Gender Differences Involved in the Pathophysiology of the Perinatal Hypoxic-Ischemic Damage

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Summary
Hypoxic-ischemic encephalopathy (HIE) is a neonatal condition that occurs as a consequence of perinatal asphyxia, which is caused by a number of factors, commonly via compression of the umbilical cord, placental abruption, severe meconium aspiration, congenital cardiac or pulmonary anomalies and birth trauma. Experimental studies have confirmed that male rat pups show a higher resistance to HIE treatment. Moreover, the long-term consequences of hypoxia in male are more severe in comparison to female rat pups. These sex differences can be attributed to the pathophysiology of hypoxia-ischemia, whereby studies are beginning to establish such gender-specific distinctions. The current and sole treatment for HIE is hypothermia, in which a reduction in temperature prevents long-term effects, such as cerebral palsy or seizures. However, in most cases hypothermia is not a sufficient treatment as indicated by a high mortality rate. In the present review, we discuss the gender differences within the pathophysiology of hypoxia-ischemia and delve into the role of gender in the incidence, progression and severity of the disease. Furthermore, this may result in the development of potential novel treatment approaches for targeting and preventing the long-term consequences of HIE.

Key words
Gender differences • Hypoxia • Hypoxic-ischemic encephalopathy • Immature brain

Introduction to the concept of the perinatal hypoxia and ischemia

The term perinatal hypoxia-ischemia (HI) describes oxygen deprivation in immature, developing cerebral tissue. It is the 5th leading cause of mortality among children under five years and it is responsible for 23% of neonatal deaths worldwide (Bryce et al. 2005, Lawn et al. 2005). More than two thirds of children who survive suffer from neurological impairments such as motor disabilities, seizures and developmental delays, where most of these signs are seen in the first few days of life (Millar et al. 2017). The newborn central nervous system develops robustly towards the adulthood and it is extremely sensitive to hypoxic conditions (Cuaycong et al. 2011). Mild hypoxic damage induced perinatally may remain unrecognized until adolescence (Inder and Volpe 2000). The pathophysiology of HI is accompanied by multiple processes, such as inflammation (Serdar et al. 2019), oxidative stress (Solevåg et al. 2019) and excitotoxicity (Doble 1999). These processes generally occur simultaneously and are one of the many reasons why the treatment of HI (or encephalopathy following these processes) is near impossible. One of the leading causes of HI is perinatal asphyxia – the reduction of blood flow to the immature brain (i.e. deprivation of oxygen). It initiates various processes in order to compensate for this oxygen deprivation, for example, redistribution of the blood flow to the vital organs (Giussani 2016). However, prolonged asphyxia (Fig. 1)
exhausts and decompensates such mechanisms (Rainaldi and Perlman 2019). In addition, there are other predisposing factors that subject a neonate to a HI injury such as prolonged delivery, sepsis and shock. Subsequently, this can lead to cerebral palsy, cognitive deficits, respiratory distress and death (Allen and Brandon 2011). There is currently one established and main line therapy for HIE, which is the hypothermia. This involves cooling the neonatal brain to approximately 33 °C to slow the spread of cellular injury and minimize permanent damage (Shankaran et al. 2012, Pfister and Soll 2010). It is administered using either a cooling cap for head cooling or the whole-body-cooling for 72 h. This will slow the metabolic rate, allowing cells to recover and prevent further brain damage (Gluckman et al. 2005, Shankaran et al. 2005, Zhou et al. 2010). Despite the beneficial effect, one of the main issues is that hypothermia is limited by time; it should begin within 6 h following birth to minimize the spread of damage within the brain (Azzopardi et al. 2009, Diaz et al. 2017). Treatment delay from the diagnosis of HIE to the initiation of hypothermia may result in diminished therapeutic outcomes.

\[\text{Cellular changes caused by asphyxia}\]

**Differences in metabolism between immature and adult brain**

The immature brain of a neonate differs from an adult brain in response to HI injury. In addition to the role of gender, these attributes provide different modalities for potential treatment of HI. Firstly, the rate of metabolism is considerably lower in the immature neuronal tissue compared to an adult brain (Cremer 1982). Other metabolic substrates may be used as a metabolic fuel during the maturation of the brain; instead of glucose, ketone bodies and lactate should be used. These substrates cover more than half of the fuel that is required to satisfy the metabolic demands (Cremer 1982, Nehlig and Pereira de Vasconcelos 1993). Additionally, another significant difference between the adult and neonatal brain is the blood brain barrier morphology, where the immature brain shows a higher permeability, which further enhance brain damage in neonates (Millar et al. 2017). This is supported by experimental study of Muramatsu et al. (1997), reporting that rats at postnatal day (PND) 7 have higher permeability to immunoglobulin G compared to PND 14 rats (Muramatsu et al. 1997). The immature brain tissue could cope more effectively with the lack of metabolic substrate as compared to an adult brain tissue, but if a certain point of energy deprivation is reached, the immature brain tissue would suffer from excitotoxic damage (Puyal et al. 2013, Riljak et al. 2016). Destabilization of the cellular energy management is accompanied by the release of the excitatory amino acids and their failure of effective buffering in the extracellular space, which allows amino acids to reach their toxic concentrations (Doble 1999, Johnston 2005).
The role of the excitotoxicity, oxidative stress and inflammation in the development of HIE

Excitotoxicity, oxidative stress and inflammation are simultaneous processes during the setting of HI and each process can exacerbate neurological dysfunction resulting in encephalopathy, which is a non-specific response of the brain to injury (Vannucci et al. 1994, Riljak et al. 2016).

The production of reactive oxygen species (ROS) is a crucial outcome during ischemia (Folbergrová et al. 2016), which in turn, can trigger an inflammatory response. Reduction in blood flow, which last seconds to minutes, causes switching of the cell from aerobic to anaerobic metabolism, resulting in lactic acid formation, while fuel reserves decrease. This leads to the release of excitatory amino acids such as glutamate, whereby the intracellular concentration of calcium increases (Ferrer and Planas 2003). Glutamate binds to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), N-methyl-D-aspartate (NMDA) and kainate receptors resulting in tonic activation of these receptors. Consequently, more calcium can enter the cells, reaching a toxic level and an overexcitation of the cells. Excitotoxic amino acids (EAA) re-uptake mechanisms are failing and may be accompanied by cell depolarization and functional overload of the neurons. This can lead to rupturing and lysis of cells, which subsequently would initiate apoptotic cell death. Generation of free radicals, ROS, nitrogen species (NOS) and the release of calcium dependent enzymes such as caspases intensify the apoptotic activity of the cells (Velasco et al. 2017).

In addition, inflammatory cells can release other cytotoxic substances such as metalloproteinases, which serve as a source of ROS and cytokines such as IL-1, IL-6. This exacerbates the damages to the blood brain barrier and as a result, the disrupted blood brain barrier allows for other neurotoxic substances to enter leading to the formation of brain oedema (Kumar et al. 2008). Pro-inflammatory cytokines such as IL-1β can induce a rapid increase in excitability by activating its receptor, resulting in seizures (Vezzani et al. 2013, Youn et al. 2013), which is one of the long-term consequences of HIE.

Reduction in the partial pressure of oxygen (pO₂, Fig. 2) and an increase in the partial pressure of carbon dioxide affects significantly the ventilation. Only if the ventilation is restored, cerebral blood flow is matched with pO₂ and reperfusion prevents the exacerbation of oxidative stress and inflammation. If the ventilation is not

Fig. 2. Gender differences in apoptotic cascade triggered by HI.
restored adequately, the worsening of HI leads to the disruption of the blood brain barrier and consequently to excitotoxicity (Greer 2006, Kawabori and Yenari 2015, Kleman et al. 2010, Thorton et al. 2012).

Gender differences in severity of HIE

The unfavorable outcome of HIE in male infants compared to female infants have been established in ongoing research and demonstrated by radiological methods such as magnetic resonance imaging (MRI) and ultrasound (Gorelik et al. 2016, Jarvis et al. 2005, Johnston and Hagberg 2007, Zhu et al. 2006, Du et al. 2009, Hill and Fitch 2012, Hill et al. 2011a,b). The mortality rate of male infants is much higher than females, which suggests that one of the risk factors is gender. Stillbirths and respiratory distress syndrome are seen more in the male population (Zhao et al. 2017, Stephenson et al. 2000). Furthermore, male infants have a higher risk for blindness, deafness, developmental disorders including Autistic Spectrum Disorder and learning disabilities such as dyslexia, attention deficit disorder and cerebral palsy (Donders and Hoffman 2002, Hintz et al. 2007, Jarvis et al. 2005, Johnston and Hagberg 2007). Male infants are identified to be two times more likely to have prenatal anoxia, hemorrhages and an infection risk caused by cerebral birth trauma (Hill and Fitch 2012). The reason why males have a higher risk of cerebral birth trauma compared to females has not been investigated yet. However, MRI imaging has yielded positive results, showing premature male infants with a higher proportion of white matter injury compared to females, where the latter have their cerebral grey matter more vulnerable to hypoxia (Thompson et al. 2007). This was evident from rodent models of HIE where no sex difference was reported in the case of severe insult, however, in case of moderate insult females display less injury, which was attributed to differences in apoptosis (Zhu et al. 2006). MRI and ultrasound have shown that males following HI exhibited a higher brain volume loss, disrupted myelination and pronounced behavioral deficits, further supporting an exacerbated clinical picture in contrast to female neonates (Mayoral et al. 2009, Lan et al. 2011, Takeoka et al. 2002).

Contradictory to previous data, there is a study demonstrating lower mortality rate in males in comparison to females. A four-year study of newborns delivered in South-East Nigeria, demonstrated that females have higher mortality rate due to asphyxia. However, this study suffers from some limitations, as e.g. more male than female neonates were incorporated in this study (Ekwochi et al. 2017).

Cellular mechanisms coping with HI in both genders

On a cellular level, sexual dimorphism can be attributed to hormone-related actions, the apoptotic cascade differences and the so-called gene linked advantages. Higher levels of testosterone in males during the first year of life may enhance the process of neurotoxicity (Vannucci and Hurn 2009). The secretion of testosterone, which is the highest between gestational week 10-20, impacts brain development, providing a possible reason of a higher mortality rate in male neonates following cerebral injury. In addition, rat pups displayed a benefit effect from the depletion of testosterone following an injury, since the presence of testosterone increases glutamate toxicity (Yang et al. 2002, Hawk et al. 1998). This hypothesis is further supported by a study of Hill et al. (2011b) using the Rice-Vannucci rat model, where male and female rat pups received testosterone propionate (TP) from PND1 to PND5. There was a clear insufficiency in auditory processing of males, in both, treated with TP and without TP (Hill et al. 2011b). Female rat pups treated with TP had significantly worse auditory processing as compared to females without TP. Furthermore, there was a significant decrease in brain weight in males and TP treated females compared to their hypoxic and only vehicle counterparts, concluding that testosterone has detrimental consequences of early HIE (Hill et al. 2011b).

Estrogen has also demonstrated protective abilities against hypoxia and ischemia. Experimental studies have shown that female rodents have a lower incidence of stroke and less tissue damage than males (Yamori et al. 1977, Nuñez et al. 2007). This is further supported by a study from Fukuda et al. (2000), who have reported higher incidence of stroke in females following a reduction in estrogen levels induced by either ovariectomy or blockade of estrogen receptors, and also by age-related decline in estrogen production (Fukuda et al. 2000). This is, however, limited by the neonatal development, where there is a minimal level of circulating estrogen due to latent activation of ovaries in neonates. Thus, there must be additional protective factors leading to a lower severity of HIE in females (Johnston and Hagberg 2007).
**Apoptotic cascades triggered by HI – gender effects**

An encouraging line of investigation is directed at exploring gender differences in the mechanisms of cell death in response to brain injury. This has led to explore factors such as differences in apoptotic cascade, which consist of two pathways that may be preferentially activated depending on gender (Hill and Fitch 2012, Haast et al. 2012, Arambula et al. 2019, Hagberg et al. 2009). Programmed cell death is triggered by HI following deprivation of oxygen and glucose in neuronal cells (Nordling et al. 2011). The range of damaging signals is initiated by a decrease in ATP and activation of neuronal nitric oxide synthase (nNOS). Along with release of excess EAA, there is prolonged activation and depolarization of NMDA and AMPA receptors, resulting in sodium and calcium influx, leading to cellular swelling and rapid cell death (Riljak et al. 2016, Zhu et al. 2006).

There are two major pathways of apoptosis: the caspase-dependent pathway and caspase-independent pathway (Fig. 3). Following an increase in nNOS activation, the caspase-dependent pathway involves the signal of apoptotic protease activating factor 1 (APAF-1) and the formation of an apoptosome, which in turn, binds with caspases (3, 6, 7 and 9) resulting in chromatin condensation and DNA fragmentation. The caspase-independent pathway involves a reduction in nicotinamide adenine dinucleotide (NAD) and the activation of poly(ADP-ribose) polymerase-1 (Parp-1), leading to release of the apoptosis inducing factor (AIF) and endonuclease G from the mitochondrial compartments and ultimately, causing cell death (Zhu et al. 2006). Male or female gender favors either one of these pathways, but further research is required to determine why females rely more on the caspase-dependent pathway and males rely on the caspase-independent pathway following HIE (Lang and McCullough 2008, Renolleau et al. 2007, Zhu et al. 2006, Wang et al. 2004). On the contrary, previous studies showed that following HIE injury, there was a difference in the number of activated caspases. Males tend to have more caspases activated, which is subsequently associated with a wider apoptotic event and this may be attributed to a higher vulnerability in males, thus causing early brain damage (Netto et al. 2016, Liu et al. 2009, Hill and Fitch 2012).

Inhibitors of apoptosis, in particular, Parp-1, has been extensively studied in relation to the caspase-independent pathway of apoptosis. Male rodents that were deficient in this enzyme, presented with a reduction in cerebral damage following stroke (Yuan et al. 2009). This is supported by another study, reporting lower inflammatory response in males, but not in females, following the inhibition of Parp-1 (Mabley et al. 2005). Thus, males preferentially undergo the caspase-independent pathway. Parp-1 and AIF were also found in higher concentration in the brains of male rodents at PND9 in comparison to female brains (Zhu et al. 2006). In female rodents, following a middle cerebral artery occlusion, the activation of a caspase-dependent apoptosis is preferred. Additionally, the inhibition of caspases-3 and the release of cytochrome-c resulted in a certain level of neuroprotection, observed in animal models (Zhu et al. 2006).

Moreover, the advantage of gene inheritance of endogenous apoptotic inhibitors such as X-linked...
inhibitor of apoptosis protein (IAP) has been reported in females. Higher expression of X-linked IAPs in females may be beneficial during a HI insult, therefore cerebral damage is less extensive than in males (Deveraux and Reed 1999). The apoptotic cascade during development is highly regulated, thus, the need for anti-apoptotic signals promote the survival of neuronal cells. The IAPs are the most potent inhibitors that can effectively bind caspase-9 and prevent the down streaming of caspases, hindering cell death. The expression of X-linked IAPs has been observed in preclinical and clinical studies following HI injury (Askalan et al. 2011, Deveraux and Reed 1999). In comparison to males, females with a higher expression of X-linked IAPs preferentially activate apoptosis via caspases (Askalan et al. 2011).

Treatment – should it be gender targeted?

Despite the extensive research on sex differences in studies of neonatal HIE novel treatment approaches are not fully explored so far (Mayoral et al. 2009, Lan et al. 2011, Saraceno et al. 2010). Furthermore, a vast majority of studies (Hill and Fitch 2012, Yang et al. 2002) continue to utilize only male rodent models. However, this could be a possible source of bias reporting the worse clinical picture in males (Hill and Fitch 2012).

Hypothermia is the first line therapy of the HIE. Unfortunately, it is limited by sexual dimorphism as indicated by the high HIE mortality rate in male patients (Bona et al. 1998, Davidson et al. 2015, Hoehn et al. 2008).

Erythropoietin (EPO) is a new additive treatment currently used for premature infants as a treatment of anemia. EPO modulates NMDA excitotoxicity, reduces free radical toxicity, inflammation and improves the cognitive responses in primates and rats (Traudt et al. 2013, Davidson et al. 2015).

Melatonin is another perspective substance, which has been extensively tested within preclinical and clinical studies (Muller and Marks 2014). It has garnered some interest over the years due to being a free radical scavenger that can act in synergy with hypothermia to reduce oxygen deprivation in cerebral structures such as the hippocampus, where melatonin receptors are expressed to regulate myelination (Paprocka et al. 2019). Remarkably, the half-life of melatonin in preterm neonates is 15 h compared to adult brains which last up to 60 min. This significant difference in half-life makes melatonin a promising target for neuroprotection since it can possibly limit white matter injury, particularly in male rats (Park et al. 2014). It was proven beneficial if given synergistically with hypothermia as well (Park et al. 2014, Garg 2019).

Allopurinol is a xanthine oxidase inhibitor that can reduce the production of ROS, resulting in neuroprotection and improvement of HI (Rodriguez-Fanjul et al. 2017). In combination with hypothermia, this could be another potential treatment for HIE and its associated consequences. It has been shown (Fan et al. 2013, Nijboer et al. 2007), that following HI brain injury, females appear to benefit more from neuroprotective interventions (hypothermia, EPO and allopurinol). Nevertheless, studies also demonstrated that EPO and melatonin have a positive effect in males, providing a promising area of research into novel treatment for HI injury (Wen et al. 2006, Fang et al. 2013). There are many other substances that are now being considered to be as potential treatments in the therapy of HIE, such as topiramate, xenon and N-acetyl cysteine, however, more clinical and preclinical evidence are needed (Ozyener et al. 2012, Noh et al. 2006).

Conclusions

It is clear that gender is a crucial factor in hypoxia, its severity and the clinical outcome in neonates. Male neonates are at a higher risk of cerebral palsy as they showed more pronounced motor deficits than females. Moreover, the main line therapy with hypothermia may be affected by sex, since the outcome of HI is worse in males than females. Other treatments in combination with hypothermia may be promising at least in the rodent models and could be a potential treatment in clinical trials (e.g. EPO, melatonin and allopurinol). Thus, more therapies and novel gender-specific targets need to be investigated.

A vast majority of preclinical studies utilize male rodent models only, which consequently shows a worse clinical picture when compared to females. However, if there will be more female animal models used in experimental studies, it would bridge our understanding of sex-specific differences in hypoxia. Additionally, more research is required on the inherited role of IAPs and possibly on other apoptotic inhibitors in HI. Future studies would be focused on the relationship between steroid hormones and HI as there is a clear distinction in the concentration of sex hormones between males and females. Gene expression, along with the
apoptotic cascade represent a few of the many factors, which may play role in sex differences and are responsible for the disparities in severity of HIE. Future research might be focused on finding sex-specific neuroprotectants improving the outcome of neonatal HIE.

Conflict of Interest
There is no conflict of interest.

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