

**Evaluating a Premature Piglet Model to Assess the Nutritional Needs of the Human Neonate**

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## **ABSTRACT**

Research suggests that the premature neonate is unable to make use of necessary enteral nutrition due to an immature gastro-intestinal tract. Therefore, a more in-depth understanding of total parenteral nutrition is required. Currently, the effects of specific nutrients on the development of the small intestine of the premature infant remain largely unknown. In response to this lack of knowledge, the main objective of this project was to evaluate the effects of increased arachidonic (AA) and docosahexaenoic (DHA) acid supplementation in total parenteral nutrition (TPN) on the pre-term gastro-intestinal tract. The hypothesis was that AA and DHA supplementation would lead to an increase in mucosal surface area of the small intestine supporting supplementation of AA and DHA as vital components of pre-term infant formulas for proper development of digestive function, which is essential for the wellbeing of the newborn. Piglets were collected by caesarean section at 106 d of gestation and equipped with an arterial umbilical catheter for TPN delivery, as well as maintained in a heat and humidity controlled incubator for 6 d. Piglets were fed one of three diets: a control diet, a low DHA: AA diet (0.3 and 0.69% of total fatty acids as DHA and AA, respectively) and a high DHA: AA diet (6 and 14% of total fatty acids as DHA and AA, respectively). In the first trial, premature piglets displayed symptoms of feed intolerance and failed to survive, while in the second trial only premature piglets receiving control TPN reached equivalent full-term age. In response, piglets were removed by cesarean section at 114 d of gestation and administered the same dietary regimens as premature pigs. Three piglets were viable for tissue collection. Histological analysis of the jejunum and ileum indicated a marked difference in the intestinal morphology of the piglet receiving the high AA/DHA TPN formula, however, it cannot be concluded that this is due to a treatment effect.

## **INTRODUCTION**

The average gestation in humans is 40 wk, however, approximately 14%<sup>1</sup> of all pregnancies result in pre-term birth. The majority of preterm infants have not developed sufficiently in-utero to survive without intensive care and one of the most critical aspects in their post-natal development is the nutrition they receive. However, the pre-term infant, deprived of the development it would have experienced within the last several weeks in utero, is born with an immature gastro-intestinal tract which is sensitive to nutritional strategies<sup>2</sup>.

In contrast to full term infants, where enteral nutrition promotes intestinal growth and maturation, premature infants may be unable to utilize nutrients delivered via the gastro-intestinal tract and may develop necrotizing enterocolitis (NEC) when fed enterally<sup>8</sup>. Necrotizing enterocolitis is one of the most common emergencies associated with nutritional management of neonates and can lead to a plethora of debilitating problems and ultimately death.<sup>3</sup> Instead, many preterm infants are fed via total parenteral nutrition (TPN), which administers nutrients intravenously. Although the use of TPN is a widely accepted practice for nutritional management of the premature neonate, several potential problems exist. Because the intestinal tract is by-passed during TPN and is not being directly stimulated by nutrient delivery, atrophy of the gastrointestinal tract can result. As a direct result of this atrophy, the infant's ability to digest food and absorb nutrients may be seriously affected, which further compromises the premature infant.<sup>4</sup> For infants who are prematurely removed from the maternal environment that provides essential nutrients which aid in the development of the fetal GI tract before birth, it is necessary to create the right balance of nutrition in order to stimulate post-natal gastro-intestinal growth and development. As a result, the infant can eventually be transitioned to enteral nutrition and avoid dangerous conditions such as NEC.

Current research indicates that certain polyunsaturated fatty acids, or PUFAs, may have beneficial effects on intestinal growth and repair<sup>8</sup>. In adults, PUFAs are provided by either dietary fat or synthesized de novo. Although most can be synthesized naturally, linoleic and  $\alpha$ -linolenic fatty acids must be obtained from the diet and are collectively known as essential fatty acids (EFA). Linoleic and  $\alpha$ -linolenic acids serve as precursors

in the synthesis of other PUFA, such as arachidonic (AA) and docosahexaenoic acid (DHA). During the last trimester of pregnancy, these PUFA are delivered across the placenta and accumulate rapidly in the brain.<sup>5</sup> Evidence suggests that infants cannot synthesize these fatty acids at rates required for optimal growth and development<sup>6</sup>, necessitating the supplementation of these fatty acids in the infant's diet. When infants are subjected to a decreased amount of these two PUFA, there are implications on the health and proper development of the newborn.<sup>5</sup>

Although these two fatty acids work together to promote proper growth of the neonate, arachidonic and docosahexaenoic acid each have individual roles. While DHA accumulates in greatest concentrations in the developing brain and is suggested to play a role in neural function<sup>7</sup>, AA is especially important in the formation of eicosanoids, which are compounds that include prostaglandins, leukotrienes and thromboxanes.<sup>8</sup> Prostaglandins and the other eicosanoids have hormone-like properties and may be important for intestinal development. Studies suggest the prostaglandins derived from arachidonic acid, play a role in proper membrane and cellular development in the small intestine<sup>13</sup>. They work specifically by stimulating mucous secretion, which in turn helps to protect the sensitive epithelial lining of the digestive tract.<sup>9</sup> Current fatty acid research indicates that a diet rich in long chain PUFA promotes marked recovery and growth in intestinal mucosa. For example, reduced intestinal surface area and absorptive abilities, as a consequence of malnourishment, are improved in piglets fed a formula containing increased amounts of long chain PUFA.<sup>10</sup> While results of this study imply that the long chain PUFA used in the diet had a beneficial effect on the recovery of the GI tract, the benefits of individual long-chain PUFA remain unknown as a mixture of fatty acids was

included in the diet. Furthermore, the impact of these fatty acids on gastrointestinal maturity in the premature neonate, which may also suffer from reduced surface area and absorptive abilities, is not known.

Currently, the effects of specific nutrients, including PUFA, on the development of the small intestine of the premature infant remain largely unknown. As research suggests that the premature neonate is unable to make use of necessary enteral nutrition due to an immature gastro-intestinal tract, a more in-depth understanding of total parenteral nutrition is required, however, the lack of a suitable animal model limits advances in this area. In response to this lack of knowledge, the main objective of this project was to evaluate the effects of increased AA and DHA supplementation in TPN on the gastro-intestinal tract in a premature piglet model. The hypothesis was that AA and DHA supplementation would lead to an increase in mucosal surface area of the small intestine supporting supplementation of AA and DHA as vital components of pre-term infant formulas for proper development of digestive function, which is essential for the wellbeing of the newborn. The comparable level of maturity at birth between the piglet and human neonate and anatomical similarities between the two make the piglet a useful model for this study<sup>11</sup>.

## **MATERIALS AND METHODS**

**Animals:** Pregnant sows were sedated using ketamine (11-15 mg/kg) and maintained under general anesthesia (isoflurane) for delivery of piglets via cesarean section. Sows were positioned in right lateral recumbency with the left rear limb elevated and retracted caudally. While under isoflurane gas anesthesia, an incision was made in the paralumbar

fossa and piglets were removed manually through an incision in one of the uterine horns. Immediately following delivery, the umbilicus of piglets was affixed with an umbilical clamp, air passages cleared, and piglets were stimulated to commence respiration. After towel drying, piglets were placed into an infant incubator (Air Shields 100C), which maintained a temperature of 35 C and 100% humidity, with extra oxygen to ensure arterial blood oxygen saturation.

Premature piglets were delivered for the first two studies (106 d of gestation, n=8/study). In addition a third study was conducted with full term piglets (113 d of gestation, n=16). Piglets were catheterized according to Odle et. al., (1992). Briefly, piglets were placed in a lateral recumbency, the umbilical area cleaned serially three times with betadine, and following ligation with a cotton thread to prevent bleeding, the umbilicus was cut to a length of approximately one inch. To locally anesthetize the umbilical area, lidocaine was injected into multiple sites of the umbilical stump. The dorsal aorta was dilated and a 3.5- F polyvinyl catheter affixed with a three-way stop cock was inserted 18 cm for premature or 22 cm for full-term piglets. The catheter was flushed with heparanized saline and subsequently sutured to the cord stump and the skin. Triple antibiotic ointment was applied to the area and the catheter was further secured using elastic tape around the piglet's midline. Piglets were returned to the incubator and monitored to ensure proper recovery from anesthesia. Premature piglets were maintained in the incubators for the length of the study (6d) to achieve thermoneutrality (35 C) and additional oxygen supply. Full term piglets were moved to individual cages and maintained at 30 C. To reduce the risk of infection, gentamicin (2.0 mg/kg) and penicillin (5000 U/kg) were administered through IV once daily for the first three days.

For studies two and three, maternal blood was collected via cardiac puncture while the sow was under general anesthesia. Serum was aseptically prepared and 5 mL was infused through the umbilical catheter at 6, 12, and 24 h after birth to provide maternal antibodies to the immunodeficient piglets according to Sangild and colleagues<sup>4</sup>.

**Dietary Treatments:** Total parenteral nutrition was formulated according to Wykes and colleagues<sup>12</sup> for full-term pigs and modified according to Sangild and colleagues<sup>4</sup> for premature piglets. Piglets were fed either a control TPN diet with no supplemental AA: DHA, a low diet, which consisted of an AA: DHA ratio of 0.69 to 0.3%, or a high diet, in which the AA: DHA ratio was 14.0 to 16.0% (Table 1). Diets consisted of a mixture of commercial products (Hospira Lakeforest, IL), in addition, AA and DHA lipid emulsions were developed and supplemented with the commercial products. The AA emulsion was derived from a spray dried algal source (ABN; Columbia, MD), and DHA was utilized in an oil form (donated by Martek; Winchester, KY). Lipid emulsions were formulated using 20% lipid, 1.2% egg phosphatide, and 2.5% glycerol. Diets were prepared aseptically in a fume hood and used within 24 h of preparation. For premature piglets, TPN was delivered via volumetric infusion pumps at a rate of 90 mL/kg body weight per day during the first 24 h of infusion and gradually increased to a maximal rate of 180 mL/kg body weight per day by d 3 postnatal to promote moderate growth. For full term piglets, TPN was infused initially at a rate of 112 mL/kg body weight and subsequently increased to 225 mL/kg.

Table 1: TPN Diet Formulations

Ingredient, %	Control (-AA/-DHA)	Low AA/DHA	High AA/ DHA
AA Emulsion <sup>1</sup>	0	0.1	2
DHA Emulsion <sup>1</sup>	0	0.2	5.5
Liposyn II, 20%	15.5	15.2	8
Aminosyn 8.5% <sup>2</sup>	53	53	53
Dextrose <sup>2</sup>	14.4	14.4	14.4
MVI-Pediatric	0.5	0.5	0.5
MTE-4 <sup>3</sup>	0.01	0.01	0.01
Ca gluconate	0.075	0.075	0.075

<sup>1</sup> Emulsion were made using 20% lipid, 1.2 % egg phosphatide, and 2.5 % glycerol

<sup>2</sup> Aminosyn 8.5% and glycerol were increased to 62.5 % and 20%, respectively for full-term pigs

<sup>3</sup> Additional Na<sub>2</sub>SeO<sub>3</sub> and KIO<sub>3</sub> were added to meet nutrient requirements of pigs

**Tissue Collection:** Piglets were euthanized by IV injection of sodium pentobarbital and tissues collected. Following euthanasia, piglets were situated dorsally and an incision was made in the thoracic cavity in order to remove the length of the small intestine. The small intestine was excised by securing a hemostat at the pyloric sphincter to mark the beginning of the small intestine. The intestine was then followed while removing it from surrounding viscera until the ileo-cecal junction was reached. A second hemostat was used to clamp this junction, and the length of the small intestine was removed and looped six times in order to determine the general location of the three segments: duodenum, jejunum and ileum<sup>13</sup> (Fig 1).



Figure 1

Two one inch jejunal segments were removed from the midpoint of the fourth loop, rinsed with saline, and placed in Prefer<sup>®</sup> fixative (Anatech; Battle Creek, MI). Ileal samples were removed from the midpoint of the sixth loop and placed in fixed. Samples remained in fixative for 24 hours, and were subsequently dehydrated using increasing concentrations of ethanol according to protocol modified by Ottobre<sup>16</sup>.

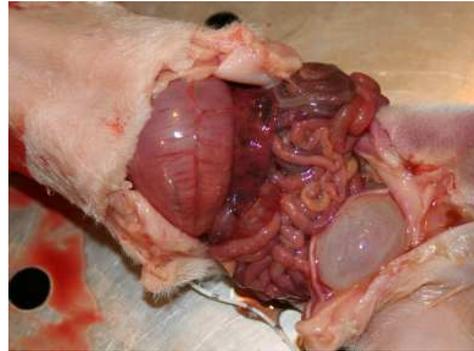
Intestinal samples were then embedded using Paraplast<sup>®</sup> paraffin (Kendall; Mansfield, MA). Embedded intestinal samples were sliced transversely at 7  $\mu\text{m}$  in thickness using a microtome. Eight total slides were made for each piglet, which included two slides from each jejunal and ileal section. Slides were then stained using Hematoxylin and Eosin technique<sup>14</sup>.

**Intestinal Morphology:** A villus height: crypt depth ratio measurement was averaged for each individual piglet jejunal and ileal sample. Measurements were taken using a micrometer with an average of five measurements per slide prepared for each intestinal sample. Crypt depth and villus height measurements were done according to Nunez, et al<sup>15</sup>. Measurements of the villus height were taken by situating the micrometer at the villus-crypt junction and measuring to the tip of the villi. This measurement was repeated five times on different villi throughout the slide. Crypt depth measurements were taken starting at the nadir of the crypt to the villus crypt junction, and were also repeated five times on different crypts throughout the slide. Proper care was taken to only measure intact villi and crypts in order to ensure the most representative measurement.

## RESULTS

In the first trial, observation of edema within 2 d of premature birth and subsequent blood chemistries (elevated blood glucose and plasma urea nitrogen) suggested pigs were unable to tolerate initial rates of TPN infusion, which resulted in mortality. In response to the increased mortality during the first trial, nutrient and fluid intakes were gradually increased from the initial rate of 80 mL/kg, to 120 mL/kg for d 2

and then a maximal rate of 180 mL/kg by d 3 to promote moderate growth. Optimization of the nutritional regimen corresponded to reduced mortality rates, however, five of the initial eight pigs exhibited signs of infection and subsequently died. Surviving piglets were transitioned to an enteral diet, but showed clinical signs of necrotizing enterocolitis (NEC) upon introduction of the diet and were euthanized. In response to the low success rate of the premature piglets, full term piglets were administered the formulated TPN diet for the third study.



**Figure 2**

Unexpectedly, clinical symptoms of NEC including distention and discoloration of the lower abdomen were observed (Fig 2). Pigs were subsequently euthanized and samples from viable piglets were collected to examine the condition of the small intestine.

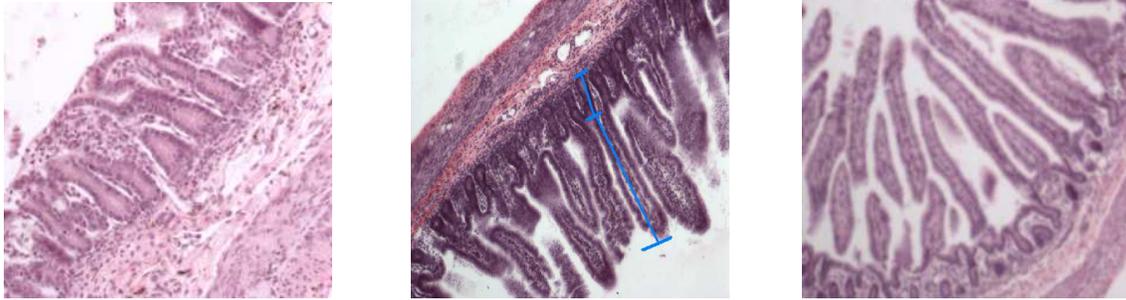
The pig (n=1) receiving the TPN formulated to contain high concentrations of AA and DHA (14 and 6%, respectively) that showed clinical signs of NEC had a decreased villus: crypt ratio (Table 2) compared to control pigs (n=2).

**Table 2. Mean Ratio ( $\pm$  SE) of villus height to crypt depth.**

Treatment	Pig	Jejunum	Ileum
High	1	5.17 $\pm$ 0.66	2.32 $\pm$ 0.17
Control	6	6.35 $\pm$ 0.84	7.00 $\pm$ 1.10
Control	8	5.64 $\pm$ 0.54	3.85 $\pm$ 0.14

In addition, the villi appeared attenuated (Fig 3a) which suggested reduced absorptive capacity. Assessment of the ileal and jejunal samples indicated increased crypt depth, and possibly attempted repair of the necrotizing intestine. Intestinal sections from the control piglets suggest an increased villus height: crypt depth ratio in

comparison to the piglet receiving the high AA:DHA diet, which is characteristic of a increased absorptive ability (Fig 3b and 3c).



**Figure 3: a.**

**b.**

**c.**

## **CONCLUSION**

Because of their striking similarities to human neonates as well as their availability for study piglets are an ideal model for the human infant, however, limited studies have utilized the piglet as a model to assess the role of AA and DHA in postnatal development of the gastrointestinal tract . As research continues to highlight the importance of these PUFA for the neonate<sup>5</sup>, a more in-depth understanding of total parenteral nutrition and its lipid components were the basis for this study.

In order to study the effects of these PUFA and their role in the development of the pre-term infant's GI tract, AA and DHA were supplemented to mimic concentrations supplied by breast milk or reported to be delivered to the fetus while in utero during the last third of gestation. Results of the initial study showed a decreased survival rate. In accordance with research conducted by Sangild and colleagues<sup>4</sup> increased plasma urea-nitrogen levels of premature piglets were indicative of a feed intolerance as a result of initial infusion rates of the TPN diets. In response, the infusion protocol was adjusted so that diets were infused at a reduced rate during the second trial. A reduced rate of initial infusion corresponded to an increase in survivability, however, optimal growth and

development were not achieved and 60% of the piglets died from unknown causes. Surviving piglets were transitioned to an enteral diet but were euthanized due to declining health and symptoms of infection. .

As a result of the complications encountered with utilizing pre-term piglets, full term piglets were used to evaluate the TPN diets. The full term piglets were subjected to the same protocol as the prior studies, including the experimental diets. Full term pigs were able to tolerate maximal rates of infusion (225 ml/kg body weight) by d 2 following initial rates of 112 mL/kg body weight during the first 24 h of life. A loss of eight of the full term piglets occurred within 24 h of removal from the sow due to infusion pump malfunction. Progressive loss of five pigs which displayed symptoms (lethargy, discoloration of lower limbs) similar to the premature pigs, occurred over the course of the 6 d trial. Surviving piglets were subsequently euthanized at the end of the trial due to clinical observations of NEC.

Histological measurements made from ileal and jejunal samples collected from full term pigs following clinical observation of NEC indicated a marked difference in the intestinal morphology of the pig receiving the high AA:DHA formula. However, it cannot be concluded that this was due to treatment effect as only one pig was available for measurement. A difference in the crypt depth; villus height ratio was observed, and villi in the high diet pig appeared attenuated.

The symptoms of lethargy, abdominal distension, gastrointestinal lesions and hemorrhage, as well as histological analysis of the intestine noted in the current study are in agreement with the onset of clinical NEC reported by Sangild and colleagues,<sup>16</sup>. Future research, however, will be necessary in order to understand the underlying causes

of morbidity associated with the current study. A greater understanding of the factors which influence survivability of pigs receiving TPN will be necessary to establish the pig model for assessing AA and DHA in postnatal development.

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