

# Understanding the timing of brain injury in fetal growth restriction: lessons from a model of spontaneous growth restriction in piglets

Hannah Musco, Julie A. Wixey\*

Fetal growth restriction (FGR) is one of the most common contributors to increased risk of mortality in the fetal/neonatal period and long-term morbidity in the infant (Malhotra et al., 2019). FGR can arise from many pathophysiological processes associated with maternal, fetal, genetic, or placental compromise; however, placental insufficiency is the most common cause of FGR. Placental insufficiency during pregnancy results in chronic fetal hypoxia where a lack of oxygen and nutrients supply to the developing fetus impacts normal development of the fetus. The fetal brain is especially vulnerable to FGR conditions. Neuronal and white matter injury are major pathophysiological features of FGR with clinical imaging studies identifying lower grey and white matter complexity in FGR preterm infants compared with both preterm and term infants (Esteban et al., 2010). These structural abnormalities remain at one year of age and are associated with neurodevelopmental disabilities (Esteban et al., 2010). Unfortunately, there are currently no interventions for the prevention or treatment of these structural brain alterations in the FGR infant. Determining mechanisms of brain injury and the timing of these injury processes (i.e., when they are initiated) would greatly assist with the identification and timing of treatment options for FGR to improve brain outcomes.

FGR can be broadly classified into two groups, early- (occurs prior to 32 weeks gestation) and late- (occurs during the third trimester) onset. Early-onset FGR (symmetrical FGR) occurs in around 20–25% of FGR fetuses and is characterized by global growth restriction throughout pregnancy. Late-onset FGR is the most common form of growth restriction occurring in around 70–80% of FGR fetuses (Sharma et al., 2016). This is where a fetal circulatory redistribution response is mounted and blood flow is preferentially redistributed away from peripheral organs to favor the heart and brain, termed ‘brain sparing’ or asymmetric growth restriction. Although brain-sparing is considered to protect the FGR brain, this may not be the case as several recent studies have reported asymmetric FGR infants have worse neurodevelopmental outcomes compared with symmetric FGR cohorts.

Magnetic resonance imaging studies have indicated neuronal and white matter disruption in FGR fetuses through adolescence, but the detail on when this disruption is initiated is lacking. However, at a histological level, a study in human

fetuses observed no differences in a number of brain cells prior to 27 weeks of gestation between FGR and normally grown fetuses (Samuelsen et al., 2007). Though when comparing cell counts from fetuses after 27 weeks gestation, lower cell numbers were observed in what would be the future cortex of FGR fetuses (Samuelsen et al., 2007). This suggests neuronal and white matter injuries may manifest at approximately 27 weeks gestation in the FGR fetus. This was an important starting point for further studies into mechanisms that cause and/or exacerbate neuronal and white matter injury and determining the timepoint when these mechanisms are initiated. Animal models of FGR are crucial to unravel and explore mechanisms of brain injury however, there are pros and cons for each animal species. Brain growth spurt occurs postnatally in the rodent, prenatally in the sheep, while piglet brain growth trajectory is like the human newborn occurring around birth. Unlike other FGR animal models requiring intervention, FGR occurs spontaneously in the piglet by placental insufficiency resulting in asymmetric growth restriction. FGR piglets are classically identified by their lower birth weight (< 10<sup>th</sup> centile of cohort), however, other measurements include relative brain to body weight and brain to liver weight ratio. Due to asymmetrical growth, FGR piglets can also be recognized by their head shape and gaunt body composition. Determinants of head morphology include ‘dolphin-like’ head shape, bulging eyes, and wrinkles perpendicular to the mouth. The FGR piglet displays neuronal and white matter injury similar to reported in the human FGR brain (Wixey et al., 2019a) demonstrating the piglet is appropriate to explore human neonatal brain disorders for not only timing of brain growth spurt but also similarities in neuronal development and myelination (Pond et al., 2000).

Disturbances to neurons, neuronal cytoskeletons, and myelination in early brain development can have long-term impacts on the FGR neonate. Disruption to these components have been identified at multiple gestational time points in the FGR piglet arising at 104 days gestation (equivalent to 26–28 weeks of gestation in humans), but not before (Kalanjati et al., 2017). A finding confirming the human FGR results mentioned above (Samuelsen et al., 2007). In the FGR piglet, a loss of neuronal somatodendrites is evident from 104 days gestation onwards (Kalanjati et al., 2017) suggestive of disruption to the neuronal

cytoskeletal architecture and normal cortical developmental processes in the FGR brain due to chronic growth restriction. Loss of axonal fibers indicates impairment of myelination in white matter for FGR piglets from 104 days gestation until at least postnatal day 7 (Kalanjati et al., 2017). At 104 days, gestation brain growth and myelination are occurring at a rapid rate and therefore disruption to white matter and neurons at this vulnerable time point may result in ongoing neuropathological effects in the FGR neonate.

Multiple mechanisms may mediate brain injury in the FGR neonate, such as excitotoxicity, oxidative stress, necrotic and apoptotic degeneration, blood-brain barrier (BBB) disruption, and inflammation. One mechanism of particular interest is the role of inflammation due to its association with neuronal and white matter injury in other neurological disease states. Many processes are involved in neuroinflammation including the activation of microglia, increased production of proinflammatory cytokines and astrogliosis (Wixey et al., 2017). The dynamic and complex processes involved in neuroinflammation continue for days after birth in FGR (Wixey et al., 2019a). In the FGR piglet, elevated inflammatory responses are observed at postnatal days 1 and 4 with increases in activated microglia, reactive astrocytes, and elevated levels of proinflammatory cytokines with corresponding decreases in anti-inflammatory cytokines (Wixey et al., 2019a). This inflammatory response is also associated with white and grey matter disruption in the FGR piglet. Not only is there a decrease in neuronal cell counts in FGR piglets at both time points, but also an association with neuronal degeneration and cell death (Wixey et al., 2019a). This suggests an impairment, rather than a delay, to neuronal development in the FGR piglet (Wixey et al., 2019a). These findings indicate that birth, as a mechanism for removal from the adverse environment, does not prevent further damage but, instead, disruption and damage to brain development continues in the days following birth (Wixey et al., 2019a). It is also suggestive of a key role of inflammation in neuronal and white matter damage observed in the FGR brain (Wixey et al., 2019a). Inflammation may also be a main mechanism through which the BBB is disrupted in the FGR brain (Chand et al., 2021a). In the FGR piglet brain, activated microglia at the vasculature appears to act in a phagocytic manner where they are seen to engulf blood vessels and are in close contact with astrocytic end-feet. Astrocytic end-feet should almost completely ensheath brain microvessels under non-pathological conditions. However in the FGR brain astrocytic end-feet retract from blood vessels altering BBB integrity with a concurrent influx of peripheral immune cells (Chand et al., 2021a). This disruption to the BBB could result in infiltration of systemic proinflammatory cytokines causing further damage to the developing FGR brain. However, following anti-inflammatory treatment with ibuprofen on the first day of life (for 3 days), a marked reduction in inflammation is apparent in the FGR piglet



brain at postnatal day 4 (Chand et al., 2021a). This FGR piglet study reported reduced numbers of activated microglia and enhanced astrocytic end-feet interaction with the vasculature, with a reduction in immune cell infiltration in to the FGR brain demonstrating improved BBB integrity (Chand et al., 2021a). These results suggest that anti-inflammatory treatment administered postnatally may be effective in reducing damage to the FGR neonatal brain.

Therapeutically targeting both inflammation and the BBB may provide additive neuroprotection in the FGR neonate. A recent study showed that one dose of a combination of mesenchymal stromal cells (anti-inflammatory) and endothelial colony-forming cells (ECFCs; true vascular stem cells), collectively identified as cECFCs, administered intravenously on the first day of life assists in both reducing inflammation in the FGR brain as well as repairing the BBB (Chand et al., 2021b). This combination stem cell treatment, sourced from the human placenta, not only decreased pro-inflammatory cytokines, but resulted in marked increases in anti-inflammatory cytokines in the FGR piglet brain (Chand et al., 2021b). In addition to their effect on inflammation and the vasculature, treatment with cECFCs in the FGR piglet also resulted in maintained neuronal and white matter integrity (Chand et al., 2021b). These beneficial effects were not apparent with mesenchymal stromal cell treatment alone. Further studies on the long-term impacts of cECFC treatment is crucial for determining effectiveness and safety for clinical use.

Evidence indicates neuronal and white matter injury may be initiated during the third trimester of pregnancy. However, as previously mentioned FGR can be early- or late-onset. Brain injury could occur at different time points depending on FGR classification and therefore many FGR fetuses may have impaired brain development prior to the third trimester; especially if in the early-onset category. The current method to detect brain injury in the fetus via magnetic resonance imaging has limitations due to reduced sensitivity in detecting subtle evolving neuronal and white matter injury, especially in the fetus. Therefore, this injury could occur earlier but is not detectable using current clinical methods. Nonetheless, it has been demonstrated in the FGR piglet that treating at birth (after the injury has been initiated) still offers the ability to improve brain outcomes (Wixey et al., 2019b; Chand et al., 2021a, b). Although it would be ideal to treat the FGR fetus in utero to prevent neurological injuries, up to 50% of FGR neonates are not diagnosed until around the time of birth (Sovio et al., 2015). Therefore, postnatal therapies are a practical solution. Further research will determine whether there is a wider therapeutic window of opportunity to intervene to improve neurodevelopmental outcomes for these neonates.

In summary, studies have demonstrated sustained proinflammatory response in the FGR brain is associated with brain injury. However, there is a knowledge gap on when the inflammatory event is initiated in the FGR brain. We have seen disruption to neurons and white matter commences prior to birth and is sustained until at least postnatal day 7 in the FGR piglet. Though when exactly this disruption commences in utero and for how long it persists is undetermined. Further, whether the inflammatory event occurs prior to birth or birth itself is the trigger for the inflammatory cascade is unknown. How long this inflammatory event is sustained for is also unknown. As previously mentioned, there are many mechanisms of injury that affect the developing FGR brain. Determining the profile of these mechanisms (*in utero* to *ex utero*) and their correlation with neuronal and white matter injury in the FGR brain is important for providing the information to underpin selection and timing of treatments for the FGR newborn to improve neurodevelopmental outcomes and give children the best opportunity to reach their full potential.

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**Hannah Musco, Julie A. Wixey\***  
UQ Centre for Clinical Research, Faculty of Medicine, University of Queensland, Queensland, Australia

\*Correspondence to: Julie A. Wixey, PhD,  
j.wixey@uq.edu.au.  
<https://orcid.org/0000-0002-9716-8170>  
(Julie A. Wixey)

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