The effect of glutathione supplementation on intestinal morphology and liver antioxidant status in parenterally fed piglets

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Abstract

Total parenteral nutrition (TPN) causes deleterious changes to the gastrointestinal tract (GIT), in part related to the decrease in blood supply that occurs without enteral stimuli. Nitric oxide (NO) regulates blood flow, but in situations of oxidative stress, as with the delivery of oxidized nutrients in TPN, it can react with superoxide and thus limit its function as a vasodilator. We hypothesized that supplementing the antioxidant glutathione to PN would protect NO's function as a vasodilator, leading to greater blood flow to the GIT. Piglets (8-12 days old) underwent surgery and were randomized to one of three treatment groups for 7 days: control TPN (PN, n=10), control TPN diet with 10 μM glutathione disulfide (GSSG, n=9), or the control TPN diet delivered enterally (EN, n=10). Blood flow ($p<0.0001$), mucosa weight ($p<0.01$) and crypt cell proliferation ($p<0.01$) were higher in the EN compared to both parenterally fed groups. Interestingly, villus height was greater in EN versus PN ($p<0.01$) but not different from GSSG; liver weight in the GSSG was similar to EN, while PN had a higher liver weight, typical of TPN feeding. The parenteral groups (PN and GSSG) had greater liver antioxidant capacity and less liver lipid peroxidation than EN. These data suggest that glutathione supplementation to TPN delays the changes in intestinal morphology and the route of feeding has a greater effect on antioxidant status than glutathione supplementation in TPN.

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List of Abbreviations

2-VP	2-vinylpyridine
γ-GL	γ-glutamyl-leucine
ANOVA	Analysis of Variance
AOPP	Advanced Oxidation Protein Products
AP-1	Activator Protein 1
ARE	Antioxidant Response Factor
ATP	Adenosine Triphosphate
BHT	Butylated Hydroxytoluene
BSA	Bovine Serum Albumin
BPD	Bronchopulmonary Dysplasia
CREAIT	Core Research Equipment & Instrument Training Network
dH_2O	Distilled Water
DAB	3,3'-Diaminobenzidine
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EN	Enteral Group
eNOS	Endothelial Nitric Oxide Synthase
FRAP	Ferric Reducing Antioxidant Power
FSR	Fractional Synthesis Rate
GCL	Glutamate-Cysteine Ligase
GCLC	Glutamate-Cysteine Ligase Catalytic Unit

GCLM	Glutamate-Cysteine Ligase Modifier Unit
GC-MS	Gas Chromatography Mass Spectrometry
GIT	Gastrointestinal Tract
GLY	Glycine
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GS	Glutathione Synthase
GSH	Glutathione (Reduced Glutathione)
GSSG	Glutathione Disulfide (Oxidized Glutathione)
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HClO	Hypochlorous Acid
H & E	Hematoxylin and Eosin
HPLC	High-Performance Liquid Chromatography
IG	Intragastric
iNOS	Inducible Nitric Oxide Synthase
IV	Intravenous
KCl	Potassium Chloride
Keap1	Kelch-like ECH-associated protein 1
LC-MS/MS	Liquid Chromatography Mass Spectrometry
MAT	Methionine Adenosyl Transferase
Mg^{2+}	Magnesium Ion
Mn^{2+}	Manganese Ion

NAC	N-acetylcysteine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NEC	Necrotizing Enterocolitis
NEM	N-ethylmaleimide
NFκB	Nuclear Factor Kappa B
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
$NO^{\bullet}$	Nitric Oxide Radical
NOS	Nitric Oxide Synthase
NPBI	Non-Protein Bound Iron
NRC	National Research Council
Nrf2	NF-E2 Related Factor 2
$O_2^{\bullet -}$	Superoxide Anion
OH^-	Hydroxyl Radical
$ONOO^-$	Peroxynitrite
PBS	Phosphate Buffered Saline
PCA	Perchloric Acid
PFBBr	Pentafluorobenzyl Bromide
PN	Control Parenteral Nutrition Group
PNAC	Parenteral Nutrition-Associated Cholestasis
PNALD	Parenteral Nutrition-Associated Liver Disease
PO_2	Partial Pressure of Oxygen
PTFE	Polytetrafluoroethylene

RBC	Red Blood Cell
ROS	Reactive Oxygen Species
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SIM	Selected Ion Monitoring
SMA	Superior Mesenteric Artery
SOD	Superoxide Dismutase
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TBS	Tris-Buffered Saline
TH	Total Hydroperoxides
TLR	Toll-like Receptors
TMP	2,2,6,6-Tetramethylpiperidine
TPN	Total Parenteral Nutrition
UHPLC-UV-MS/MS qTOF	Ultra High Performance Liquid Chromatography Ultraviolet Tandem Mass Spectrometry Quadrupole Time-Of-Flight

Chapter 1.0 Introduction

1.1 Total Parenteral Nutrition (TPN)

TPN is the intravenous (IV) administration of daily nutritional requirements in their elemental forms. Parenteral nutrition was first used as an alternate feeding method for an infant in 1944, published in the report by Helfrick and Abelson. In this case, a 5-month old male infant was given an IV infusion of glucose, fat and amino acids for five days because of poor weight gain following birth (Helfrick & Abelson, 1944). The use of parenteral nutrition was soon applied to infants with gastrointestinal injuries and low birth weights (Heird & Gomez, 1996). The benefits of TPN are to maintain life and give premature infants' gastrointestinal tracts (GIT) time to mature or to heal to the stage where they can digest and absorb adequate nutrients enterally. These benefits do not come without a detrimental impact on the intestinal tract and accompanying secondary organs. The associated complications can be divided into hepatic diseases and intestinal diseases. The hepatic diseases include hepatic steatosis and hepatic cholestasis (Guglielmi *et al*., 2006) (Section-1.1.3). The intestinal complications include a decline in blood flow to the intestinal tract, which contributes to changes in gut morphology, decreased cell proliferation, and impaired intestinal barrier integrity (Section 1.1.4) (Guglielmi *et al*., 2006; Niinikoski *et al*., 2004; Burrin *et al*., 2000). The composition of the parenteral diet is not only vital to the survival of the infant, but should optimize normal growth and development; however, the ideal composition remains elusive, as evidenced by the associated complications. The work presented in this thesis addresses how glutathione supplementation affects the GIT in piglets on TPN.

1.1.1 The nutrient composition of TPN

The TPN diet is composed of glucose, vitamins, minerals, amino acids, and lipids. All are required for a complete diet; however, two very important components regarding the development of complications are the amino acids and lipids.

One of the more complex components of the TPN diet are the amino acids. The gold standard for protein intake for newborns is human milk proteins (Walker, 2010). In the case of TPN, amino acids are provided as free amino acids, which is further complicated by the fact that TPN is provided intravenously, therefore bypassing the gut. A large proportion of dietary amino acids are extracted by the gut and used for synthesis of proteins, conversion into other amino acids, or synthesis into other metabolic products; but with intravenous delivery of nutrients, many of these processes cannot occur (van Goudoever *et al*., 2006). The essential amino acids that are required in TPN include histidine, threonine, valine, isoleucine, leucine, phenylalanine, lysine, methionine and tryptophan. Infants have an additional five amino acids that are essential to their development: cysteine, taurine, tyrosine, arginine and glycine (Rassin *et al*., 1994). Cysteine appears to be essential for the preterm neonate because of low activity of the cystathionase enzyme that is required to convert methionine into cysteine (Gaull *et al.*, 1972; Vina *et al*., 1995).

Another component of TPN diets is a lipid emulsion. Two commercial lipid emulsions that are widely used in Canada are Intralipid® and SMOFlipid® (Fresenius Kabi, 2020). Intralipid® has been available for many decades and is primarily composed of soybean oil (Fresenius Kabi, 2020). SMOFlipid® is the most recent emulsion approved for use in Canada and is composed of soybean oil, medium chain triglycerides, olive oil and fish oil (Fresenius Kabi, 2020). Growing evidence has demonstrated that Intralipid®

is not ideal for the neonate. In a study of preterm neonates comparing morbidities with the use of Intralipid® versus SMOFlipid®, a greater incidence of late onset sepsis was reported with Intralipid® (Choudhary *et al*., 2018); however, there were no differences in the rate of bronchopulmonary dysplasia (BPD) or necrotizing enterocolitis (NEC) on the different lipid emulsions (Choudhary *et al*., 2018). A recent study of infants with intestinal failure used an Intralipid®-treated historical cohort and compared morbidity outcomes to a group of infants treated with SMOFlipid®. Infants given SMOFlipid® had a lower rate of TPN-associated liver disease (Belza *et al*., 2020; Unal *et al.,* 2018). The use of SMOFlipid® should improve outcomes for TPN-treated infants; however, it will likely not eliminate the occurrence of co-morbidities associated with TPN use (Belza *et al.*, 2020; Deshpande *et al.*, 2014; Unal *et al.*, 2018). In Newfoundland, at Eastern Health, SMOFlipid® is the primary lipid emulsion used in the neonatal intensive care unit and this practice was implemented in 2015 (Eastern Health - Division of Newborn Medicine, personal communication, September 6, 2019).

1.1.2 Sources of oxidants in TPN

The elemental composition of TPN provides neonates with the required nutrients; however, nutrients when combined in solution at a TPN diet, can form pro-oxidants. Delivering parenteral solutions directly into the circulation may substantially increase the oxidant load that must be metabolized by the neonate. Helbock *et al*. (1993) and Pitkanen *et al*. (1991) first demonstrated that the use of Intralipid® as the lipid emulsion in TPN resulted in significant levels of hydroperoxides and free radicals. Following these studies, the multivitamin admixtures were determined to contribute significantly to the formation of peroxides within TPN (Lavoie *et al*., 1997a). This research group reported that both

ascorbylperoxide and hydrogen peroxide (H_2O_2) are produced in the TPN solution due to the oxidation of ascorbic acid and riboflavin in the presence of light (Elremaly *et al*., 2014; Lavoie *et al*., 1997a). Moreover, mixing the multivitamin preparation with the lipid emulsion, in this case Intralipid®, increased the concentration of peroxides in the TPN solution (Lavoie *et al*., 2008). A simple method proposed to reduce peroxide formation in TPN was to protect the TPN diet from light. Light protection reduced the concentration of peroxides in TPN and therefore, resulted in a more negative redox potential of glutathione in the animals that received the light protected diet (Elremaly *et al*., 2014; Lavoie *et al*., 2008). This indicates that there is more GSH available to reduce pro-oxidant molecules, reducing their ability to damage tissue. While light protection is beneficial, the ability to apply this practice in the clinical setting is impractical and therefore has not been widely implemented; this is surprising, because a number of studies have demonstrated that the oxidant load delivered with parenteral nutrition is linked to many serious diseases that arise during the neonatal period in preterm infants (Aceti *et al.*, 2018; Belli *et al.*, 2003; Chessex *et al.*, 2002; Elremaly *et al.*, 2014; Mohamed *et al.*, 2017).

1.1.3 Diseases associated with TPN in the preterm neonate

The diseases commonly associated with TPN use are hepatic steatosis, hepatic cholestasis, NEC and increased oxidative stress in the lungs leading to BPD.

1.1.3.1 Hepatic steatosis

Hepatic steatosis is defined as the accumulation of fat in the liver, which is associated with co-morbid states, such as obesity, type 2 diabetes, and alcohol abuse. Long-term TPN use has also been shown to lead to hepatic steatosis in neonatal animals (Guglielmi *et al*., 2006; Wang *et al*., 2006). In a TPN-fed piglet model, treatment for only one week led to clear

evidence of hepatic steatosis (Wang *et al*., 2006). A subsequent study found that long-term use (16 days) of TPN in piglets resulted in hepatic steatosis with significant liver inflammation (Stoll *et al*., 2010). Photooxidation of multivitamin preparations that include riboflavin and are used in TPN have also been correlated to the development of hepatic steatosis (Chessex *et al*., 2002). When the multivitamin preparation is exposed to light, a component of the preparation becomes hepato-toxic resulting in the hepatic dysfunctions observed with TPN (Chessex *et al*., 2002). In children who have been on TPN for an average of 7.9 ± 0.8 years (mean ± SEM), 41% of the children in the study developed a form of hepatic steatosis (Peyret *et al.*, 2011). However, hepatic steatosis is not a common condition found in preterm human neonates on short-term TPN (Kelly, 1998).

1.1.3.2 Hepatic cholestasis

The most commonly occurring hepatic dysfunction in children on TPN is hepatic cholestasis. Parenteral nutrition associated cholestasis (PNAC) is defined clinically as a rise in serum-conjugated bilirubin equal to or greater than 0.111 mM (Guglielmi *et al*., 2008; Guglielmi *et al*., 2006). In addition to high bilirubin, there are also increases in activities of γ-glutamyl transpeptidase, alkaline phosphatase and serum transaminase, all indicators of hepatic dysfunction (Guglielmi *et al*., 2008, Guglielmi *et al*., 2006). In the review by Guglielmi *et al*. (2008), they reported that the incidence of hepatic cholestasis in infants on TPN ranged from 7.4% to 84%, depending on the study cohort (Guglielmi *et al*., 2008). An older study by Beale *et al.* (1979) found a 23% incidence of hepatic cholestasis in TPN-fed neonates born weighing less than 2000 g. Beale *et al.* (1979) also found that the incidence of hepatic cholestasis was 50% in neonates on TPN with a birth weight of less than 1000 g. In a larger study comprising 624 infants who were treated with TPN in the first 30 days

of life, Bell *et al.* (1986) found an incidence of 7.4%, meaning 46 neonates out of 624 neonates developed hepatic cholestasis. More recent findings indicated that both birth weight and length of time on TPN were predictive risk factors for developing cholestasis in preterm neonates (Alkharfy *et al*., 2014; Veenstra *et al*., 2014). Another study conducted in rats, a TPN solution that included lipids further decreased bile flow when compared to TPN that contained only dextrose and amino acids, suggesting that lipid within TPN is another factor contributing to the development of hepatic cholestasis (Belli *et al*., 2003). Belli *et al.* (2003) hypothesized that the addition of homocysteine to TPN would reduce oxidative stress in the liver, as homocysteine is a precursor for GSH, an antioxidant. The addition of homocysteine to TPN did result in greater bile flow in the rats on a TPN diet with lipids; however, it did not improve hepatic oxidative stress as indicated through higher lipid and protein oxidation levels (Belli *et al*., 2003). This suggests that homocysteine is either involved in the pathway for bile secretion or is a precursor for a molecule involved in the bile secretion pathway.

An earlier study investigated the possible link between NEC and hepatic cholestasis in infants (Moss *et al*., 1996). It was found that preterm neonates who had received TPN and developed NEC had significant hepatic injury consistent with PNAC, while those infants that did not receive TPN and developed NEC had mild, non-specific hepatic injury (Moss *et al*., 1996). This suggests that the development of NEC following the administration of TPN may increase the risk of hepatic injury, specifically PNAC (Moss *et al*., 1996). Another study found that there was no specific component of the TPN diet that increased the risk of developing PNAC following the onset of NEC (Veenstra *et al*., 2014).

These studies associate the development of hepatic cholestasis with a previous diagnosis of NEC, the most common intestinal dysfunction observed in neonates on TPN.

1.1.3.3 Necrotizing enterocolitis and oxidative stress

NEC is described by many as one of the most devastating diseases that occurs in neonates (Neu and Walker, 2011; Aceti *et al*., 2018). NEC is an inflammatory condition caused by anaerobic bacterial invasion of the intestinal wall that leads to tissue necrosis and may progress to perforation (Rich and Dolgin, 2017). Approximately 1-3 in 1000 newborn infants develop NEC (Aceti *et al*., 2018; Lim *et al*., 2015). NEC has a mortality rate of approximately 15-30% (Aceti *et al*., 2018; Grishin *et al*., 2016). Many studies have investigated possible factors that increase an infant's risk of developing NEC. Low birth weight and preterm birth/lower gestational age increase the likelihood of developing NEC (Neu and Walker, 2011; Fitzgibbons *et al.*, 2009; Lim *et al.*, 2015). Related factors such as an immature intestinal tract, genetic predisposition and undesirable bacterial colonization at birth also lead to increased risk of NEC (Neu and Walker, 2011). More recently, other factors involved in the pathogenesis of NEC have been identified, including oxidative stress and the involvement of nitric oxide (NO) (Perrone *et al*., 2012; Grishin *et al*., 2016; Robinson *et al*., 2018; Drucker *et al*., 2018). Perrone *et al*. (2012) were attempting to decipher whether oxidative stress markers in the cord blood of newborn infants could be used as an indicator of possible NEC development. Oxidative stress markers, such as advanced oxidation protein products (AOPP), total hydroperoxides (TH) and non-protein bound iron (NPBI), were useful in identifying neonates at higher risk of developing the disease (Perrone *et al*., 2012). Another study outlined a mechanism of pathogenesis in which NO promotes bacterial infiltration of the intestinal barrier leading to the development

of NEC (Grishin *et al*., 2016). In contrast, a study conducted in piglets found that a decrease in the concentration of citrulline and decreased expression of enzymes in the arginine-citrulline-NO pathway preceded the onset of NEC (Robinson *et al*., 2018). Similar to the study by Robinson *et al*. (2018), Drucker and colleagues found that a decrease in endothelial nitric oxide synthase (eNOS), which is a part of the arginine-citrulline-NO pathway, resulted in greater intestinal injury and thus suggested that higher eNOS activity is important to prevent NEC (Drucker *et al*., 2018). These studies suggest that NO may be involved in enhancing the onset of NEC or may afford protection from the disease and indicates a need for more research on the pathogenesis of this disease. The study by Drucker *et al*. (2018) also found that the loss of eNOS and the development of NEC resulted in greater lung injury in mouse pups (Drucker *et al*., 2018). This association indicates a correlation between intestinal health and lung health in neonates.

1.1.3.4 Oxidative stress in the lung

The lungs are another organ that can be affected by the use of TPN. In guinea pigs, ascorbylperoxide, a contaminant in TPN diets, was correlated with the development of BPD in a dose-dependent manner (Elremaly *et al*., 2014). BPD was characterized by a decrease in alveolar number and a decrease in vascular development in the lungs and has a 50% incidence rate in premature neonates (Elremaly *et al.*, 2014; Jobe and Bancalari, 2001). Prior to this study, Lavoie *et al.* determined that light exposed TPN and the duration of TPN greatly increased the redox potential of glutathione (*i.e*., greater concentration of oxidized glutathione (GSSG)) and a lower number of alveoli in guinea pig pups (Lavoie *et al*., 2008). In addition, co-administration of the parenteral lipid emulsion with the multivitamin preparation was able to partially protect against the development of lung

fibrosis while not improving alveolarization in 3-day old guinea pig pups (Lavoie *et al*., 2005). All these studies confirmed an increase in oxidant load within TPN; however, an increase in total body oxidative stress is associated with oxygen supplementation in infants. Chessex *et al.* (2010) have shown that premature neonates (mean gestational age of 26 ± 1 weeks) on fractional inspired oxygen >25% had a more oxidized redox potential (meaning more GSSG compared to GSH) compared to infants on <25% fractional inspired oxygen. This finding suggests that an increased oxidant load to the lungs, meaning more oxygen supplementation, can induce systemic oxidative stress shown through less available GSH, the antioxidant form of glutathione (Chessex *et al*., 2010). In conjunction with this knowledge, oxygen supplementation following a period of hypoxia in guinea pig pups resulted in increased GSSG, the by-product of reactive oxygen species (ROS) reduction (Fokkelman *et al*., 2007). In rabbits, during the last five days of gestation, the activity of glutathione peroxidase increases approximately 200% and the activities of superoxide dismutase and catalase increase approximately 110% in the lungs (Frank and Groseclose, 1984). Although these data are not directly transferrable to human neonates, this suggests that prematurity results in a possible lack of antioxidant capacity in the lungs to combat the increased oxidant load they receive when supplemental oxygen is required and/or when TPN administration is necessary. Similar to the data from rabbits reported by Frank and Groseclose (1984), Nassi *et al.* (2009) demonstrated that premature infants born at the mean gestation age of 34.5 weeks had significantly lower glutathione peroxidase activity in venous blood until 20 days of life. The high oxidant load administered to premature neonates in critical care likely affects the lungs, which will then have an impaired capacity to combat the oxidant load. A novel strategy that proved to mitigate some of the lung

damage that occurred with the use of TPN in 3-day old guinea pig pups was the addition of GSSG to TPN (Elremaly *et al*., 2016). These guinea pigs were not exposed to supplemental oxygen and were treated with TPN as the model insult, to assess damage to the liver and lungs (Elremaly *et al.*, 2016). The addition of 10 µM GSSG to TPN reduced alveolar loss and apoptosis in the lungs compared to control TPN (Elremaly et al., 2016). The addition of pre-formed GSSG to TPN ameliorated some of the lung damage; however, the effect of the glutathione on other organ systems susceptible to oxidative damage was not investigated. Therefore, it is an important objective to determine whether there might be advantages to the GIT when TPN is required.

1.1.4 Intestinal changes associated with TPN

1.1.4.1 Decreased blood flow to the intestinal tract

The first measurable change that occurs following the initiation of TPN combined with the cessation of enteral feeding, is a decrease in blood flow to the intestine. This impacts blood flow through the superior mesenteric artery (SMA), the major vessel that is responsible for supplying blood to the upper intestinal tract. After 8 hours of TPN, the SMA blood flow decreased by approximately 30% when compared to enterally fed piglets (Niinikoski *et al*., 2004). Blood flow is regulated by nitric oxide (NO), a vaso-regulatory compound (Guan *et al*., 2003). The endogenous synthesis of NO is from L-arginine which is converted to citrulline and NO by the activity of NOS (reviewed by Moncada *et al*., 1991). NOS is found in 3 forms: the inducible (iNOS), endothelial (eNOS) and neuronal (nNOS) forms. A study that investigated iNOS activity in piglets reported a significant decrease in activity following 48 hours of TPN treatment when compared to an enterally fed control group (Niinikoski *et al*., 2004). The reduction in iNOS activity is a possible

mechanism that may be responsible for the decrease in blood flow through the SMA observed when human neonates are placed on a TPN diet, however this has yet to be confirmed.

A study conducted by our research group aimed to determine whether the administration of a high concentration of intravenous (IV) or enteral arginine as a precursor for NO would abolish the decline in blood flow through the SMA that occurs with TPN (Dinesh *et al*., 2014). High IV arginine induced less of a decline in blood flow from baseline compared to the concentrations of arginine administered enterally (Dinesh *et al*., 2014). The study by Dinesh *et al*. (2014) speculated that modified TPN diets (i.e., higher in arginine concentration) could reduce the deleterious effects of a TPN diet, and this may be a result of L-arginine availability for the synthesis of NO (Dinesh *et al*., 2014; Guan *et al*., 2003).

A detrimental endogenous effect of NO is that it can be diverted from functioning as a vaso-regulatory compound to a damaging pro-oxidant compound (Radi, 2018). NO can react with superoxide anion ($O_2^{-\bullet}$) to produce peroxynitrite ($ONOO^-$), a pro-oxidant compound (Radi, 2018). $ONOO^-$ causes the activation of caspases which leads to apoptosis. It can also cause lipid peroxidation, which induces cell damage and tissue damage (Pacher *et al.*, 2007).

1.1.4.2 Changes in intestinal morphology

The rapid decline in blood flow that occurs with parenteral feeding likely contributes to dramatic changes in intestinal morphology that have been reported with TPN. Burrin *et al.* (2000) found that after 6 days of TPN or enteral diet administration to piglets, the mean villus height in TPN groups was 478 µm compared to 642 µm in the enterally fed piglets.

In addition, the crypt depth in TPN versus enterally fed piglets was 79.8 µm and 96.4 µm, respectively (Burrin *et al.*, 2000). Subsequently, another study reported similar changes in piglets after an even shorter period of time on TPN (Niinikoski *et al.*, 2004). After 48 hours of TPN administration, villus height in TPN piglets was 488 µm compared to 583 µm in the enteral group (Niinikoski *et al.*, 2004). These studies demonstrate how quickly TPN and the lack of enteral feeding affect the morphology of the intestinal tract in a relevant model for human neonates.

1.1.4.3 Cell proliferation

The gross changes in intestinal morphology reported with the use of TPN and the lack of enteral feeding are likely mediated by a rapid decline in cell proliferation. Several studies in piglets have reported a lower rate of cell proliferation in the jejunum of TPN-fed piglets compared to piglets on an enteral diet (Burrin *et al*., 2000; Niinikoski *et al*., 2004; Fitzgerald *et al*., 2005). One study investigated a possible mechanism involved in the regulation of intestinal cell proliferation using a TLR4 knockout mouse model (Freeman *et al.*, 2015). As described in the study, one of the regulatory pathways for the secretion of TNF-α, a cytokine involved in the mediation of inflammation and apoptosis that is upregulated when TPN is administered, is the TLR4 pathway (Feng & Teitelbaum, 2012; Freeman *et al.*, 2015). Freeman *et al.* (2015) administered TPN to the TLR4 knockout mice to elucidate whether knocking out the pathway would reduce atrophy and apoptosis in the intestinal tract. The knockout of the TLR4 pathway resulted in less TNF-α secretion, greater cell proliferation and deeper crypts compared to the wild type mouse on TPN (Freeman *et al.*, 2015). Freeman *et al.* (2015) demonstrated that intestinal atrophy and cell proliferation

in the intestinal tract is, in part, mediated by TLR4 signalling pathways (Freeman *et al*., 2015).

1.1.4.4 Integrity of the intestinal barrier

An important consequence of reduced cellular proliferation in the gut is an impairment of the intestinal barrier function. Phenotypic alterations in intestinal lymphocyte populations have been reported in association with TPN diet administration (Kiristioglu *et al*., 2002; Ganessunker *et al*., 1999). One study conducted in mice reported decreases in $CD4^+$ and $CD44^+$ lymphocytes, indicating a decrease in the number of mature lymphocytes (Kiristioglu *et al*., 2002). In contrast, a previous study found that TPN-fed piglets had an increase in inflammatory markers within the intestinal tract including CD4+ and CD8+ T-lymphocytes and major histocompatibility complex II mRNA expression. This same study showed that as inflammatory markers increased in TPN fed piglets, the villus height and overall gut weight decreased (Ganessunker *et al*., 1999). These studies indicate that lymphocyte populations in the intestinal tract changed when TPN was used, and enteral feeding was stopped. Higher lymphocyte populations are an indicator of inflammation in the intestinal tract and as it progresses, inflammation will damage the intestinal mucosa and weaken the integrity of the intestinal barrier (Kiristioglu *et al.*, 2002). Following the establishment of the inflammatory process and thus changes to the intestinal barrier, investigations into cytokine differences was conducted. A study of cytokine concentrations in TPN-fed animals reported an increase in intestinal epithelial IFN-γ which led to greater apoptosis (Yang *et al*., 2003). Subsequent to this finding, it was found that TPN induces a decrease in IL-10, a cytokine responsible for regulation of the integrity of the intestinal barrier through maintaining tight-junction proteins (Sun *et al*., 2008). Sun *et al.* (2008)

found that TPN administration in mice resulted in a lower expression of IL-10 compared to the control and resulted in a lower expression of tight-junction proteins. When IL-10 was provided to TPN mice exogenously, it restored the expression of tight-junction proteins when compared to the control mice (Sun *et al.*, 2008). The intestine undergoes changes in the expression of certain toll-like receptors (TLR) when TPN is administered (Ikeda *et al*., 2010). These changes in TLR alter the intestines' ability to fight infection, as specific TLR signal the release of cytokines within the intestinal tract (Ikeda *et al.*, 2010). The different cytokine expression when TPN is administered can result in the weakening of the intestinal barrier through signalling of apoptosis and reduced expression of protective proteins like tight-junction proteins (Ikeda *et al.*, 2010; Sun *et al.*, 2008).

1.2 Oxidative Stress

Oxidative stress is a term that has been manipulated and misused in the popular press and by nutritional supplement manufacturers. It is defined as an imbalance in the production and degradation of reactive oxygen species (ROS), favouring the production of ROS (Pisoschi *et al*., 2015; Sies, 1991). The overproduction of ROS can result in damage to DNA, proteins and lipids (Kohen and Niska, 2002;). ROS is an overarching term describing compounds composed of oxygen that are typically highly reactive (Kohen and Niska, 2002;). It includes compounds such as the hydroxyl radical ($OH^{\cdot}$), nitric oxide radical ($NO^{\cdot}$), superoxide anion radical ($O^{\cdot}{}_2^{-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and peroxynitrite ($ONOO^{-}$) (Kohen and Niska, 2002). The increase in ROS has been linked to cancer (Meng *et al*., 2018; Liao *et al*., 2019), cardiovascular disease (Ochoa *et al*., 2018), neurodegenerative diseases (Cheignon *et al*., 2018), diabetes (Incalza *et al*., 2018) and inflammatory diseases such as asthma (Kohen and Niska, 2002). Oxidative

stress has also been implicated in the TPN-associated injury to the liver, as demonstrated by elevated lipid peroxidation and hepatocellular steatosis (Sokol *et al*., 1996). To further understand oxidative stress, it is important to define the sources of ROS, both exogenously and endogenously.

1.2.1 Sources of Reactive Oxygen Species

ROS can be generated both exogenously and endogenously. The exogenous sources include radiation, cigarette smoking, narcotic drug use, anesthetizing gases as well as pathogenic bacteria and viruses (Kohen and Niska, 2002). The largest exogenous source of pro-oxidants (i.e. ROS) is food. One study has attempted to supplement an antioxidant (i.e., 500mg glutathione (GSH) twice daily) orally to improve oxidative stress markers in healthy volunteers, however, no changes were observed in the oxidative stress biomarkers, in blood and urine, of the participants after a 4-week study period (Allen *et al*., 2011). In another study, participants were also given a supplement of an antioxidant (i.e., either 250 mg daily of glutathione (GSH) or 1000 mg GSH daily) resulting in an increase in specific tissue (*i.e.* erythrocytes, plasma, lymphocytes, buccal mucosal cells) glutathione levels compared to the baseline values after 1-month, 3-months and 6-months (Richie *et al*., 2015). The values continued to increase each month where samples were collected and dropped after a 1 month washout period (Richie *et al.*, 2015). These results are contradictory and that may be due to the methods used and the study length of each study. More research is required to understand the effect of oral supplementation on antioxidant status and oxidative stress.

The largest endogenous source of ROS is the mitochondria. The production of ATP through the electron transport chain results in the production of many oxygen derivatives and, in turn, results in the leakage of ROS into the intracellular space (Kohen and Niska,

2002). Within the mitochondria, uncoupling proteins have been shown to control the production of ROS (Cadenas, 2018). Other endogenous sources of ROS include enzymes that produce ROS as a by-product of their function and leukocytes that use ROS to combat pathogenic invaders (Kohen and Niska, 2002). Many studies have investigated how these responses differ when exposed to different pathogens and how they differ between adults and neonates (Kabanov *et al*., 2019; Destin *et al*., 2009).

1.2.2 Oxidative stress in the neonate

The oxidative balance is critical for neonates starting before birth, with differences in pro-oxidant and antioxidant production being observed during birth (Thompson *et al*., 2012; Diaz-Castro, 2016). Compared to the gestational period, parturition changes the exposure to oxygen drastically for neonates (Frank and Groseclose, 1984). Under normal conditions, the fetus is in a relatively hypoxic environment (arterial PO_2 ~20-25 mm Hg) and at birth is exposed to a hyperoxic environment (21% oxygen or PO_2 160 mm Hg) (Frank and Groseclose, 1984). Clearly, neonates require mechanisms to prevent negative consequences resulting from this transition. In response, lung antioxidant enzymes undergo maturation 3 to 5 days prior to birth to prepare for the transition to an oxygen-rich environment (Frank and Groseclose, 1984; Frank and Sosenko, 1987). The change to a hyperoxic environment results in a large increase in ROS production. The lungs must be able to combat this surge in production at birth and therefore the maturation of these enzymes is critical.

A population at high risk of inadequate adaptation to the post-natal hyperoxic environment is premature neonates. Prematurity introduces an additional challenge of an immature antioxidant defense system (Nassi *et al*., 2009). Glutathione peroxidase activity has been reported to be significantly lower in preterm compared to term-born infants up to

20 days after birth (Nassi *et al*., 2009). In the same study, the activity of superoxide dismutase was significantly lower even at 100 days after birth (Nassi *et al*., 2009). The lower activity of these critical enzymes in the antioxidant system places preterm infants at a greater danger of developing medical complications, especially when interventions are required. Many infants born prematurely are placed on a TPN diet that is contaminated with pro-oxidants (Section 1.1.2 above). In addition to TPN administration, premature neonates commonly require supplemental oxygen to maintain normal oxygen saturation (Mohamed *et al*., 2017; Elremaly *et al*., 2014). Both TPN and supplemental oxygen represent sources of oxidative stress for infants (Lavoie *et al*., 1997a; Chessex *et al*., 2010).

In addition, the ability to combat oxidative challenges has been shown to have sex differences. In female rats, the mitochondria have a higher expression of antioxidant genes resulting in lower levels of oxidative damage compared to male rats (Borras *et al*., 2003). Endothelial tissue from the umbilical cords of female infants had higher glutathione reductase activity compared to male infants, following the administration of an organic peroxide, tertbutylhydroperoxide (TBH) (Lavoie *et al.*, 1997b). Higher glutathione reductase activity in the female infants suggests a higher capacity to recycle glutathione, allowing it to function as an antioxidant (Lavoie *et al*., 1997b).

With all the challenges faced by premature neonates, understanding the endogenous system responsible for mitigating oxidative stress is vital.

1.3 Glutathione

Glutathione (GSH) is a non-protein tripeptide composed of glutamate, cysteine and glycine (**Figure 1.1**). Glutathione disulfide (GSSG) is the oxidized form of glutathione and results from the reduction of reactive oxygen species (**Figure 1.2**). The primary localization site is in the cytosol and the secondary sites are the mitochondria and endoplasmic reticulum (Lu, 2009).

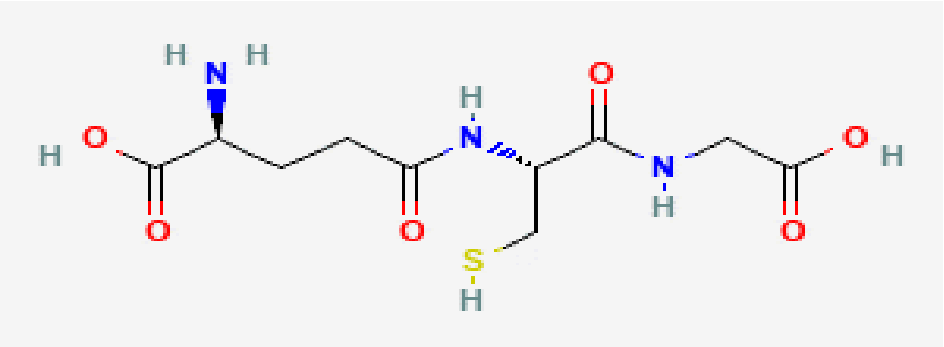

Figure 1.1: Structure of glutathione (reduced form). Structure retrieved from the National Center for Biotechnology Information. (2022a).

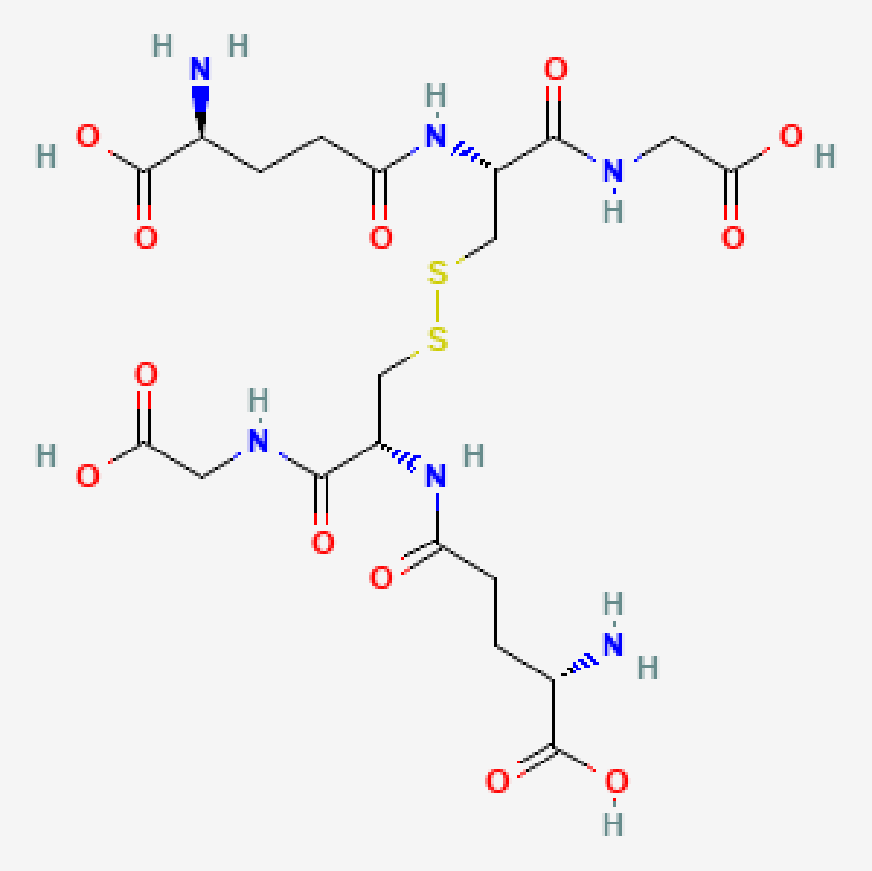

Figure 1.1: Structure of glutathione disulfide (oxidized form). Structure retrieved from the National Center for Biotechnology Information. (2022b).

1.3.1 Biosynthesis

The endogenous synthesis of GSH is a two-step reaction. The first is the rate-limiting reaction between glutamate and cysteine catalyzed by glutamate-cysteine ligase (GCL) (**Figure 1.3**). GCL has a requirement for Mg^{2+} or Mn^{2+}. It is composed of a catalytic subunit and a modifier subunit. This first step is highly dependent on the concentration of cysteine (reviewed by Lu, 2009). This enzyme is regulated by a non-allosteric competitive feedback inhibition by GSH and regulated by the availability of L-cysteine (Lu, 2009; Lu, 2013). The second step of GSH synthesis is the reaction between the newly formed γ-glutamylcysteine and glycine in the presence of glutathione synthase (GS) (**Figure 1.3**). Glutathione biosynthesis occurs in most cells in the body (Lu, 2009).

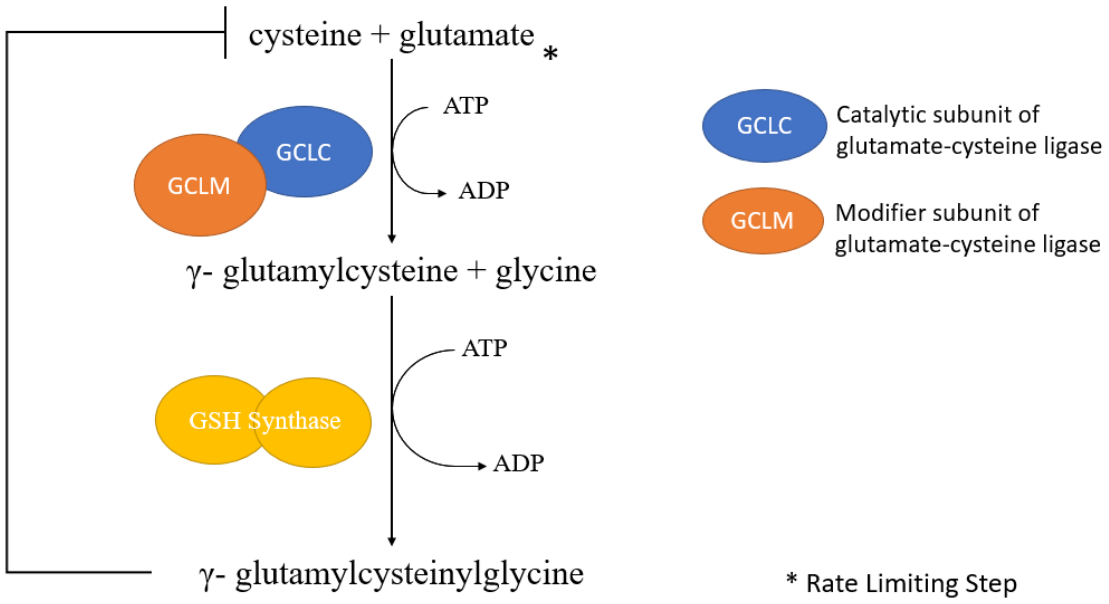

Figure 1.2: Two-step reaction mechanism to synthesize glutathione in the reduced form (GSH; γ-glutamylcysteinylglycine) endogenously. Glutamate-Cysteine Ligase Catalytic Unit (GCLC) is the catalytic unit of glutamate-cysteine ligase (GCL). Glutamate-Cysteine Ligase Modifier Unit (GCLM) is the modifier unit of GCL. Adapted from Lu (2009).

The regulation of GSH synthesis occurs through the synthesis of the enzymes, GCL and GSH synthase, involved in the two-step reaction mechanism to synthesize glutathione (shown in **Figure 1.3**) and based on the availability of the amino acid subunits, specifically cysteine (Lu, 2009). Lu (2009) describes how GSH synthesis is regulated during transcription of the subunits of GCL (GCLC and GCLM), during the post-transcriptional stage of GCLC synthesis, and regulated during transcription of GS. The regulatory mechanisms are detailed in the review articles by Lu in 2009 and 2013.

Though the regulation of GCL and GS occurs at multiple stages in their synthesis, one of the main regulatory pathways is through the binding of the NF-E2 related factor 2 (Nrf2) transcription factor to the antioxidant response element (ARE) located in the promoter region of the human GCLC gene (Lu, 2013). Nrf2 is found in the cytosol bound to Keap1 under non-stressful physiological conditions (Lu, 2013). However, in situations of oxidative stress, Keap1 or Nrf2 are modified thus releasing Nrf2 and allowing it to enter the nucleus to then induce the transcription of antioxidant genes (GCLC, GCLM and GS, if overexpressed) (Lu, 2013). Other regulators of GCL and GS synthesis include the binding of AP-1 and NFκB family members to the respective binding sites in the promoter regions of GCLC, GCLM and GS genes (Lu, 2013). The binding of these transcription factors takes place under conditions of oxidative stress (Lu, 2013).

1.3.2 Antioxidant function

The antioxidant function of glutathione is cyclical. Hydrogen peroxide will be the peroxide used in the description of the cycle. However, the peroxides that can be reduced in this cycle include lipid peroxides and glutathionylation of proteins (Lu, 2013). The cycle begins once hydrogen peroxide is formed through the reaction between peroxides and superoxide

dismutase. Two GSH molecules reduce hydrogen peroxide in the presence of glutathione peroxidase (GPx) to form water. Following the reduction of hydrogen peroxide, glutathione is found in its oxidized form of GSSG. GSSG must react in the presence of glutathione reductase (GR) and NADPH to reconstitute GSH. GSH can function once again in its role to reduce peroxides and prevent damage caused from reactive oxygen species.

1.3.4 Glutathione status in premature neonates

It is well established that the GSH antioxidant pathway is impaired in premature neonates. In the neonatal rabbit lung, a 200% increase in glutathione peroxidase activity was observed in the last 5 days of gestation (Frank and Groseclose, 1984). In human preterm infants, erythrocyte glutathione peroxidase activity is significantly lower compared to term infants up to 20 days post-birth (Nassi *et al*., 2009). In addition, 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress, was significantly higher in preterm infants compared to term infants (Nassi *et al*., 2009). These studies demonstrate that the activity of one of the critical enzymes in the cyclical pathway of GSH is reduced and therefore the antioxidant capacity within premature neonates is reduced.

A method used to increase glutathione capacity in premature infants has been through supplementing cysteine, as poor cysteine status will impair the synthesis of glutathione. In human neonates, birth prior to 32 weeks gestation resulted in high plasma cystathionine concentrations and lower plasma cysteine concentrations compared to infants born at greater than 37 weeks gestation (Vina *et al*., 1995). Importantly, in erythrocytes, glutathione synthesis from methionine was significantly lower in premature infants (<32 weeks gestation) indicating that endogenous cysteine synthesis was impaired and in turn, may impair glutathione synthesis (Vina *et al*., 1995). The study by Vina *et al.* (1995) was

conducted using venous blood collected from infants born at different gestational ages and within the first 24 hours of life. In addition, glutathione synthesis was measured after the cells had been depleted of endogenous glutathione, using diethyl maleate, because high glutathione concentrations inhibit glutathione synthesis (Vina *et al.*, 1995). Vina *et al.* (1995) were able to determine that methionine was not being converted to cysteine through the analysis of plasma amino acids because cysteine was in low concentrations in infants born prior to 32 weeks gestation, compared to infants born after 37 weeks gestation. They also compared the results of glutathione synthesis when methionine was provided and when *N*-acetylcysteine (NAC) was provided, finding no differences in glutathione synthesis when NAC was provided as a precursor (Vina *et al.*, 1995). Preformed cysteine as a precursor for glutathione was assessed in a study by Te Braake *et al*. (2009). In this study, 20 preterm infants weighing less than 1500 g were randomly assigned to either the standard-dose cysteine (45 mg/kg per day) parenteral nutrition diet or the high-dose cysteine (81 mg/kg per day) parenteral nutrition diet that was provided exclusively for 2 days. On day 2, the infants were given a stable isotope infusion of [1-^{13}C] glycine to measure its incorporation into glutathione and measure glutathione synthesis rates. Te Braake *et al.* (2009) found that increasing cysteine concentrations in a parenteral diet did not increase the rate of glutathione synthesis or the concentration of glutathione in erythrocytes and therefore, was not an effective method of increasing glutathione concentrations and synthesis in premature neonates.

Premature neonates have low enzyme activity and a poorly developed capacity to produce endogenous glutathione. These insufficiencies introduce a need to devise strategies

to improve the premature neonate's ability to manage oxidative stress and enhance antioxidant capacity.

1.4 Cysteine requirements in neonates

1.4.1 Sources of cysteine

Cysteine is a sulfur-containing amino acid that may be produced endogenously from methionine through the transsulfuration pathway; alternatively, the other sources of cysteine are from diet and protein breakdown (Lu, 2009). It is a precursor for the synthesis of taurine, sulfates, and glutathione (Bauchart-Thevret *et al*., 2009; Shoveller *et al*., 2005). The primary site of cysteine metabolism is in the liver; however, metabolism of sulfur-containing amino acids in the intestinal tract is vital for gut health (Shoveller *et al*., 2005). Intestinal metabolism of these amino acids is bypassed with the use of TPN. Thus, the requirement for sulfur-containing amino acids is altered with TPN administration (Shoveller *et al*., 2003a). Neonatal piglets require approximately 30% less dietary methionine when supported by TPN (Shoveller *et al*., 2003a). In addition, cysteine added to TPN resulted in a sparing of dietary methionine in both parenterally and enterally fed piglets (Shoveller *et al*., 2003b). These studies illustrated how the route of feeding and amino acid supplementation can alter sulfur-containing amino acid requirements.

Cysteine is very unstable in aqueous solution and can precipitate at relatively low concentrations in a TPN diet. Dimerization of cysteine to cystine limits the concentration of cysteine that can be added to a TPN diet. However, NAC has been successfully included as a source of cysteine in a TPN diet fed to piglets due to its greater solubility and stability (Shoveller *et al*., 2006). Approximately 85% of infused NAC was retained (Shoveller *et al*., 2006). Because the instability of cysteine prevents it from being directly supplemented

in TPN, another strategy is to improve the endogenous synthesis of cysteine from its precursor, methionine.

1.4.2 Cysteine availability, oxidative stress and GSH synthesis

The first enzyme involved in the conversion of methionine to cysteine, methionine adenosyltransferase (MAT), is inhibited by the presence of peroxides (Elremaly *et al*., 2012). Elremaly *et al.* (2012) studied the effects of TPN administration in a guinea pig model on the activity of MAT, the concentrations of GSH and GSSG, and on the hepatic redox potential. The results of TPN administration on the above parameters were compared against a control group receiving regular guinea pig food, a sham group that underwent surgery and received regular guinea pig food, and a group that received an infusion of H_2O_2 with dextrose who also had free access to regular guinea pig food (Elremaly *et al.*, 2012). The reduction in the activity of MAT and the lower GSH concentration in both the TPN and H_2O_2 compared to the control and sham groups indicate that the presence of peroxides inhibits the MAT enzyme and in turn, reduces the concentration of GSH produced in the liver (Elremaly *et al.*, 2012). The multivitamin preparations used in TPN diets also contribute a major source of peroxides because of the exposure to ambient light and room temperature (Lavoie *et al.*, 1997a). A study in guinea pigs infused with solutions containing varying concentrations of ascorbylperoxide and H_2O_2 for 4 days investigated the effects of peroxides on GSH and GSSG concentrations and the effect on alveolarization in the lung (Elremaly *et al.*, 2014). Ascorbylperoxide has been shown to be produced within TPN at a concentration of 36 ± 1 μM (Elremaly *et al.*, 2014). The infusion of peroxides resulted in higher concentrations of GSSG compared to GSH indicating that the glutathione system was responding to oxidative challenge and became depleted of GSH (Elremaly *et al*., 2014).

As such, infants on TPN have to deal with peroxides directly received with the diet; in addition, they must also endogenously synthesize GSH to be able to combat the high oxidant load intracellularly. However, GSH synthesis may also be inhibited by the presence of those same peroxides in the TPN diet due to the inhibition of MAT (Elremaly *et al.*, 2012). The lower GSH concentration and the inhibition of MAT by peroxides indicates that the reduced activity of the enzyme may lead to lower cysteine synthesis from methionine and as a result, lower endogenous GSH synthesis (Elremaly *et al*., 2012; Elremaly *et al.*, 2014).

1.4.3 Problems measuring glutathione

As a vital compound in the endogenous antioxidant system, the ability to measure glutathione in its various forms is critical. However, there are multiple challenges that arise when measuring glutathione. A review from 2016 outlines these many challenges which begin with the structure of glutathione (GSH). GSH contains a sulfhydryl/thiol group that is readily oxidized to glutathione disulfide (GSSG). Endogenously, this readily occurs, as described above. However, when the goal is to measure the ratio of GSH to GSSG, oxidation can drastically alter the results. Giustarini *et al*. (2016) stated that if 1% of GSH is oxidized, there would be, approximately, 150% bias on the measurement of GSSG. This difference in measurements resulted in new methods being developed to accurately measure GSH and GSSG. A common method is treating biological samples with N-ethylmaleimide (NEM). NEM protects GSH from reacting by binding to the sulfhydryl/thiol group required for redox reactions. NEM passes through the cell membrane and reacts with GSH within seconds of interacting (Giustarini *et al*., 2016). NEM is also able to inhibit glutathione reductase (GR). This ensures that no GSSG is converted back to

GSH and therefore the ratio remains stable for analysis. Other derivatizing agents have been used, such as 2-vinylpyridine (2-VP), however, they did not prove to be as effective as the NEM method.

1.5 Rationale

TPN is the administration of all daily nutrient requirements provided intravenously and is used in the treatment of premature infants when normal enteral feeding is not possible. The administration of TPN has been shown to result in a decrease in blood flow through the SMA within 4 hours of initiation, due to bypassing the gut (Niinikoski *et al.*, 2004). Consequently, changes in intestinal morphology are visible within 48 hours of TPN administration and continue to be apparent after 6 days of TPN administration (Niinokoski *et al.*, 2004; Burrin *et al.*, 2000). Nitric oxide induces vasodilation and is synthesized by the enzymes called NOS. Niinikoski *et al.* (2004) found that iNOS decreased in activity within 24 hours of TPN administration. In addition, a decrease in the activity of eNOS has been shown to result in greater intestinal injury preceding the onset of NEC (Drucker *et al.*, 2018). These enzymes are responsible for the production of the vasodilator, NO, and therefore a decrease in their activity would result in a decrease in the production of NO. Greater NO production is important in maintaining blood flow to the gut during TPN administration, thus lowering inflammatory markers within the mucosa due to the maintained blood flow; but a problem with NO is that it can react with superoxide to produce a pro-oxidant compound, peroxynitrite (Ikeda *et al.*, 2010; Kiristioglu *et al.*, 2002; Pacher *et al.*, 2007; Radi, 2018). Peroxynitrite induces lipid peroxidation which leads to cell damage and possible necrosis (Pacher *et al.*, 2007). To prevent the overproduction of peroxynitrite and other pro-oxidant compounds, the endogenous antioxidant system

counteracts these compounds by reducing them. However, in neonates, it has been shown that the antioxidant system specifically related to the production of glutathione is likely impaired (Elremaly *et al.*, 2012; Vina *et al.*, 1995). Premature infants have a decreased production of glutathione from its precursor methionine, and this may be due to limited activity in one of the enzymes in the pathway, cystathionase (Vina *et al.*, 1995). Due to the lower synthesis of glutathione in premature infants and the common requirement for TPN as a support strategy, research is required to elucidate whether exogenous supplementation of glutathione in TPN will be beneficial to manage oxidative stress in neonates.

1.6 Hypothesis

Premature infants lack the ability to synthesize enough glutathione to combat pro-oxidant compounds that are present in the TPN diet as well as those produced endogenously. In addition, they are at risk of developing conditions like NEC due the decrease in blood flow to the gut. In the TPN-fed neonatal piglet, a well-established model for preterm infants, we hypothesized that:

1. Supplementing glutathione in TPN would result in greater superior mesenteric artery blood flow.
2. Glutathione-supplemented TPN would enhance the antioxidant status (i.e., greater antioxidant concentration) in the liver and small intestine.
3. Glutathione supplementation in TPN would help prevent the loss of small intestinal mucosal cell mass and structure and reduce inflammation in the small intestinal mucosa.

1.7 Objectives

1. To measure changes in blood flow to the gut (via the SMA) after the initiation of TPN, and compared to enteral feeding.
2. To determine whether glutathione-supplemented TPN enhances SMA blood flow compared to standard TPN.
3. To determine if glutathione-supplemented TPN diet enhances antioxidant status in the liver and small intestine compared to a standard diet treatment.
4. To determine if the glutathione-supplemented TPN diet helps maintain small intestinal mucosal mass and structure, more similar to enterally fed piglets, and decreases inflammation markers in the small intestinal mucosa compared to piglets treated with standard TPN.

Chapter 2.0 Materials and Methods

2.1 Animal Study Protocol

2.1.1 Animal Model

The study was conducted using Yucatan miniature piglets. The piglet model is the best non-primate model for studies concerning human infants due to similar organ systems and developmental physiology (Miller *et al.*, 1987). Piglets between 8 to 12 days of age were obtained from a breeding herd at Memorial University of Newfoundland (St. John's, Canada). Animal protocols were approved by the university's Institutional Animal Care Committee according to the guidelines of the Canadian Council on Animal Care (ethics approval number 18-01 JB).

2.1.2 Surgical Procedures

Upon arrival, the piglets were given a dose of ketamine (0.22 mg/kg), acepromazine (0.5 mg/kg), buprenorphine hydrochloride (0.03 mg/kg; Temgesic, Canada) and atropine (0.05 mg/kg). Ketamine and acepromazine were administered intramuscularly in the same syringe. Buprenorphine was administered intramuscularly. Atropine was administered subcutaneously. The piglet was then intubated and placed on 1.5% isoflurane as the gas anesthetic carried in oxygen (1.5 L/min). The first incision was 2 cm in length made along the left side of the sagittal plane on the piglet's back, perpendicular to the front left condyle of humerus (elbow). The trocar was used to tunnel under the skin to the neck, above the front leg and to the left of the midline of the piglet. Once the trocar has punctured the skin, jugular vein catheter was threaded through the trocar and pulled through to the location along the neck. The trocar was used once again to tunnel under the skin from the incision along the back to the top of the left lateral condyle of femur (stifle).

The femoral vein catheter was threaded through the trocar and pulled through to the top of the left lateral condyle of femur (stifle). Returning to the incision along the back, grommets on both catheters were secured to muscle under the skin. The incision was sutured around the externalized catheters and care was taken to allow for fluids to move through the catheters. The piglet was then turned onto its back, exposing the neck and leg to allow for the insertion of the catheters into the respective veins.

To insert the jugular vein catheter, a 2 cm incision was made on the neck to the left of the midline of the piglet. Using the straight forceps, the muscle layers were separated to expose the jugular vein. Using the curved forceps, the vein was cleaned of fat and separated from surrounding tissue. Using the 90° forceps, two threads were inserted under the vein and positioned at the extremities of the visible portion of the vein and clamped using curved clamps. The threads, once in position, caused hemostasis. To insert the catheter, a 22-gauge needle was bent to a 90° angle and used to puncture the vein. A vein prick was then inserted under the needle to open the puncture site. The catheter tip, cut to a point, was inserted under the vein prick and slowly advanced to the superior vena cava using thin forceps and curved clamps. Once in the vein, the thread located around the vein and catheter was tightened to secure the catheter. The thread around the section of the vein located cranially, is tightened to prevent blood from filling the cavity. The grommet located around the jugular vein catheter was sutured to the muscle preventing the catheter from moving once in the vein. The incision site was sutured closed.

The next step in the surgical procedure was the insertion of the femoral vein catheter**.** A 1-2 cm incision was made from the last nipple of the piglet to the medial side left lateral condyle of femur (stifle). The femoral vein was isolated and separated from

surrounding tissue between the femoral nerve and the femoral artery. Using the 90° clamps, two threads were placed along the extremities of the visible portion of the femoral vein causing hemostasis. Using the 22-gauge needle with the 90° bend, a whole was punctured into the vein. A vein prick was inserted under the needle to open the puncture whole. The catheter with a pointed end was inserted under the vein prick and slowly advanced to the inferior vena cava using thin forceps and clamps. Once the catheter was in the vein, the thread around the vein and catheter was tightened while ensuring the maintenance of blood flow. The thread around the vein at the opposing extremity was tightened to maintain hemostasis. The grommet around the catheter was secured to the muscle. The incision site was sutured closed. The piglet was then turned to expose the left side.

The next part of the surgery involved inserting the ultrasonic probe around the superior mesenteric artery (SMA). A 3 cm incision was made along the line of the ribcage caudally. The muscle layers were separated to locate the kidney. To ensure the renal vasculature was not damaged, the dissection started above the kidney and moving cranially around the top of the kidney. Once located ventral to the kidney, retractors were used to move the kidney out of the line of vision. Using a haemostat, dissection of tissues continued medially ensuring no punctures through the peritoneum into the abdominal cavity. Once the vena cava and aorta became visible, dissection ceased. The SMA is located between the vena cava and the aorta. Careful isolation of the SMA was made, ensuring a tool could be inserted under the vessel. Once the vessel was isolated, the 4 mm ultrasonic probe (Transonic) could be implanted around the SMA. Using haemostats clamped to the moveable section of the probe, the probe was implanted around the vessel.

A second pair of haemostats were used to hold the probe in place while the first haemostats are used to push the moveable section down around the vessel. The first pair of haemostats are removed from the window while the second pair of haemostats were holding the probe in place. A screwdriver was then used to fasten the moveable section in place and securing the probe in place. Using a 1 mL syringe, ultrasonic gel was injected around the probe and vessel to ensure the blood flow rate could be read. The flow rate was then tested using the accompanying flow meter (Transonic®, New York, USA). Once a reading was confirmed, the wire of the probe was tunnelled under the skin to an incision site along the back ventral to the first incision on the back. At the incision site along the ribcage, a loop in the probe wire was created and tied together to have excess length under the skin, in the case that the probe was pulled. The muscle layers were sutured back together. A pocket was created under the skin to house the loop in the probe wire. The loop was secured to the muscle to prevent movement under the skin. The final step was to suture the incision site and close it.

The piglets that were randomized to enteral feeding underwent an additional procedure to insert a gastric catheter into the stomach. Through the same incision along the back where the wire of the SMA probe exits the skin, the gastric catheter was tunneled under the skin to the ventral side of the piglet between the nipples. A 3 cm incision was made from between the second set of nipples (counting down to the caudal end of the piglet) to the cranial side of the umbilicus. To ensure no damage to the organs, forceps were placed under the skin to lift the skin and extend the incision if needed. The stomach was then isolated and held above the skin with wet gauze. On the cranial side of the stomach, a purse-string suture was placed and using an 18-gauge needle, a hole was

punctured into the lumen of the stomach at the center of the suture. The gastric catheter was clamped with haemostats and pushed through the hole into the stomach. The first grommet along the gastric catheter was pushed into the stomach and the second grommet remained outside the stomach wall. The purse-string suture was tightened to secure the catheter in the stomach. Once secured, the stomach is put back into the body cavity. The peritoneum and muscle layers were sutured to close the abdominal cavity. The final step was to suture the skin to close the incision site. The port for the gastric catheter could then be secured to the exterior end of the gastric catheter.

All piglets were given 20 mg trimethoprim and 100 mg sulfadoxine antibiotics (Borgal, Intervet Canada Ltd., Canada) diluted with sterile saline in a 5 mL syringe. The antibiotic was given into the femoral catheter to reduce the probability of infections following the surgery. The jackets were then put on the piglets and the catheters were threaded through the jacket to prevent any occlusion in the catheters. Each suture incision site was treated with an antibacterial/antifungal ointment (1% Chlorhexidine acetate; Hibitane, Canada). The piglets were given a second dose of buprenorphine hydrochloride (Temgesic, Canada) at 0.03 mg/kg between 8 to 12 hours following the first dose. The piglets were subsequently placed in the metabolic cage wearing a cotton jacket that was attached to a swivel and tether system (Lomier Biomedical, Canada) that allowed for free movement while facilitating diet infusion. The room was lit from 0700h to 1900h, and the temperature was maintained between 28°C and 32°C with additional heat lamps.

2.1.3 Daily Animal Care

The piglets were housed individually in metabolic cages next to another piglet with which they could see and interact with during the study period. Starting on Day 2, the piglets

were removed from the metabolic cage to be weighed, the incision sites were treated with the antibacterial/antifungal ointment (1% Chlorhexidine acetate; Hibitane, Canada) and the jugular and femoral vein catheters were flushed to check for any blockage or resistance. The piglets were given another dose of buprenorphine hydrochloride on Day 1 and antibiotics (trimethoprim and sulfadoxine; Borgal, Intervet Canada Ltd., Canada) on Day 1 through Day 4. After Day 1, buprenorphine hydrochloride (Temgesic, Canada) was administered when piglets displayed pain behaviour.

2.1.3.1 Diet Flow Rate

On Day 0, after the completion of the surgical procedure, all piglets were provided a complete intravenous diet at 6 mL/kg/h corresponding to 50% of the total nutrient requirements. On the morning of day 1 for the piglets randomized to complete parenteral feeding, the diet flow rate was increased to 9 mL/kg/h, corresponding to 75% of the final diet flow rate of 12 mL/kg/h. On the evening of Day 1, the diet flow rate was increased to 12 mL/kg/h. For piglets randomized to enteral feeding, on the morning of Day 1 the IV flow rate remained at 6 mL/kg/h and the piglet was also provided with an intragastric (IG) diet infusion at a flow rate of 6 mL/kg/h. On the evening of Day 1, IV flow rate for the enteral group was lowered to 3 mL/kg/h and the IG flow rate was increased to 9 mL/kg/h. On the morning of Day 2, the IV diet was stopped, and the IG rate was increased to 12 mL/kg/h which corresponded to 100% of requirements.

2.1.3.2 SMA Blood Flow Rate Measurement

The first SMA blood flow measurement was taken on the morning of Day 1 and was recorded every minute over a 10-minute period prior to the administration of the analgesic and antibiotic (described in 2.1.3.3). Approximately 5 hours after the first SMA

blood flow reading on Day 1, a second reading was taken every minute over a 10-minute period. The final SMA blood flow rate measurement for Day 1 was taken 5 hours after the second reading. This procedure was repeated for Days 2 to 6 of the study. One final SMA blood flow reading was taken on Day 7.

2.1.3.3 Daily Blood Sampling

A 2 mL blood sample was collected daily; 1 mL was placed into an EDTA BD Vacutainer (BD Biosciences, Mississauga, Canada) to measure total GSH content in whole blood. The other 1 mL was also placed in an EDTA tube to which 100 μL of 310 mM NEM (Sigma-Aldrich, Canada) was added to allow the measurement of GSH and GSSG concentrations in whole blood.

2.1.4 Stable Isotope Infusion

On Day 7, a primed then constant delivery of [D_5]-glycine (prime 175 $\mu mol \cdot kg^{-1}$; constant 100 $\mu mol.kg^{-1}.h^{-1}$) (Cambridge Isotopes Laboratories, Inc., USA) was infused intravenously over 5 hours to measure the incorporation of [D_5]-glycine into GSH and GSSG pools in the RBC and tissues. Prior to the start of the infusion, three baseline blood samples of 1 mL each were collected within an hour. A 1 mL blood sample was collected on hour 1, 2, 3, 3 ½, 4, 4 ½, and 5 hours. The 1 mL sample was split in half between two 1.7 mL Eppendorf tubes (0.5 mL of blood per tube) pre-treated with 10 μL of 0.26 mM EDTA. The blood samples were centrifuged at 12,100 *g* for 2 minutes. The plasma was removed and placed in a clean Eppendorf tube. One sample was placed on ice. The other sample was subsequently treated with 200 μL of 100 mM NEM (Sigma-Aldrich, Canada) and 10 μL of 10 mM γ-glutamyl-leucine. At 4.5 hours into the [D_5]-glycine infusion, a dose of 0.15 $mmol \cdot kg^{-1}$ L-[ring-D_5]-phenylalanine (Cambridge Isotopes Laboratories,

Inc., USA) and of 1.35 mmol·kg^{-1} of unlabelled phenylalanine (Sigma-Aldrich, Canada) was infused in 5 minutes to measure tissue-specific protein synthesis rates (Lamarre et al., 2015).

2.1.5 Necropsy

The piglets were removed from the metabolic cage following the isotopic infusion and transported to the surgical suite where they were anaesthetized using 2.5% to 3% isoflurane in oxygen for the terminal surgery. Using a cauterizing iron, the abdominal wall was opened exposing the intestinal tract and liver. The small intestine was removed, the total length was measured, and the total weight of small intestine was recorded. To prepare a histology sample, 2 cm sampled from the proximal jejunum was cut and immersed immediately in neutral buffered 10% formalin (Fisher Scientific, Canada) to fix the tissue. The next 50 cm of the proximal jejunum was flushed with cold saline and used to harvest the mucosa. The intestinal section was cut longitudinally and positioned on a glass plate on ice. The mucosa was scrapped using two glass slides with even pressure. The mucosa was weighed and approximately 0.5 g of the mucosa was immediately placed in 3 mL of 100 mM NEM (Sigma-Aldrich, Canada) and 50 μL of 10 mM H-Glu (Leu-OH)-OH (Bachem Holdings, Switzerland) to be homogenized. The remainder of the mucosa was flash-frozen with liquid nitrogen. The next organ sampled was the liver. The whole liver was weighed, and the left lateral lobe was cut and weighed. Approximately 0.5 g of liver sample from the lateral lobe was cut and placed in 3 mL of 100 mM NEM (Sigma-Aldrich, Canada) and 50 μL of 10 mM H-Glu(Leu-OH)-OH (Bachem Holdings, Switzerland) to homogenize the liver sample. The remainder of the left lateral lobe was flash-frozen in liquid nitrogen. The left lung was removed next and weighed.

Approximately 0.5 g of the left lung was removed and immediately placed in 3 mL of 100 mM NEM (Sigma-Aldrich, Canada) and 50 μL of 10 mM γ-glutamyl-leucine (γ-GL) to homogenize the sample. The remainder of the left lung was flash-frozen in liquid nitrogen. The right kidney was removed and cut in half along the sagittal plane. The kidney was subsequently flash-frozen in liquid nitrogen. The final samples taken were from the longissimus dorsi muscle and the gastrocnemius muscle and were immediately flash-frozen in liquid nitrogen.

2.2 Treatment Groups

The study consisted of three treatments; two were delivered as parenteral nutrition, and one was an enteral control group with the diet delivered into a gastric catheter. The control parenteral group (PN; n=10) was provided a diet that was nutritionally complete and based on commercially available total parenteral nutrition solutions for infants that was adapted for piglets (Wykes *et al.*, 1993). The glutathione-supplemented (GSSG) parenteral group (GSSG; n=9) was the same nutritionally complete diet as the PN group with GSSG supplemented at a concentration of 10 μM, which has been shown to support physiological concentrations in plasma (Elremaly *et al.*, 2016). The control enteral group (EN; n=10) was provided the same nutritionally complete diet as the PN group however, the diet was administered enterally through the gastric catheter implanted during the surgery described above.

All diets were prepared in the laboratory and were cold sterilized by filtering through a AcroPak^{TM} 200 Supor® Membrane filter (Pall Corporation, Switzerland) using a peristaltic pump (EMD, Millipore, Darmstadt, Germany). Diets were pumped into empty sterile IV bags (Baxter Corporation, Mississauga, ON, Canada) using an 18G 1-

inch needle (BD – Canada, Mississauga, ON, Canada) in a laminar flow hood. Each diet bag was weighed to ensure 750 mL (795 g) of diet was added to the bag and then stored at 4 °C in a dark environment until they were used.

2.2.1 Diet Preparation

The composition of elemental diets is summarized in Table 1 and Table 2. None of the diets contained dietary fibre, including the EN diet. The complete diet was delivered at 12 mL kg^{-1} h^{-1} to provide 12.9 g of amino acids kg^{-1} d^{-1} (Ajinomoto, Canada, Evonik Industries AG, Germany or Sigma Aldrich, Canada) and non-protein energy delivery of 1.1 MJ kg^{-1} d^{-1} from dextrose (Sigma Aldrich, Canada) and lipids (SMOFlipid; Baxter, Canada). The amino acid profile was based on a commercially available parenteral nutrition solution for infants (Vaminolact, Germany) that was modified for piglets (Wykes et al., 1993). A mixture of vitamins was prepared in the laboratory, filtered through a sterile AcroPak™ 200 Supor® Membrane filter (Pall Corporation, Switzerland), and added to the diet bags just prior to feeding. Iron dextran (Fe, 3.0 mg/kg; Vetoquinol Canada Inc., Quebec, Canada) and trace minerals (Sigma Aldrich, Canada) (NRC,1998) were also added to each diet bag immediately before use. The diets were continuously infused (24 h) via pressure-sensitive peristaltic pumps.

Table 2.1: Amino acid profile of the elemental diets.

	PN and EN diet	GSSG diet
Amino Acids	**g/kg/day**	
Alanine	1.41	1.41
Arginine	0.87	0.87
Aspartate	0.80	0.80
Cysteine	0.18	0.18
Glutamate	1.37	1.37
Glycine	0.35	0.35
Histidine	0.40	0.40
Isoleucine	0.60	0.60
Leucine	1.36	1.36
Lysine-HCl	1.07	1.07
Methionine	0.25	0.25
Phenylalanine	0.72	0.72
Proline	1.08	1.08
Serine	0.74	0.74
Taurine	0.06	0.06
Tryptophan	0.27	0.27
Tyrosine	0.11	0.11
Valine	0.69	0.69
Threonine	0.53	0.53
Glutathione Disulfide	0	1.47E-03

Table 2.2: *Non-amino acid components of the elemental diet.*

	PN, EN and GSSG diet g/kg/day
Dextrose	21.62
K_2HPO_4 Trihydrate	0.38
KH_2PO_4 Monobasic	0.26
Potassium Acetate	0.35
NaCl	0.52
$MgSO_4$	0.19
$ZnSO_4$	0.02
Calcium Gluconate	1.53
$ZnSO_4 \cdot 7H_2O$	9.74
$CuSO_4 \cdot 5H_2O$	0.75
$MnSO_4 \cdot H_2O$	0.44
$CrCl_3 \cdot 6H_2O$	0.01
SeO_2	0.01
NaI	0.01

2.3 Analysis Procedures

2.3.1 Tissue Specific Protein Synthesis

Frozen liver and mucosa samples were pulverized in liquid nitrogen. Approximately 200 mg of the pulverized liver sample, or 100 mg of pulverized mucosa sample, were homogenized in 4 mL of distilled water (dH_2O) for one minute (1:20 dilution). 0.5 mL of 2 M PCA (Fisher Scientific, Canada) was added to the homogenate and vortexed thoroughly. The solution was centrifuged for 20 minutes at 4°C at 1107 *g*. Following centrifugation, the supernatant was analyzed for phenylalanine isotope enrichment in the **tissue free amino acid pool** and the pellet was analyzed for the concentration of phenylalanine isotopes in **tissue bound amino acid pool**.

Tissue-Free Amino Acid Pool in Supernatant

The supernatant was filtered through a C18 bond elut column (**Table 2.3).**

Table 2.3: Tissue free pool conditioning process that was run through a bound elut filter.

Step	Volume	Solution	Eluted Solution
1	1 mL	100% Methanol	Discard
2	1 mL	0.1 M PCA	Discard
3	0.5 mL liver 1 mL mucosa	Tissue Free Supernatant Sample	Discard
4	1 mL	0.1 M PCA	Discard
5	1 mL (tissue free); 2 mL (protein bound)	100% Methanol	Collect into glass vial with a screw cap.

After filtering, the eluent vial was frozen with liquid nitrogen and freeze dried for approximately 10 hours. Subsequently, 500 µL of HPLC water was added to resuspend the sample and it was vortexed thoroughly. The derivatization process required 50 µL of sample and is described below.

Tissue-Bound Pool in Pellet

The protein pellet was mixed with 5 mL of 0.2 M PCA (Fisher Scientific, Canada), vortexed and then centrifuged for 20 minutes at 4°C at 1100 *g*. The supernatants were discarded, and the pellets were kept. The above washing with PCA (Fisher Scientific, Canada) was repeated for a total of 3 times. Using the screw cap tubes**,** 4 mL of 6 M HCl was added to break up the pellets. The tubes were capped and was incubated at 110°C for 18 hours. After the incubation period, the samples were filtered through a 0.45 µm filter. The filtered samples were then filtered using a bond elut column as described in **Table 1**. The final sample (step 5) was collected in a screw capped glass vial. The sample was dried in the vacuum oven for approximately 10 hours. To resuspend the dried sample, 500 µL of HPLC water was added. The derivatization process required 50µL of sample.

Derivatization of tissue free and protein bound amino acids

In the screw-capped 2 mL GC-MS vials (screw cap liner was silicon/PTFE), 21.5 µL of 0.5 M phosphate buffer, 50 µL of the resuspended sample, and 133 µL of 100 mM pentafluorobenzyl bromide (PFBBr; Sigma Aldrich, Canada) in acetone were added in that order. The tubes were capped and vortexed thoroughly. The vials were incubated in a heating block at 60 °C for 1 hour. The samples were cooled to room temperature for approximately 5 minutes. Once at room temperature, 333 µL of hexane (Sigma Aldrich, Canada) was added to the vials that were immediately recapped. The solution was then

vortexed for approximately 1 minute. To allow for the two clear layers to form, the glass tubes were left to sit for approximately 2 minutes. In a glass vial insert, 200 µL of the top layer formed in the glass vials was added and the insert was placed into the glass GC-MS vials. The tubes were immediately recapped and were ready to be analyzed on the GC-MS.

GC-MS Program

The isotopic enrichment of L-[ring-D5] phenylalanine in tissue-free and protein-bound fractions was determined by GC-MS with a model 6890N GC system linked to a 5973 quadrupole MSD (quadrupole) (Agilent Technologies; Mississauga, Canada) operating in the electron ionization mode (Lamarre et al., 2015). The GC oven was preheated to 50°C, and 1 µL of the sample was injected into a DB-5ms column (0.25 mm x 30 m x 0.25 µm) (Agilent Technologies; Mississauga, Canada). Helium was used as a carrier gas at a constant flow rate of 1.1 mL/min. The programme run time was 14.67 minutes. The settings for the method are summarized in Table 4. The mass selective detector was operated in selected ion monitoring (SIM) with a mass-to-charge ratio (m/z) of 300 and 305 for Phe and D5-phe, respectively. Tissue-specific protein synthesis was calculated as previously described (Lamarre et al., 2015).

Table 2.4: The settings of the GC-MS method for the analysis of tissue specific protein synthesis.

Parameter	**Setting**
Initial Oven Temperature	50°C
Final Oven Temperature	280°C
Rate of temperature increase	30°C/min for 4 min
Flow rate through column	1.1 mL/min
Pressure in inlet	8.79 psi
Solvent Delay	6.50 min
Carrier Gas	Helium
Column	DB-5ms (0.25 mm x 30 m x 0.25 μm)
Run time	14.67 min
Electron impact collision energy	70 eV

The percent molar enrichment (mol%) was determined and the fractional synthesis rate (%/day) of protein was calculated as follows:

$$FSR = \frac{IE_{bound}}{IE_{free}} \times \frac{1440}{t} \times 100$$

where IE_{bound} and IE_{free} are the isotopic enrichments (mol%) of L-[D5]-phenylalanine of the tissue bound and the tissue free phenylalanine pool; *t* is the time of tracer incorporation in minutes; 1440 are the number of minutes in a day.

2.3.2 Intestinal Parameters

The small intestine fixed in neutral buffered 10% formalin (Fisher Scientific, Canada) was embedded in paraffin wax. Three tissue cassettes prepared per gut sample and were labelled with a letter designating the different slices of tissue (i.e A through C). Three slices of 2-3 mm in thickness were cut per sample. The first slice of tissue from the end of the sample was discarded. Each slice of small intestine was loaded into the cassettes and the lid was closed. The remainder of the tissue was placed back into fresh neutral buffered 10% formalin (Fisher Scientific, Canada). The loaded cassettes were then placed into the basket of the tissue processor to be submerged in the neutral buffered 10% formalin (Fisher Scientific, Canada) bath. The tissue processor was set to program C, outlined in **Table 2.5**, and started.

Table 2.5: Program C from the tissue processor used to prepare small intestine samples for embedding in wax.

Position	Reagent	Time (h:min)
1	Formalin	00:01
2	Formalin	00:01
3	70% Ethanol	00:45
4	80% Ethanol	00:45
5	100% Ethanol	00:45
6	100% Ethanol	00:45
7	100% Ethanol	01:00
8	100% Ethanol	01:00
9	Xylene	01:00
10	Xylene	01:00
11	Hot Wax	01:30
12	Hot Wax	01:30

Once the cassettes had run through program C, they were ready to be embedded in wax. One hot plate was heated, and the metal histology base moulds were placed on the hot plate. The wax was heated in a coffee dispenser to allow for easy dispensing of the wax. A full ice bucket was obtained to allow the wax to cool following embedding. The well of the histology base moulds was filled with hot wax and placed back on the hot plate. The basket of the tissue processor was raised out of the wax and using the forceps, one cassette was removed, and the remaining cassettes were lowered back into the wax. The lid of the cassette was discarded. Using the forceps, the tissue sample was placed in the center of the well of the histology base moulds. The base mould was then removed from the hot plate and the bottom of the cassette was placed in the mould. The cassette-mould was filled with hot wax and placed on ice to harden. This process was repeated until all the tissues were embedded in wax. The embedded tissues were sent to the Histology, Medical Laboratories (Faculty of Medicine, Memorial University of Newfoundland, St. John's, Canada) to prepare sections for immunohistochemistry (section 2.2.3.1) and stain sections for hematoxylin and eosin (H & E) staining (section 2.2.3.2).

2.3.2.1 Immunohistochemistry for Ki-67 Cell Proliferation Marker

Slide-mounted sections were deparaffinized using the method highlighted in **Table 2.6**.

Table 2.6: Deparaffinization of tissue sections for immunohistochemistry staining.

Step	Solution	Time
1	Xylene (2x)	3 minutes each
2	Xylene 1:1 Absolute Ethanol	3 minutes
3	Absolute (100%) Ethanol (2x)	3 minutes each
4	95% Ethanol	3 minutes
5	70% Ethanol	3 minutes
6	50% Ethanol	3 minutes
7	Vessel of cold tap water	Rinse

Following deparaffinization, the slides were incubated in a sodium citrate buffer at 95°C for 20 minutes and allowed to cool in tap water for 10 minutes. This process allows for antigen retrieval. The slides were washed with gentle agitation twice for 5 minutes in tris-buffered saline (TBS) (Fisher Scientific, Canada) with 0.025% Triton X-100 (Sigma Aldrich, Canada). Using a Dako Pen (Agilent Technologies; Mississauga, Canada), a hydrophobic barrier was drawn around each section. The sections were then blocked with 10% normal goat serum (Abcam, Cambridge, UK) with 1% bovine serum albumin (BSA; Rockland Immunochemicals, Inc., Limerick, PA, USA) in TBS (Fisher Scientific, Canada) for 2 hours at room temperature. The slides were drained, and excess blocking solution was dabbed away, making sure not to disturb the tissue section. Next, the anti-Ki-67 (ab15580; Abcam, Cambridge, UK) primary antibody (2.45 µg/mL) diluted in TBS (Fisher Scientific, Canada) with 1% BSA (Rockland Immunochemicals, Inc., Limerick, PA, USA) was added to the tissue sections. The slides were incubated overnight at 4 °C and in darkness. The following day, the slides were rinsed with gentle agitation twice for 5 minutes each in TBS (Fisher Scientific, Canada) with 0.025% Triton X-100 (Sigma Aldrich, Canada). The slides were incubated in 0.3% H_2O_2 in TBS (Fisher Scientific, Canada) for 15 minutes. The conjugated secondary antibody (1µg/mL), a goat anti-rabbit IgG (HRP) (ab205718; Abcam, Cambridge, UK), diluted with TBS (Fisher Scientific, Canada) with 1% BSA (Rockland Immunochemicals, Inc., Limerick, PA, USA) was applied to each section and incubated for 1 hour at room temperature. Each section was developed for 10 minutes at room temperature using the DAB Substrate Kit (ab64238; Abcam, Cambridge, UK). The sections were left to sit in tap water for 5 minutes. After 5 minutes, the slides were counterstained with Mayer's hematoxylin (Sigma Aldrich,

Canada) for 2 minutes. They were then rinsed again in tap water by dipping the slides 10 times. The slides were then placed in the bluing reagent, Scott's Tap Water (VWR™, Canada), for 30 seconds and rinsed again by dipping the slides 10 times. The slides were then dehydrated in 95 % ethanol, 100% ethanol (2x) and xylene (3x). The slides were cleared for at least 1 hour. Finally, the cover slips were mounted using limonene mounting medium (ab104141; Abcam, Cambridge, UK).

Each section was examined under the microscope for complete crypts. Images were captured using AmScope/ToupView Software (Hangzou ToupTek Photonics Co., Ltd., P.R. China). The dark cells in the crypts were the Ki-67 positive cells. The total number of positive cells were counted per crypt and at least 10 measurements were taken for each animal. Villous height and crypt depth were measured if complete villi were visible and added to the measurements for the H&E stained slides.

2.3.2.2 Hematoxylin and Eosin Staining

The staining of sections began with deparaffinizing in xylene for 5 minutes. The sections were then rehydrated by placing them in decreasing concentrations of ethanol (Absolute, 95%, 80%, 70%) for 2 min each. After rinsing the sections for 1 minute, they were placed in Mayer's hematoxylin for 30 minutes. The sections were rinsed in water and then placed in the bluing reagent, Scott's Tap Water substitute (VWR™, Canada), for 3 minutes until the sections were blue. The sections were rinsed for 5 minutes and placed in eosin for 3 minutes. The sections were then quickly dehydrated in 95% ethanol and then absolute alcohol. Finally, the sections were cleared in xylene and cover slips were immediately mounted. Once the cover slips were dry, the slides were viewed under the

microscope and using the AmScope/ToupView software (Hangzou ToupTek Photonics Co., Ltd., P.R. China), the villus height and crypt depths were measured.

2.3.3 Antioxidant Capacity and Lipid Peroxidation in the Liver

2.3.3.1 Ferric Reducing Antioxidant Power (FRAP) Assay

The extraction method outlined in Katalinic et al. (2005) was used to treat and prepare the liver samples. 100 mg of frozen liver was homogenized in 1.0 mL of 1.15% KCl (10% w/v) using a mechanical homogenizer. The homogenate was centrifuged at 277 *g* for 10 minutes at 4°C. The supernatant was then diluted at a ratio of 1:5 with deionized water. The diluted supernatant was measured for antioxidant capacity using the Ferric Reducing Antioxidant Power (FRAP) assay kit (ab234626; Abcam, Cambridge, UK).

2.3.3.2 Thiobarbituric Acid Reactive Substances Assay

To measure lipid peroxidation in the liver, the thiobarbituric acid reactive substances were measured. The standards were prepared by diluting 200 µM of TMP with varying volumes of absolute ethanol. 100 mg of liver sample was homogenized in 1 mL of phosphate buffered saline (PBS, pH 7.4) using a mechanical homogenizer. After the liver sample was homogenized and standards prepared, 100 µL of homogenate or standard was transferred into their respective lockable 1.7 mL Eppendorf tube. In the following order, these solutions were added to the Eppendorf tube; 100 µL of 0.9% NaCl, 200 µL of 8% TCA, 100 µL of 2.5 mM BHT (dissolved in 1 part water and 1.27 parts 95% ethanol), 150 µL of 29 mM TBA and 60 µL pf 8.1% SDS. The tubes were then vortexed for 15 minutes and then heated at 95°C ± 1°C in a water bath for 60 minutes. After 60 minutes, the tubes were left to cool on ice for 10 minutes. 750 µL of n-butanol was added to each tube and vortexed. The tubes were then centrifuged at 2400 *g* at 4°C for 20 minutes. The

supernatant was subsequently filtered through a 0.45 µm syringe filter. 200 µL of filtered supernatant was transferred to a 96-well plate and immediately read at a wavelength of 532 nm. A set of standards in duplicate were prepared for each plate. All samples were prepared in duplicate.

2.4 Statistical Analyses

Data were analyzed by one-way ANOVA with Bonferroni post-hoc tests or two-way repeated measures ANOVA with Bonferroni post-hoc tests with treatment as the dependent variable. Two-way repeated measures ANOVA with Bonferroni post-hoc test was used to analyze the blood flow through the superior mesenteric artery. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, USA). Data are presented as mean ± standard deviation (SD). $p<0.05$ was considered to be significant.

Chapter 3: Results

In total, there were 33 piglets that underwent surgery to be placed in the study. Only 29 piglets survived for the duration of the study period (7 days). The 4 piglets that died within 24 h after surgery were randomly placed into the GSSG treatment group. Of the 4 piglets, 3 were replaced in the study; the 4th piglet was not replaced because animals were not available. As a result, the GSSG group had a sample size of 9 piglets with 10 piglets for the EN and PN groups. Throughout the study period, there were some challenges that arose, including instances of vomiting for reasons unknown and in the EN group, bouts of diarrhea. As previously mentioned, the EN diet did not contain any dietary fibre. Despite these adverse events, the piglets remained very interested in their environment and readily interacted with toys found in their cage and with people.

3.1 Animal Weights

Upon arrival to the facilities, the piglets were weighed and randomly assigned to one of the three treatment groups that were organized in a predetermined order. Towards the end of the study, piglets were placed into groups to replace piglets who died prematurely in the study. There were no significant differences in the initial body weight of the piglets between treatment groups (Figure 3.1(a)). The final body weight of the piglets measured on day 7, was different among treatments; the PN group had a significantly lower final body weight compared to the EN group (Figure 3.1 (b)). No significant differences were apparent in the final body weight of the piglets in the EN group compared to the GSSG group. Although the final body weights differed between the PN and EN groups, all groups had similar weight gain patterns over time (Figure 3.2).

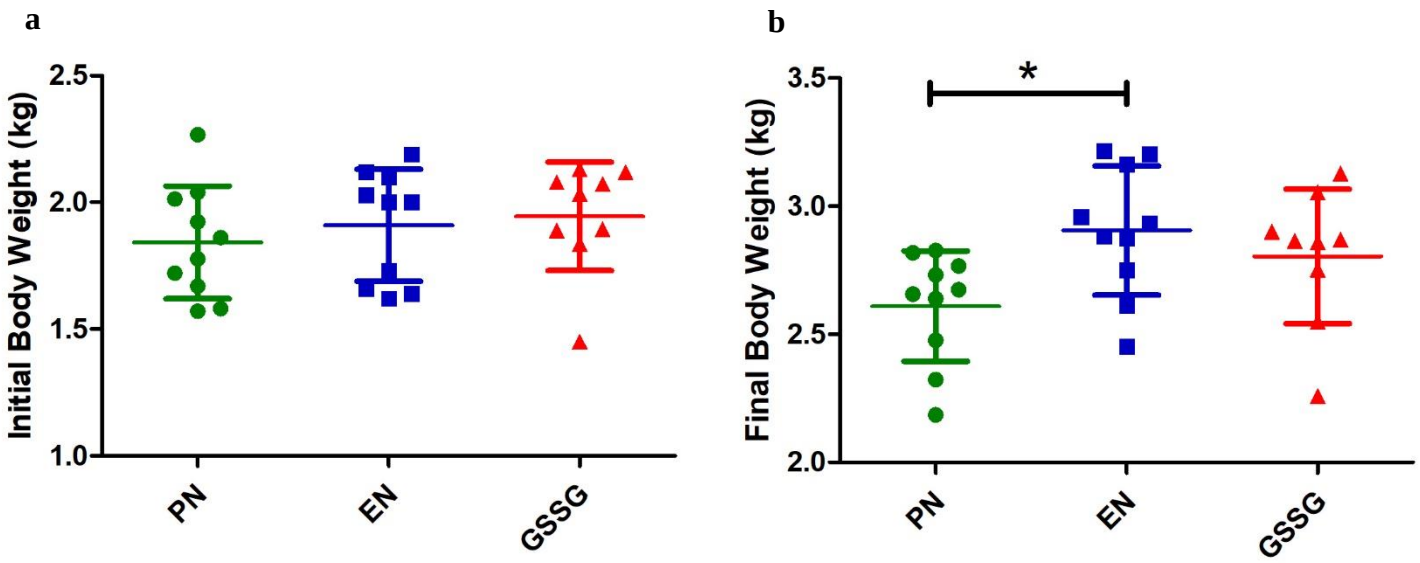

Figure 3.1: (**a**) Initial body weight, (**b**) final body weight of piglets on day 7 (n= 10 (PN, EN), 9 (GSSG)). Data are means ± SD. Treatment effect by ANOVA for final body weight, p=0.036; means compared by Bonferroni's post-hoc test, *p<0.05.

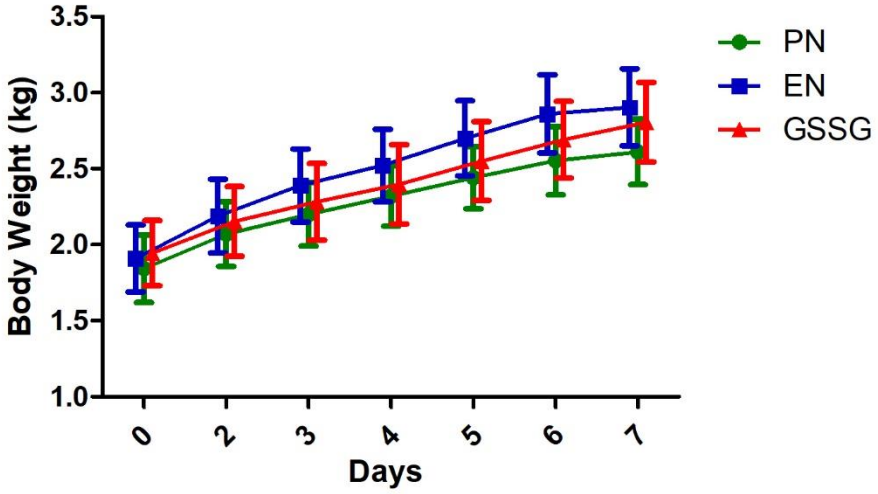

Figure 3.2: Weight change over the 7-day study (n= 10 (PN, EN), 9 (GSSG)).

When total body weight gain over the 7-day study period was compared, there was significantly lower body weight gain in the PN group compared to the EN group (Figure 3.3). No significant differences were observed between EN and GSSG supplemented parenteral feeding (Figure 3.3).

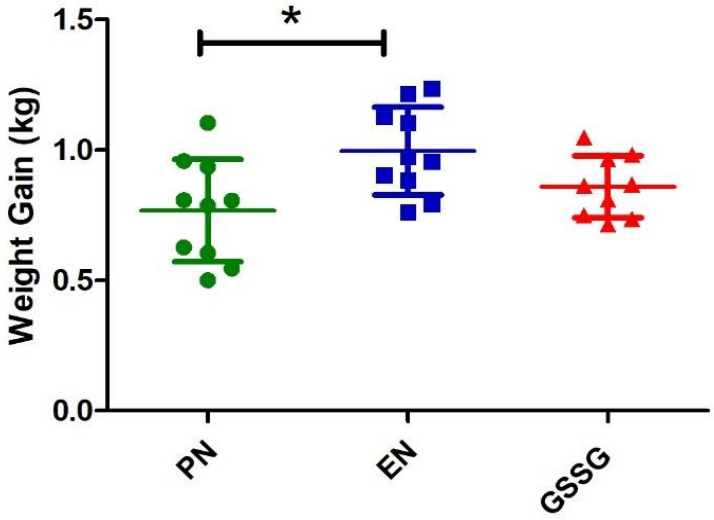

Figure 3.3: Total weight gain over study period of 7 days (n= 10 (PN, EN), 9 (GSSG)). Treatment effect by ANOVA, p=0.02; means compared by Bonferroni's post-hoc test, *p<0.05.

3.2 Organ Morphology

There was significantly lower small intestinal weight in the PN group compared to the EN group and this same effect was apparent between the EN and GSSG groups (Figure 3.4 (a)). Mucosa weight was significantly lower in the PN group compared to the EN group; similarly, the GSSG piglets had lower mucosa weight compared to the EN animals (Figure 3.4 (b)).

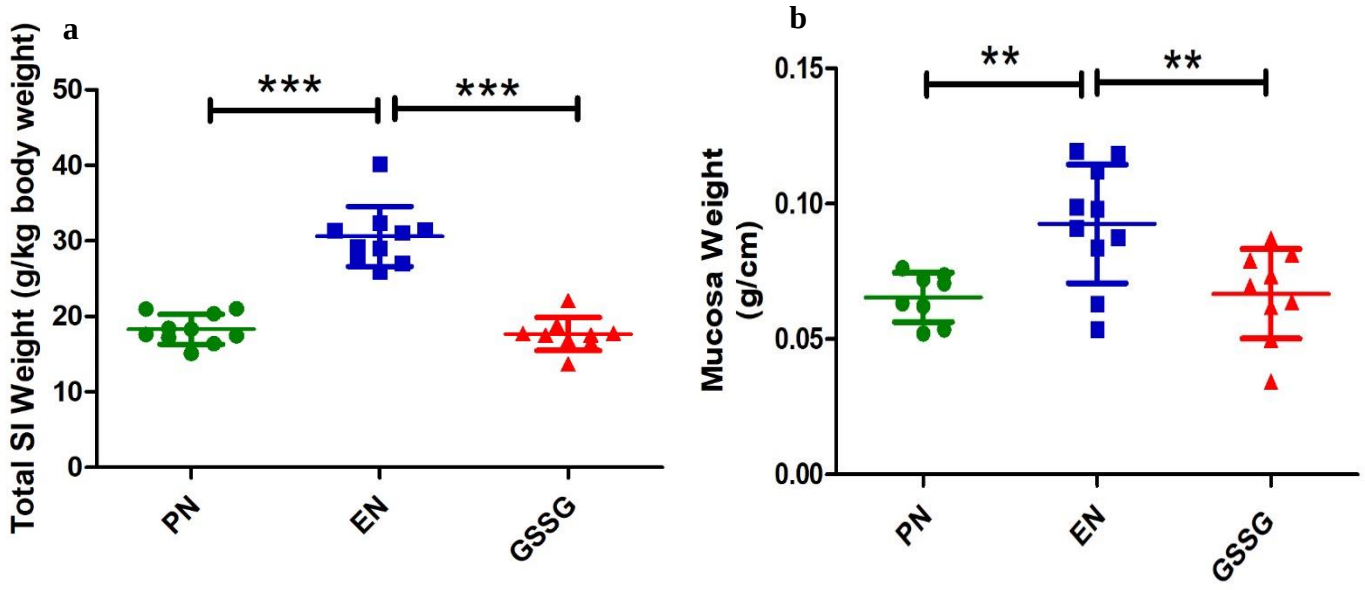

Figure 3.4: (**a**)Total small intestine (SI) weight normalized to body weight (g/kg body weight) (n= 10 (PN, EN), 9 (GSSG)). Treatment effect by ANOVA, $p<0.0001$. (**b**) Mucosa weight normalized to body weight (g/cm) (n= 8 (PN), 10 (EN), 9 (GSSG)). Treatment effect by ANOVA, $p=0.003$; means compared by Bonferroni's post-hoc test, ***$p<0.001$, **$p<0.01$.

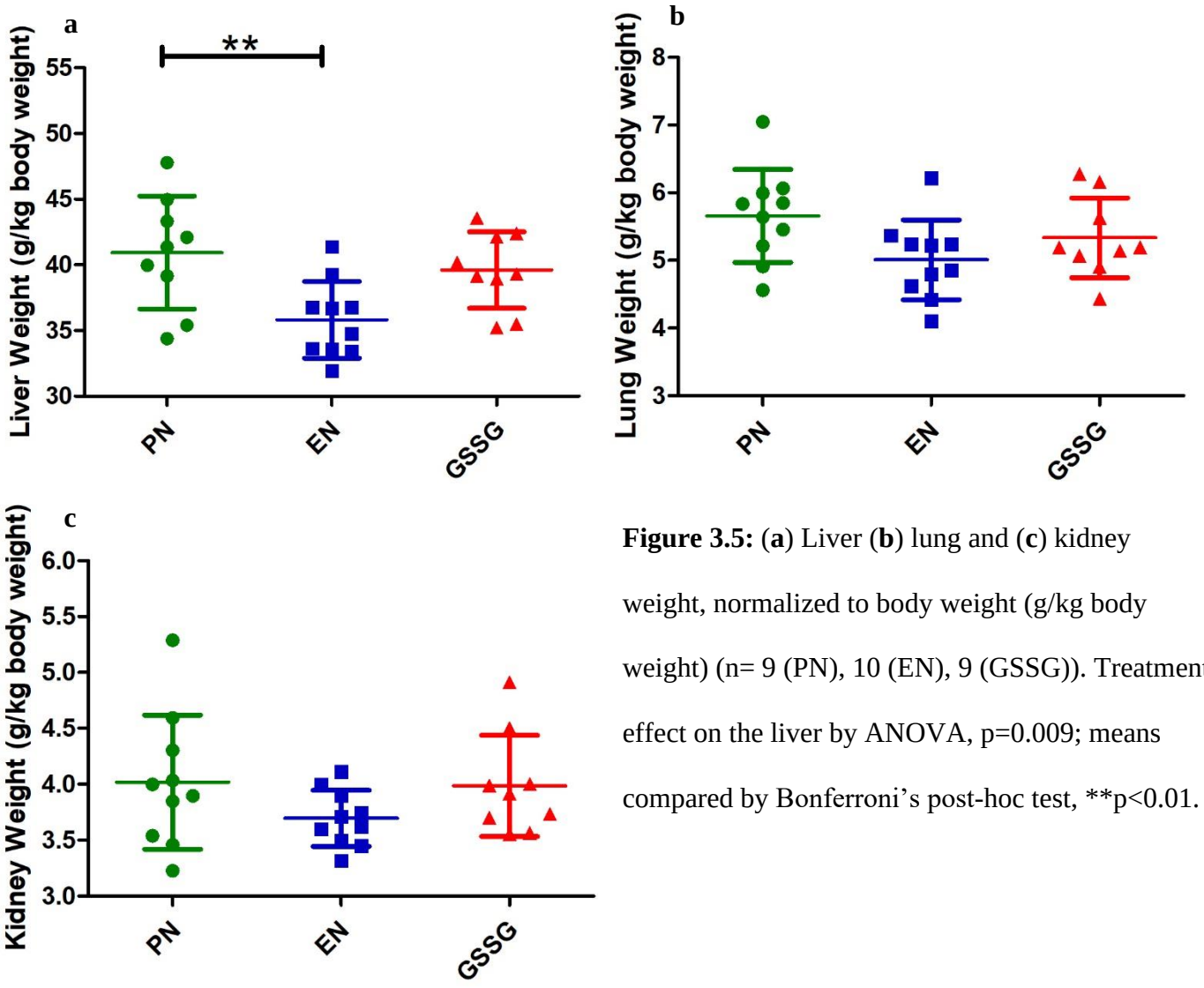

Figure 3.5: (**a**) Liver (**b**) lung and (**c**) kidney weight, normalized to body weight (g/kg body weight) (n= 9 (PN), 10 (EN), 9 (GSSG)). Treatment effect on the liver by ANOVA, p=0.009; means compared by Bonferroni's post-hoc test, **p<0.01.

The liver weight was significantly greater in the PN group compared to the EN group; however, there was no significant difference in liver weight between the EN and GSSG supplemented parenteral group (Figure 3.5 (a)). Overall, the effect of the diet on the weight of the lungs did not reach significance and therefore, no statistically significant differences were observed in lung weight between the groups (Figure 3.5 (b)). There were no significant differences in kidney weight among any of the diet treatments (Figure 3.5 (c)).

3.3 Blood Flow through the Superior Mesenteric Artery

Once 100% of diet delivery was achieved by the EN group at 32 hours, blood flow rose to a mean flow rate of 45.6 ± 5.0 mL/min/kg for the remainder of the study period (Figure 3.6). Parenteral nutrition induced a significant decrease in blood flow compared to the EN group (Figure 3.6). In the first 58 hours, blood flow through the SMA in the GSSG group resulted in maintained blood flow with a mean of 39.9 ± 1.6 mL/min/kg compared to a mean of 43.7 ± 8.4 mL/min/kg in the EN group. At approximately 39 hours, blood flow between the PN group and the EN group became significantly different with a 39% greater blood flow in the EN group (at 39 hours, PN mean: 32.3 ± 5.1 mL/min/kg; EN mean: 53.5± 13.3 mL/min/kg). After 58 hours, the blood flow dropped in the GSSG group to a mean of 23.3 ± 4.0 mL/min/kg and became very similar to the blood flow in the PN group with a mean of 24.3 ± 2.6 mL/min/kg.

The lower sample number for blood flow is due to only having one chance of inserting the blood flow probe around the SMA resulting in a higher failure rate. If the probe failed, no measurements were taken from that animal and thus a lower sample number for blood flow.

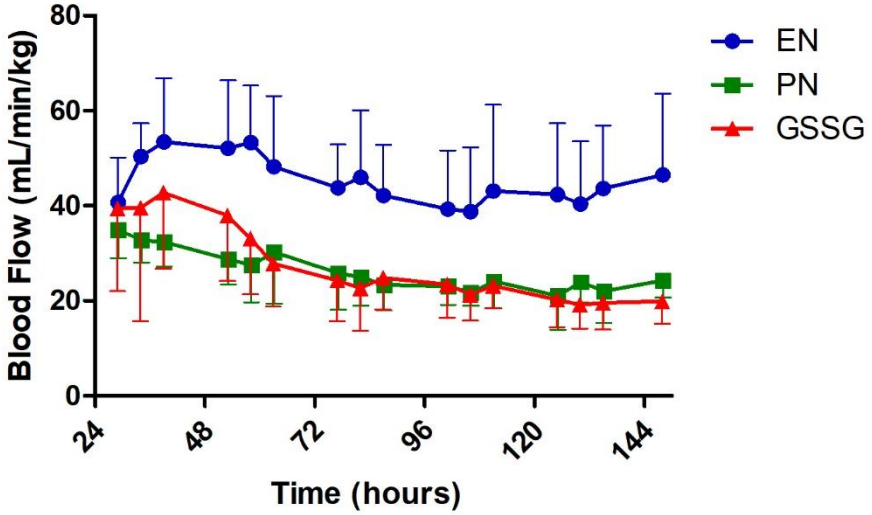

Figure 3.5: Superior mesenteric artery blood flow (n= 7 (PN), 8 (EN), 7 (GSSG)). Treatment effect by two-way repeated measures ANOVA, p=0.004. Means compared by Bonferroni's post-hoc test. At 39 hours, a significant difference in blood flow between the EN and PN group was found through the analysis described above.

3.4 Tissue Specific Protein Synthesis

The fractional protein synthesis rate in the liver, reported as percent per day, was not significantly different between treatment groups (Figure 3.7 (a)). The fractional protein synthesis rate in the mucosa was greater in the EN group (121.2 ± 18.20 %/day) compared to both the PN (34.3 ± 6.34 %/day) and GSSG (53.6 ± 8.27 %/day) groups (Figure 3.7 (b)). In addition to these differences, the fractional protein synthesis rate in the mucosa was greater in the GSSG group (53.6 ± 8.27 %/day) compared to the PN group (34.3 ± 6.34 %/day) (Figure 3.7 (b)).

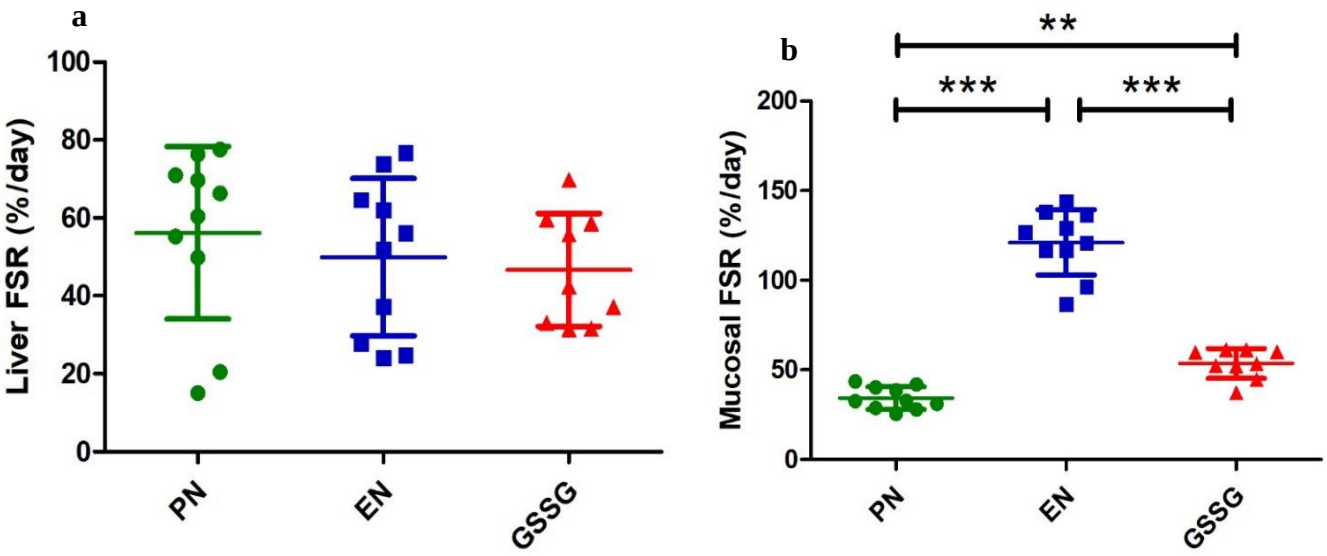

Figure 3.6: Fractional synthesis rate of (**a**) the liver and (**b**) the mucosa as percent per day (n= 10 (PN, EN), 9 (GSSG)). Treatment effect by ANOVA for the mucosa, $p<0.0001$. Means compared by Bonferroni's post-hoc test, **$p<0.01$, ***$p<0.001$.

3.5 Cell Proliferation in the jejunum

The control PN treatment resulted in less ki-67 positive cells in crypts compared to the EN group (Figure 3.8). This same difference was apparent between the EN and GSSG group (Figure 3.8). This indicated that there was less cell proliferation occurring in the crypts of the piglets that were provided with parenteral nutrition, when compared to the enterally fed animals.

The lower sample number was due to a lack of visibility of the mucosal structures on the prepared slides (e.g., sample being in the wrong orientation to view the structures) resulting in data not being collectable. In addition, repeating the slides was not possible due to the integrity of the samples degrading over time as it was stored in neutral buffered 10% formalin (Fisher Scientific, Canada).

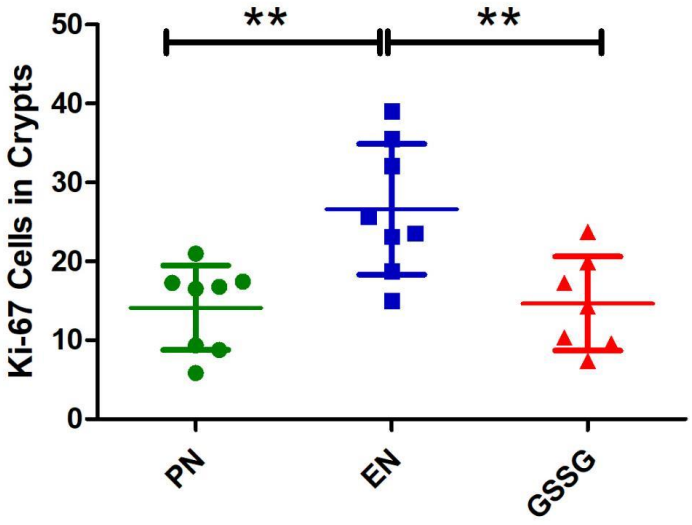

Figure 3.7: Total number of proliferating cells in the crypts measured through the Ki-67 cell proliferation marker using immunohistochemistry (n= 8 (PN, EN), 7 (GSSG)). Treatment effect by ANOVA, p=0.002. Means compared by Bonferroni's post-hoc test, **p<0.01.

3.6 Intestinal Morphology

3.6.1 Villus Height and Crypt Depth

There were significantly shorter villi in the PN group compared to the EN group (Figure 3.9 (a)); however, no significant difference in villus height was observed between the EN and GSSG groups.

The diet also had a significant effect by ANOVA on crypt depth; however, no significant differences in the group mean crypt depths were apparent by post-hoc test (Figure 3.9 (b)).

The lower sample number was due to a lack of visibility of the mucosal structures on the prepared slides (e.g., sample being in the wrong orientation to view the structures) resulting in data not being collected. In addition, repeating the slides was not possible due to the integrity of the samples degrading over time as it was stored in neutral buffered 10% formalin (Fisher Scientific, Canada).

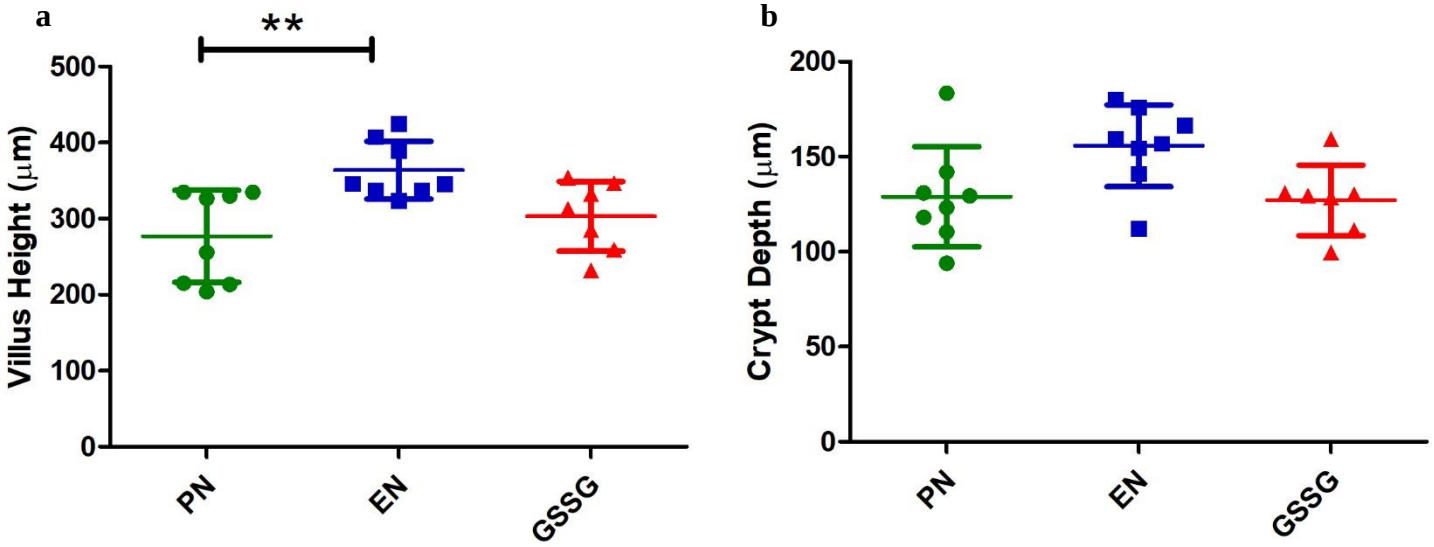

Figure 3.8: (**a**) Villus height and (**b**) crypt depth in the jejunum (n= 8 (PN, EN), 7 (GSSG)). Treatment effect by ANOVA for villus height, p=0.0062 and for crypt depth, p=0.04. Comparisons of means by Bonferroni's post-hoc test, **p<0.01.

3.7 Antioxidant Assays in Liver and Lung Tissues

3.7.1 Ferric Reducing Antioxidant Power (FRAP) Assay

Surprisingly, the antioxidant reducing power of the liver of the animals that were fed enterally was significantly lower compared to both of the parenterally fed groups (PN and the GSSG) (Figure 3.10 (a)). In the lung, there was no difference in antioxidant reducing power between the PN, EN and GSSG groups (Figure 3.10 (b)) (data acquired by honours student: Druken *et al.* (2021))

The lower sample number in the lungs was due to values not being within a physiological norm. Samples could not be repeated due to time restrictions and a delay in accessing more reagents.

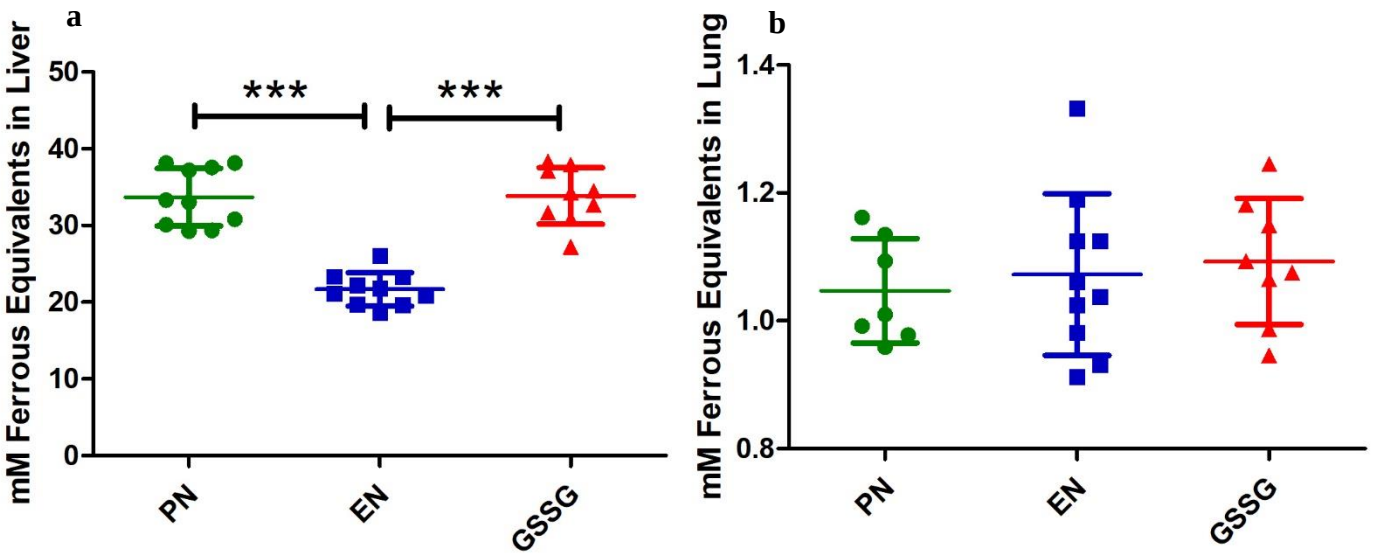

Figure 3.9: Ferric reducing antioxidant power of the (**a**) liver and (**b**) lung (liver n= 10 (PN, EN), 9 (GSSG); lung n= 7 (PN), 10 (EN), 8 (GSSG)). Treatment effect in the liver by ANOVA, $p<0.0001$. Comparison of means by Bonferroni's post-hoc test, ***$p<0.001$. Lung tissue analyzed by A. Druken (Druken, 2021).

4.3.2 Thiobarbituric Acid Reactive Substances (TBARS) Assay

The TBARS assay was conducted to measure the amount of lipid peroxidation in the liver. Interestingly, greater lipid peroxidation occurred in the livers of the EN piglets compared to both the PN and GSSG groups (Figure 3.11 (a)). When analyzing the lung tissue, lipid peroxidation was greater in both the PN and GSSG groups compared to the EN group (Figure 3.11 (b)) (Analyzed by honours student: Druken *et al.* (2021))

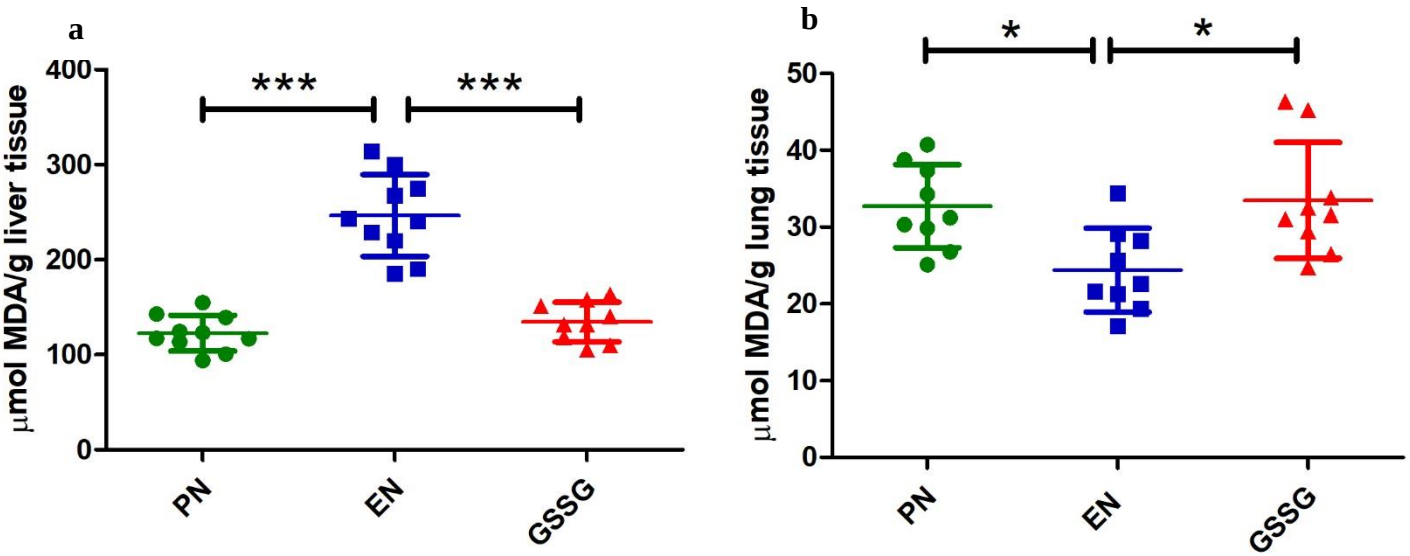

Figure 3.10: Lipid peroxidation in (**a**) the liver and (**b**) the lung measured through the thiobarbituric acid reactive substances (TBARS) assay (liver n= 10 (PN, EN), 9 (GSSG); lung n= 9 (PN, EN, GSSG)). Treatment effect by ANOVA for the liver, $p<0.0001$ and for the lung, $p=0.008$. Means compared by Bonferroni's post-hoc test, *$p<0.05$, ***$p<0.001$. Lung tissue analyzed by A. Druken (Druken, 2021).

Chapter 4: Discussion

Human milk is unequivocally the gold standard for feeding preterm neonates. It contains the required nutrition for the human neonate and provides protective effects through immunological factors (Castellote *et al.*, 2011; Walker, 2010). For the preterm neonate that cannot consume human milk due to immaturity and/or complications related to medical management, many are treated with TPN to provide adequate nutrition to sustain growth and ideally to continue on the trajectory of normal development. However, with the use of TPN, the intestinal tract is bypassed, which results in a change in how nutrients are taken up and metabolized. This lack of intestinal stimulation by TPN feeding is also accompanied by a drop in blood flow through the SMA, effectively reducing blood supply to the gut. This may predispose infants to TPN-associated diseases like NEC and hepatic cholestasis. Studies have investigated how the administration of enteral feeding with TPN can affect the onset of conditions such as NEC. Fang *et al.* (2001) and Elgendy *et al.* (2021) demonstrated that early enteral feeding stimulated an increase in SMA blood flow which led to a greater tolerance of full enteral feeds, compared to infants introduced to enteral feeding at a later age (8-30 days of age). TPN also introduces substantial oxidative stress to the neonate, and this could exacerbate the impaired blood flow by interacting with NO activity. The primary intracellular antioxidant is GSH and prematurity in human neonates has been associated with lower GSH synthesis from methionine, and therefore, a possible impairment in the endogenous antioxidant system within the premature neonate. In this study, we found that the administration of GSSG in a TPN diet, resulted in an almost 40% higher daily fractional intestinal protein synthesis rate compared to PN group. However, the higher protein synthesis rate did not translate to

a difference in villus height or a difference crypt cell proliferation in the GSSG group compared to the PN group. GSSG supplementation in TPN did however result in a similar liver weight to that of the EN-treated piglet group indicating possible protection by GSSG in the liver. When investigating total antioxidant capacity in the liver and lungs, the route of feeding may have impacted the organs' response to oxidative stress.

4.1 The effect of GSSG supplementation on SMA blood flow

Blood flow through the SMA has been shown to drop following the initiation of parenteral nutrition (Niinikoski *et al.*, 2004). In our study, blood flow through the SMA declined by 26% from the first measurement after the initiation of parenteral feeding to the final measurement on the morning of day 7. These results align with results published by Niinikoski *et al.,* indicating a drop in blood flow after parenteral nutrition administration. Within our study, we suspect that we missed measuring the largest drop in blood flow post-surgery because we did not record blood flow in the intra-operative period when piglets were technically in an enterally fed situation; therefore, we are missing an enteral baseline measurement prior to the initiation of the respective TPN diets. Comparing the blood flow in the parenteral group to that of the enteral group, the enteral group had a different blood flow pattern in that blood flow increased following the initiation of 100% enteral diet administration and the blood flow rate was sustained over time. The PN and EN groups were provided with identical diets but via different routes, so these data confirm that the route of feeding did affect blood supply to the gut.

We hypothesized that supplementation of PN with GSSG would preserve the blood flow through the SMA. Over the long term, there was no sustained benefit in terms of blood flow; however, GSSG supplementation appeared to have an early acute effect on blood

flow through the SMA. During the first 58 hours of parenteral feeding, the blood flow in the GSSG piglets was only 9% lower than that of the EN piglets and 15% greater than that of the PN piglets; however, these differences were not significant. At 39 hours, blood flow was 39% greater in the EN group compared to the PN group and this difference was significant. The blood flow in the GSSG piglets compared to the EN piglets began to show a difference at 58 hours of diet administration with a 38% lower blood flow in the GSSG piglets. Over the length of the study period (7-days), GSSG was not able to maintain blood flow through the SMA when compared to the EN group. The supplementation of GSSG only appeared to be effective early following the initiation of the TPN diet in maintaining blood flow. Evidence to support a functional benefit of this early preservation of blood flow was the similar outcome of villus height between the GSSG and EN piglets. It may be that short term GSSG-sustained blood flow had an impact on preventing villus atrophy, even when measured days later. Our hypothesis was that supplementing glutathione would maintain SMA blood flow by protecting NO, allowing it to function as a vaso-regulatory compound. With the current analysis and results, this mechanism of action could not be elucidated. To further investigate whether this mechanism was involved, future studies could employ a stable isotope of L-arginine to quantify the rate of synthesis of NO via the conversion of arginine to NO and citrulline. The quantification of peroxynitrite could also provide more information on the reaction between NO and superoxide. Finally, enzyme assays could be used to understand how enzyme activities may have impacted the results. Quantifying superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities would provide some insight into whether the antioxidant capacity in the gut was influenced by either higher or lower enzyme activity thus possibly masking

the impact of the treatment groups. Measuring nitric oxide synthase (NOS) activity in the arterial endothelial tissue could provide information on localized activity. The use of stable isotopes will provide information on whole body NOS activity. Calorimetric or fluorometric assays could be performed on the arterial endothelial tissue to investigate local NOS activity within the blood vessel that supplies blood to the intestines. Analyzing results from whole body NO synthesis and the results from the local arterial endothelial NOS activity assay would help elucidate NO production capacity within the artery that supplies blood to the gut and how this production is affecting whole-body NO synthesis.

4.2 The effect of GSSG on liver parameters

Parenteral nutrition is known to be hepatotoxic and one of the gross markers of liver damage is an enlarged liver. This adverse outcome of TPN feeding was demonstrated in a study that compared TPN-fed to sow-reared piglets (Turner *et al.*, 2016). Piglets on a parenteral diet that included SMOFlipid fed for 14 days had liver weights (per kg body weight) that were almost twice that of sow-reared piglets (Turner *et al*., 2016). Higher liver weights were also measured in our PN groups when compared to the EN piglets. In line with what has been reported in the literature, we found that the liver weight in the PN group was greater than that of the EN group. However, when GSSG was supplemented in the parenteral diet, the liver weight was similar to that of the EN-treated piglets, indicating that glutathione supplementation in parenteral nutrition may have protected the liver to some extent. Dinesh *et al.* (2018) used the TPN-fed miniature piglet model and provided IV feeding for 14 days. Interestingly, the liver weight in that study was over 50 g/kg body weight, which is 20% higher than in our PN group that were fed for 7 days. Thus, PN

feeding results in rapid, progressive enlargement of the liver in this neonatal model. The relationship between time on TPN and liver weight has not been published in the literature.

The rate of protein synthesis in the liver did not differ significantly among treatment groups and ranged from 46 to 56%/day. These values were similar to that reported in the study by Dinesh *et al.*, (2018) which was just below 60%/day. As the rates of protein synthesis were not different by treatment, it seems unlikely that protein synthesis would contribute to the difference in liver weights among our treatment groups. A well described outcome of prolonged parenteral nutrition that contributes to an increased liver weight is parenteral nutrition-associated liver disease (PNALD), which encompasses parenteral nutrition-associated cholestasis (PNAC) (Guthrie & Burrin, 2021). A histological marker of PNALD within infants on TPN is hepatic steatosis, the accumulation of lipids within the liver (Guthrie & Burrin, 2021). The use of different lipid emulsions within TPN have been shown to increase total hepatic fatty acid content outside the normal range in piglets on TPN for 14 days (Isaac *et al.*, 2019). As such, more analysis will be required to investigate fat deposition and lipid composition in the liver of the piglets within this study. We conducted further analyses to understand what may be occurring in the liver of the piglets by investigating antioxidant capacity and lipid peroxidation.

The measurement of reducing power or antioxidant capacity in the liver was conducted using the ferric reducing antioxidant power (FRAP) assay. There was no difference in the antioxidant capacity in the liver between the PN piglets and the GSSG piglets. The supplementation of GSSG in parenteral nutrition did not measurably increase the ability of the liver to combat pro-oxidant compounds when compared to the control PN diet. This result was surprising because the purpose of supplementing GSSG, a more stable

form of the antioxidant GSH, was to increase antioxidant concentrations and in turn, increase the antioxidant capacity of the piglets. However, based on the results, supplementing GSSG did not raise the antioxidant capacity within the liver. A surprising result was the finding that the lowest liver antioxidant capacity was found in the EN group. A reason for this difference must be related to the route of feeding, because the diet compositions were identical. The EN piglets received the standard elemental TPN diet which is considered a highly reactive formulation, containing abundant free pro-oxidant compounds. After the gut absorbs nutrients and diet components, the next organ to encounter the high oxidant load from the diet is the liver. Due to the high oxidant load going from the gut to the liver, it may be that the antioxidant systems become depleted, resulting in the lower antioxidant capacity measured through the FRAP assay. The parenteral diet was administered into the superior vena cava so the first organ to be exposed to the high oxidant load was the heart. In this study we did not collect the heart however, we did collect the lungs, the second organ to encounter the high oxidant load. Later analyses of the lungs (as part of an honours thesis) showed no differences in antioxidant capacity in the lungs (Druken *et al.*, 2021). The lungs are organs that encounter high oxidant loads as part of their role in respiration and therefore have the innate ability to combat high oxidant loads. It is important to note that our piglet model is not under the same level of oxidative stress in the lungs as is common in preterm infants who are placed on supplemental oxygen because of developmentally immature lungs at birth. The lungs' ability to handle oxidation may be a reason for not seeing a difference in total antioxidant capacity and may explain why the liver in the parenterally fed animals had a higher antioxidant capacity than enterally fed pigs. Once the diet had entered the arterial system, it became diluted and by the time it

reached the liver, the demands on the liver to combat the pro-oxidant compounds that were delivered in the diet may have been reduced. Future studies could investigate whether effects on the liver are present with long-term TPN feeding.

To understand the extent of pro-oxidant delivery and status in the liver, we investigated lipid peroxidation within the liver through the thiobarbituric acid reactive substances (TBARS) assay. The TBARS assay measures the level of lipid peroxidation within a tissue and is an indication of oxidative damage (Niki, 2008). In line with the FRAP data, the EN piglets also had a greater level of liver lipid peroxidation than both the PN and GSSG piglets. The parenterally fed animals (PN and GSSG) appeared to be able to combat pro-oxidant compounds, indicated by the lower lipid peroxidation levels, perhaps due to the greater liver antioxidant capacity. However, the lower level of lipid peroxidation in the liver of the parenterally fed animals could also be another indication that the lungs (and maybe the heart) were dealing the pro-oxidant load delivered in the diet, as the first organs exposed. Indeed, the lungs of the parenterally fed piglets did have greater lipid peroxidation compared to the EN piglets (Druken *et al.*, 2021). These results indicate that the route of feeding has a significant impact on organ susceptibility to oxidative stress, and that the addition of glutathione to parenteral nutrition did not ameliorate lipid peroxidation in the lungs or liver.

4.3 The effect of GSSG on mucosal mass and structure

One of the most interesting findings from this study was the enhanced rate of intestinal protein synthesis in the TPN-fed animals that were supplemented with GSSG. The daily fractional rate was almost 40% higher with the addition of the antioxidant to the parenteral diet. It should follow that enhanced protein synthesis would lead to measurable

differences in intestinal morphology. This was apparent in villus height, as the EN group had significantly longer villi than the control PN group, corresponding to the 3-fold higher intestinal protein synthesis. But the villus height in the GSSG and PN groups were not different despite the difference in rate of protein synthesis. It may be that the study period of 7 days was not long enough to discern a positive effect of enhanced protein synthesis in mucosal morphology. If the study had carried on for longer, advantages of GSSG on intestinal morphology may have become more apparent. Since protein synthesis is expressed as percent/day, small differences early in the study can result in overall large differences in gross morphology at the end of the study as such, a longer study period could have lessened these large morphological changes. Enteral feeding sustained a rate of protein synthesis that was over double that measured in the GSSG animals, and more than triple the rate in the control group. Alternatively, GSSG may have maintained villus height at the start of the study however, at 7-days, the mucosal morphology began to look similar to that of the PN group. As such, a shorter study period may also elucidate the advantages of GSSG supplementation on mucosal morphology since all the piglets are assumed to have a healthy gut and thus high protein synthesis at the start of the study.

Another factor that likely affected outcomes related to intestinal morphology was intestinal blood flow. In section 3.3, SMA blood flow was summarized, and it showed that in the GSSG group, blood flow through the SMA was not different than in the EN group during the first 58 hours of study. From this study, it is not possible to know what sort of protection this early effect might afford the neonate, but others have clearly demonstrated rapid changes in gut morphology following the initiation of parenteral feeding. Niinikoski *et al.* (2004) found that after only 24 hours of TPN, the villus height in the jejunum

shortened significantly. Sustaining blood flow during this early transition to parenteral nutrition would most certainly delay or ameliorate gross changes to gut structure and may also provide benefits such as enhanced immune function. Such advantages could be of critical importance to fragile infants at high risk for developing NEC. It would be interesting to try to quantify potential benefits, by shortening the period on TPN and analyzing immune markers in the intestinal tract and evaluating the integrity of tight junction proteins. This would allow us to understand if short-term glutathione supplementation in TPN is protecting the gut and perhaps the infant from developing NEC.

Over the longer term, Kansagra *et al.* (2003) found that villus height was 43% greater in enterally compared to parenterally fed piglets after a 7-day study period; however, there was no difference in crypt depth between groups. Deeper crypts provide greater opportunity for cell proliferation, the driver of cell movement along the villus (Parker *et al.*, 2017). However, an indicator of a greater absorptive capacity in the intestinal tract is a crypt depth accompanied by a greater villus height (Seyyedin & Nazem, 2017). Crypt depths may be similar between groups, however, if greater cell proliferation in the crypts and a greater villus height is associated to crypt depth, this indicates greater capacity for nutrient absorption (Seyyedin & Nazem, 2017). In our study, the better villus height outcome in the EN piglets was supported by the cell proliferation data. EN piglets had greater crypt cell proliferation than both the PN and GSSG piglets, indicated by more Ki-67 positive cells. One might expect that greater crypt cell proliferation would be accompanied by deeper crypt measurements. We found an overall effect of diet on the crypt depths, with a trend towards deeper crypts in EN pigs, but no significant differences in the group means were detected with the Bonferroni's post-hoc test. When comparing

cell proliferation and mucosal protein synthesis rates, a similar result is observed as was seen with the villus height and the protein synthesis rate. Although the GSSG group had a higher rate of mucosal protein synthesis compared to the PN group, this did not translate to higher cell proliferation in the crypts. This could be attributed to the 7-day study period, as previously explained when discussing the villus height in the PN and GSSG groups. Future studies could investigate different study periods to determine the advantageous effect of GSSG on mucosal morphology.

Putting together the information from the intestinal tract and intestinal mucosa, GSSG supplementation in parenteral nutrition was able to reduce the villus atrophy which was likely supported by greater protein synthesis. The effect of lower protein synthesis in the GSSG group compared to the EN group may have been observed in the intestinal morphology if the study period had been extended past 7 days. The short time period, approximately 58 hours, where blood flow was not different between the GSSG and EN groups, may have provided the intestinal tract with required nutrients that allowed for protein synthesis to be higher and to reduce villus atrophy. However, it was not able to maintain the gut weight, mucosa weight and crypt cell proliferation for the study period. A longer study period may have resulted in all intestinal parameters being different between the enterally fed group and the parenterally fed groups, irrespective of GSSG supplementation.

4.4 Rationale for Treatment Groups

Our standard, well established parenteral nutrition diet was used as an appropriate control group, to gain information on how GSSG supplementation might alter outcomes measured in the gut and liver.

The GSSG treatment was based on work published by Elremaly *et al.* (2015). Elremaly and colleagues used a neonatal guinea pig model and demonstrated that 10 μM GSSG in TPN was able to protect lungs from damage induced by ascorbylperoxide that was produced within parenteral nutrition solution. GSSG was used, as opposed to GSH, because of its low reactivity with other components of the parenteral nutrition solution (Elremaly *et al.*, 2015; Elremaly *et al.*, 2016). If GSH had been used as opposed to GSSG, it would have likely reacted with components in the parenteral nutrition solution and in turn, reduced the concentration of GSH available to function endogenously. In addition, the concentration of 10 μM GSSG was selected because in the guinea pig model, it supported normal plasma concentrations of glutathione (Elremaly *et al.*, 2015). We chose the same concentration and form of glutathione based on the results from the studies in guinea pigs, because to our knowledge, glutathione supplementation had not been studied in any other neonatal animal model (Elremaly *et al.*, 2015; Elremaly *et al.*, 2016; Morin *et al.*, 2019). Based on our study and the challenges encountered, we now surmise that a pilot study to determine baseline concentrations of glutathione in piglets could have been beneficial in determining whether this concentration was supporting glutathione levels at a normal or high normal range. It would have also allowed us to determine the most accurate method of measuring glutathione. If the ideal concentration of glutathione supplementation was greater in the piglet model than what we used, effects within the intestinal tract may have differed.

Our third treatment group was established to isolate the effects of route of feeding on our study parameters. These piglets were given the standard parenteral nutrition diet; however, the diet was provided enterally. In previous studies, parenterally fed piglet data

were compared to a standard sow-fed piglet group, rather than a group on a parenteral diet administered enterally (Dinesh *et al.*, 2018; Brunton *et al.*, 2012; Myrie *et al.*, 2012). By providing the parenteral diet enterally, we were able to make direct comparisons about the route of feeding. It also allowed us to compare how the same diet with the same concentration of oxidized nutrients would be handled by the gut. In a review article by Aw from 1999, the effect of lipid peroxides from the diet were described, including the influence on the activity of NF-κB that resulted in either cell proliferation, apoptosis or necrosis. The effect of dietary lipid peroxides is concentration dependent and higher concentrations can lead to apoptosis and necrosis of the intestinal tract (Aw, 1999). Since the gut is the first tissue to encounter pro-oxidants from the diet, the gut would have the role of dealing with the pro-oxidants and thus diminish the systemic impact. Providing the parenteral diet to piglets enterally allowed us to identify the gut and liver as the first to encounter and thus combat pro-oxidants within the diet. Further analysis is required to understand the antioxidant status in the mucosa and the effect of an oxidant-rich diet, like the parenteral diet, on the gut.

4.5 Detection of GSSG: Challenges and Future Directions

An important objective of this study was to use isotope kinetics to measure whole body glutathione synthesis as an indicator of the neonate's capacity to metabolize the pro-oxidant compounds that are delivered with TPN. The antioxidant status was going to be determined through the analysis of GSH, GSSG and its derivatives in order to determine how GSSG supplementation affected the antioxidant capacity of the piglets. Measurements of glutathione and its derivatives in the PN and EN groups will provide clarification on the endogenous antioxidant capacity and the effect of diet and route of feeding on antioxidant

status. Comparing the antioxidant capacity and status within the different treatment groups would provide greater understanding of how neonates handle pro-oxidants and whether supplementation of glutathione would be beneficial in their treatment process. Analysis of isotope enrichments related to glutathione synthesis required the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) which was available to us, however the method had not been established in our facility. The protocol was based on a method by Vassilyadi *et al.* (2016), with modifications. Our study utilized a different precursor isotope, [D_5]-GLY, rather than the [^{15}N, $^{13}C_2$]-GLY isotope used by Vassilyadi *et al.* (2016). Development of the protocol began on the new LC-MS/MS that had been recently acquired by Dr. R. Brown in the Department of Biochemistry. The first challenge encountered was that the communication between the liquid chromatography portion of the equipment and the mass spectrometry portion had not been established and therefore, the LC-MS/MS was not fully operational. Standards for GSH, GSSG and its derivatives were run through the mass spectrometer to identify peaks. The next step was to determine whether our samples could be run through the mass spectrometer (excluding the separation of ions through chromatography); however, this was halted because of the COVID-19 pandemic. As such, we turned to the CREAIT Network and used their mass spectrometry laboratory. We began to use the UHPLC-UV-MS/MS qTOF available in the CREAIT Network; however, the pandemic restrictions only allowed for the supervisor of the mass spectrometry laboratory to work on the development of the method for the study.

Standards for GSH and its derivatives were run on the UHPLC-UV-MS/MS, and elution times were determined. An observation of the total ion chromatogram was that the peaks for each ion of interest were not distinct and it was through searching for the peaks

that they were found. However, we moved forward with running an individual sample. When a blood sample was run, no peak was visible for the GSH-NEM (m +5) ion, indicating that our isotope was not incorporated into glutathione or that the protocol was not optimized enough to detect the peak. With this information, and the restrictions in place to problem solve the protocol ourselves, it was decided to set this outcome aside and to focus on the remaining outcomes that were close to completion. In addition to not being able to detect an important peak, the development of the protocol for the derivatization of glycine in the second half of the blood samples was required. Limited time because of the pandemic and availability of equipment prevented protocol development for glycine derivatization.

The other samples that required more protocol development were the daily whole blood samples for the detection of GSH and GSSG. The method chosen was the spectrophotometric detection of GSSG and total GSH published in the article by Giustarini *et al.* (2013). This method allows for quick detection of GSH and GSSG to determine the antioxidant status of the piglets throughout the 7-day study period. The limitation with this method is that once the samples have been processed, there would only be a limited number of attempts to run the samples due to the small blood volume collected from the piglets. More time was required to make this protocol functional and effective before risking our blood samples.

Moving forward, these protocols will require the time and dedication to make them reliable and reproducible. Currently, we have a different protocol that has been developed on our HPLC system and could be used as an alternative to the LC-MS/MS method and spectrophotometric methods. The challenge is that the samples were not prepared with the

HPLC method in mind and therefore would require careful oversight and troubleshooting to ensure that the method on the HPLC functions properly for both sample sets.

4.6 Summary and Future Directions

Previous evidence has shown that glutathione supplementation in parenteral nutrition protected lung tissue from damage incurred from components of the parenteral nutrition diet (Elremaly *et al.*, 2015). Based on this previous research, we hypothesized that supplementing glutathione in parenteral nutrition would result in greater superior mesenteric artery blood flow by sparing NO, allowing it to function as a vaso-regulatory compound, and improving the antioxidant status in the small intestine, liver, and lungs. We concluded that glutathione supplementation in parenteral nutrition did not maintain blood flow over an extended period of time, however, it may be beneficial in the short-term. Glutathione did not improve mucosal mass and cell proliferation; however, it was able to prevent villus atrophy as shown by the finding that the villus height was not significantly different from the enterally fed group; greater protein synthesis within the mucosa was also found with GSSG supplementation in PN-fed animals.

We also demonstrated that glutathione in TPN benefited the liver as the weight was not different from that measured in the enterally-fed group. As well, glutathione did not alter the antioxidant capacity within the liver or the level of lipid peroxidation, an indicator of oxidative damage, and did not affect the rate of hepatic protein synthesis.

Research to further elucidate the effects of glutathione supplementation in parenteral nutrition will aid in understanding whether the piglets are better able to deal with pro-oxidant compounds or if they are experiencing oxidative stress. This can be achieved by studying the GSH to GSSG ratio in blood and tissues (Jones, 2002). In addition, enzyme

assays investigating the activity of catalase, glutathione reductase and glutathione peroxidase would provide more information on the endogenous antioxidant capacities within the tissues of piglets exposed to the different treatments.

Chapter 5 References

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