ARGININE AND SMALL INTESTINAL ATROPHY IN PARENTERALLY-FED NEONATAL PIGLETS

By

O. Chandani Dinesh

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Abstract

Piglets that are fed via total parenteral nutrition (TPN) experience profound gut atrophy and rapid decline in splanchnic blood flow compared to enterally-fed piglets. Sixty percent of total nutrient intake as enteral feeding is required to sustain "normal" small intestinal metabolism and morphology, which is likely not feasible with sick neonates. Whether the potential benefit of complete or partial enteral nutrition is related to single or multiple nutrients is not yet fully understood. However, the luminal presence of specific nutritive factors such as amino acids may act as trophic factors for intestinal recovery during TPN or enteral re-feeding. Arginine is a good candidate to investigate potential trophic effects as it is involved in many important metabolic pathways such as nitric oxide synthesis, ureagenesis, polyamine synthesis and protein synthesis. TPN-induced gut atrophy reduces intestinal de novo synthesis of arginine in neonates which may then limit arginine availability. We hypothesized that delivery of a high amount of dietary arginine alone into the gut could induce partial recovery from TPN-induced gut atrophy in neonatal piglets, while improving superior mesenteric artery (SMA) blood flow. We also hypothesized that enteral delivery of low amounts of arginine would result in attenuated responses compared to the high enteral arginine. Thus, our objectives were to assess the effect of route of intake and dietary concentration of arginine alone on tissue protein synthesis, SMA blood flow and gut morphology in TPN-fed neonatal Yucatan miniature piglets with small-intestinal atrophy. Twenty-four piglets (14 - 17 d old) were fed complete TPN, containing 1.0 g arginine/kg/d until study day 4 to induce atrophy, then switched

to arginine-free TPN and randomized to receive a continuous intragastric infusion of either low arginine (0.6 g/kg/d) (IG-L Arg) (n = 6) or high arginine (1.6 g/kg/d) (IG-H Arg) (n = 6). A third group was randomized to receive high arginine intravenously (1.6 g/kg/d in TPN) (IV-H Arg) (n = 6). A group of sow-fed littermates (SF Reference) (n = 6) was also included. On study day 7, crypt cell proliferation and tissue specific protein synthesis rates were measured. This thesis demonstrated for the first time that the delivery of arginine alone intragastrically, irrespective of the amount provided, stimulated hepatic protein synthesis (P = 0.01) compared to intravenous delivery of arginine. SMA blood flow declined continuously during the period when all animals were receiving the same complete TPN. It further decreased following the initiation of the test diets and reached a plateau approximately 48 hours after the test diets were initiated. At steady state, SMA blood flow was significantly different among all treatment groups (P = 0.002). IV-H Arg treatment had the smallest reduction in blood flow (22% lower than baseline). The provision of high arginine enterally attenuated some of the reduction of blood flow, whereas the IG-L Arg piglets demonstrated the greatest reduction, at ~40% lower than baseline at study end. In piglets fed solely by TPN, some of the plasma indispensable amino acids were very high compared to the IG-H Arg piglets. Remarkably, the provision of only arginine into the gut resulted in a plasma profile of some of these amino acids that was very similar to sow-reared piglets. In spite of experiencing a greater amelioration of the reduction of SMA blood flow, the small intestinal morphological characteristics were not significantly improved compared to the other treatment groups. Although IG-H

Arg was not as effective as IV-H Arg in sustaining SMA blood flow, morphological outcomes were not significantly worse than in the IV-H Arg piglets; indeed, the small intestine was significantly longer in the IG-H Arg piglets compared to IV-H Arg group, and all other outcomes were similar among treatment groups. These results suggest that IG delivery of arginine was likely beneficial as a trophic factor in gut-atrophied neonatal piglets.

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Abbreviations

AAO	Amino Acid Oxidation
Arg	Arginine
ASL	Argininosuccinate Lyase
ASS	Argininosuccinate Synthase
ATP	Adenosine Triphosphate
BH4	Tetrahydrobiopterin
BrdU	5-Bromodeoxyuridine
BW	Body Weight
cNOS	Constitutive NOS
CPS-1	CarbamoylPhosphate Synthetase-1
DAB	3, 3'- Diaminobenzidine
DNA	Deoxyribonucleic Acid
eNOS	Endothelial NOS
FSR	Fractional Synthesis Rates
GDH	L-Glutamate Dehydrogenase
GI	Gastrointestinal
GLP-2	Glucagon-Like Peptide 2
HPLC	High Performance Liquid Chromatography
IAAO	Indicator Amino Acid Oxidation
IG	Intragastric
IG-H Arg	Intragastric High Arginine
IG-L Arg	Intragastric Low Arginine
iNOS	Inducible NOS

IV	Intravenous
IV-H Arg	Intravenous High Arginine
α-KG	Alpha-Ketoglutarate
MeOH	Methanol
NAGs	N-Acetylglutamate Synthetase
NEC	Necrotizing Enterocolitis
nNOS	Neuronal NOS
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NRC	National Research Council
OAT	Ornithine Aminotransferase
ODC	Ornithine Decarboxylase
OTC	Ornithine Transcarbamylase
P5C	Pyrroline-5-Carboxylate
P5CDH	P5C Dehydrogenase
P5CS	P5C Synthase
PBS	Phosphate-Buffered Saline
PDV	Portal–Drained Viscera
PEN	Partial Enteral Nutrition
Phe	Phenylalanine
PITC	Phenylisothiocyanate
PPHN	Persistent Pulmonary Hypertension
PYY	Peptide YY
SAv-HRP	Streptavidin Horse Radish Peroxidase

SF Reference	Sow-Fed Reference
SI	Small Intestinal
SMA	Superior Mesenteric Artery
SRA	Specific Radioactivity
TEA	Triethylamine
TEN	Total Enteral Nutrition
TFA	Trifluoroacetic Acid
TPN	Total Parenteral Nutrition
VLDL	Very Low Density Lipoprotein

1.0 Review of Literature

1.1 Total Parenteral Nutrition

Total parenteral nutrition (TPN) refers to the provision of a nutritionally complete, elemental formula via an intravenous route; the provision of nutrients into the gastrointestinal tract is known as enteral nutrition. TPN is frequently necessary for the nutritional management of preterm or premature neonates who cannot tolerate complete enteral feeding. It is common for newborn preterm infants to require short term TPN for nutritional support, often due to severe respiratory distress syndrome that precludes oral feeding (Hay 2008). Long term TPN support is sometimes required by infants born with congenital gastrointestinal (GI) malformations, or those who develop necrotizing enterocolitis (NEC) which can lead to short bowl syndrome (Quiros-Tejeira et al, 2004). In neonatal intensive care units, providing small amounts of enteral feeding, known as partial enteral nutrition, in parallel with TPN feeding is a common practice to avoid complications that are known to develop with prolonged TPN feeding. Partial enteral feeding may also be beneficial to minimise the feeding intolerance that commonly occurs during the re-introduction of enteral feeding. It is not yet clear whether the potential benefits related to partial enteral feeding are related to the overall composition of the nutritive substance provided, or whether a single specific nutrient is important. Therefore, the luminal presence of specific nutritive factors, such as amino acids, may be beneficial as trophic factors during TPN feeding or when enteral re-feeding is initiated.

1.2 Amino Acids in Parenteral Nutrition

One of the main aims of TPN support is to provide adequate but not excessive

amounts of indispensable amino acids to fulfill the requirement for whole body protein synthesis and growth. Excess amounts of indispensable amino acids or their by-products could stress immature metabolic systems in neonates, and thus lead to the accumulation of toxic end products such as ammonia. For human neonates, the amino acid profiles of the various commercial parenteral products currently available were devised based on the amino acid profiles in human milk, infant plasma or cord blood (Brunton et al, 2000).

Ideally, the specific enteral or parenteral requirement for each indispensable amino acid should be determined in neonates; currently, they are not well defined. What is currently known about the requirements for the indispensable amino acids has been determined by a number of different methods in adults and to a lesser extent in children. In the past, nitrogen balance was used to determine individual amino acid requirements of adult humans (Zello et al, 1995). However, more precise metabolic approaches such as plasma amino acid concentrations, amino acid oxidation (AAO) and the indicator amino acid oxidation (IAAO) techniques were subsequently introduced to estimate amino acid requirements (Zello et al, 1995). The AAO technique is based on the concept that amino acids in excess of the amounts needed for protein synthesis are preferentially oxidized. An important methodological issue with AAO is that all of the excess amino acids are not always oxidised completely, but catabolised to other metabolic products (Zello et al, 1995). The IAAO technique was developed to address such issues. The IAAO technique is based on the partitioning of a single indispensable amino acid between oxidation and

protein synthesis, and the balance is sensitive to the most limiting indispensable amino acids in the diet. The

provision of excess amounts of the previously limiting amino acid will reduce the oxidation of the indicator amino acid to a low and constant level. Recently, this method has been adapted to be minimally invasive and has been employed to measure the requirement for some amino acids in parenterally-fed neonates (Bertolo et al, 1998; House et al, 1998).

An important concept when considering amino acid requirements of infants is that requirements are not the same for the parenteral versus enteral route of feeding for many amino acids. Wykes et al (1992) demonstrated that plasma amino acid concentrations were very different in human neonates fed the same elemental formula via the two different routes, suggesting that a significant role is played by the splanchnic organs in amino acid metabolism. A series of studies in neonatal piglets has demonstrated that the requirements for lysine, threonine, branched-chain amino acids and methionine are substantially lower during TPN feeding compared to enteral feeding (House et al, 1998; Bertolo et al, 1998; Elango et al, 2002; Shoveller et al, 2003). The methionine, threonine and lysine requirements for post-surgical intravenously fed human neonates estimated by IAAO technique were also lower than the amounts provided in commercially available pediatric TPN products (Courtney-Martin et al, 2008, Chapman et al, 2009; Chapman et al, 2010). These studies have demonstrated the importance of considering the concentration of individual amino acids in the diet when the route of feeding by-passes the splanchnic tissues.

1.3 Neonatal piglet as a model for human infants

The piglet is an appropriate animal model to investigate questions related to nutritional metabolism in human neonates because of similar gastrointestinal tract morphology, physiology and metabolic changes during development (Pond et al, 1978b). The gross body composition of the newborn piglet is more similar to the preterm infant than the term-born infant which has greater fat accretion at birth (Shulman et al, 1993). Piglets are also developmentally immature compared to human, term-born infants (Shulman et al, 1993). Therefore, the neonatal piglet is a clinically relevant model to study the complications associated with TPN feeding in prematurely born infants.

The neonatal miniature piglet has not been widely used for studies of nutrient metabolism compared to the domestic piglet. Shulman et al (1988) reported that the small intestinal mucosal enzyme activities of 6-wk-old miniature piglets were similar to this those found in young human infants. The mean growth rate of sow-fed Yucatan miniature piglets is less rapid during first month of life, compared to domestic piglets, growing at a rate of ~45 g/kg/d (Myrie et al, 2011) compared 79 g/kg/d (Wykes et al, 1993). In contrast, low birth weight premature infants grew at ~20.5 g/kg/d during first month of their life when fed fortified human milk (Kashyap et al, 1990). Thus, studies using a piglet model may quickly identify nutrient deficiencies or metabolic changes because of the accelerated growth rate; however, the miniature pig model may represent a better model for nutrient metabolism in infants, growing at a rate that is only half the growth rate of domestic piglet and more similar to infants.

For the study of amino acid metabolism and requirements, there are now very good data to demonstrate that the piglet is an ideal model for the human infant. There has been a series of studies in piglets which determined specific amino acid requirements, and subsequent studies in human infants that confirmed the piglet

findings. One of the first was a study to determine the optimal proportion of aromatic amino acids in TPN. House et al (1997a; 1997b) established the requirement and demonstrated that the aromatic amino acids should be provided as 59% phenylalanine and 41% tyrosine in the neonatal piglet model. The ratio was later established in human infants to be 56:44 (Roberts et al, 2001). The absolute amino acid requirement values for human infants were also predicted from the piglet data by dividing the piglet value by five, to accommodate the differing growth rates. Using this correction, the similarity between the predicted (from piglets) and measured requirements in humans has now been established for threonine (Bertolo et al, 1998; Chapman et al, 2009), methionine (Shoveller et al, 2003; Courtney-Martin et al, 2008), and tyrosine (House et al, 1997a; Roberts et al, 2001). It is now clear that estimates of amino acid requirements derived from piglet data are transferable to human infants (Chapman et al, 2009). Unfortunately, the requirement for arginine, a metabolically important amino acid for the neonate, has not yet been determined for the human infant. An attempt was made to determine the arginine requirement for TPN-fed neonatal piglets using the IAAO technique. However, the researchers were not successful in determining a breakpoint estimate of requirement, likely because arginine is a conditionally indispensable amino acid for neonates, and there

was variability in de novo synthesis of arginine amongst the piglets (unpublished data from Brunton et al).

1.4 Arginine

Arginine is an aliphatic straight chain amino acid with a guanidine group at the distal end of the chain. As the guanidine group is positively charged in neutral, acidic and even somewhat basic environments, arginine imparts basic chemical properties.

1.4.1 Biological importance of arginine

Arginine is an important, abundant amino acid. It constitutes approximately 6.9% of total body proteins in fetal and neonatal piglets (Williams et al, 1954) and 7.7% in the human foetus (Widdowson et al, 1979). Arginine is also involved in the synthesis of other biologically important molecules such as nitric oxide (NO), creatine and polyamines. Arginine plays a critical role as a urea cycle metabolite, thus is important for ammonia detoxification (Figure 1). Two well-described pathways of arginine metabolism include the conversion of arginine to NO and the breakdown of arginine to urea and ornithine by arginase. The fate of arginine catabolism via the urea cycle or NO synthesis depends on the activities of the enzymes involved in the different catabolic pathways and the provision of substrates. As such, higher arginase activity in the liver leads to urea synthesis, with no release of arginine.

Arginine is also an allosteric activator of N-acetylglutamate synthetase (NAGs) (Kawamoto et al, 1982). NAGs catalyses the synthesis of N-acetylglutamate from L-glutamate and acetyl CoA in mitochondria of liver cells. The N-acetylglutamate is an allosteric activator of carbamoyl phosphate synthetase-1 (CPS-1) in the liver of ureotelic

animals (Kawamoto et al, 1982). The CPS-1 is the first enzyme in the urea cycle that converts ammonia and bicarbonate into carbamoyl phosphate which is the substrate for ornithine transcarbamylase (OTC). Therefore arginine is an activator of the urea cycle by a mechanism other than as a direct intermediary amino acid in the urea cycle.

Nitric oxide is an end product of arginine metabolism whereby arginine is converted into citrulline and nitric oxide. NO acts as a vasodilator which modulates blood pressure and thus blood flow. NO also acts as a neurotransmitter and mediator of the immune response (Homer & Wanstall, 2000). The amount of arginine utilised for NO synthesis accounts for only 0.6 μmol arginine/kg/h in young adults (Castillo et al, 1994). Although the fractions of whole body arginine flux associated with urea (15%) and NO synthesis (1.2%) in healthy humans are small, arginine leaving the plasma compartment serves as a major precursor pool (54%) for whole body daily NO synthesis (Castillo et al, 1996). Currently, there are no data available for neonates to describe the proportion of arginine flux dedicated to the various metabolic pathways.

Arginine is also a precursor of ornithine, which is the amino acid precursor for polyamine synthesis. Polyamines (putrescine, spermidine and spermine) regulate gene expression, DNA and protein syntheses, apoptosis, cell proliferation and differentiation, and thus are essential for growth and differentiation of cells in many tissues including intestinal epithelial cells (Johnson, 1988; Blachier et al, 1995). It is accepted that highly proliferative cells such as gastrointestinal tissues in growing animals demand higher amounts of polyamines.

Recently, it has been suggested that de novo creatine synthesis places a significant demand on arginine partitioning (Brosnan et al, 2009). Creatine and creatine phosphate function as buffers for ATP synthesis in tissues with high energy demand. They also serve as an energy shuttle between the sites of ATP synthesis and ATP utilization (Wyss & Kaddurah-Daouk, 2000*)*. However, once again, the proportion of arginine flux required to meet the demands for creatine synthesis has not yet been empirically determined in human infants.

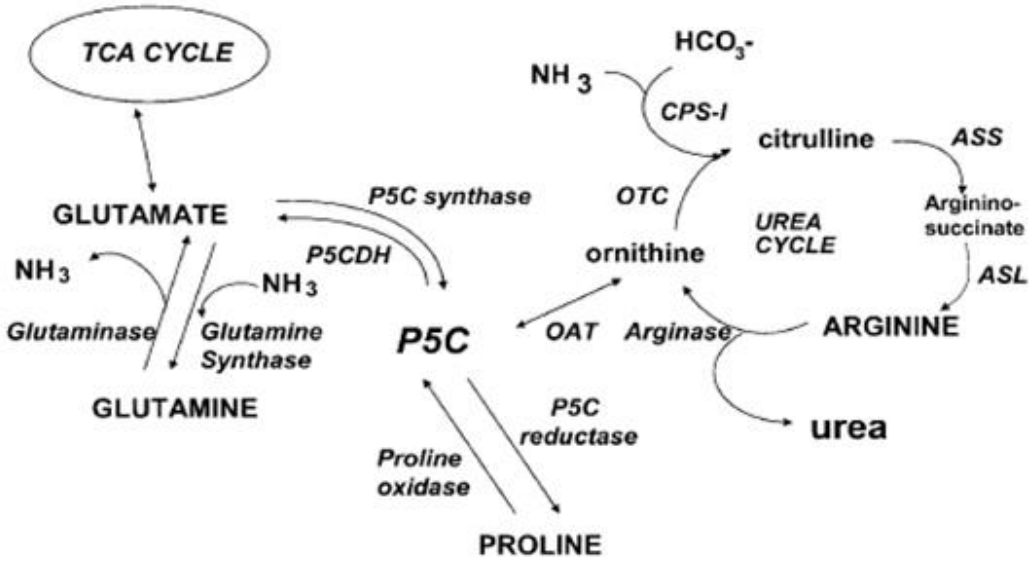

Figure 1.1: Metabolic pathway of arginine Bertolo & Burrin, 2008

This figure represents arginine metabolism in adult human (Small intestinal-renal axis). Abbreviations in the figure indicate enzymes as; ASL: argininosuccinate lyase; ASS: argininosuccinate synthetase; CPS-I: carbamoyl phosphate synthetase; OAT: ornithine aminotransferase; OTC: ornithine transcarbamoylase; P5C: pyrroline-5-carboxylate; P5CDH: pyrroline-5-carboxylate dehydrogenase. P5C synthase does not occur in adult human liver while the functional urea cycle occurs only in liver. OTC expressed widely in small intestine while ASS and ASL expressed widely in kidney.

In summary, arginine is a metabolically important amino acid for neonates during the rapid growth period, and recently, the consequences of limited arginine availability have captured the attention of clinicians and researchers.

1.4.2 General arginine metabolism

1.4.2.1 Arginine de novo synthesis

a. De novo arginine synthesis in adults

According to studies in vitro, proline, glutamate and glutamine in adults are effectively converted into citrulline in the small intestine as the activities of pyrroline-5-carboxylate synthase (P5CS) (Jones, 1985), ornithine aminotransferase (OAT), CPS-1 and OTC are relatively high in intestinal mucosal tissues (Wu, 1998) (Figure 1). However, a recent study by Marini et al (2010) demonstrated that the dietary glutamine

and proline made only a minor contribution to the synthesis of citrulline in mice, even when the mice were fed an arginine-free diet. The extraction of plasma arginine and ornithine by the small intestine was adequate to support citrulline synthesis during the arginine-free diet feeding. In another study in mice, using multiple isotope tracers, Marinin et al (2012) showed that dietary and plasma arginine were the main precursors for citrulline synthesis in the gut during feeding and feed-deprivation respectively (Marini et al, 2012). Low intestinal activities of argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), and higher activity of arginase also results in the net synthesis of citrulline and release into the portal vein (Cynober et al, 2002). In contrast, appreciable ASS and ASL enzyme activities in the kidney in conjunction with very low renal arginase activity facilitate net arginine synthesis and release in to the peripheral circulation (Brosnan et al, 2003;

Dhanakoti et al, 1990). Therefore, small intestinally-derived citrulline is the major precursor for renal de novo arginine synthesis; thus, the small intestinal–renal axis is considered as the major pathway for arginine de novo synthesis in adult humans. Using an in vivo amino acid tracer technique, Ligthart-Meliset al (2007; 2008) reported that arginine was synthesised from glutamate and glutamine in adult humans. However, those results were obtained using nitrogen-labelled tracers, which have been criticized because the movement of the tracer could potentially reflect transamination. Recently, a study by Tomlinson et al (2011b) used multiple carbon labelled tracers (arginine and proline) to determine whether dietary proline is a precursor for de novo arginine synthesis in adult humans. They demonstrated that 40% of newly synthesised arginine was derived from

dietary proline. Thus, proline is now considered an important dietary precursor for arginine synthesis in the adult human. A subsequent study by the same research group also clearly identified glutamine as a precursor for arginine, by using multiple glutamine tracers (^{13}C and ^{15}N). Based on the ^{13}C tracer enrichments and flux, 50% of newly synthesised arginine was derived from glutamine (Tomlinson et al, 2011c).

b. Arginine de novo synthesis in neonatal piglets

Arginine concentration in sow milk is relatively low (4.4% of total amino acids at d 21 of lactation) compared to the concentration of some other indispensable and conditionally indispensable amino acids such as proline (11.7%), leucine (8.9%), lysine (7.9%) or glutamate and glutamine (20.8%) (Davis et al, 1994). Mature sow milk provides approximately 0.47 g/kg/d of arginine to the suckling neonatal piglet (Moughan et al, 1992). According to the NRC recommendations (1998), the dietary requirement for arginine for a healthy neonatal piglet is 0.38 g/kg/d; as such, it is met by the arginine concentration in sow milk. However, Brunton et al (2003) measured protein synthesis rates as a functional determinant of the adequacy of arginine intake in TPN-fed piglets; the IV intake necessary to maximize muscle protein synthesis was 1.2 g Arg/kg/d. Although sow milk is a relatively poor source of arginine, it contains abundant amounts of arginine precursors. Thus, sow-reared, neonatal piglets must synthesize at least some of their daily requirement of arginine to maximize growth.

De novo arginine synthesis in neonates does not follow the intestinal-renal axis as has been described in adults. A series of studies in neonatal piglets have clearly shown that arginine synthesis occurs in the small intestine (Brunton et al, 1999; Bertolo et al,

2003; Wilkinson et al, 2004). Brunton et al (1999) were the first to demonstrate in neonatal piglets that feeding an arginine-free diet intravenously resulted in the rapid onset of severe hyperammonemia, despite the presence of proline in the diet. The same diet delivered intragastrically resulted in only moderate hyperammonemia after eight hours of feeding, indicating that de novo arginine synthesis occurred from dietary proline only when delivered into the gut. Bertolo et al (2003) used isotope kinetic analyses to isolate the metabolic roles of small intestine in the inter-conversions of arginine, proline and ornithine in neonatal piglets. This study isolated intestinal metabolism from hepatic and peripheral metabolism by infusing isotopes into the portal vein. They observed an absence of proline to ornithine conversion and very low ornithine tracer conversion into arginine during intraportal compared to intragastric tracer infusions. Furthermore, there was no difference in arginine to ornithine or ornithine to proline conversion between the two routes of

feeding. Only 10% of proline requirement is synthesised in the small intestine while 50% of arginine requirement is synthesised in the gut (Bertolo et al, 2003). Accordingly, near absence of small intestinal proline de novo synthesis facilitated a net positive release of arginine into the portal blood. Bertolo et al (2003) were the first to use multiple tracers to estimate the arginine de novo synthesis in the small intestine. Wilkinson et al (2004) also found 42–63% of whole body arginine synthesis occurred during the first pass intestinal metabolism in neonatal piglets. Later, Urschel et al (2005) demonstrated that the hepatic contribution to net arginine synthesis is negligible. Indeed, they found that net arginine de novo synthesis is dominated by the small intestinal mucosa of neonatal piglets by

isolating the first pass hepatic metabolism through the infusion of intraportal and intravenous tracers. Based on the enzyme activities (in vitro) measured in mucosal tissues in humans and piglets (Kohler et al, 2008; Wu, 1998), and in vivo multitracer studies using various routes of administration in neonatal piglets (Bertolo et al, 2003; Wilkinson et al, 2004), it is clear that arginine de novo synthesis occurs predominantly in the small intestinal enterocytes in neonates.

c. Arginine as a semi-indispensable amino acid for sow-reared neonatal piglets

In contrast to healthy adults, healthy neonatal piglets have been shown to require at least part of their whole body arginine requirement from diet. Apparently, the capacity for de novo synthesis is not sufficient to meet the whole body requirement during rapid growth and development (Wilkinson et al, 2004; Kim & Wu, 2004). A recent study by Kim & Wu (2004) demonstrated that even under normal feeding circumstances, dietary arginine intake from sow milk might be inadequate. In

this study, a sow-milk replacer was used to feed piglets. The formulas contained arginine at either the sow milk level, or supplemented with arginine at 0.2% and 0.4%. The piglets fed arginine at the sow-milk level had significantly lower plasma arginine, citrulline and ornithine concentrations by days 14 and 21 of age; this was accompanied by significantly higher plasma ammonia (Kim & Wu, 2004). Arginine supplementation enhanced daily weight gain by 28 and 66% in the two groups, compared to control piglets who received arginine at the sow milk level. The authors suggested that even normal, healthy neonatal piglets cannot synthesize adequate amounts of arginine to meet the requirement for rapid growth during early life. However, it is important to note that colostrum and sow milk are

also important sources of polyamines and other growth factors (Kelly et al, 1991; Motyl et al, 1995; Cheng et al, 2006) as well as providing exogenous creatine (Brosnan et al, 2009). Thus, a formula diet containing arginine at the concentration found in sow milk may not produce a rate of growth that is truly reflective of the sow-reared piglet.

d. Precursors for arginine de novo synthesis

Sow milk contains abundant glutamate and glutamine, which were once thought to be potential precursors for arginine synthesis. However, in vivo and in vitro evidence suggests that glutamate cannot act as a precursor for arginine due to negligible activity of P5C synthase in the neonatal intestine (Wu 1998). P5C synthase catalyzes the conversion of glutamate to P5C, which can then be converted into ornithine via OAT, or proline via P5C reductase. Bertolo et al (2003) demonstrated the localisation of the OAT to the small intestinal mucosa of the neonatal piglet by measuring the conversion of labelled ornithine to proline and glutamate when infused

intravenously versus intragastrically. However, they observed no differences in proline to glutamate or in ornithine to glutamate conversions between intraportal and intragastric tracer infusions (Bertolo et al, 2003). This was likely due to low P5C dehydrogenase (P5CDH) activity (Wu, 1998), which converts P5C into glutamate (Figure 1). Low P5CDH activity would increase the availability of proline as a precursor for arginine de novo synthesis in the neonatal gut. Wilkinson et al (2004) also demonstrated the conversion of intragastrically-delivered proline tracers into arginine, demonstrating arginine synthesis from dietary proline. However, they did not detect the glutamate tracer in arginine, indicating that there was biologically insignificant conversion of glutamate

into arginine. These findings are in contrast to the data found in adults, where 50% of de novo arginine synthesis was derived from the carbons of glutamine (Tomlinson et al, 2011c). Thus, it is clear that dietary proline, but not glutamate or glutamine, is the precursor for arginine de novo synthesis in neonatal piglets.

e. Arginine de novo synthesis in human neonate

Until recently, it was only speculation that human neonates would have the capacity for de novo arginine synthesis, based on the knowledge that mammalian milk (including human milk) has a low concentration of arginine relative to the requirements for growth. Furthermore, proline is abundant in human milk (Davis et al, 1994). In 2008, Kohler et al, reported that the fetal and perinatal human intestine has the ability to synthesise arginine, based on the enzyme activities measured in small intestinal enterocytes. A recent landmark study by Tomlinson et al (2011a) employed a multi-tracer stable isotope design in human neonates, and demonstrated

that the precursor for arginine synthesis was proline but not glutamine. In that study, the researchers measured products of the proline isotope tracer (arginine, ornithine) in the urine of infants during isotopic plateau. However, the arginine product of a glutamate tracer, and the glutamate tracer itself, were not detected in the urine of the enterally-fed premature infants, indicating neither glutamate nor glutamine were precursors for arginine synthesis.

The combined evidence from two neonatal piglet studies (Bertolo et al, 2003; Wilkinson et al, 2004) and the one human study by Tomlinson et al (2011a) clearly demonstrate that enterally-derived proline, but not glutamine or glutamate, is a major

dietary source for the de novo arginine synthesis in the small intestinal mucosa of neonates.

1.4.2.2 Arginine and NO synthesis

Arginine is the precursor for NO synthesis. The fraction of whole body arginine flux associated with NO synthesis (1.2%) in healthy adult humans is small (Castillo et al, 1996). However, the plasma arginine compartment serves as a major precursor pool (54%) for whole body daily NO synthesis (Castillo et al, 1996). In healthy adults, the first-pass splanchnic use of dietary arginine is about 38% (Castillo et al, 1993a). However, the dietary arginine metabolized to NO during first pass splanchnic metabolism represents 16% of the whole-body nitrate production (Castilloet al, 1993b). Furthermore, in the healthy adult human, dietary arginine intake did not have an effect on NO synthesis, as both fed and fasted situations in adults resulted in approximately 1.2% of arginine turnover used for NO synthesis (Castillo et al, 1996). In contrast, dietary L-arginine supplementation stimulated endothelial NO

synthesis in adult rats (Kohli et al, 2004). Currently, there are no such data available for healthy human neonates. In sick neonates recovering from persistent pulmonary hypertension (PPHN), NO synthesis accounted for less than 0.5% of plasma arginine flux, which was less than the proportion of arginine converted into NO in healthy adults. In the same study of infants with PPHN, Castillo et al (1995) reported that the rate of plasma arginine conversion to NO synthesis was higher (45.6 μmol/d) when dietary arginine intake was 36 μmol/kg/h compared to the rate of NO synthesis (10.3 μmol/d) with a dietary arginine intake of 16 μmol/kg/h arginine. Urschel et al (2007) studied the

proportion of NO synthesis from dietary arginine in healthy neonatal piglets. In contrast to 0.5% of plasma arginine flux converted into NO in sick human neonates, Urschel et al (2007) demonstrated that NO synthesis accounted for 13% of whole body arginine flux in enterally fed neonatal piglets irrespective of the amount of arginine in the diet. In the Urschel study, the isotope tracers were infused into a gastric catheter, and as such included small intestinal NO synthesis, as opposed to Castillo's study which used IV infusion. The difference between the two studies in the amount of arginine flux directed to NO synthesis is quite large, which may be due to the route of infusion, or the model used. Urschel et al (2007) fed either generous arginine (1.8 g Arg/kg/d) or a deficient diet (0.2 g Arg/kg/d) intragastrically to piglets, and found significantly higher whole body arginine conversion into NO with the higher intake. Accordingly, piglets fed the generous arginine diet had significantly higher NO synthesis compared to piglets fed the deficient arginine diet, but the proportion of whole body arginine converted into NO was not different. Thus, greater arginine intake led to greater NO production in both sick human neonates and neonatal piglets; however arginine deficient piglets could not up-regulate the NO synthesis.

1.4.3 Arginine metabolism in pathological situations

a. Arginine de novo synthesis in pathological situation: human neonate

TPN feeding reduces plasma arginine in human neonates: Arginine is considered to be a conditionally indispensable amino acid for human neonates. It is unlikely, based on current evidence, that the healthy neonate can synthesise adequate arginine in the small intestinal mucosa from abundantly available precursors, suggesting

that arginine is semi-indispensable. Early in the history of TPN use for neonates, it was clear that there was a requirement for arginine in TPN solutions. Hypoargininemia in parallel with hyperammonemia was observed in three infants receiving total parenteral nutrition with a synthetic amino acid mixture (Heird et al, 1972). Interestingly, the hypoargininemia and accompanying hyperammonemia resolved with the administration of arginine intravenously, clearly demonstrating the inadequacy of arginine in neonatal TPN. However, the absolute requirement for arginine was not known; nor was it known that bypassing small intestinal metabolism would impair de novo synthesis of arginine. Later studies by Batshaw et al (1984) reported that premature neonates had elevated plasma ammonia and significantly lower plasma arginine and ornithine concentrations within the first two months of their life, compared to normal birth weight neonates. Again, elevated plasma ammonia was corrected by the provision of additional arginine in TPN. These findings suggest that the concentration of arginine in the intravenous diets used for premature human neonates was not sufficient to maintain ammonia detoxification, even with full TPN feeding.

Prolonged TPN feeding, low plasma arginine and development of NEC: Prolonged TPN-feeding of human neonates places them at high risk of developing a clinical complication known as necrotizing enterocolitis (NEC). NEC is an aggressive, anaerobic infection that develops rapidly in the gastrointestinal tract of premature neonates during TPN feeding. This is the most common gastrointestinal emergency in premature infants, occurring in approximately 10% of all premature babies in North America (Di Lorenzo et al, 1995). Despite the fact that NEC has been a longstanding

medical issue in premature infants in the hospital setting, the pathophysiology of this infectious process is not well understood. Plasma amino acid analysis in premature neonates suffering from NEC showed that for most of the NEC cases, plasma arginine and glutamine concentrations were low at diagnosis (Di Lorenzo et al, 1995; Zamora et al, 1997). It is not yet clear whether NEC in the premature neonates interferes with arginine synthesis, or whether inadequate arginine availability to the small intestine contributes to the development of NEC. Becker et al (2000) followed premature infants from birth to identify factors that might contribute to the development of NEC. They reported significantly lower plasma arginine and glutamine concentrations at day 7 of life in infants who later developed NEC, compared to those who did not develop the disease (controls). The majority of NEC infants had parenteral amino acid intake that was double the intake of control infants. The infants who developed NEC were experiencing greater feeding intolerance at 7 days of age. Furthermore, irrespective of the higher arginine intake from TPN in the NEC group, plasma concentrations of arginine, glutamine and essential amino acids were still significantly lower even at 14 days of life. Accordingly, it is likely that lower plasma arginine concentrations in

premature neonates may pre-dispose them to the development of NEC. This concept is further supported by the work done by Amin et al (2002). In that study, premature infants received either TPN supplemented with additional arginine, or at a standard arginine (control) concentration. Only 6.7% of premature infants on the higher arginine intake (398 mg/kg/day) developed NEC by day 20 of life. In comparison, 16.9 % of the infants on the lower arginine intake (181 mg/kg/day) developed NEC by day 10 of life. Plasma

arginine concentrations were also significantly higher in arginine-supplemented infants compared to the control group. This clearly indicates that TPN feeding with a standard commercial formulation results in sub-optimal arginine status in premature infants, predisposing them to NEC.

b. Arginine metabolism and TPN feeding in neonatal piglets

According to the NRC (1998), the dietary requirement of arginine for healthy neonatal piglets is 0.38 g/kg/d. However, hypoargininemia and hyperammonemia occurred during a TPN feeding trial in piglets provided arginine at the NRC requirement (Brunton et al, 1999). Even with slightly more than double the amount of arginine in the NRC recommendation, low plasma arginine and high ammonia concentrations were observed. This amount of arginine (1.0 g arginine/kg/d) is mid-range for the concentration delivered by the various commercial pediatric products. In the neonatal piglet, it is apparent that the concentration is not adequate to meet the metabolic demands for arginine during TPN feeding. Brunton et al (2003) demonstrated that 1.0 g/kg/d of arginine in TPN did not maximise muscle protein synthesis in TPN-fed neonatal piglets, which implies that although ureagenesis might be maintained, the demand for arginine for creatine biosynthesis, NO synthesis and growth may not be fulfilled. In the same study, plasma arginine concentration increased with increasing dietary arginine, and reached a plateau at a concentration similar to sow-fed reference piglets only when the piglets received greater than 1.2 g/kg/d of arginine. The plasma ammonia concentration was low and stable at this arginine intake while the muscle protein synthesis was maximised. Therefore, they suggested that metabolic arginine requirement for TPN-fed, neonatal

piglets must be 1.2 g/kg/d or higher. Arginine de novo synthesis becomes compromised during TPN feeding, and as such, the whole body arginine requirement must be met by the dietary (IV) intake. In infant studies that provided arginine in TPN at concentrations similar to human milk, hypoargininemia and hyperammonemia were reported among premature neonates (Batshaw et al, 1984). Therefore, de novo arginine synthesis in the neonatal intestine seems to be impaired in both humans and piglets during TPN feeding, as both experience hypoargininemia and hyperammonemia during exclusive IV feeding (Brunton et al, 1999; Batshaw et al, 1984).

1.5 Blood flow in the small intestine

1.5.1 General information on blood flow to the gut in humans

The celiac artery, the cranial or superior mesenteric artery and the caudal or inferior mesenteric artery are three direct branches of the aorta that supply blood to the GI tract. The celiac artery supplies blood to the stomach, liver, and spleen, while the superior mesenteric artery, the largest single branch of the abdominal aorta, supplies the entire small intestine, proximal portions of the colon, and the pancreas. The inferior mesenteric artery delivers blood to the distal colon. In the small intestine, the duodenum is supplied by the duodenal branch of the superior mesenteric artery while

the jejunum is supplied by jejunal branches of the superior mesenteric artery. The ileum and the distal end of the intestine are supplied by the ileal, right colic, ileocolic, and appendiceal branches of the superior mesenteric artery. The blood flow to the intestinal tissues is then distributed into the various layers of the small intestine known as localised blood flow. Total blood flow through the aortic branches which supply the small intestine

is increased when food is ingested. This increase in blood flow is transferred to the mucosal layer of which 60% is directed towards the villi while 40% reaches crypts (Chou et al, 1976).

When the absorption of nutrients from a meal is complete, blood flow to the intestine returns to the food-deprived level. During digestion, blood flow increases and reaches a peak (15 – 20 % above the control) and stays elevated for 2 to 3 hours before returning to the unfed state (Chou et al, 1976).The portal vein collects nutrient-rich blood from intestinal tissues and supplies the liver before the nutrients are delivered to the post-hepatic systemic circulation. In general, blood flow to tissues is auto-regulated by metabolic factors such as decreased PO_2, pH, or osmolarity and increased PCO_2 or adenosine. These mechanisms serve to maintain blood flow to meet the intestinal tissue requirements for oxygen and nutrients and to facilitate nutrient delivery and waste removal, thus preserving the function and the integrity of the intestine during digestion and absorption processes (Sparks, 1980; Matheson et al, 2000). The pig as a model has been widely used in experimental studies of human cardiovascular diseases as pig is similar to human in cardiovascular anatomy and physiology (Pond et al, 1978a). The figure 2.0 illustrates the blood circulation of the pig.

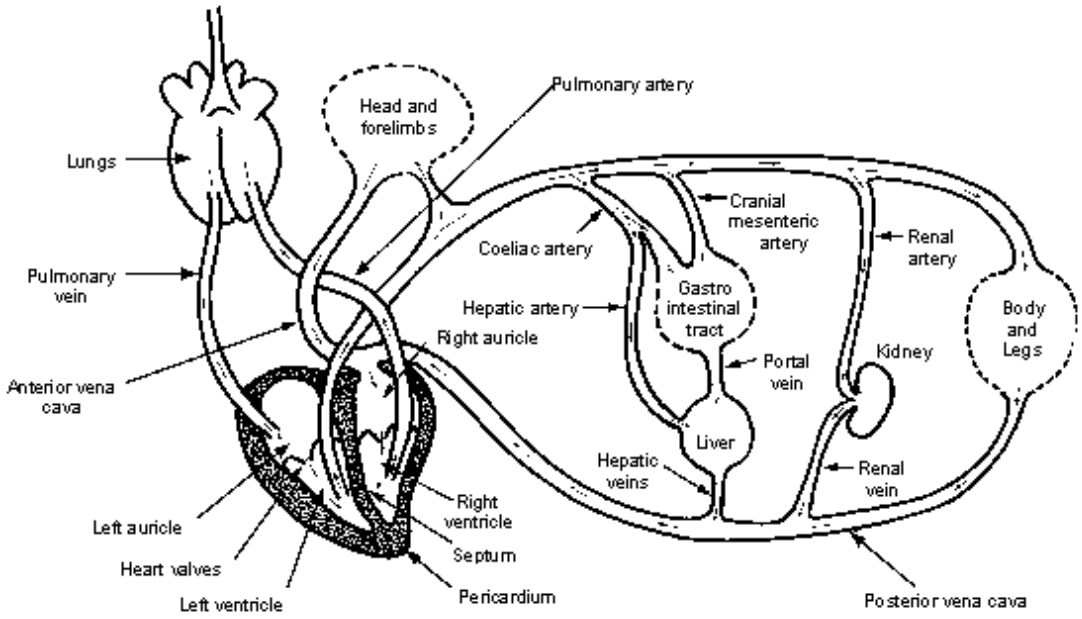

Figure 1.2: Blood circulation of the pig

1.5.2 Factors affecting blood flow

There are differences between adults and neonates in blood flow to gastrointestinal tissues in response to macronutrient intake. In the neonatal piglet, the small intestine demonstrated increased blood flow to all the layers of the proximal half of the small intestine with gastric infusion of a lower concentration of glucose than was required in the adult animals (Dudgeon et al, 1981; Bohlen, 1980). The response by the healthy, neonatal small intestine indicated an exaggerated response to luminal nutrients compared to the adult gut. An increase in blood flow to the small intestine in response to a high fat diet has also been well established; however, the findings related to proteins have been less clear. One research study reported an increase in blood flow to the small intestine with a high protein diet (Siregar & Chou, 1982) while other studies reported little or no response to amino acids or dipeptides (Chou et al, 1978; Kvietys et al, 1980). The impact of the ingestion of free amino acids on small intestinal blood flow had not been investigated, to our knowledge, when we were planning the research described in this thesis.

1.5.3 Arginine & NOS isoforms

As arginine is the precursor for NO synthesis, inadequate arginine availability to vascular endothelial cells may negatively affect blood flow. The specific function of NO depends on which isoform of the NO synthase enzyme is responsible for its synthesis. Nitric oxide synthase (NOS) has three isoforms; the two constitutive isoforms are endothelial NOS (eNOS) and neuronal NOS (nNOS), and the one inducible form is

iNOS. The constitutive forms are expressed at low levels in a variety of tissues and cells while the inducible form does not express in significant levels

under normal physiological situations (Garvey et al, 1997; Ford et al, 1997). An excessive level of NO production associated with iNOS expression is considered detrimental because its expression is associated with inflammation and ischemic injury (Alican & Kubes, 1996; Di Lorenzo & Krantis, 2001; Gookin et al, 2002). eNOS, which is concentrated in the endothelial cells (closer to the vascular smooth muscle cells), is responsible for the vascular diameter and thus, hemodynamic properties of the vessel. NO synthesised from eNOS is the most potent vasodilator in the newborn intestine (Nankervis et al, 2001). The NOS isoforms are unique in their function and their location. The NO produced by iNOS does not substitute for NO synthesised from eNOS. Thus, iNOS-derived NO does not participate in vascular regulation, nor does eNOS-derived NO participate in the inflammatory response (Nowicki et al, 2007).

1.6 TPN feeding & small intestinal atrophy

Small intestinal atrophy has been characterised by reduced small intestinal mucosal weight, villous height, mucosal protein mass, protein synthesis, crypt cell proliferation and polyamine synthesis secondary to reduced ornithine decarboxylase (ODC) activity and small intestinal goblet cell expansion. Intestinal atrophy, induced by TPN feeding, has been shown to occur in neonatal animals, and to a lesser extent in human neonates (Rossi et al, 1993; Dudley et al, 1998; Bertolo et al, 1999a & b; Conour et al, 2002; Niinikoski et al, 2004). Indeed, intestinal atrophy has been well documented in TPN-fed neonatal piglet models (Goldstein et al, 1985; Bengmark & Gianotti, 1996;

Bertolo et al, 1999a & b; Conour et al, 2002; Kansagra et al, 2003; Burrin et al, 2003; Niinikoski et al, 2004). During a 3 week feeding study, 6 week old intravenously-fed piglets had significantly lower total small intestinal weight compared to piglets that were fed with the same formula into a gastric catheter **(**Goldstein et al, 1985)**.** Furthermore, small intestinal length, villous height, crypt depth and epithelial cell number were significantly lower compared to values obtained for piglets that were fed an oral chow diet for 3 weeks. Dudley et al (1998) observed a significant reduction of fractional, absolute and total mucosal protein synthesis after 6 days of parenteral feeding in 3 d old neonatal piglets, compared to enterally-fed piglets provided the same formula. Bertolo et al (1999a & b) induced extensive gut atrophy in neonatal piglets within 7 days of TPN-feeding. They reported significantly lower small intestinal weight compared to controls. In addition, the mucosal wet weight was significantly lower in all sections of the small intestine, and the villous height was shorter in the duodenum. Total body nitrogen content and nitrogen retention were also significantly low in intravenously-fed compared to intragastrically-fed neonatal piglets. The small intestinal mucosal nitrogen content was also significantly lower in the intravenously fed animals, but the nitrogen content in the liver and kidney were not significantly different between treatment groups. This suggests that the body as a whole is affected by gut atrophy. It is also clear that the absence of luminal nutrients contributes to small intestinal atrophy, despite the provision of adequate parenteral nutrients.

Conour et al (2002) also used the neonatal piglet model to demonstrate TPN-induced gut atrophy. They killed a group of animals at 1 day old (study day 0) to get

base-line measurements, while two other groups were fed with either TPN or TEN (Total Enteral Nutrition) for 3 or 7 days. They reported significantly lower jejunal villous heights in piglets that were fed TPN for 3 days or 7 days compared to piglets that received TEN. The jejunal crypt depth was also significantly lower in 7 d TPN-fed neonatal piglets compared to piglets fed TEN for 7 days. Both of these histomorphometric characteristics were significantly lower in the TPN group compared to the values obtained from reference piglets killed at 1 d old (base-line).

In neonatal piglets, the enterocyte life span is 3-5 d, depending on the location along the small intestine (Fan et al, 2001). As described above, Conour et al (2002) found that after only 3 days of TPN feeding, newborn term piglets demonstrated small intestinal morphologies that were inferior to measurements obtained from animals killed at study day 0. However, the differences between 3 and 7 days of TPN feeding were negligible; thus, it likely takes only 3 days of TPN to induce intestinal atrophy. Niinikoski et al, (2004) investigated TPN-induced gut atrophy using neonatal piglets that were studied at 18-22 days old. All piglets were enterally fed and then randomized to TPN feeding or were continued on the enteral diet. The researchers observed reduced jejunal mass and jejunal villous height after only 24 h of TPN feeding, when compared to enterally-fed piglets. Mucosal fractional protein synthesis was also significantly reduced by 24 h of TPN. Jejunal crypt cell proliferation, rates of villus and crypt epithelial cell apoptosis in the jejunum and DNA mass were not different at 24 h of TPN feeding compared to enteral feeding. However, they were significantly reduced by 48 h of TPN feeding. Thus, the magnitude of the reduction of mucosal morphometric characteristics was profound

even by 48 h of TPN. The data are clear from the studies by Conour et al (2002) and Niinkoski et al (2004) that the process of TPN-induced intestinal atrophy is well established within 2-3 d of TPN feeding in neonatal piglets.

1.7 TPN feeding and reduced blood flow: piglet studies

An important question related to TPN-induced intestinal atrophy is how atrophy affects arginine metabolism and de novo arginine synthesis. It is well established that arginine becomes an indispensable amino acid in neonatal piglets during TPN feeding, as the de novo synthesis is compromised without first pass SI metabolism. Therefore, if there is inadequate arginine available during TPN feeding for NO synthesis in the blood vessels, the consequence may be reduced blood flow to the small intestine. Niinikoski et al, (2004) investigated portal and superior mesenteric arterial (SMA) blood flow during the transition from a food-deprived state to enteral feeding and then to TPN feeding in 18-22 d old neonatal piglets. SMA blood flow was measured during food deprivation, and then during 8 h of duodenal feeding followed by 20 h of TPN infusion. With the initiation of enteral feeding, the SMA flow increased by 30% compared to the food-deprived baseline. Four hours after the transition to TPN feeding, the SMA flow then significantly decreased compared with the mean 8-h enteral infusion rate. The flow appeared to stabilize at a rate that was 40% lower than the enteral blood flow rate, which was 10% lower than the food deprived rate. The portal blood flow was also expressed as the change from the mean blood flow during the period of food deprivation. Portal flow increased significantly after the start of duodenal feeding and remained 30% above the baseline level in piglets administered the continuous duodenal infusion. In TPN-fed piglets, portal

flow decreased to the baseline values after ~7 h of TPN. These data demonstrate clearly that both portal and SMA blood flow changed within a few hours of the initiation of enteral feeding (after food deprivation) and dramatically reduced after the transition from enteral to TPN feeding. Based on these results, it is evident that TPN acutely reduces blood flow to the intestine relative to enteral feeding. It is also apparent from the above study that the significant declines in intestinal blood flow in response to TPN feeding occurred quickly (less than 8 h), and likely preceded the morphological changes observed by others. However, this information is not adequate to elucidate whether the reduction of SMA blood flow is responsible for the gut atrophy, or whether rapid gut atrophy occurs independent of blood flow in TPN feeding (Niinikoski et al, 2004).

Reduced eNOS expression, which is the isoform responsible for the NO synthesis in the vascular endothelial cells, may be a reason for the reduced blood flow during prolonged TPN feeding. Reber et al (2002) studied 1, 3 and 10 d old fed and non-fed piglets in order to investigate the effect of enteral feeding on eNOS mRNA level and eNOS protein expression in mesenteric artery. They observed significantly higher eNOS protein expression in 1 d old fed piglets compared to 1 d old non-fed piglets. In piglets that were fed, the eNOS expression was significantly higher in the 10 d compared to 1 d old piglets. They also observed significantly higher blood flow in the mesenteric artery of 1 d old fed piglets compared to 1 d old non-fed piglets. Similarly, blood flow was significantly higher in 3 d old fed piglets than in 1d old fed piglets. This suggests a connection between increased eNOS expression in small intestinal mesenteric artery and blood flow to the small intestine during postnatal enteral feeding. In contrast, Niinikoski

et al (2004) investigated NOS activity in intestinal tissues taken from 3 week old neonatal piglets after TPN feeding. Despite a

reduced SMA blood flow, they measured no change in cNOS activity (eNOS and nNOS) or eNOS abundance. However, Niinikoski did not measure the eNOS activity or abundance in the mesenteric artery. Therefore, the data from the study by Niinikoski et al (2004) suggests that reduced SMA blood flow during TPN feeding may not be related to eNOS enzyme activity and/or eNOS protein abundance in small intestinal tissues. Instead, the feeding-induced change in SMA blood flow may be a systemic effect rather than a local effect.

1.8 Partial Enteral Nutrition and SI atrophy in neonatal piglet

It is evident that enterally derived nutrients are more readily available for small intestinal metabolism compared to systemic extraction, including most of the amino acids. However, full enteral feeding is not always possible in sick and/or premature neonates. The practice of giving small amounts of diet enterally has been adopted clinically, which is referred to as minimal enteral nutrition. However, the minimum amount or the nutrient composition that is needed to sustain small intestinal growth and function in neonates is not clear. Burrin et al (2000) quantified the total amount of the nutrient intake that was required enterally for the stimulation of small intestinal growth and normalisation of gut function. In that study, 7 day old neonatal piglets received the same total nutrient intake, of which 0, 10, 20, 40, 60, 80 or 100% was given enterally; the balance of intake was given intravenously. The animals were fed continuously for 7 days. Compared to TPN-fed controls, the piglets that received 40% of total nutrient intake

enterally had greater small intestinal mucosal mass, protein mass and villus height. However, 60% of the diet given enterally was required to sustain normal mucosal cell proliferation, small intestinal crypt depth and villi height,

and ODC (ornithine decarboxylase) activity. Plasma concentrations of glucagon-like peptide 2 (GLP-2) and peptide YY (PYY), which are considered to be good candidates for intestinal growth regulators, were also significantly higher with 60% of the diet fed enterally (Burrin et al, 2000). However, we do not know which dietary components, delivered enterally, were responsible for eliciting the responses in small intestinal morphological outcomes.

1.9 Rationale and Objectives

Absence of enteral feeding is considered the underlying reason for small intestinal mucosal atrophy. Burrin et al (2000) demonstrated that at least 60% of the daily nutrient intake provided enterally was required to avoid compromising small intestinal metabolism induced by TPN feeding. Providing more than 60% of all the nutritional requirements orally or enterally may not be possible with sick neonates. The mechanisms by which luminal nutrients influence small intestinal mass are not fully understood, nor is it known whether single or multiple specific nutrients are more important for regulating small intestinal mucosal development. However, the luminal presence of specific nutritive factors such as amino acids may act as trophic factors to sustain intestinal growth and integrity during TPN feeding or when enteral re-feeding is initiated.

Arginine is a good candidate to investigate potential trophic effects as it participates in many important metabolic pathways including NO synthesis, ureagenesis,

polyamine synthesis and protein synthesis. Numerous studies have shown that TPN feeding attenuates SMA blood flow in parallel with intestinal atrophy. Arginine is the precursor for NO synthesis, which is a key vasodilator in vascular

endothelial cells. Therefore, limited arginine availability to the endothelial cells of gut-atrophied neonatal piglets may lower SMA blood flow. Arginine also represents 6.7% of total body protein in neonatal piglets. Thus limited arginine availability in neonatal piglets may adversely affect the fractional protein synthesis in tissues which are highly proliferative such as small intestinal mucosa. For these reasons, arginine may have a particularly important role in preventing or ameliorating intestinal atrophy.

Arginine is synthesised in the small intestinal enterocytes in healthy neonates. TPN-induced gut atrophy is well established in the neonatal piglet model. Arginine becomes an essential amino acid in TPN-fed neonates as the atrophied gut has reduced metabolic activity and is deprived of enteral proline as a dietary precursor. Thus, the entire requirement for arginine needs to be provided by endogenous synthesis during TPN feeding. Therefore, we investigated whether a high concentration of dietary arginine, delivered as the sole nutrient into the gut, could facilitate recovery from TPN-induced intestinal atrophy in neonatal piglets.

We hypothesised that in neonatal piglets, enteral delivery of high compared to low arginine would act as a trophic factor, resulting in greater small intestinal fractional protein synthesis, greater crypt cell proliferation and greater SMA blood flow compared to TPN-fed piglets. We also hypothesised that enteral delivery of low arginine to gut-

atrophied piglets would produce attenuated responses compared to enteral delivery of high arginine.

Objectives:

In piglets that were provided high or low concentrations of arginine either enterally or parenterally, our objectives were to:

1. assess the effects of dietary arginine concentration and route of delivery on small intestinal morphology, small intestinal mucosal protein synthesis rate and SMA blood flow.
2. assess the effect of dietary arginine concentration and route of delivery on liver, skeletal muscle and kidney protein synthesis rates
3. assess the effect of arginine concentration and route of delivery on plasma urea, ammonia, and amino acid concentrations, as well as tissue free amino acid concentrations.

2.0 Methodology

2.1 Animals

The Institutional Animal Care Committee (Memorial University of Newfoundland) approved all the procedures used in this study. Yucatan miniature piglets, aged 14 to 17 d old, were obtained from Animal Care Services, Memorial University of Newfoundland. The study was designed to obtain animals as litter mates, with four animals used from each of six litters. However, blocking by litter was incomplete because of unplanned mortalities during the study (described in detail in section 3.0).

In this study, we calculated that we would need a sample size of n = 6 per group (with $\alpha = 0.05$ and $\beta = 0.8$). This sample size allowed for detection of a 20% difference in the rate of mucosal protein synthesis and would detect a 30% increase mucosal weight per cm of mucosal length.

2.1.2 Surgical procedure

Piglets were transported from the Vivarium (the Animal Care Services breeding and housing facility) to the Biotechnology building. Upon arrival, piglets were pre-anesthetised with an intramuscular injection of ketamine hydrochloride (22 mg/kg, Bimeda Canada, Cambridge, Ontario) and acepromizine (0.5 mg/kg, Vetoquinol, Canada, Inc, Quebec) as a single dose. Atropine (Rafter &Products, Canada) (0.05 mg/kg) was also given as sub-cutaneous injection to reduce air-way secretions during surgery. The animals were intubated to assist the piglet in maintaining respiration during surgery. The anaesthesia was maintained with 1.5 – 2 % isoflurane (Abbott Laboratories Inc., Canada) delivered with oxygen at 1.5 L/min

via the endotracheal tube. The heart rate and oxygen saturation were continuously monitored using a pulse-oximeter. Body temperature was maintained via a homeothermic blanket system that automatically adjusted heat output in response to a rectal temperature probe.

Eighteen of the 24 piglets were subjected to diet intervention, and as such, were designated as “experimental animals”. The experimental animals were implanted with a silastic catheter into the left jugular vein, which was advanced to the superior vena cava cranial to the heart. The jugular catheter was used for diet infusion. Another silastic catheter was implanted into the left femoral vein and advanced to the inferior vena cava caudal to the heart. The femoral vein catheter was used for the blood sampling and drug infusion. A silastic gastric catheter was also implanted using the Stamm gastrostomy technique (Rombeau et al. 1984), but only into piglets that received an intra-gastric (IG) infusion of arginine (n = 12). To facilitate chronic blood flow measurements, a 4 to 6 mm ultrasonic perivascular blood flow probe (Transonic Systems Inc., USA) was implanted on the SMA. Once the SMA was isolated, and the ultrasonic probe was in place, the space between the vessel and the reflector bracket of the probe was filled with ultrasonic gel (Aquasonic, Parker Laboratories, Inc., USA) mixed with non-heparinized blood. The quality of the signal from the probe was tested before the site was closed. The lead wire of the ultrasonic probe was tunnelled under the skin, and exteriorized on the back to facilitate chronic blood flow measurements without removing the piglet from its cage.

A group of piglets that remained with the sow for the duration of the study (sow-fed, SF Reference) (n = 6) also underwent surgery for the implantation of only a

jugular venous catheter that was fitted with a subcutaneous port. This allowed for daily blood sampling with lower risk of infection or damage; immediately following recovery from surgery, these SF piglets were returned to the sow and remained with the litter until d 7 of study.

2.1.3 Animal housing

The experimental animals were fitted with jackets and were placed in individual metabolic cages. A swivel and tether system (Lomir Biomedical Inc., Canada) allowed the piglets to have free movement in the cages while maintaining continuous diet infusions. The cages were fitted with heat lamps and the room temperature was maintained at 25^{o}C. A 12:12-h light-dark cycle was maintained.

2.1.4 Post surgical care

Immediately following surgery, piglets were given 0.5 mL of Borgal (40 mg trimethorprim and 200 mg sulfadoxine/mL) (Intervet Canada Ltd., Canada) and analgesic (0.03 mg/kg buprenorphine hydrochloride) (Temgesic, Reckitt Benckiser Healthcare, UK). The analgesic was given every 12 h for two days post-operatively. Subsequent doses were given if piglets showed signs of discomfort, such as limited movement. Antibiotics were also continued until study day 3. Again subsequent doses were given as needed if piglets exhibited signs of infections such as fever, lethargy, poor wound healing with pus, vomiting or altered breathing. The incision sites were covered with an antibacterial veterinary ointment each morning until they healed recovered. Immediately following surgery, all piglets received a continuous infusion of total parenteral nutrition

(TPN) that contained a moderate arginine concentration, providing 1.0 g/kg/d until the morning of day 4. Parenteral nutrition (diets described

below) was initiated at 50% of the maximal rate and increased to 75% on the morning following surgery, and was advanced to full TPN (13.5 mL/kg/h) by the evening of d 1. This allowed the piglets to adapt to the fluid load and to reduce the metabolic stress that accompanies intravenous feeding.

2.2 Study protocol

Small intestinal atrophy was induced by TPN feeding ("adaptation period") until the morning of d 4 (Conour et al, 2002).Subsequently, the diets were changed such that 12 piglets were randomized to receive a low dietary concentration of arginine via the gastric catheter (IG-L Arg) (0.6 g /kg/d), or a high concentration of arginine (IG-H Arg) (1.6 g /kg/d). These two groups were also provided with ***arginine-free*** TPN solution intravenously, to meet the remainder of their nutrient requirements. The third group continued on exclusive TPN with no intra-gastric infusion, but the TPN was modified to contain a high concentration of arginine (1.6 g/kg/d) (IV-H Arg). Thus the IG-H Arg and IV-H Arg received an identical concentration of arginine; only the route of delivery differed. The test diets were continued until the end of the study on d 7. The experimental protocol is described in Figure 2.

The low dietary concentration of arginine (0.6 g Arg/kg/d) was selected based on a number of factors. A previous study of intragastrically-fed neonatal piglets demonstrated that 0.6 g Arg/kg/d was adequate to avoid hyperammonemia, but resulted in low plasma arginine concentrations (Brunton et al, 2003). Also, this intake level is 50%

greater than the NRC recommendation for young piglets of 0.4 g/kg/d, which we believe is inadequate. The high dietary concentration (1.6 g Arg/kg/d) was

chosen based on data that suggested that the whole body dietary requirement for arginine is greater than 1.2 g Arg/kg/d in TPN-fed neonatal piglets (Brunton et al, 2003). Therefore, the amount selected for this study (1.6 g/kg/d) was likely in excess of requirements. A moderate amount of arginine (1.0 g/kg/d) was used during the adaptation TPN feeding period (days 0 to 4). This concentration is equivalent to a commercially available amino acid product used in TPN solutions for human neonates (Vaminolact-Fresenius Kabi, Germany), and is likely marginally deficient, according to previous findings (Brunton et al, 2003).

The intragastric infusion of arginine was provided at 2 mL/kg/h continuously using a syringe pump. This provided 0.6g/kg/d and 1.6 g/kg/d of arginine alone into the gastric catheters.

2.2.2 Preparation of diet

a. TPN preparation

All the diets were prepared in the laboratory. The amino acid concentrations for each TPN diet are presented in Table 2.1. The amino acids were sourced from either Sigma (St. Louis, MO, Canada) or from LV Lomas (Ajinomoto Company Inc, Japan, via LV Lomas, Brampton, Canada).Free L-arginine was used to make the diets. The amino acids were weighed on an analytical balance, and then mixed thoroughly as dry ingredients before adding to HPLC grade water in 4 L beakers on stir plates. The water was heated to 55^{o}C-65^{o}C prior to the addition of small aliquots of the amino acid mixture to facilitate

dissolving. The amino acids were mixed and dissolved under a blanket of nitrogen gas to protect amino acids from oxidation. All the diets were designed to provide 15 g amino acids/kg/d and 1.1 MJ metabolizable energy/kg/d with glucose and lipid each supplying 50% of non-protein energy. All of the experimental diets were isonitrogenous, via the manipulation of serine. In general, the amino acid pattern was based on that of a commercial total parenteral nutrition solution (Vaminolact-Fresenius Kabi, Germany) with slight modifications. Once the amino acids were completely dissolved, D-glucose and minerals were added (Table 2.2). Finally, the solution was brought up to the final total volume by adding HPLC-grade water. The diets were sterilized via filtering through a 0.22 µm filter (AcroPak^{TM}, Pall Life Sciences, USA) in a laminar flow hood. The filtered diet was infused directly into sterile intravenous bags (Baxter, Canada), and refrigerated until needed.

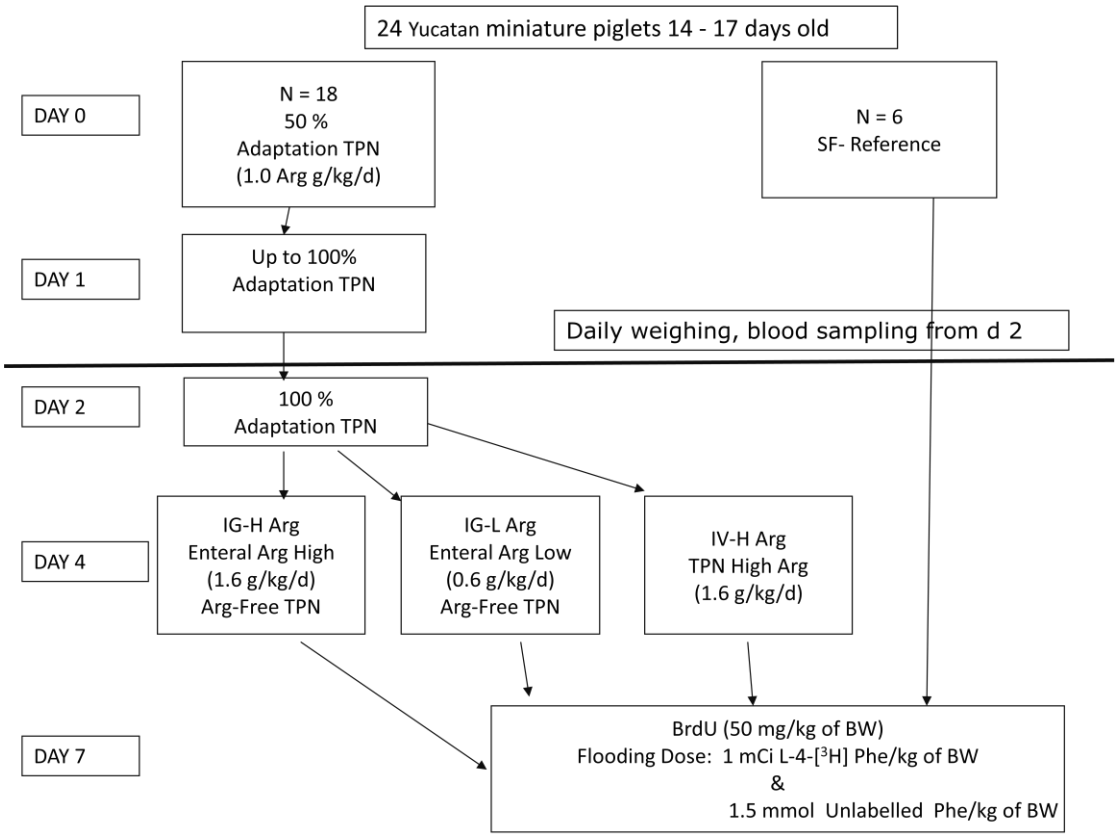

Figure 2.1: Experimental design

Table 2.1: Amino acid profiles (g/L)*of TPN diets

	Adaptation**	IV-H Arg	IG-H Arg	IG-L Arg
alanine	4.50	4.50	4.50	4.50
arginine*	***3.68***	***5.88***	***0***	***0***
aspartate	3.36	3.36	3.36	3.36
cysteine	0.80	0.80	0.80	0.80
glutamate	5.81	5.81	5.81	5.81
glycine	1.51	1.51	1.51	1.51
histidine	1.71	1.71	1.71	1.71
isoleucine	2.55	2.55	2.55	2.55
leucine	5.76	5.76	5.76	5.76
lysine-HCL	5.73	5.73	5.73	5.73
methionine	1.07	1.07	1.07	1.07
phenylalanine	3.03	3.03	3.03	3.03
proline	4.58	4.58	4.58	4.58
serine	***5.33***	***0***	***0***	***8.90***
taurine	0.25	0.25	0.25	0.25
tryptophan	1.17	1.17	1.17	1.17
tyrosine	0.43	0.43	0.43	0.43
valine	2.93	2.93	2.93	2.93
threonine	2.24	2.24	2.24	2.24

*The diets not including lipid, but containing amino acids, D-glucose, minerals, trace elements, vitamins and iron were infused at a rate of 272 mL/kg of body weight/day
**Adaptation TPN fed to all experimental piglets until d 4, to induce intestinal atrophy
***Arginine was not included in the IG-H Arg and IG-L Arg TPN diets, but was infused enterally. The amount of arginine given enterally would be equivalent to 5.88 g/L (IG-H Arg) or 2.21 g/L (IG-L Arg) if included in the TPN solutions described in this table.

Table 2.2: Composition of the TPN diets

	g/L
Amino acids	55.3
D-Glucose	90.3
Trihydrate K_2HPO_4 (g)	1.57
Monobasic KH_2PO_4 (g)	1.09
Potassium acetate (g)	1.47
NaCl (g)	2.17
$MgSO_4$ (g)	0.78
$ZnSO_4$ (g)	0.09
Calcium gluconate (g)	6.41

b. TPN Admixture Components

The salts listed in the Table 2.3 were dissolved in HPLC-grade water and then filtered through a 0.22 µm filter (Millipore Ireland ltd., Ireland) into sterile vacuum sealed bottle (Baxter, Canada) and stored at 4^{o}C. Just prior to feeding the intravenous diets, each bag containing 750 mL of diet was injected with 3 mL of Multi-12/K_1 pediatric multivitamin commercial solution designed for TPN feeding (Table 2.4) (Baxter, Canada), 1 mL of iron dextran that provides 3 mg of iron (ferric hydroxide) per kg of body weight (Vetoquinol Canada Inc., Canada), 3 mL of trace element mix (Table 2.3) and 145 mL of 20% Intralipid (Fresenius Kabi, and Uppsala, Sweden). The vitamin mix provided all vitamins at more than 100% of the NRC (1998) requirement for neonatal piglets, and the trace element mix provided a minimum of 200% according to the NRC.

c. Intragastric arginine infusions

The intragastric infusion solution of arginine was prepared similar to the procedure for TPN diets, except only arginine was added to heated water so that the base arginine solution contained 12.5 g Arg/L and 33.34 g Arg /L for the IG-L Arg and IG-H Arg infusions, respectively. The infusates were filtered and stored in sterile intravenous bags (Baxter, Canada) at 4^{o}C as described above.

Table 2.3: Trace element mix

Element	Minerals supplied as:	Mineral Salt (g/L)	SingleElement Dose (mg/kg of BW/d)
Zinc	$ZnSO_4.7H_2O$	40.69	10.07
Copper	$CuSO_4.5H_2O$	3.12	0.86
Manganese	$MnSO_4.H_2O$	1.86	0.66
Chromium	$CrCl_3.6H_2O$	0.05	0.01
Selenium	SeO_2	0.06	0.05
Iodide	NaI	0.02	0.02

BW – Body weight

Table 2.4: Commercial multi-vitamin mix* designed for TPN feeding

Vitamins	Amount in 5 mL of vitamin mix *	Dose (per kg of BW/day)
Ascorbic acid (mg)	80	17.41
Vitamin A (IU)	2300	500.48
Vitamin D (IU)	400	87.04
Thiamine (as hydrochloride) (mg)	1.2	0.26
Riboflavin (as phosphate) (mg)	1.4	0.30
Pyridoxine hydrochloride (mg)	1	0.22
Niacinamide (mg)	17	3.70
d-Panthenol (mg)	5	1.1
Vitamin E (*dl*-alpha tocopheryl acetate) (IU)	7	1.52
Vitamin K1 (mg)	0.2	0.04
Biotin (µg)	20	4.35
Folic Acid (µg)	140	30.46
Vitamin B12 (cyanocobalamin) (µg)	1	0.22

*Multi-12/K_1Pediatric Multivitamin Mix (Baxter, Canada); Vitamins are provided as two separate solutions, that are mixed just prior to use (final volume 5 mL). 3 mL of the prepared solution was added to each 750 mL diet bag, to deliver the vitamin doses described above. BW – Body weight

2.3 Daily care

A daily care record was maintained for each animal during the study that included total weight of diet infused, daily piglet weight, drug records and general comments about the piglets' wellbeing.

2.3.1 Blood sampling, animal weighing and adjusting diet infusion rates

The blood volume of a neonatal piglet is approximately 10% of body weight. A 2 week-old piglet weighs approximately 2.5 kg, therefore the total blood volume is ~250 mL. A guideline for blood sampling without affecting the health of the piglet is 2% of the total blood volume (5 mL) every second day, as young pigs are capable of producing erythrocytes at a rate proportional to their increase in body weight if adequate iron is available (Talbot & Swenson, 1970). In this study, we sampled 2.5 mL of blood every morning prior to flushing the lines with heparinized saline. The plasma was separated and stored at -20^{o}C until needed for analyses. Animals were weighed every morning and the diet infusion rate was adjusted to accommodate daily weight gain. The pump efficiency was also calculated daily and the rates of diet infusion were adjusted accordingly.

2.3.2 Blood flow reading

Blood flow rate was measured every 12 h beginning from d 3 and was recorded for a 15 min interval each time. The data were averaged for the period, and converted from a voltage measurement to mL/min using a conversion factor supplied by the manufacturer (Transonic Systems Inc., USA). A baseline blood flow was established as the mean blood flow of individual animals during the 36 h period prior

to initiation of the test diets (ie the average of the blood flow measures on study d 3

morning and afternoon and study d 4 morning). Subsequently, blood flow after the initiation of the test diets was expressed as the percentage of the baseline blood flow. Therefore, the baseline blood flow represented blood flow while all the piglets were fed a moderate amount of arginine in TPN.

2.4 Isotope infusion protocol and necropsy procedure

On study d 7, a bolus IV dose of 5-bromodeoxyuridine (BrdU) (50 mg/kg body weight) (Sigma Aldrich, Oakville, Canada) was infused intravenously 4 h prior to the necropsy to facilitate the measurement of in vivo small intestinal crypt cell proliferation index(described below in section 2.5.2).

A flooding dose of unlabelled phenylalanine (1.5 mmol/kg) (Sigma Aldrich, Oakville, Canada) was administered over 5 minutes, along with tritiated phenylalanine (1 mCi of L-[4-^{3}H] phenylalanine/kg body weight) (GE Biosciences/Amersham Biosciences, Oakville, Canada) via the jugular vein catheter. This was done 30 minutes before the necropsy to measure the tissue specific fractional protein synthesis rates (described in section 2.5.1.2). The intravenous TPN infusion was stopped during the 5 minute infusion.

Thirty minutes following the phenylalanine infusion, piglets were anaesthetized by mask (halothane/oxygen). The abdomen was opened and the entire gut weight and length were recorded. A 60 cm long section of the proximal jejunum was flushed with ice-cold saline, and the mucosa was scraped from the muscle layers using a glass microscope slide. The mucosa was weighed, and frozen in liquid nitrogen and stored at -

70°C. Samples of liver, left kidney and skeletal muscle (gastrocnemius) were also frozen in liquid nitrogen, and the exact time the tissues

entered the liquid nitrogen was recorded. Tissues were stored at -70°C. A section of proximal jejunum was formalin-fixed for histological analyses. The positions of the SMA blood flow probe and all the catheters were verified at necropsy. The piglets were killed while still under anaesthesia via exsanguination after the removal of the liver.

2.5 Analytical procedures

2.5.1 Biochemical analysis of plasma, tissue amino acids and protein synthesis

2.5.1.1 Plasma amino acid analysis

a. Plasma preparation and derivatization for amino acid analysis via HPLC

Plasma amino acids were analysed by reverse-phase high-performance, liquid chromatography (HPLC) using phenylisothiocyanate derivatives (PITC, Waters, Woburn MA) as per the method of Bidlingmeyer et al (1984). HPLC is a technique that can be used to separate and then to identify and/or quantify individual amino acids. The eluent A used in the HPLC Waters Pico Tag method contains HPLC grade water and sodium acetate trihydrate (Fisher Scientific, Whitby, Canada) and acetonitrile (Fisher Scientific, Whitby, Canada) at pH ~6.55. The eluent B contains acetonitrile, HPLC water and MeOH. Both of the eluents were filtered using 0.45 µm Magna nylon filter paper (Thermos Scientific, Canada). Eluent A allows separation of hydrophilic amino acids while eluent B allows separation of hydrophobic amino acids. In detail, 20 µL of 2.5 µmol/mL norleucine standard (Sigma Aldrich, Oakville, Canada) was added to 100 µL of plasma with 1 mL of 0.5% trifluoroacetic acid (TFA) (Sigma Aldrich, Oakville, Canada)

in methanol (MeOH) (Fisher Scientific, Whitby, Canada) and vortexed to precipitate proteins. The samples were centrifuged at 4200 *g* to separate the plasma proteins. The resulting supernatant was frozen in liquid nitrogen and dried on the freeze dryer (Thermo Savant, Canada) overnight. 50 µL of a solution of 20:20:60 of triethylamine (TEA) (Sigma Aldrich, Oakville, Canada): MeOH: water was added to each dried sample, followed by ~1 hour freeze drying. This step was to adjust the pH for the derivatization. Samples were then incubated with 20 µL of 10:10:70:10 solution of water: TEA: water: MeOH: PITC (Thermo Scientific, USA) for 35 minutes at room temperature. This derivatization step allows PITC to bind with free amino acids to form phenylthiocarbamyl amino acids. The phenylthiocarbamyl amino acids in solution are relatively stable in neutral pH. The labelling with PITC was stopped by freezing with liquid nitrogen and the samples were placed on a freeze dryer overnight. Prior to measuring on the HPLC, the samples were re-suspended in 200 µL of sample diluent (710 mg of Na_2HPO_4 (Sigma Aldrich, Oakville, Canada) in 1 L of HPLC water, titrated to pH 7.4 with 10% H_3PO_4 acid and diluted with acetonitrile so that the acetonitrile equals 5% by final volume). Samples were centrifuged and the supernatant was transferred to 0.25 mL Eppendorf tube.

c. HPLC analysis of plasma samples

The HPLC system is fitted with an auto-sampler such that up to 48 samples may be injected sequentially. A 40 µL aliquot of sample was injected into the HPLC column. The reverse phase C18 column temperature was maintained at 46^{o}C to facilitate the separation procedure. The phenylthiocarbamyl amino acids were separated on the column during a 90 min run, at 1 mL/min and quantified by UV absorbance at 254 nm. The peaks in the

chromatogram for each amino acid were integrated using Breeze Software (Waters,Version 3.3, 2002, Waters Corporation, Woburn MA, USA), and the amino acid concentrations in the plasma were determined by comparing peak areas to the area produced by the internal standard (norleucine).

2.5.1.2 Fractional rates of tissue specific protein synthesis

a. Tissue preparation

One gram of tissue was homogenized with 3 mL of 2% perchloric acid at 50% speed using a mechanical homogenizer for ~45 seconds. The homogenates were centrifuged at 3000 *g* for 15 min to separate acid-soluble, tissue-free amino acids from acid-insoluble, protein-bound amino acids. This was repeated 3 times for the complete separation of tissue-free amino acids (supernatant) from the protein-bound pool.

Tissue-free amino acids: The standard, 100 µL norleucine (25 µmol/mL) (Sigma Aldrich, Oakville, Canada) was added to the supernatant containing the tissue-free amino acids. Prior to analyzing the tissue free amino acid concentrations, 9 mL of the supernatant was neutralised with 375 uL of 2M K_2CO_3 and the samples were centrifuged to separate the supernatant from precipitate. One mL of filtered supernatant was dried down and derivatized as described above (2.5.1.1 a), with the exception that larger volumes of the pre-derivatization solution (TEA:water:MeOH, 100 µL) and the derivatization solution (TEA:water:MeOH:PITC, 50 µL) were used. In the final step, the derivatized samples were re-suspended in 300 µL of sample diluent.

Protein-bound amino acids: The internal standard, norleucine (400 µL of 25 µmol/mL) was added to the acid-insoluble protein pellet that remained after separating

the tissue free component. Subsequently, the pellet was hydrolysed with 6M HCl for 24 h at 110ºC. The resulting hydrolysate solution was cooled overnight and then diluted to 25 mL with HPLC water. The resulting solution was filtered using 0.45 µm syringe filters (Life Sciences, Canada) and 1mL of the filtrate was placed in a vacuum oven until dry. The dried pellet was derivatized via the same method as the tissue free amino acids (2.5.1.2 b).

b. HPLC and fraction collection

Phenylalanine concentration in tissues was determined as described above for plasma amino acids. The radioactivity associated with tissue free and bound phenylalanine was determined by fraction collecting the eluent that was associated with the phenylalanine peak. Three minute windows were collected that were timed to correspond to the phenylalanine peak, which resulted in 3 mL fractions. The radioactivity in the phenylalanine fraction was determined by liquid scintillation counting using biodegradable scintillant (10 mL) (Fisher Scientific, Whitby, Canada).

c. Calculations

A fractional rate of tissue-specific protein synthesis (FSR) was determined using the following formula:

$$Ks\ (\%/day) = (S_b/S_a) \times (1{,}440/t) \times 100$$

Where S_b is the SRA of the protein-bound, labelled amino acid, S_a is the SRA of the precursor pool and the t is the duration of labelling in minutes (Garlick et al, 1980).

2.5.2Immunohistochemistry - Crypt cell proliferation Index

a. Immunohistological staining of BrdU incorporated nuclei

Formalin fixed sections of jejunum were assigned with random numbers by a person not associated with the study. Subsequently, the sections were paraffin-embedded. A microtome (Thermos Scientific, USA) was used to cut 5 µm thickness sections, which were mounted on gelatine-coated slides that were prepared in the laboratory. Plain slides (Bio-nuclear Diagnostics Inc., Canada) were coated using a mixture of 0.3 g of gelatine (Sigma Aldrich, Oakville, Canada) in 290 mL of dH_2O and 0.03 g of chromium potassium sulphate (Sigma Aldrich, Oakville, Canada) in 10 mL of dH_2O. The mounted slides underwent microwave pre-treatment at 60^oC overnight to make the cells permeable for antibody labelling of BrdU. The sections were stripped of paraffin using xylene (Fisher Scientific, Whitby, Canada), 100% ethanol and then 95% ethanol. The sections were outlined with liquid repellent slide marker pen (Daido Sangyo Ltd., Tokyo), then the sections were incubated with 3% H_2O_2 in phosphate-buffered saline (PBS) at room temperature for 10 minutes to inhibit endogenous peroxidase activity. After rinsing the slides with fresh PBS, slides were heated with antigen retrieval system at 89^oC in a coplin jar for another 10 minutes. This procedure unmasks the antigenic sites leading to improved immunostaining. The slides were cooled at room temperature and rinsed 3 times with fresh PBS before they were incubated overnight with biotin anti-BrdU monoclonal antibody in a humidified chamber at 4^oC. The PBS-rinsed sections were again incubated for 1 hour with secondary antibody, streptavidin horse-radish peroxidase (SAv-HRP) enzyme complex (BD Biosciences, Mississauga, Canada). The resulting complex was stained with 3, 3'- diaminobenzidine (DAB) substrate system (Vector Laboratories Inc., Burlington, Canada) to visualize the BrdU labelled DNA (BrdU In-Situ

Detection kit, BD Pharmingen, Mississauga, Canada). The slides were counterstained with hematoxylin and eosin (Fisher Scientific, Whitby, Canada) and then rinsed thoroughly with water by allowing tap water to run through the container which held the slides. These slides were dehydrated using 95% and 100% ethanol, each for 10 minutes. Again xylene was used to clear the slides. Sections were covered with cover slips using permount (Fisher Scientific, Whitby, Canada). The slides were dried overnight in the fume hood, and were then ready for light microscopic visualization.

b. Counting BrdU-labelled nuclei

The crypt cell proliferation index was quantified by counting the number of BrdU labelled nuclei in 15 well oriented crypts, and was expressed as the percentage of total nuclei per crypt. This procedure was done by the author of this thesis (OCD) who remained blinded until the entire sample set was completed.

2.5.3 Colourimetric analysis of plasma urea, ammonia and liver protein mass

2.5.3.1 Plasma urea concentrations

Plasma urea concentration was assayed on d7 plasma via colourimetric analysis (Quantichrome Urea Assay kit-BioAssay Systems, USA). This assay kit is designed to measure urea directly in biological samples without any pre-treatment. The colour produced by urea with chromogenic reagent was measured at 520 nm and this intensity is directly proportional to the urea concentration of the samples.

2.5.3.2 Plasma ammonia concentrations

Plasma ammonia concentration was also assayed quantitatively using enzymatic reduction method (Ammonia Assay Kit-Sigma Aldrich, Oakville, Canada). The d 7

plasma, which had been stored at -20^{o}C, was used. The ammonia present in the plasma reacts with Alpha-Ketoglutarate (α-KG) and reduces NADPH in the presence of L-glutamate dehydrogenase (GDH) to form L-glutamate and oxidized $NADP^{+}$. The decrease in absorption (measured at 340 nm) measured after adding the enzyme is due to the oxidation of NADPH which is proportional to the ammonia concentration in the plasma.

2.5.3.3 Liver protein mass

Liver protein content was assayed by Biuret reaction as per the Biuret method (Gornall et al, 1949).

2.5.4 Statistical analyses

For most outcomes, data were analysed using one-way ANOVA. If a significant outcome was determined, then treatment groups were compared by Bonferroni multiple comparisons test (GraphPad Prism 4, Graph Pad Software, San Diego, California, USA). For the blood flow measurement, data were analysed by repeated measures ANOVA for the whole time period and then again just for the last 3 measures when the values reached plateau (determined by a slope not different than zero). Plasma amino acid concentrations were analysed by repeated measures ANOVA using values from study days 4, 5 and 6. All data were expressed as mean ± SD. Data collected from the sow-fed reference group were not included in the statistical analyses, as they did not receive experimental diets. Rather, these data are presented as a range that represents the group mean ± 1 SD, and used for comparison purposes.

3.0 Results

Over the course of the trial, three piglets did not complete the study. Two of the piglets that did not complete the study belonged to the first set of animals introduced to the study protocol, and died after the initiation of the enteral arginine infusion. The piglets were hyperammonemic, and post-mortem examination indicated that the gastric catheter had slipped out of the stomach. The catheter design and surgical procedure were modified to avoid this issue. These piglets were replaced by animals obtained from subsequent litters; thus the randomization by blocking for litters was incomplete. The third animal (IG-L Arg) did not complete the study because it was showing signs of hyperammonemia on d 7, and was euthanized prior to obtaining the final measurements. This animal was not replaced. The rest of the piglets used in this study were active, healthy and were interested in their environment during the study period

3.1 Growth during study period

The dietary treatments did not affect the mean weight gain of the piglets, and all three treatment groups gained weight at a rate that was well below the sow-fed littermates (Figure 3.1).

3.2 Small intestinal morphology

3.2.1 Small intestinal length and weight

The mean small intestinal weight of all the experimental groups was 30 - 50% lower compared to the sow-fed reference animals, but the treatment groups were not significantly different from each other. However, the IG-H Arg piglets had significantly greater small intestinal length growth compared to IV-H Arg group ($P = 0.04$) (Table

3.1).Thus, the enteral infusion of high arginine partially ameliorated the small intestinal atrophy that was induced by TPN feeding.

3.2.2 Jejunum crypt cell proliferation

A sample of BrdU staining of crypt cell nuclei is presented in Figure 3.2. Neither the route of feeding of arginine, nor the amount fed resulted in significant differences in crypt cell proliferation index in the jejunum among treatment groups (Table 3.1).

3.3 Tissue specific rates of protein synthesis and free amino acid concentrations

3.3.1 Small intestinal proximal jejunal mucosa

Independent of the route of delivery and the amount of arginine provided, our experimental treatments did not induce significant differences in the rate of mucosal protein synthesis. TPN feeding of arginine (IV-H Arg) resulted in a mean mucosal protein synthesis rate that was more than 2 SD below the SF Reference mean (Table 3.2).

3.3.2 Liver

Regardless of the amount of arginine provided, both groups given enteral arginine had significantly higher rates of liver protein synthesis compared to the IV-H Arg group (Table 3.2). In contrast, there was no significant difference in the tissue free arginine concentration in the liver between IG-H Arg and IV-H Arg groups; instead, liver free arginine was significantly lower in the IG-L Arg compared to IV-H Arg (Figure 3.3).

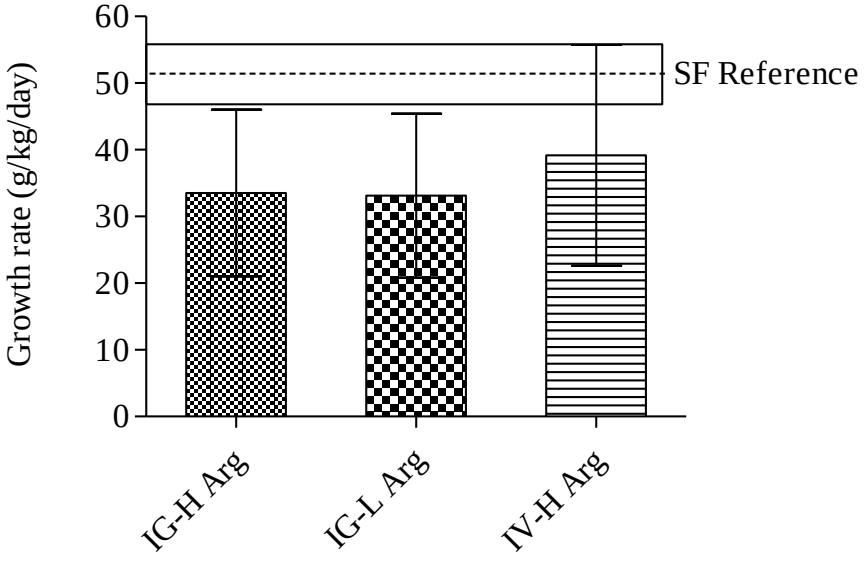

Figure 3.1: Daily growth rates of piglets fed intragastric high arginine (IG-H Arg), intragastric low arginine (IG-L Arg) or intravenous high arginine (IV-H Arg). Values are mean ± SD, n = 6 per treatment. The box represents the sow-fed reference mean ± 1 SD, derived from a group of sow-fed piglets (n = 4) (SF Reference) that were littermates to the treatment groups.

Table 3.1: Small intestinal (SI) morphology at necropsy

	IG-H Arg	IG-L Arg	IV-H Arg	P value	SF Reference
SI weight (g/kg body weight)	17 ± 2	16 ± 2	15 ± 2	0.39	28± 5
SI length (cm/kg body weight)	164 ± 11^{a}	148 ± 14^{ab}	141 ± 17^{b}	0.04	191± 21
Crypt cell proliferation index (% of BrdU positive cells)	15.9 ± 1.7	12.7 ± 4.1	12.0 ± 3.8	0.13	16± 4

Values are means ± SD; n = 6 (all treatment groups) or 5 (SF Reference) for SI weight and length. For BrdU, n = 5 (IG-L Arg) or 6 (all other groups). P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Bonferroni's multiple comparisons test. SF Reference values are derived from a group of sow-fed piglets that were littermates to the treatment groups.

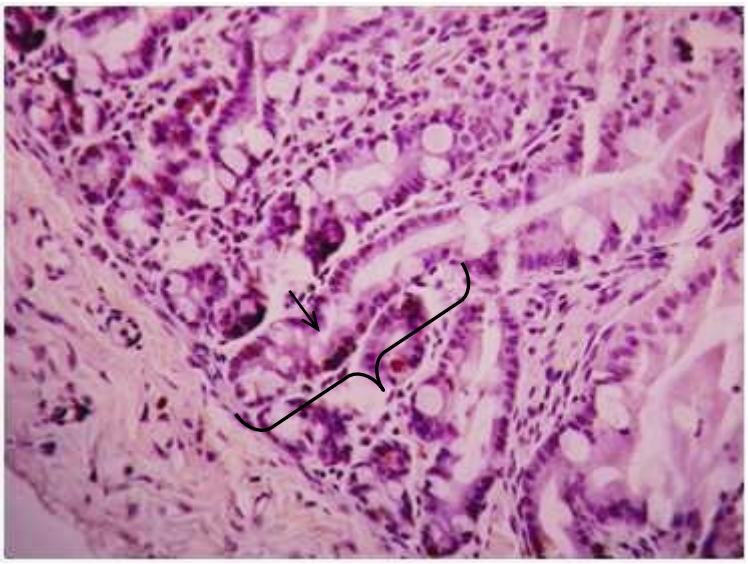

Figure 3.2:5-Bromo 2-deoxyuridine labelling of SI crypt cells

Bracket indicates full crypt in proximal jejunum. Arrow indicates BrdU labelled nuclei (brown staining).

3.3.3 Kidney

We also analysed the rate of protein synthesis in kidney tissue, however, we found no difference among treatment groups (Table 3.2).

3.3.4 Skeletal muscle

Protein synthesis in skeletal muscle sampled from gastrocnemius also was not affected by dietary arginine or the route of arginine delivery (Table 3.2).

3.4 SMA blood flow

All the experimental animals experienced a steady decline in SMA blood flow following surgery, during the adaptation period while being fed parenterally with moderate arginine-containing TPN (1.0 g/kg/d of arginine). The decline continued until the morning of day 4 (data not shown), at which point the test arginine infusions were initiated. Blood flow further declined, irrespective of the route of arginine delivery, or the amount of arginine provided (Figure 3.4). The decline in blood flow reached plateau in all groups by 48 h after the test diets were initiated (48 h on Figure 3.4), as determined by a slope not different than zero by linear regression (data not shown). From 48 to 72 h of treatment, the mean blood flow values were significantly different among all treatment groups (repeated measures ANOVA, $P = 0.002$). IV-H Arg treatment had the smallest reduction in blood flow (22% lower than baseline). It is interesting that the provision of high arginine alone (IG-H Arg) attenuated some of the reduction of blood flow, whereas the IG-L Arg piglets demonstrated the greatest reduction at ~40% lower than baseline at study end.

Table 3.2: Tissue specific fractional rates of protein synthesis (%/d)

	IG-H Arg	IG-L Arg	IV-H Arg	P value	SF Reference
Liver	130 ± 26^{a}	126 ± 23^{a}	85 ± 22^{b}	0.01	135± 41
Mucosa	71 ± 14	60 ± 28	57 ± 17	0.4	110 ± 23
Gastrocnemius muscle	17 ± 4	18 ± 6	17 ± 7	0.9	15 ± 5
Kidney	32 ± 11	44 ± 18	35 ± 11	0.5	51 ± 21

Values are means ± SD. Liver and mucosa: n = 5 (IG-L Arg) or 6 per group. Kidney: n = 3 (IG-L Arg) or 4 per group. Gastrocnemius: n = 4 (IV-H Arg) or 5per group. P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Bonferroni's multiple comparisons test. SF Reference values were derived from a group of sow-fed piglets that were littermates to the treatment groups.

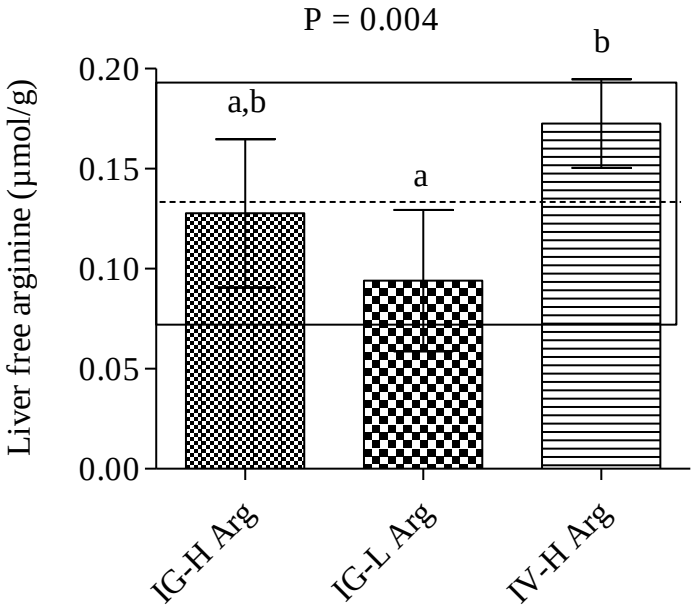

Figure 3.3: Liver free arginine concentrations. Values are means ± SD; n = 5 (IG-L Arg) or 6 per group. P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.01$ using Bonferroni's multiple comparisons test. The broken line within the box represents the mean ± 1 SD, derived from a group of sow-fed piglets (n = 6) (SF Reference) that were littermates to the treatment groups

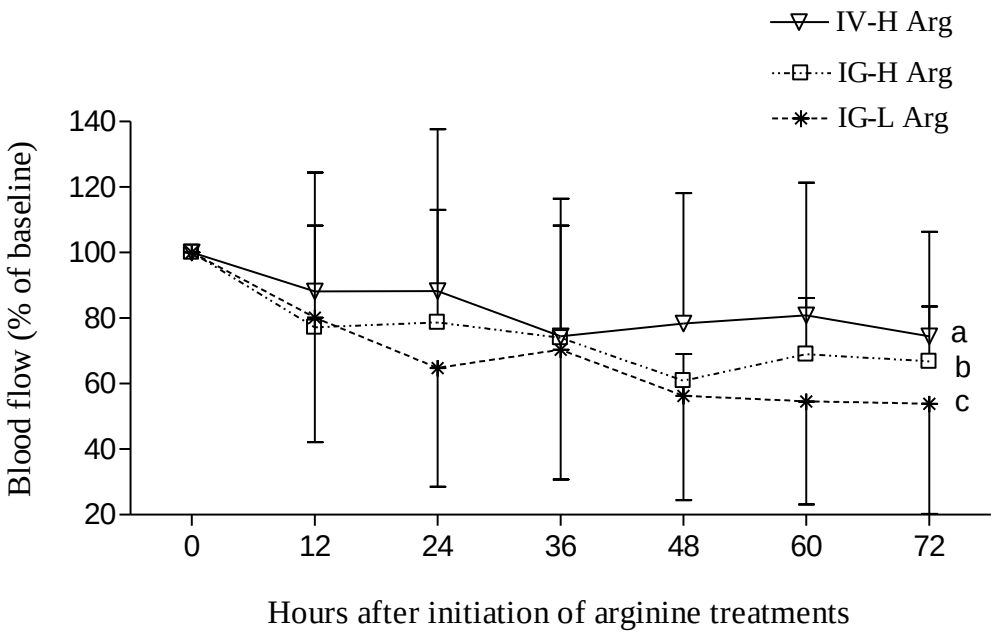

Figure 3.4: Superior mesenteric artery blood flow. Data are expressed as the percentage of baseline, which was the mean blood flow of individual animals calculated from measurements during the 36 h period prior to initiation of the test diets (baseline). Values are means ± SD, n = 5 (IG-H Arg) or 6 (IG-L Arg, IV-H Arg) piglets per treatment. Data from 48 to 72 h were analyzed by repeated measures ANOVA using Bonferroni's multiple comparisons test, a vs b, $P < 0.05$; a vs c, $P < 0.01$, b vs c, $P < 0.05$.

3.5 Plasma amino acids

Plasma amino acid concentrations were highly variable among treatment groups. Only the significant results are presented below. The complete data set for plasma free amino acid concentrations is presented in Appendix I.

3.5.1 Arginine family of amino acids

Enteral provision of low arginine (IG-L Arg) led to lower concentrations of plasma arginine compared to intravenous provision of high arginine (IV-H Arg) (Figure 3.5). In IG-H Arg treatment, mean plasma proline was similar to the SF Reference mean while IG-L Arg and IV-H Arg groups were more than 1 SD above the SF Reference mean (Figure 3.5); however, the treatment groups were not significantly different from each other. Plasma ornithine concentrations were not significantly different between experimental groups; however mean concentrations follow the similar pattern as mean plasma arginine concentrations. In our animals, plasma mean citrulline concentrations were not differed by the treatment.

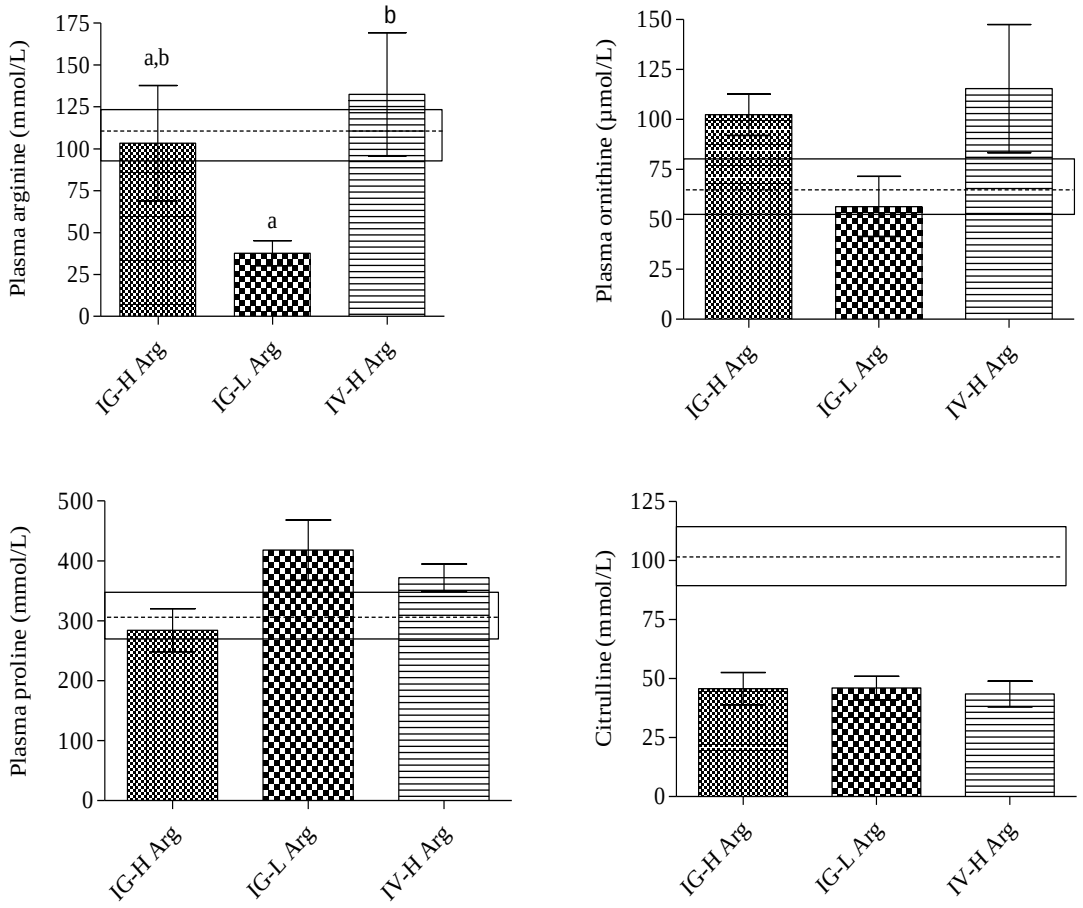

Figure 3.5: Plasma arginine, ornithine, proline and citrulline concentrations. Data are expressed as mean ± SD using plasma amino acid concentrations from d 4, 5 and 6; n = 6 (IG-H Arg and IG-L Arg) or 5 (IV-H Arg) per group. P values were determined by repeated measures ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Bonferroni's multiple comparisons test. The broken line within the box represents the mean ± 1 SD derived from a group of sow-fed piglets (n = 3) (SF Reference) that were littermates to the treatment groups

3.5.2 Plasma indispensable amino acids

In plasma, the lysine concentration was altered by the mode and the amount of arginine infused, as IV-H Arg group had significantly higher plasma lysine concentration compared to both IG groups (Figure 3.6). Interestingly, the mean lysine concentration for the IG-H Arg group fell within the SF Reference range, whereas for the IV-H Arg group, lysine was well above the SF Reference range. Plasma histidine was significantly lower in the IG-H Arg group compared to IV-H Arg group; the mean value of histidine in the IG-H Arg and IG-L Arg groups fell below or at the lower margin of the SF Reference range, whereas the mean histidine concentration of the IV-H Arg group was at the highest margin of the SF Reference range. Threonine, a major amino acid in the mucin component of mucus secreted in the intestine, was significantly affected by dietary treatment, as determined by the repeated measures ANOVA ($P = 0.04$), but no difference between groups was determined by the multiple comparisons test. The most remarkable differences were in the aromatic and some branched-chain amino acids. Plasma phenylalanine and tyrosine were significantly lower in both IG groups compared to IV-H Arg group. Altering the route of delivery resulted in more than five-fold and fourteen-fold difference in plasma mean concentrations of phenylalanine and tyrosine concentrations respectively, where the mean concentration of IV-H Arg group was more than seven-fold and three-fold higher than the SF Reference values. For branched-chain amino acids, both isoleucine and valine were significantly lower in IG groups compared to the IV group, and again were more similar to the SF Reference data, with the IV-H Arg group being

extremely high(more than three-fold higher than the SF Reference mean concentrations for both

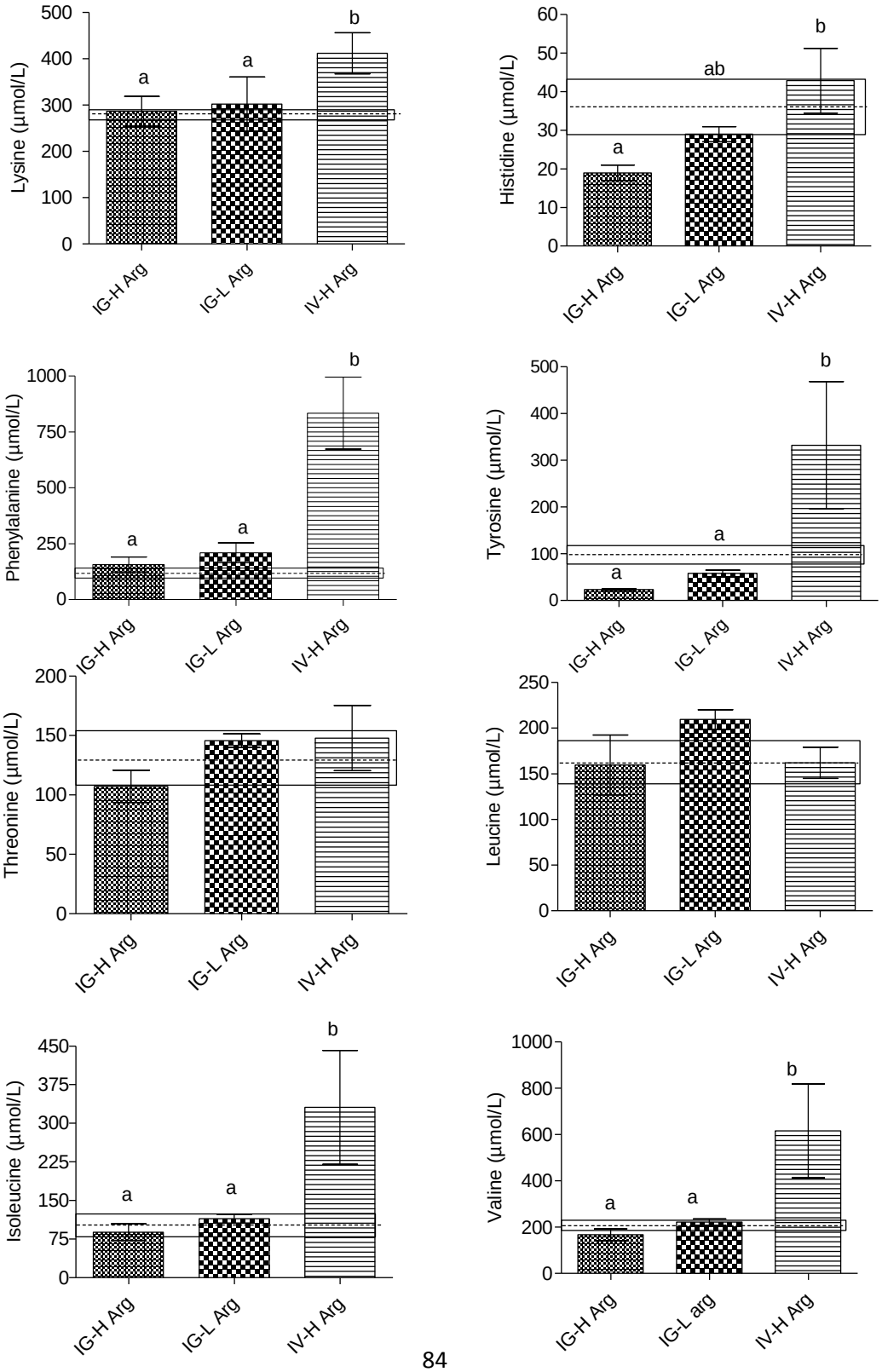
Lysine (µmol/L)
500
400
300
200
100
0
a
a
b
IG-H Arg
IG-L Arg
IV-H Arg
Histidine (µmol/L)
60
50
40
30
20
10
0
a
ab
b
IG-H Arg
IG-L Arg
IV-H Arg
Phenylalanine (µmol/L)
1000
750
500
250
0
a
a
b
IG-H Arg
IG-L Arg
IV-H Arg
Tyrosine (µmol/L)
500
400
300
200
100
0
a
a
b
IG-H Arg
IG-L Arg
IV-H Arg
Threonine (µmol/L)
200
150
100
50
0
IG-H Arg
IG-L Arg
IV-H Arg
Leucine (µmol/L)
250
200
150
100
50
0
IG-H Arg
IG-L Arg
IV-H Arg
Isoleucine (µmol/L)
450
375
300
225
150
75
0
a
a
b
IG-H Arg
IG-L Arg
IV-H Arg
Valine (µmol/L)
1000
800
600
400
200
0
a
a
b
IG-H Arg
IG-L arg
IV-H Arg

Figure 3.6: Plasma indispensable amino acid concentrations. Data are expressed as the mean ± SD using plasma amino acid concentrations from day 4, 5 and 6; n = 6 (IG-H Arg and IG-L Arg), or 5 (IV-H Arg) per group. P values were determined by repeated measures ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Bonferroni's multiple comparisons test. The broken line within the box represents the mean ± 1 SD derived from a group of sow-fed piglets (n =3) (SF Reference) that were littermates to the treatment groups.

of isoleucine and valine) (Figure 3.6). It was interesting to note that plasma leucine concentrations did not differ among groups, despite the large differences in the other branched chain amino acids.

3.6 Plasma ammonia and urea

There was no significant difference in plasma ammonia concentration among treatment groups. All the values were high, including SF Reference (Figure 3.7) (~ 199 uM) when compared to the values obtained for freshly sampled plasma from domestic piglets using the same TPN amino acid profile (Brunton et al, 1999). This is likely related to the prolonged freezer storage of our samples as opposed to assaying freshly sampled plasma (Brunton et al, 1999). Similarly, plasma urea concentrations were not different among treatments (Figure 3.8), but IV-H Arg tended to have higher mean values compared to the SF Reference mean value (9.8 mmol/L).

3.7 Liver weight and protein mass

There were no differences in liver wet weight or protein mass among experimental animals (Table 3.3). Interestingly, mean liver wet weights were more than two standard deviations higher than the SF Reference mean in all three experimental groups. There was a trend towards lower liver protein mass in piglets fed solely by the intravenous route compared to the animals fed only arginine into the gastric catheter ($P = 0.09$).

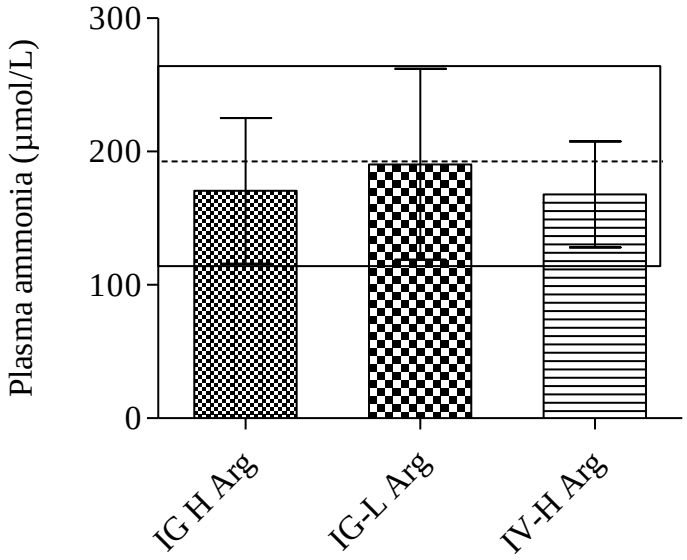

Figure 3.7: Plasma ammonia concentrations. Values are means ± SD; n = 6 (IG-H Arg), or 5 (IG-L Arg and IV-H Arg) per group. P values were determined by one-way ANOVA. The broken line within the box represents the mean ± 1 SD derived from a group of sow-fed piglets (n = 4) (SF Reference) that were littermates to the treatment groups

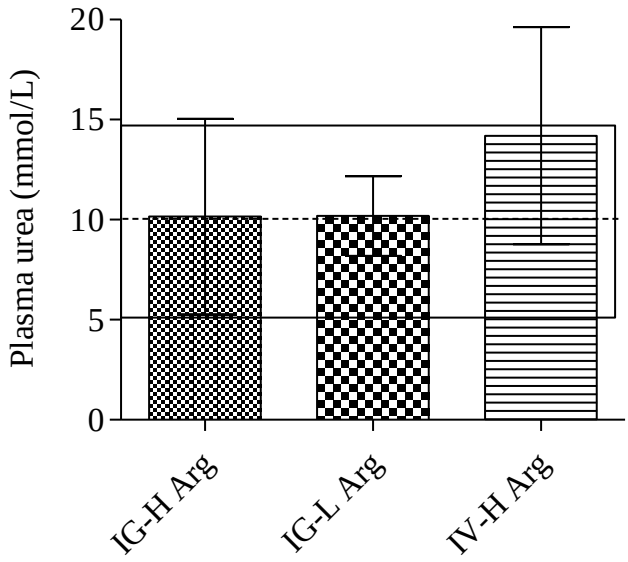

Figure 3.8: Plasma urea concentrations. Values are means ± SD; n = 6 (IG-H Arg), 5 (IG-L Arg and IV-H Arg) per group. P values were determined by one-way ANOVA. The broken line within the box represents the mean ± 1 SD derived from a group of sow-fed piglets (n = 4) (SF Reference) that were littermates to the treatment groups

Table 3.3: Liver wet weight and protein mass

	IG-H Arg	IG-L Arg	IV-H Arg	P value	SF Reference
Liver weight g/kg of BW	44 ± 3.5	45 ± 7.3	41 ± 8	0.6	33 ± 2.5
Liver protein mg/kg of BW	2943 ± 787	3018 ± 944	2069 ± 481	0.09	2378 ± 975

Values are means ± SD; n= 6 (IG-H Arg and IV-H Arg) or 5 (IG-L Arg) per group. P values were determined by one-way ANOVA. SF Reference values were derived from a group of sow-fed piglets (n = 5) that were littermates to the treatment groups. BW – Body weight.

4.0 Discussion

We hypothesised that lack of arginine availability for small-intestinal, first-pass metabolism during TPN feeding may contribute to the etiology of TPN-induced gut atrophy in neonates. The atrophied neonatal small intestine is likely incapable of adequate de novo arginine synthesis due to the lack of substrate and reduced metabolic activity. Thus, we were interested in the effects of infusing a high amount of arginine enterally to accommodate the requirement during such a physiological situation. To that end, we infused a high concentration of arginine either enterally or parenterally, and compared it to low enteral arginine to assess the effects of the arginine concentration and the route of delivery on small intestinal morphology, tissue protein synthesis and SMA blood flow.

4.1 Hepatic protein synthesis in response to enteral arginine

One of the most interesting results of our study was that changing the route of delivery of only one amino acid altered the rate of liver protein synthesis. We demonstrated significantly higher rates of liver protein synthesis in both groups given enteral arginine compared to the IV-H Arg group. The two major sources of amino acids for metabolism in the liver are extracellular from arterial and portal blood supplies and intracellular from protein breakdown and from intracellular metabolism. However, evidence from previous studies supports preferential utilization of extracellular amino acids as precursors for hepatic protein synthesis during the fed state (Berthold et al, 1995; Jahoor et al, 1994; Reeds et al, 1992; Cayol et al, 1996; Lichtenstein et al, 1990; Parhofer et al, 1990). In our study, there were no differences in liver free arginine concentrations between groups that received intragastric versus

intravenous arginine. The liver free arginine that we measured in our study represented arginine extracted from the hepatic artery, portal vein and from hepatic protein breakdown. Therefore, total liver free arginine likely did not contribute to the observed differences in liver protein synthesis in our animals.

In a study by Stoll et al (1997), ^{2}H-phenylalanine was infused via a peripheral vein and ^{13}C-phenylalanine was given via the stomach along with a milk-replacer diet under steady state conditions. They found a greater ratio of $^{13}C/^{2}H$ phenylalanine in portal blood compared to the arterial blood. However, there was no difference between portal blood and plasma VLDL apoB-100 enrichments. Furthermore, the enrichment values for portal blood and apoB-100 were two-fold higher than the enrichments in the hepatic free pool. This indicated that portal phenylalanine was the preferred precursor source for the hepatic synthesis of secretory proteins in the fed state. In a second study by Stoll et al (1998b), amino acid labelling was measured in arterial and portal blood, in hepatic-free and protein-bound pools, and in hepatic secretory proteins. This was accomplished by feeding ^{13}C-labelled algal protein, in which multiple amino acids were intrinsically labelled. The protein was infused intragastrically for 6 h along with a high protein diet. The isotopic enrichment of amino acids in apoB-100 was higher than in the respective hepatic-free amino acid pools. They demonstrated that dietary amino acids are the preferred source for hepatic constitutive and secretory protein synthesis rather than amino acids in the systemic circulation (Stoll et al, 1998b). Thus, Stoll et al (1997; 1998b) clearly showed that the portal vein amino acid pool was the preferred source for hepatic-

origin plasma protein synthesis in the fed state. Unfortunately, we did not collect the plasma from the portal

vein to measure the portal amino acid concentrations; therefore we cannot be certain that the gastrically infused arginine appeared in the portal vein. However, it follows that the intravenous delivery of arginine would result in lower portal concentrations compared to IG delivery, as the only net addition of arginine to portal blood must be from tissue protein breakdown in the complete TPN piglets. Thus, our finding that liver protein synthesis is greater with enterally delivered arginine is consistent with the idea that portal amino acids provide the amino acid precursor pool for hepatic protein synthesis; arterial and liver-origin amino acids seem less important since the same amount of parenteral arginine had a lesser effect on hepatic protein synthesis. It is interesting that only one amino acid could induce such an effect, and seems likely that other mechanisms are involved, which have not yet been identified.

Arginine is a known secretagogue for insulin when delivered intravenously, but whether it affects insulin secretion following oral ingestion in neonates is not known. In adults, oral intake of arginine alone did not affect insulin secretion (Gannon et al, 2002). Therefore, if insulin was involved in stimulating protein synthesis in our model, it is more likely that the IV-H Arg group would have been most affected. Davis et al (2001) demonstrated that amino acids and insulin or insulin alone stimulated peripheral tissue protein synthesis in neonatal piglets, but in the liver, the effect of amino acids was independent of insulin. Two other recent studies with neonatal piglets have demonstrated that amino acids provided into the carotid artery stimulated hepatic protein synthesis and

that this response did not require insulin (Suryawan et al, 2009; O'Connor et al, 2004). Thus, our finding that intragastric arginine infusion induced hepatic protein synthesis was likely not mediated by insulin.

4.2 Superior mesenteric artery blood flow in response to arginine

We hypothesised that the provision of high arginine enterally would ameliorate the reduction of SMA blood flow that is induced by prolonged TPN feeding, via increased synthesis of nitric oxide. In contrast to our hypothesis, SMA blood flow further declined after the initiation of treatments, irrespective of the route of delivery or the amount of arginine provided. The decline in blood flow reached plateau approximately 48 h after the test diets were initiated. From 48 to 72 h of treatment, mean blood flow values were significantly different between all treatment groups. Animals that received the low intragastric arginine demonstrated the greatest decline in blood flow, being ~40% lower than baseline at study end. Provision of the high concentration of arginine into the gastric catheter attenuated some of the reduction in blood flow. However, surprisingly, the smallest reduction in blood flow was observed in piglets given the IV-H Arg treatment. It is well known that fasting and TPN feeding result in the reduction of blood flow to the small intestine. Alternatively, an increase in blood flow is observed immediately after oral feeding in neonatal piglets (Van Goudoever et al, 2001). Fasting mesenteric artery blood flow reached a peak within one hour after feeding in term-born, healthy infants that were either breast-fed or bottle-fed (Coombs et al, 1992; Gladman et al, 1991). In a recent study of TPN-fed neonatal piglets by Puiman et al (2011), they reported almost a

doubling of the SMA blood flow with the initiation of partial enteral-feeding (40% of total intake) compared to piglets maintained on complete intravenous feeding. Moreover, a previous study involving chronic TPN feeding of piglets showed that 4 h following the transition from enteral to TPN feeding, a 50% reduction of SMA blood flow was observed

(Niinikoski et al 2004). This cumulative evidence suggests that contact with luminal digesta or absorbed nutrients by the mucosal cells must trigger this increase in SMA blood flow. Infusing only arginine into the gastric catheter in the present study was not nearly as effective as infusing a complete diet, as the decline in blood flow continued following the initiation of enteral arginine.

Interestingly, arginine availability did play a role in mediating blood flow, as we observed an attenuation of SMA blood flow decline with high compared to low arginine. In our study, we cannot directly link arginine availability to NO synthesis. However, in enterally-fed, arginine-deficient piglets, Urschel et al (2005; 2007) reported significantly lower whole body arginine synthesis and significantly lower nitric oxide synthesis when compared to piglets fed generous arginine. The proportion of arginine flux converted into nitric oxide was not different between groups (13%), even though the whole body arginine flux was significantly lower in piglets fed arginine-deficient diets (Urschel et al, 2005; 2007). These data indicate that the capacity to synthesize nitric oxide is at least partially dependent on precursor availability in neonatal piglets. In our study, the best outcome in terms of blood flow occurred when we delivered arginine intravenously, despite identical intakes in the IG-H Arg and IV-H Arg groups. This finding does not

necessarily indicate that intragastric provision of high arginine was ineffective, or that the degree of intestinal atrophy is solely a consequence of reduced blood flow. It is highly likely that some of the arginine provided intragastrically was extracted by the splanchnic bed, which may have resulted in a lower concentration of precursor available in the superior mesenteric arterial circulation to ultimately synthesize endothelial NO. Small intestinal mucosal growth parameters such as crypt cell proliferation, small intestinal weight and mucosal fractional protein synthesis consistently tended to be higher in the IG-H Arg group compared to the other groups, although not significantly different. Indeed, none of the outcomes (either small intestinal mucosal fractional protein synthesis or morphological outcomes that we measured) were significantly worse in the IG-H or IG-L Arg groups, compared to the IV-H Arg group, despite greater reductions in blood flow. Thus, the utilization of enterally-delivered arginine by the intestinal mucosa was likely occurring in both IG-H and IG-L Arg animals, and perhaps less was getting into the systemic circulation. While not significantly different, plasma arginine in the IG-H Arg group tended to be ~23% lower than the TPN group; plasma arginine was 71% lower in the IG-L Arg group than TPN group, which was significantly different. Thus, it is possible that two things were going on that were in conflict. On one hand, reduced SMA blood flow might have encouraged intestinal atrophy in IG Arg groups, while luminal arginine availability to the intestinal mucosa was stimulating recovery.

Recently, a study was published with a design that was quite similar to our study (Puiman et al, 2011) as both studies assessed the effects of arginine infused intragastrically to piglets with low SMA blood flow induced by TPN feeding. However,

the arginine protocol used was different between the two studies. Surprisingly, arginine infused intragastrically as the sole amino acid for three hours at more than five times the requirement (3.35 g/kg/d) did not induce an increase in SMA blood flow (Puiman et al, 2011). In contrast to the Puiman study, the concentration of arginine given to our IG-H Arg animals was estimated to meet the requirement for

piglets (Brunton et al, 2003), but was only 48% the concentration used in the Puiman study (Puiman et al, 2011). Furthermore, Puiman's animals received the intragastric arginine dose over 3 h, thus a bolus dose, whereas we infused the treatments continuously and recorded blood flow over 3 days. The blood flow response we observed was different than the response measured by Puiman et al (2011) as Puiman did not find a measurable change in SMA blood flow. The differences in the outcomes between the two studies need to be interpreted with caution. If the effect of arginine as a precursor for nitric oxide is systemic (as opposed to local), it may take time to observe a measurable effect at the systemic level, especially if induction of NOS enzymes is necessary**.** Also, there may be a maximal effect; thus providing more arginine during a short period of time may not be beneficial. Therefore, it is possible that it takes much longer than 3 hours to produce a measurable difference in SMA blood flow after initiation of an intragastric infusion of arginine. The fact that Puiman did not see at least some stimulation of SMA blood flow in complete TPN-fed animals with the infusion of large amounts of arginine is likely due to time as a limiting factor.

The change in SMA blood flow that is induced by full enteral feeding may be a systemic effect rather than a local effect. A study by Niinikoski et al, (2004) measured

changes in blood flow in neonatal piglets, following the re-introduction of enteral feeding. They found that the increase in SMA blood flow occurred much earlier than the rise in portal blood flow (1 vs. 4 h). They also assessed the portal blood flow during the transition from enteral to parenteral nutrition, in which the portal blood flow declined to the food-deprived level by 8 h after the transition. SMA blood flow took only 4 h to stabilize to the food-deprived level (Niinikoski et al,

2004). This suggests that the change in SMA blood flow occurs more rapidly than the corresponding change in portal blood flow and thus may be a systemic effect rather than a local, post-intestinal effect. Indeed, that must be the reason why we saw the greatest amelioration of the reduction of SMA blood flow with complete IV feeding of high arginine compared to the IG-H Arg group. If it is a systemic effect rather than a local effect, it may be worthwhile to target arginine availability in the superior mesenteric arterial (SMA) circulation. We did not collect arterial blood; instead we sampled blood from the inferior vena cava just caudal to the heart, which is similar to arterial blood except for the effects of lung metabolism. In piglets, 13% of arginine flux is used for NO synthesis which is rather significant (Urschel 2005; 2007). In our study, the pattern of arginine concentrations in the three diet groups paralleled the blood flow response even though the plasma arginine was not significantly different among all the groups, with the exception of the IG-L Arg and IV-H Arg groups. The plasma arginine concentrations were measured in vena cava blood, which may not accurately represent the concentration in the SMA. However, the data from our study and the results from the Puiman et al (2011) suggest that both systemic availability of arginine for NO synthesis, and the

duration of the continuous infusion of arginine, likely contributed to the maintenance of SMA blood flow during TPN feeding.

The SMA response that we observed may be attributed to the capacity of eNOS and the availability of arginine precursor for NOS activity in our piglets. Castillo et al (1996) determined that 50% of the substrate for NOS came from the plasma compartment, in healthy men. There is evidence of channelling of extracellular (plasma) arginine directly to eNOS by the arginine transporter CAT-1 (Arnal et al,
1995; Norris et al, 1995; Bune et al, 1995). Enzyme kinetics studies have demonstrated that the Km of eNOS for plasma-derived arginine was 73 µm in one study (Granger et al, 1990) and 150 µM in another study (Iyengar et al, 1987). The plasma concentration in our individual IG-L Arg animals ranged between 30 to 45 µM, lower than the estimated Km values of eNOS for extracellular arginine. Therefore, it is possible that plasma arginine availability, but not intracellular arginine levels, affected endothelial NO synthesis capacity in our IG-L Arg piglets, resulting in the lower SMA blood flow compared to the other two experimental groups.

Data from the current study combined with studies by Puiman et al (2011) and Niinikoski et al (2004) suggest that arginine alone could not induce the magnitude of response that is stimulated by full feeding enterally. Thus, other factors may explain the muted response of intragastric arginine on SMA blood flow. TretrahydrobiopterinBH4 is an essential cofactor for the activity of all NOS isoforms and is synthesized in endothelial cells. Arginine can stimulate an increase in BH4 synthesis (Wu & Meininger, 2009); thus, greater arginine in endothelial cells would result in greater availability of BH4. Therefore,

NO production by NOS may be lower due to reduced availability of BH4 in endothelial cells in our IG Arg piglets.

4.3 Plasma and tissue free indispensable amino acids

In the present study, a pattern emerged of higher plasma indispensable amino acids in the group of piglets fed solely by TPN compared to the sow-fed reference group. In contrast, the mean values for the two intragastric groups were generally within the sow-fed range. The amino acid intakes for the IG-H Arg and the IV-H Arg groups were identical; thus, differences in the plasma amino acid pools could only be

the result of the route of feeding of one amino acid. Bertolo et al (2000) reported a number of differences in plasma amino acid concentrations in piglets fed an identical diet intravenously, intragastrically or intraportally. Furthermore, they observed differences in organ amino acid concentrations in the same study, suggesting that the route of feeding alters the plasma and organ amino acid concentrations. We also observed significantly higher liver free phenylalanine and valine concentrations in IV-H Arg piglets compared to the IG groups. The liver free phenylalanine concentration was well outside the sow-fed reference range in IV-H Arg piglets. In total, these findings support the idea that there is either greater protein breakdown or less amino acid utilization, or both occurring in the IV-H Arg piglets. Remarkably, the provision of *only* arginine into the gut partially "normalized" the plasma profile of many indispensable amino acids in our animal model. Thus, an important finding from this study is that enterally-delivered arginine induced a response in plasma amino acids similar to the response induced by complete or partial enteral feeding.

4.4 Small intestinal morphology

In our study, the enteral provision of arginine at 1.6 g/kg/d resulted in greater length of the small intestine, compared to a similar intake provided parenterally. However, none of the other small intestinal morphological characteristics that we measured (small intestinal weight, crypt cell proliferation, and mucosal protein synthesis) reached statistical significance between treatments. Bertolo et al (1999a & b) reported that feeding identical elemental diets via intragastric, intraportal or intravenous routes did not affect small intestinal length in neonatal piglets over an 8 day feeding period, suggesting that small intestinal atrophy is not characterised by

reduced small intestinal length growth. Thus, it was surprising that enteral arginine facilitated greater small intestinal length, and no other morphological outcome in our model. To my knowledge, our study is the first to investigate mucosal growth specifically in response to an enteral arginine infusion in TPN-fed neonatal piglets with gut atrophy. A similar study conducted by Puiman et al (2011) investigated the effects of providing a supra-physiological concentration of arginine with partial enteral (PEN) feeding (20%) in neonatal piglets fed for 3 days. They demonstrated greater proximal small intestinal mucosal mass, protein mass and DNA mass when the enteral infusate was supplemented with 3.34 g/kg/d of arginine, compared to animals given alanine as a control. The fact that PEN stimulates mucosal growth is well recognized from an earlier study by this group (Burrin et al, 2000); 60% of total intake was required to maximise intestinal growth. This time however, they provided only 20% of nutrients enterally along with more than twice the daily arginine requirement (Puiman et al, 2011). Despite a number of differences in

morphological outcomes, they found no significant effect of arginine on mucosal protein synthesis or crypt cell proliferation, similar to our findings. Thus, our results combined with those of Puiman et al (2011), suggest that the enteral arginine has a role in stimulating recovery from TPN-induced intestinal atrophy, but more than 20% of total intake enterally is likely necessary along with high arginine. In our model, the lack of enteral amino acids and other nutrients explains why we did not see significant morphological differences. The design of our model was such that we did not expect to see major morphological differences; rather, it was a design that might allow us to isolate potential effects of arginine from that of other trophic factors.

4.5 Indicators of whole body arginine adequacy: plasma ammonia, urea and arginine concentrations

There were no significant differences in plasma urea or ammonia concentrations between treatment groups. However, the mean urea concentration in the piglets that did not receive enteral arginine was well above the sow-fed reference mean; in contrast, the mean plasma urea concentrations in the IG Arg groups were similar to sow-fed reference mean. It is possible that there was greater catabolism of amino acids ongoing in the IV-H Arg group, because of inefficient utilization for protein synthesis; alternatively, excess amino acids may have accumulated from protein breakdown in the IV-H Arg group. In support of this, we also observed significantly higher concentrations of some of the plasma indispensable amino acids in our IV-H Arg piglets compared to IG-H and IG-L Arg groups. No difference in plasma ammonia concentrations among groups indicated that even with the low intake of 0.6 g arginine/kg/d intragastrically, the urea cycle

requirements were met. In contrast, the IG-L Arg piglets had a mean plasma arginine concentration that was only one-third that of the SF Reference piglets, clearly indicating that 0.6 g/kg/d of arginine delivered enterally did not fulfill the total body arginine requirement. Plasma urea measured in our sow-fed 20 d old Yucatan miniature pigs was 9.8 ± 5mM (mean ± SD). Another recent study in our lab determined that the plasma urea concentration was much lower, at 5.3 ± 0.9 mM (mean ± SD) in 17 - 19 d old TPN fed neonatal piglets (Brunton et al, unpublished data). The high urea values in the current study may be because the plasma samples underwent freeze-thaw cycles during storage. Therefore the absolute values are suspicious.

4.6 Liver morphology: liver weight and protein mass

A noteworthy observation was the higher liver weights in all our experimental animals, compared to the SF Reference group, suggesting hepatic steatosis which is common in TPN feeding (Wang et al, 2006). There was no difference in the total liver protein content, despite significantly higher liver protein synthesis in the IG-H Arg compared to IV-H Arg piglets; this may be due in part to the secretion of newly synthesized hepatic proteins from liver. It is also possible that there were differences in liver composition, with greater lipid deposition in the IV-H Arg. However, we did not analyse liver water or lipid content in these animals.

4.7 Growth and the composition of growth

We measured the growth rate by means of weight gain per day and skeletal muscle protein synthesis. We did not see differences in growth rate related to arginine

intake, or route of infusion. Similar to our findings, most neonatal piglet studies have found that route of diet delivery did not alter the absolute weight gain during the period of study (Bertolo et al, 1999a& b; Burrin et al, 2000), with the exception of a recent study by Stoll et al (2010), that reported significantly higher daily body weight in enterally-fed piglets, over a 17 d feeding trial. According to Stoll et al (2010), TPN-feeding resulted in greater fat mass and lesser lean and bone mass compared to enterally fed piglets. Therefore, it is important to consider that absolute growth rates are difficult to interpret during short-term feeding trials.

In the present study, neither intragastric nor intravenous arginine stimulated greater skeletal muscle protein synthesis. The skeletal muscle fractional protein synthesis rates for all the experimental groups were within the range of the sow-reared reference group, but interestingly, the overall weight gain per day was below that of the sow-reared piglets. Brunton et al (2003) reported that skeletal muscle protein synthesis increased with increasing provision of arginine in intravenously-fed piglets. We hypothesized that muscle protein synthesis would be higher in our IG-H Arg and IV-H Arg fed animals compared to IG-L Arg piglets, because the intake by the IG-L Arg was estimated to be below the estimated whole body requirement. However, we did not see such a difference in the present study.

4.8 Discussion of the model

We infused only arginine (dissolved in HPLC water) into the gastric catheter in two groups of piglets, to isolate the specific effects of arginine on intestinal atrophy. When deciding on the model, we were cognizant of the fact that providing just arginine

alone would not maximize a response. The lack of many other essential dietary factors those are normally available in full or partial enteral feeding limited the potential trophic effect of intragastric arginine. This may be the reason that we did not see significant differences for some of the outcomes, but observed many "trends" suggesting an effect of enteral arginine.

5.0 Conclusions

The objective of our study was to determine whether arginine, delivered as a single nutrient into the small intestine, would act as a trophic factor and attenuate some of the features of small intestinal atrophy that accompany TPN feeding. We were the first to demonstrate that the delivery of arginine alone into a gastric catheter, irrespective of the amount provided, stimulated hepatic protein synthesis. By contrast, the provision of arginine enterally was not as effective as the parenteral delivery of

arginine to ameliorate the reduction of SMA blood flow, thus suggesting the importance of adequate arginine at the systemic level. Despite a greater sustained blood flow in the IV-H Arg piglets, none of the other small intestinal outcomes were significantly enhanced. Therefore, the greater SMA blood flow induced by high intravenous arginine did not translate into significant growth-promoting effects in the small intestine. In fact, provision of arginine into the gut must have provided an advantage since the IG Arg piglets sustained small intestinal morphology that was comparable to the IV-H Arg piglets, despite lower SMA blood flow. Finally, and perhaps the most interesting outcome, was the fact that the enteral delivery of only arginine along with TPN resulted

in a plasma amino acid profile in which a number of indispensable amino acids were "normalized" relative to sow-fed piglets, indicating a profound metabolic effect.

We are currently working to replace minimum enteral feeding (60% of TPN) in the clinical setting with a single or few amino acids which might have potential trophic effects on gut atrophy. In neonatal piglets, we have shown that enteral arginine alone has partial trophic effect on the amelioration of gut atrophy induced by prolonged TPN feeding. It is possible that some other amino acids with or without arginine might also have trophic effects on gut atrophy, thus warranting further studies. Since the early introduction of minimal enteral nutrition is not always possible, it is important to identify therapies that could replace challenging minimal enteral nutrition in clinical settings.

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Appendix I. Plasma amino acid concentrations.

	IG-H Arg	IG-L Arg	IV-H Arg	P-value	SF Reference*
Arginine family of Amino acid (µmol/L)					
Arginine	103 ± 34[ab]	38 ± 7[a]	133 ± 37[b]	0.03	94 – 124 (109)
Aspartate	14 ± 5	17 ± 6	15 ± 5	0.7	11 – 19 (15)
Citrulline	46 ± 7	46 ± 5	44 ± 5	0.8	57 – 89 (73)
Glutamine	104 ± 24	132 ± 5	128 ± 26	0.2	202 – 240 (221)
Glutamate	98 ± 1	104 ± 8	82 ± 26	0.4	50 – 94 (72)
Ornithine	102 ± 10	57 ± 15	116 ± 32	0.06	54 – 80 (67)
Proline	284 ± 36	418 ± 50	372 ± 23	0.04	271 – 349 (310)
Plasma Indispensable amino acid (µmol/L)					
Histidine	19 ± 2[a]	28 ± 2	**43 ± 8[b]**	0.02	29 – 42 (36)
Isoleucine	88 ± 16[a]	114 ± 9[a]	**331 ± 110[b]**	0.02	76 – 122 (99)
Leucine	160 ± 33	209 ± 11	162 ± 17	0.09	137 – 183 (160)
Lysine	287 ± 32[a]	**302 ± 58[a]**	**412 ± 44[b]**	0.02	274 – 288 (281)
Methionine	13 ± 2	14 ± 1	20 ± 3	0.09	13 – 23 (18)
Phenylalanine	157 ± 33[a]	210 ± 44[a]	**834 ± 161[b]**	0.02	88 – 130 (109)
Threonine	107 ± 14	146 ± 6	148 ± 28	0.04	107 – 153 (130)
Tryptophan	97 ± 23	97 ± 14	94 ± 9	0.9	54 – 74 (64)
Valine	168 ± 25[a]	222 ± 14[a]	**616 ± 203[b]**	0.02	184 – 222 (203)
Dispensable amino acid (µmol/L)					
Alanine	237 ± 46	331 ± 27	257 ± 53	0.1	269 – 403 (336)
Asparagine	14 ± 5	17 ± 6	15 ± 5	0.7	9 – 19 (14)
Glycine	360 ± 33[a]	796 ± 79[b]	372 ± 28	<0.01	439 – 609 (524)
Hydroxyproline	47 ± 16	40 ± 2	72 ± 40	0.3	53 – 73 (63)
Serine	141 ± 12[a]	730 ± 90[b]	166 ± 22[a]	<0.01	148 – 212 (180)
Taurine	97 ± 8[a]	143 ± 13[b]	132 ± 24[ab]	0.08	75 – 155 (115)
Tyrosine	23 ± 2[a]	58 ± 7[a]	**332 ± 136[b]**	0.02	78 – 116 (97)

Values are expressed as the mean ± SD using plasma amino acid concentrations from day 4, 5 & 6; n = 6 (IG-H Arg and IG-L Arg) or 5 (IV-H Arg) per group. P values were determined by repeated measures ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Bonferroni's multiple comparison tests. *SF Reference values (n = 3) presented as a range of mean ± SD, the group mean is presented in the bracket.

Appendix II. Small intestinal mucosa free amino acid concentrations.

	IG-H Arg	IG-L Arg	IV-H Arg	P value	SF Reference *
Arginine family of amino acid (µmol/g of tissue)					
Arginine	2.21 ± 2.42	1.45 ± 1.59	0.23 ± 0.06	0.15	0.21 – 0.29 (0.25)
Aspartate	0.92 ± 0.29	0.81 ± 0.15	0.89 ± 0.28	0.87	0.72 – 1.10 (0.91)
Citrulline	0.80 ± 0.33	0.67 ± 0.32	0.76 ± 0.26	0.9	0.40 – 1.02 (0.71)
Glutamine	0.24 ± 0.09	0.28 ± 0.09	0.25 ± 0.10	0.77	0.19 – 0.61 (0.40)
Glutamate	2.45 ± 0.68	1.90 ± 0.29	2.04 ± 0.26	0.19	1.84 – 2.50 (2.17)
Ornithine	0.37 ± 0.31	0.26 ± 0.20	0.17 ± 0.03	0.3	0.15 – 0.23 (0.19)
Proline	0.63 ± 0.28	0.67 ± 0.21	0.74 ± 0.12	0.8	0.47 – 0.99 (0.73)
Indispensable amino acid (µmol/ g of tissue)					
Histidine	0.09 ± 0.02[a]	0.20 ± 0.1[b]	0.14 ± 0.06[ab]	0.04	0.09 – 0.15 (0.12)
Isoleucine	0.26 ± 0.05	0.27 ± 0.07	0.53 ± 0.41	0.09	0.14 – 0.34 (0.24)
Leucine	0.37 ± 0.15	0.50 ± 0.27	0.32 ±.0.11	0.25	0.26 – 0.44 (0.35)
Lysine	0.62 ± 0.27	0.53 ± 0.20	0.59 ± 0.15	0.35	0.32 – 0.56 (0.44)
Methionine	0.80 ± 0.25	0.67 ± 0.26	0.86 ± 0.18	0.7	0.50 – 1.14 (0.82)
Phenylalanine	1.74 ± 0.22[a]	1.67 ± 0.29[a]	2.54 ± 0.73[b]	0.01	1.45 – 1.91 (1.68)
Threonine	0.32 ±.0.11	0.34 ± 0.13	0.38 ± 0.05	0.6	0.22 – 0.40 (0.31)
Tryptophan	0.07 ± 0.02	0.09 ± 0.05	0.1 ± 0.03	0.3	0.03 – 0.07 (0.05)
Valine	0.47 ± 0.16	0.60 ± 0.28	1.0 ± 0.9	0.1	0.36 – 0.58 (0.47)
Dispensable amino acid (µmol/g of tissue)					
Alanine	1.32 ± 0.44	1.71 ± 0.8	1.37 ± 0.19	0.35	1.17 – 2.35 (1.76)
Glycine	2.91 ± 0.95	3.36 ± 0.47	2.54 ± 0.51	0.18	2.85 – 3.83 (3.34)
Hydroxyproline	0.10 ± 0.06	0.09 ± 0.04	0.08 ± 0.03	0.9	0.01 – 0.15 (0.08)
Serine	0.54 ± 0.22[a]	1.33 ± 0.23[b]	0.51 ± 0.07[a]	<0.0001	0.40 – 0.90 (0.65)
Taurine	4.44 ± 0.93	4.01 ± 0.34	4.58 ± 0.69	0.4	2.8 – 3.6 (3.20)
Tyrosine	0.24 ± 0.08	0.28 ± 0.1	0.86 ± 0.72	0.05	0.24 – 0.32 (0.28)

Values are means ± SD; n = 5 (IG-L Arg) or 6 per group. P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Bonferroni's multiple comparison test. *SF Reference values presented as a range of mean ± SD, the group mean is presented in the bracket.

Appendix III. Liver free amino acid concentrations.

	IG-H Arg	IG-L Arg	IV- H Arg	P value	SF Reference *
Arginine family of amino acids (µmol/g of tissue)					
Arginine	0.13 ± 0.04^{ab}	0.09 ± 0.04^{a}	0.17 ± 0.02^{b}	0.004	0.06 – 0.38(0.22)
Aspartate	1.02 ± 0.28	0.77 ± 0.11	1.02 ± 0.24	0.16	0.73 – 1.37(1.05)
Citrulline	0.24 ± 0.12	0.28 ± 0.18	0.28 ± 0.12	0.8	0.20 – 0.50(0.35)
Glutamine	1.33 ± 0.52	1.03 ± 0.28	1.45 ± 0.41	0.27	1.66 – 2.18(1.92)
Glutamate	1.93 ± 0.30	1.71 ± 0.39	1.66 ± 0.27	0.33	1.43 – 2.59(2.01)
Ornithine	0.41 ± 0.16	0.42 ± 0.15	0.83 ± 0.47	0.06	0.35 – 0.59(0.47)
Proline	0.83 ± 0.25^{a}	1.11 ± 0.33^{a}	2.20 ± 0.96^{b}	0.005	0.76 – 1.04(0.90)
Indispensable amino acids (µmol/g of tissue)					
Histidine	0.20 ± 0.07	0.24 ± 0.06	0.27 ± 0.11	0.4	0.22 – 0.52(0.37)
Isoleucine	0.24 ± 0.05	0.24 ± 0.05	0.52 ± 0.44	0.14	0.22 – 0.50(0.36)
Leucine	0.38 ± 0.13	0.35 ± 0.06	0.26 ± 0.22	0.4	0.36 – 0.74(0.55)
Lysine	0.50 ± 0.35	0.27 ± 0.23	0.67 ± 0.47	0.2	0.01 – 0.05(0.03)
Methionine	0.14 ± 0.05	0.13 ± 0.61	0.19 ± 0.16	0.5	0.07 – 0.43(0.25)
Phenylalanin	1.78 ± 0.39^{a}	1.64 ± 0.25^{a}	2.77 ± 0.91^{b}	0.02	1.31 – 1.81(1.56)
Threonine	0.28 ± 0.08	0.23 ± 0.02	0.30 ± 0.09	0.22	0.27 – 0.47(0.37)
Tryptophan	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.03	0.6	0.04 – 0.10(0.07)
Valine	0.38 ± 0.1	0.35 ± 0.07	0.95 ± 0.88	0.05	0.35 - 1.60(1.0)
Dispensable amino acids (µmol/g of tissue)					
Alanine	1.30 ± 0.27	1.48 ± 0.25	1.59 ± 0.97	0.7	0.99 – 2.27(1.63)
Glycine	3.62 ± 0.51^{b}	5.3 ± 1.21^{a}	3.12 ± 0.52^{b}	0.001	2.86 – 4.42(3.64)
Hydroxyproli	0.16 ± 0.03	0.12 ± 0.01	0.13 ± 0.06	0.3	0.17 – 0.27(0.22)
Serine	1.93 ± 0.22^{b}	4.8 ± 2.17^{a}	2.01 ± 0.33^{b}	0.002	1.41 – 2.05(1.73)
Taurine	6.28 ± 1.25	5.43 ± 1.44	4.02 ± 1.75	0.06	6.95 – 9.45(8.20)
Tyrosine	0.34 ± 0.13	0.34 ± 0.13	0.55 ± 0.27	0.08	0.30 – 0.70(0.50)

Values are means ± SD; n = 5 (IG-L Arg) or 6 per group. P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Bonferroni's multiple comparison test. *SF Reference values presented as a range of mean ± SD, the group mean is presented in the bracket.

Appendix IV. Kidney free amino acid concentrations.

	IG-H Arg	IG-L Arg	IV-H Arg	P value	SF Reference *
Arginine family of amino acids (µmol/g of tissue)					
Arginine	0.09 ± 0.04	0.09 ± 0.04	0.16 ± 0.10	0.31	0.01 – 0.26 (0.14)
Aspartate	1.00 ± 0.15	0.97 ± 0.24	0.98 ± 0.12	0.8	0.95 – 1.31 (1.33)
Citrulline	0.34 ± 0.10	0.36 ± 0.03	0.40 ± 0.06	0.50	0.25 - 0.45 (0.35)
Glutamine	0.40 ± 0.14	0.50 ± 0.10	0.30 ± 0.04	0.07	0.58 - 0.88 (0.73)
Glutamate	2.20 ± 0.12	2.50 ± 0.44	2.30 ± 0.40	0.70	2.40 - 3.00 (270)
Ornithine	0.33 ± 0.05	0.30 ± 0.08	0.50 ± 0.11	0.045	0.17 - 0.49 (0.33)
Proline	0.60 ± 0.16[a]	0.70 ± 0.02[ab]	0.90 ± 0.05[b]	0.01	0.60 – 1.20 (0.90)
Indispensable amino acids (µmol/g of tissue)					
Histidine	0.16 ± 0.02	0.18 ± 0.04	0.21 ± 0.06	0.3	0.16 - 0.32 (0.24)
Isoleucine	0.44 ± 0.14	0.32 ± 0.04	0.43 ± 0.21	0.56	0.35 - 0.51 (0.43)
Leucine	0.47 ± 0.09	0.48 ± 0.06	0.42 ± 0.14	0.74	0.36 - 0.6 (0.48)
Lysine	0.44 ± 0.15	0.37 ± 0.05	0.60 ± 0.30	0.28	0.20 - 0.60 (0.40)
Methionine	0.21 ± 0.03	0.21 ± 0.03	0.21 ± 0.03	0.97	0.17 - 0.29 (0.23)
Phenylalanine	1.73 ± 0.33	1.72 ± 0.29	2.65 ± 0.86	0.09	1.65 - 2.05 (1.85)
Threonine	0.36 ± 0.08	0.38 ± 0.06	0.41 ± 0.06	0.63	0.34 - 0.36 (0.35)
Tryptophan	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.03	0.98	0.06 - 0.08 (0.07)
Valine	0.52 ± 0.04	0.56 ± 0.09	0.63 ± 0.22	0.58	0.54 - 0.72 (0.63)
Dispensable amino acids (µmol/g of tissue)					
Alanine	1.23 ± 0.28	1.61 ± 0.32	1.37 ± 0.06	0.19	1.40 - 2.20 (1.80)
Glycine	3.23 ± 0.76	4.23 ± 0.96	3.37 ± 0.83	0.31	4.55 - 4.95 (4.75)
Hydroxyproline	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.77	0.08 - 0.1 (0.09)
Serine	0.72 ± 0.14[a]	1.70 ± 0.57[b]	0.68 ± 0.06[a]	0.005	0.52 - 0.84 (0.68)
Taurine	6.6 ± 0.73	5.9 ± 1.3	5.3 ± 1.9	0.50	3.60 - 5.40 (4.50)
Tyrosine	0.25 ± 0.1	0.3 ± 0.06	0.99 ± 1.22	0.29	0.24 - 0.44 (0.34)

Values are means ± SD; n = 3 (IG-L Arg) or 4 per group. P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Bonferroni's multiple comparison test. *SF Reference values presented as a range of mean ± SD, the group mean is presented in the bracket.