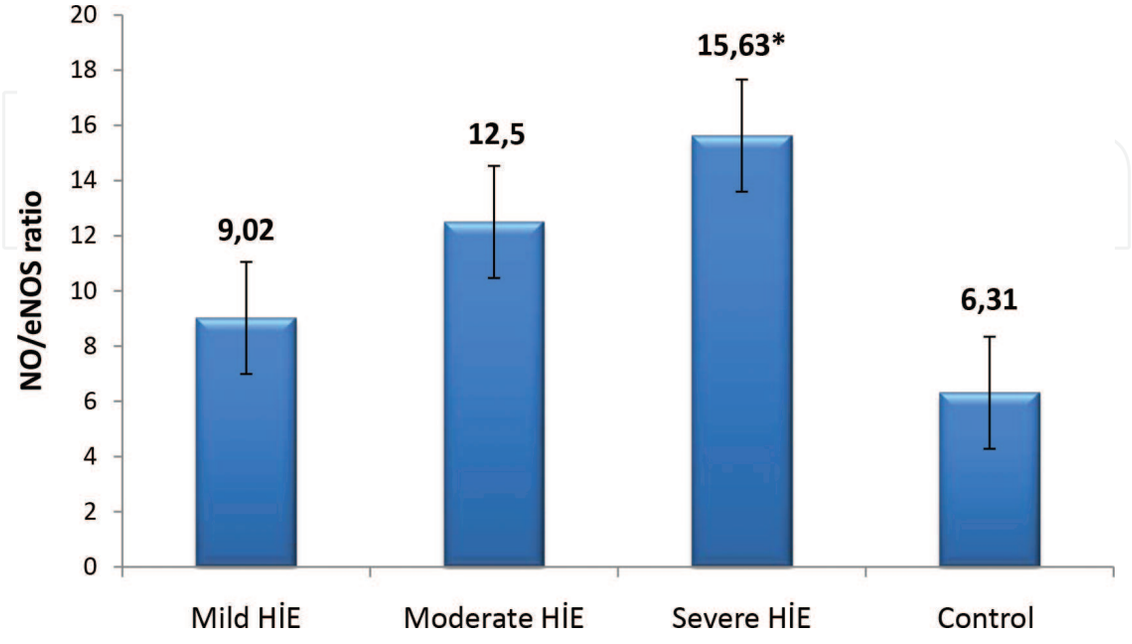


Figure 2. NO/eNOS ratios in preterm infants with HIE. *$p < 0.05$ compared with the control group.

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Significantly higher NO/eNOS ratios in preterm infants with severe HIE suggest that the activation of neuronal and inducible NO synthases is related to long-term and severe intrauterine and birth distress in infants. Moreover, increased NO in tandem with eNOS activation in infants with low risk for perinatal HIE might represent a compensatory or defensive strategy in the preterm brain. It should be noted that increased NO generation is not necessarily solely derived from areas of neuronal injury in HIE. Under hypoxic conditions, NO is also produced by the activated endothelium in all injured vasculature. Therefore, it might be difficult to accept the idea that NO/eNOS balance is a good predictor of neuronal injury. Yet, consistent with our previous investigation [53], we observed a statistically significant positive correlation between neuron-specific enolase (NSE) and NO/eNOS ratio, which suggests that decreased synthesis of NO by endothelial sources is related to more severe hypoxic changes and neuronal injury (**Figure 3**).

It was also found that growth-restricted infants are subject to significant endothelial dysfunction and eNOS depression, implicating NO in the pathogenesis of intrauterine hypoxic injury [53]. Together, these results provide a strong support for NO/eNOS balance as a marker of endothelial inflammation under hypoxic conditions. Previous experimental and clinical investigations have demonstrated that eNOS is responsible for preserving the functional integrity of the neurovascular unit [54, 55] and may have antiinflammatory effects in aging and other pathological contexts [56, 57].

The follow-up of newborn infants in the aforementioned study identified significant relationships between peripheral endothelial vasoregulatory markers in the perinatal period and the onset of neurodevelopmental disorders at an early age. It was found that, in the presence of high concentrations of NO, early eNOS activity was insufficient in infants diagnosed with cerebral palsy later in life compared to neonates who did not show neurodevelopmental delays associated with HIE (**Figure 4**). These findings suggest that depressed eNOS activity and increased non-endothelial NO synthesis play important roles in the formation of developmental impairments.

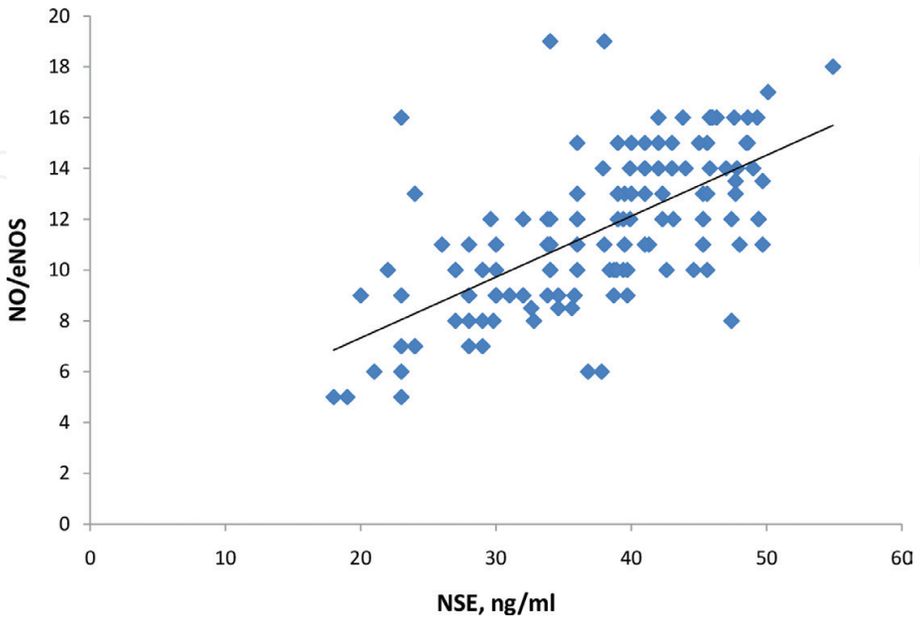


Figure 3. Spearman rank-order correlation between NO/eNOS ratio and NSE in preterm infants with HIE ($r = 0.67$; $p = 0.001$).

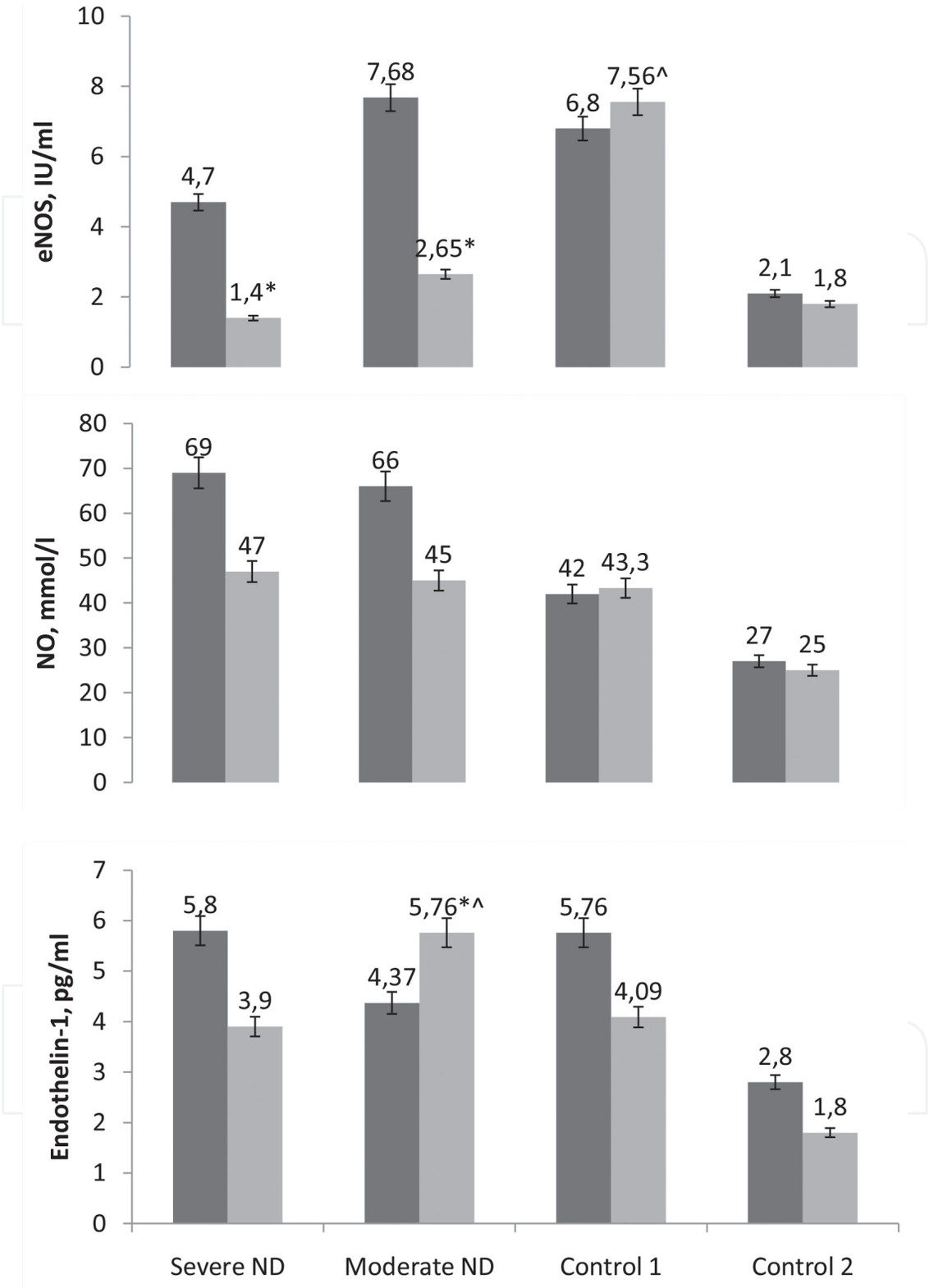


Figure 4. Peripheral vasoregulatory markers in the early neonatal period in preterm infants with neurodevelopmental disorders (NDs). Error bars indicate the standard error of the mean. Black bars show results from days 1 to 3 and grey bars show results from days 5 to 7. Control 1: data from infants with HIE who did not develop a ND; Control 2: data from healthy infants. *$p < 0.05$ compared with Control 1; ^$p < 0.05$ compared with Control 2.

As shown in **Figure 4**, cases with substantial eNOS and endothelin-1 depression during the perinatal period exhibited more profound neurodevelopmental delay and cerebral palsy. In contrast, when eNOS was depressed but vasoconstriction was maintained (i.e., increased endothelin-1 expression), functional impairments were more moderate, including mild motor and cognitive deviations and minimal brain dysfunction later in life. Therefore, insufficient eNOS activation in combination with the absence of a compensatory mechanism (e.g., peripheral vasospasm and/or the centralization of circulation in vital organs during the early stages of pathology) might ultimately drive the more serious and irreversible injury of brain tissue.

The abovementioned findings are consistent with those of several studies of different NO synthases in the pathogenesis of brain injury. In one study, chronic hypoxia decreased eNOS expression in the hippocampus and increased nNOS expression in neuronal and glial cells of the thalamus [5]. Moreover, in addition to elevated glutamate synthesis, long-term and severe hypoxemic processes have been reported to alter NO synthase enzyme activity in a manner related to DNA structure, resulting in iNOS and nNOS activation [58, 59]. Wei et al. determined that endothelial NO production by eNOS can decrease ischemic injury by inducing vasodilation, while neuronal NO production can exacerbate neuronal injury [6]. Therefore, several researchers have suggested the potential neuroprotective utility of nNOS inhibitors after brain injury [7, 8].

Many specific biochemical markers of neuronal injury are being investigated as indicators of brain damage in neonates [60]. Some neuron-specific proteins and cytokines show promise for identifying infants who are at risk for perinatal encephalopathy, although the exact value of these markers for predicting severe brain damage and neurodevelopmental disorders remains controversial [61, 62]. The early assessment of acute cerebral lesions in preterm infants may provide useful information regarding appropriate therapeutic intervention strategies and allow the prevention of future neurological complications. One possibility is that the severity of brain injury in newborns can be assessed by measuring the activity of NO synthases. Future studies are required to validate this hypothesis and better elucidate the clinical significance of NO synthesis in perinatal injury.

4. Conclusion

Autoregulation in the neonatal brain is tightly coupled with neuronal and endothelial regulatory mechanisms. Clinical and experimental investigations confirm that neuronal injury is in part mediated by the activation of endothelial and nonendothelial sources of NO synthesis. eNOS activity plays a fundamental role in the autoregulation of vascular tone in the perinatal period and is additionally involved in the formation of hypoxic brain damage during this period; however, the various roles of NO in neuroprotection and metabolism in the brain complicate our exact understanding of the relationship between NO and brain injury. Recent investigations suggest that eNOS plays a protective role in perinatal brain injury whereas other endogenous sources of NO (e.g., iNOS and nNOS) may participate in the pathogenesis of perinatal pathologies and neurodevelopmental disorders. Future studies should further delineate the molecular pathways responsible for the roles of NO synthase isoforms in brain injury and neuroprotection.

Finally, the ratio of NO/eNOS expression may indicate the severity of neuronal injury and have clinical utility for predicting long-term outcomes in infants after perinatal brain injury.

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