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A MULTI-INGREDIENT DIETARY SUPPLEMENT TO IMPROVE GROWTH AND DEVELOPMENT OF SMALL FOR GESTATIONAL AGE INFANTS: CONCEPT-TESTING USING NEWBORN PIGS

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by

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ABSTRACT

Approximately 10% of all neonates are born small for gestational age (SGA) and current feeding approaches increase adiposity and the risk of developing metabolic disorders, such as obesity and diabetes. This study determined if supplementing formula with a combination of leucine, medium chain triglyceride oil, and krill oil would promote gain of lean body mass using newborn SGA term pigs as a model for SGA infants. At birth, SGA pigs have relatively smaller livers and spleens (g/kg) than appropriate for gestational age (AGA) pigs and have higher serum values for alanine aminotransferase, creatine kinase, and lactate dehydrogenase. Newborn SGA pigs were randomly assigned to experimental and placebo groups and fed formula with and without the supplement for 20 days. Placebo pigs tended to have greater body weight ($p<0.0734$) due to significantly higher fat mass ($p<0.02$) and percent fat ($p<0.05$), and larger livers. The present findings validate the concept an experimental supplement can improve body composition of SGA term pigs and may decrease the risk of SGA neonates developing obesity and metabolic abnormalities.

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INTRODUCTION

Background

Infants that are ≥35 weeks of gestation and <10th percentile on the Fenton Growth Chart, excluding constitutional smallness are defined as small for gestational age (SGA). Approximately 10% of all births are affected by SGA(Tudehope, Vento, Bhutta, & Pachi, 2013). Intrauterine growth retardation (IUGR) is a clinical definition that applies to neonates born with characteristics of malnutrition and in-utero growth retardation, irrespective of their birth weight percentile (Sharma, Shastri, & Sharma, 2016). Therefore, SGA and IUGR are not synonymous. Being born SGA does not necessarily mean IUGR has occurred and infants who are IUGR are not necessarily SGA at birth (Iyengar et al., 2016).

Postnatal development of SGA infants is associated with higher risk of metabolic and physiological abnormalities and growth retardation, leading to short stature and obesity in adulthood, and increased risk of cardiovascular disease (CVD), and type two diabetes mellitus (T2DM) (McIntire, Bloom, Casey, & Leveno, 1999). Due to impaired growth of the brain stem and cerebellum, neurobehavioral and neurocognitive difficulties are common among SGA infants. In later life, this can lead to speech retardation, slower sensory development, and delayed motor development (Lundgren & Tuvemo, 2008; Sanz-Cortes, Egana-Ugrinovic, Zupan, Figueras, & Gratacos, 2014; Sommerfelt et al., 2000).

There have been many attempts to correct impaired growth of SGA infants by ensuring adequate nutrient intake. However, the compromised digestive functions of SGA infants complicate establishing a balance between providing a dietary intervention that promotes catch-up growth without overfeeding and causing intolerance. The most common approach is

providing SGA infants with nutrient-enriched formulas with high-protein and/or high energy to improve growth rate and nitrogen retention (Premji, Fenton, & Sauve, 2006)**.**

The higher protein intake can potentially accelerate weight gain and nitrogen accretion in formula-fed hospitalized infants, indicating enhanced postnatal growth. However, the high protein intake does pose risks. The other widely used strategy of increasing energy intake tends to increase weight gain as fat accretion (Fenton, Premji, Al-Wassia, & Sauve, 2014). Considering the special needs of SGA infants, nutritional interventions during the neonatal stage should focus on promoting postnatal growth in lean body mass without causing potential metabolic problems and the excessive accumulation of fat that increases the risk of childhood and adult obesity (Fenton et al., 2014). Therefore, it is essential to provide appropriate amounts of protein and energy in formulas fed to SGA infants, instead of simply providing more protein or energy.

In conclusion, there is a large and increasing population of SGA infants that are at risk of intellectual deficits and abnormal metabolic and physiological development later in life. Improving nutrition support of SGA infants has the potential to promote growth and development that will improve outcomes and lifelong health and well-being.

Consequences of being born SGA

a) Postnatal patterns of growth

During first two years of postnatal life, SGA infants experience catch–up or sometimes referred to compensatory growth. This specific growth type is exhibited by low birth weight premature and term SGA infants and is characterized by dramatically increased weight gain during the first 24 months of age. The rapid growth involves compensatory metabolic mechanisms that promote fast weight gain, and results in SGA infants achieving 50-75 growth and weight percentiles by the age of two years (Hwang, 2014). There are two types of catch-up

growth depending on the outcome: complete and incomplete. Incomplete catch-up growth predisposes residual height deficits in childhood and adulthood. Complete catch-up growth refers to an absence of growth deficits in adulthood. SGA infants are more likely to undergo incomplete catch-up growth that leads to permanent short stature (Cho & Suh, 2016; Karlberg & Albertsson-Wikland, 1995; Lee, Chernausek, Hokken-Koelega, & Czernichow, 2003; Leger, Limoni, Collin, & Czernichow, 1998; Leger, Noel, Limal, & Czernichow, 1996; Wit & Boersma, 2002).

There are a variety of factors influencing the intensity of catch-up growth such as hypersecretion of growth hormone (GH) (Leger et al., 1996) , initial birthweight, birth length relative to gestational age, genetic factors relating to parental height and weight (Wit & Boersma, 2002) , and nutrition. The inability of many SGA infants to achieve complete catch-up growth and have permanent short stature has been attributed to the diminished sensitivity of somatotrophic cells and resistance to pituitary GH associated with immaturity of the GH receptor (Lee et al., 2003; Leger et al., 1998).

There are two major hypotheses that have been proposed to explain the mechanism of catch-up growth:

b) Neuroendocrine hypothesis

Despite a lack of experimental evidence (Leger et al., 1998; Leger et al., 1996; Wit & Boersma, 2002), some have speculated the existence of a central mediator – possibly located in hypothalamus – for sensing actual body size and comparing it to the expected size for the current age. When the expected body size appropriate for a specific age is reached, the intensity of catch-up growth will decrease.

c) Growth plate hypothesis

The alternative theory is that age is not the limiting factor for catch-up growth, but that growth is limited by the intrinsic growth capacity of a tissue itself (Lee et al., 2003; Leger et al., 1998; Wit & Boersma, 2002) . A modification of this theory is that catch-up growth involves a delay in normal growth plate senescence. Specifically, during normal growth plate senescence, the proliferative rate of the growth plate chondrocytes diminishes with each successive stem cell cycle. Thus, growth plate senescence is not a function of time per se but rather a function of the cumulative number of divisions the stem cells have undergone (de Wit, Sas, Wit, & Cutfield, 2013).

There is a strong correlation between catch-up growth during the neonatal period and precocious puberty and metabolic syndrome later in life (Emons, Boersma, Baron, & Wit, 2005; Lui, Nilsson, & Baron, 2011) . SGA infants tend to experience early onset of puberty compared to appropriate for gestational age (AGA) infants.The precocious puberty leads to earlier fusion of the growth plates and a shorter adult height (Li et al., 2016; R. Verkauskiene, Petraitiene, & Albertsson Wikland, 2013). The pathophysiological mechanism underlying the short stature of SGA is also attributed to advanced bone maturation concomitant with lower bone density and earlier epiphyseal plate closing (Li et al., 2016; R Verkauskiene et al., 2007)

Catch-up growth of internal organs of SGA infants

a) Kidneys and renal function

Catch-up growth is a very sensitive period of neonatal and infant development and it affects not just linear growth, but influences internal organs as well; including the negative impact it has on development of the kidneys. Fetal kidney development continues from nine to thirty-six weeks of pregnancy and after term birth, no new nephrons are formed. IUGR is commonly associated with reduced kidney size and nephron number in early childhood (Schmidt

et al., 2005) and SGA infants also have lower renal mass (Hoy, Hughson, Bertram, Douglas-Denton, & Amann, 2005). These findings emphasize the vulnerability of the kidneys to IUGR and being SGA (Hoy, Rees, Kile, Mathews, & Wang, 1999; Schmidt et al., 2005).

Nowadays, ultrasound visualization of kidney volume is the gold standard for evaluation of renal size and development. Usually, overall kidney volume can be used to estimate kidney mass and is proportional to glomerular number (Lackland, Bendall, Osmond, Egan, & Barker, 2000). A small kidney on ultrasound may imply low nephron mass, but increased kidney size cannot be used to distinguish between normal growth and hypertrophy (Iyengar et al., 2016) .

Considering the impaired kidney growth in SGA infants, there is a need to focus on the critical issue of nephron protection at birth, emphasizing the importance of regular monitoring of glomerular filtration, not just reliance on ultrasound, to detect and prevent possible disturbances in renal function.

b) Catch-up growth of brain structure and neurodevelopment in SGA infants

Head circumference is considered a reliable measure of estimated brain weight and brain development during the first two years of life. Head circumference catch-up growth in SGA neonates is usually completed by the age of two years old (Geelhoed et al., 2009). However, exposure of the fetal brain to adverse factors, such as IUGR during a critical period of development can have lifelong consequences. There is increasing evidence of brain reorganization not just in severely low birth weight infants, but also in SGA infants close to term (Brandt, Sticker, & Lentze, 2003). Differences in brain metabolism, microstructure and cortical sulcation pattern have been reported in term SGA infants (Sanz-Cortes et al., 2014), including significantly larger pontine and medullar measurements compared to AGA infants. Moreover,

fetal MRI texture analysis shows cerebellar differences in SGA infants (Sanz-Cortes et al., 2013).

The cerebellum plays a crucial role not just in motor development, but also learning, memory, cognition, and behavior (Baillieux, De Smet, Paquier, De Deyn, & Mariën, 2008). The brain structural differences in SGA infants coincide with neurobehavioral differences, such as delayed neurodevelopment and lower intellectual performance. The cognitive impairment can be largely attributed to a higher incidence of neurologic abnormalities in the SGA neonates (Cruz-Martinez et al., 2009).

There are many factors contributing to intellectual status of infants such as socioeconomic status, as well as behavioral characteristics of inattention and anxiety. It has also been shown that there is a correlation between the severity of IUGR, head circumference and academic achievements. Infants with signs of placental hypoperfusion and IUGR are at greater risk of abnormal neurodevelopmental outcomes throughout life: from early infancy to the adulthood (McCarton, Wallace, Divon, & Vaughan, 1996).

Children who do not achieve full head circumference catch-up growth have lower levels of intellectual performance and show lower intelligence quotient (IQ) levels (Hack et al., 1991; O'Keeffe, O'Callaghan, Williams, Najman, & Bor, 2003). At the age of eight years, infants with smaller head circumference and lower birthweight are more likely to have lower IQ scores and even signs of neurological abnormalities. There are also differences in academic achievement and professional attainment compared with adults who were born with AGA weight (Hack et al., 1991; Larroque, Bertrais, Czernichow, & Léger, 2001; Lundgren, Cnattingius, Jonsson, & Tuvemo, 2001).Low birth weight also increases risk for the development schizophrenia (Rifkin, Lewis, Jones, Toone, & Murray, 1994; Wahlbeck, Forsén, Osmond, Barker, & Eriksson, 2001).

Metabolic concerns contributing to SGA morbidity

Changes in the growth hormone/insulin-like growth factor (GH/IGF) axis have been reported in children that were SGA infants. Notably, mean serum levels of IGF1 and IGF-binding protein-3 of neonatal SGA infants are about one standard deviation lower than those of AGA neonates (Ong, Ahmed, Emmett, Preece, & Dunger, 2000).

Concomitant insulin resistance has been reported in SGA infants and is considered to predispose for metabolic disorders such as obesity, cardiovascular disease CVD, and T2DM (Hofman et al., 1997; Soto et al., 2003).

SGA infants show signs of insulin resistance in early infancy and throughout childhood, depending on body weight and body length. There is a direct correlation between anthropometric measurements in SGA infants and insulin sensitivity and secretion: infants with lower body mass and lower body length during infancy show more signs of insulin resistance (Barker et al., 1993).

There are several theories for the development of insulin resistance in SGA infants. One of the first, "Small baby syndrome," suggests that undernutrition and unfavorable environmental factors (maternal smoking, maternal undernutrition during pregnancy) program for future metabolic abnormalities in later life (Neel, 1962).

Another theory called the Thrifty Genotype Theory was proposed in 1962 and states that genes promoting survival and growth of the fetus in an unfavorable prenatal environment also promote the development of insulin resistance in a favorable postnatal environment (Prentice, Rayco-Solon, & Moore, 2005; Vu-Hong et al., 2006). This theory suggests that under the influence of intrauterine malnutrition, SGA infants allocate the limited energy and nutrients to vital organs at the expense of somatic growth and less important organs, such as pancreas. This

leads to reduced β-cell mass and as a result, to insulin resistance and metabolic disorders. Although not supported by further research, more than changes in patterns of gene expression lead to insulin resistance and metabolic abnormalities (Cianfarani, Germani, & Branca, 1999).

Despite numerous studies of the relationship between insulin resistance and being SGA, the reasons and mechanisms leading to insulin resistance remain unclear. Regardless, the early onset of insulin resistance in SGA infants can be a potential marker for early identification and intervention to reduce the risk of developing metabolic syndromes in adulthood.

Adiposity that develops in early childhood and adulthood of former SGA infants is another keystone of insulin resistance and metabolic syndrome (Jaquet et al., 2005). Being small for gestational age is associated with less lean body mass and a higher risk of sarcopenia (Ylihärsilä et al., 2007). The ratio of lean body mass relative to fat mass is initially altered in SGA infants that suffered IUGR. SGA infants display a peculiar growth pattern of adiposity. Although severely reduced at birth (Biosca et al., 2011; Enzi, Zanardo, Caretta, Inelmen, & Rubaltelli, 1981), adiposity dramatically increases during the catch-up growth period, as evidenced by the noticeably increased body mass index (BMI) during infancy (Ezzahir et al., 2005; Jaquet, Leger, Tabone, Czernichow, & Levy-Marchal, 1999). The higher BMI persists throughout childhood into adulthood, increasing vulnerability for developing metabolic disturbances (Ibáñez, Lopez-Bermejo, Diaz, & de Zegher, 2011). In the early neonatal period, SGA infants are more prone to accumulate fat mass, specifically abdominal fat. This results in a greater central fat distribution compared to AGA infants (Fenton et al., 2014; Jaquet et al., 1999) and lowers the ratio for lean relative to fat mass, and more so when nutrient support does not meet the specific dietary needs of SGA infants [54].

Not only the extent, but also the dynamic changes during childhood in adiposity that occur in individuals who were born SGA may play a crucial role in determining the long-term metabolic outcomes (Fenton et al., 2014). After finishing catch-up growth period, SGA infants not only are characterized by having a higher percentage of fat body mass compared to AGA infants, but also have elevated levels of insulin, leptin, and significantly lower levels of adiponectin and exhibit dyslipidemia (Hediger et al., 1998).

Overview of possible nutritional interventions

There have been many attempts to correct impaired growth of SGA infants and provide adequate nutrient intake. Due to compromised digestive functions, it is very hard for the physician to choose a dietary intervention that establishes a balance between normal nutrient intake and overfeeding. The difficulty in providing adequate nutritional support for SGA newborns is one of the main reasons for postnatal growth deficits.

The most common approach of nutritional support for SGA infants is feeding high-protein/high energy formulas to improve the growth rate and nitrogen retention (20% energy-enriched and 40% to 60% more protein and minerals than term formulas). The goal is to increase body weight and length and head circumference growth during the first 18 months of age (Fenton et al., 2014). However, it has been stated that the major effect of higher energy intake (142 kcal/d vs. 120 kcal/day) in LBW infants was increased fat accretion. Studies using animal models show that protein/calorie malnutrition affects the utilization and deposition of protein and fat (Feillet-Coudray et al., 2009).

High nutrient intake in IUGR pigs causes abnormal immune functions, via lower serum concentrations of cytokines such as tumor necrosis factor and interleukin (IL) - 1β (Pollock, 1989). Furthermore, the aggressive nutritional strategy promotes excessive oxidative stress

(Décordé et al., 2009; Guoyao & Morris, 1998; Pollock, 1989; Sanz-Cortés et al., 2013), which potentially overwhelms the immature antioxidant system of SGA infants.

Another widely used approach for improving the health of SGA infants is selective enrichment of infant formula with L- arginine, L-carnitine, branched-chain amino acids, glutamine, polyunsaturated fatty acids and nucleotides. The intention is to provide nutrients that will enhance growth and maturation without excessive protein and energy intake.

a) L-arginine supplementation

Arginine is a precursor not just for protein synthesis, but also of nitric oxide, urea, polyamines, proline, glutamate, creatine and agmatine (Shah & Shah, 2007). Lower levels of these metabolites when arginine intake is limited are considered to be contributing factors in the pathogenesis of necrotizing enterocolitis (NEC). Correspondingly, supplementation with L-arginine during the neonatal period lowers the risks of necrotizing enterocolitis and improves protein synthesis (El-Shimi et al., 2015; Mitchell et al., 2014; Shah & Shah, 2007).

b) Glutamine – enriched formula

Glutamine (Gln) plays important roles in energy metabolism, maintains synthesis of arginine, nucleotides, is involved with cell signaling, and is as a "conditionally essential" amino acid. Gln supplementation (0.3 g/kg/day) of formulas can increase the growth rate of LBW infants during first 4 months of life (Korkmaz, Yurdakök, Yigit, & Tekinalp, 2007). Moreover, it increases enteral feeding tolerance in low birth weight infants and decreases duration of hospital stay in neonatal intensive care units (NICU) (Kao et al., 2016; Neu et al., 1997; van den Berg, van Elburg, Westerbeek, Twisk, & Fetter, 2005). However, Gln is unstable in solution, which limits clinical use.

c) Leucine supplementation

Recently, there has been increased interest in evaluating leucine and associated metabolites as possible adjuncts to nutritional therapies to increase muscle protein synthesis and growth. One such metabolite of leucine, β-hydroxy-β-methylbutyrate (HMB), enhances muscle growth and improves muscle strength (Columbus, Fiorotto, & Davis, 2015; Torrazza et al., 2010). Leucine itself increases anabolic rates through the mammalian target of rapamycin (mTOR) pathway. This signaling pathway stimulates protein synthesis independent of activation of the insulin signaling pathway. Extensive animal studies demonstrate beneficial effects of leucine supplementation on metabolism (Columbus, Steinhoff-Wagner, et al., 2015; Gutbrod, Wolke, Soehne, Ohrt, & Riegel, 2000; Manjarín et al., 2016; Torrazza et al., 2010).

d) Supplementation with long chain polyunsaturated fatty acids

The major long chain polyunsaturated fatty acids (LC-PUFA), like arachidonic acid (20:4n-6, ARA), eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), are essential nutrients for maintaining health, cognition, and development during fetal and early postnatal life (Meldrum & Simmer, 2016). Therefore, dietary LC-PUFA supplementation can be considered as an efficient strategy to recover the altered physiologic composition of fatty acids of IUGR neonates (Birch et al., 2010; Birch et al., 2007). Corresponding with this, DHA supplementation of infant formula at 0.32 % of total fatty acids improves visual acuity. However higher amounts of supplemental DHA do not show further improvement of visual acuity or any other health-related benefits (Innis, 2007). Although there is no doubt that DHA is critical in the developing brain, the question of whether dietary DHA is important during early neonatal human brain development remains unresolved (Gieling, Schuurman, Nordquist, & van der Staay, 2011; Jasani, Simmer, Patole, & Rao, 2017).

The pig as a model for improving nutrition support of small for gestational age infants

Intervention studies with SGA human infants are extremely difficult due to obvious ethical considerations. Furthermore, confounding factors of genetics, postnatal care, and health status complicate interpretations of responses to nutrition support. In light of this, various animal models have been used to understand the consequences of being SGA and to evaluate possible interventions. Although rodent models are commonly used, it is difficult to translate findings to SGA infants. Exemplary are the substantial differences in organ development and morphology between rodents and humans.

In this regard, the domestic pig may be an excellent model (Aigner et al., 2010). Vital organs for humans and pigs share similar anatomy, physiology, and patterns of development. This includes the lungs, kidneys, heart, liver, and gastrointestinal tract. Like humans, the major brain growth spurt in pigs extends from the late prenatal to the postnatal period. Gross anatomical features, including gyral pattern and distribution of gray and white matter of the neonatal piglet brain are similar to that of human infants (Dam, Juhl, Sangild, & Svendsen, 2017). Moreover, the size of neonatal pigs allows for neuroimaging using equipment designed for human infants. Indeed, structural magnetic resonance imaging (MRI), functional MRI, and positron emission tomography have all been conducted in pigs (Rigo et al., 1998). Finally, due to their precocial nature, newborn pigs can be fed formula from birth, raised with relative ease, and used in behavioral testing paradigms to assess learning and memory at an early age (Picaud et al., 1999). Importantly, pigs born SGA have anomalies in organ size and maturation that mimic those of SGA infants. Therefore, the neonatal pig is a highly tractable translational

animal model that can be used to investigate SGA-associated disorders and potential interventions.

Conclusion

Before providing nutritional interventions for SGA infants, neonatologists and pediatricians must consider the risks associated with metabolic abnormalities, neurodevelopment, and prevention of long-term consequences. Summarizing the existing knowledge, we can conclude that due to metabolic abnormalities of SGA neonates, nutrition strategies that are energy intensive or with high protein might not be optimal. There is a need to consider the appropriate amount of protein and energy that are required by SGA infants to support growth and development, instead of simply providing high protein or high energy supplements.

MATERIALS AND METODS

Research objectives

Previous studies indicate that formulas supplemented with fatty acids and protein dramatically increase weight gain. Additionally, SGA infants are prone to fatty acid deficiency, specifically long chain omega-3 polyunsaturated fatty acids. Together, these findings led to the concept of a multi-ingredient supplement for SGA infants. Krill oil was selected as a source of essential polyunsaturated fatty acids (DHA and EPA) because of the increased level of phospholipid rather than triglyceride. Medium- chain triglyceride (MCT) oil was chosen as a ready source of energy to reduce catabolism of protein and amino acids, and the branched chain amino acid leucine was included to activate the mTOR pathway and stimulate muscle protein synthesis. The expectation is adding this supplement to existing infant formula or to expressed

breast milk will improve growth by increasing lean body mass and promote organ development of term SGA neonates.

Based on the existing knowledge, I tested the hypothesis that newborn term SGA pigs fed for 20 days a formula (pig milk replacer) supplemented with krill oil, MCT oil, and leucine will realize better growth as greater lean body mass and accelerated organ maturation compared with siblings fed a placebo formula.

Aims

Aim 1: Determine whether supplementation improves growth and body composition

Aim 2: Determine if SGA pigs fed formula with the supplement experience accelerated organ growth.

Study design

This study included four groups that are illustrated in figure 1. The first phase of the study included two groups of 6 AGA pigs and 6 SGA pigs. These groups were examined to determine initial differences between SGA and AGA pig within 12 h after birth at term. The next phase involved obtaining newborn SGA pigs that were distributed to treatment and placebo groups. All of the pigs originated from the same commercial producer, were of the same genetic lineage, and were considered as specific pathogen free.

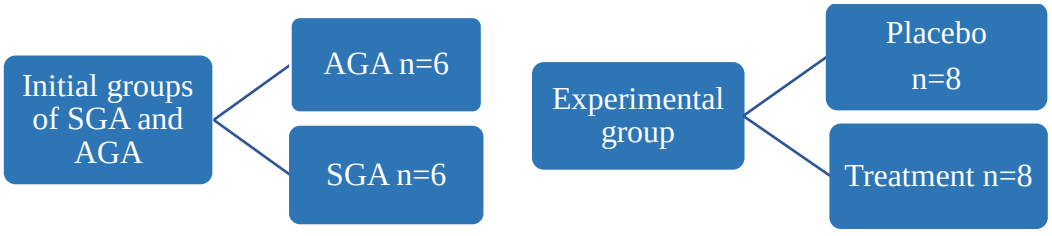

Figure 1. Study design

Animal care and housing

All phases of the research involving animal use were performed in accordance with the 8th edition of the Guide for the care and use of Laboratory Animals and have been approved by The University of Memphis Institutional Animal Care and Use Committee (IACUC).

The feeding trial used a total of 16 healthy newborn term SGA pigs of both sexes that were obtained at <24 h of age with body weights of 600 to 1200 g. Based on average birth weights, ~10% of newborn pigs are <1200 g and can be considered as SGA and therefore relevant as models for SGA human infants. The newborn pigs were allowed to suckle the sow after delivery until they were brought to University of Memphis (within 24 h after delivery) to allow for acquisition of passive immunity. The pigs were randomly assigned to placebo and treatment groups, and housed in pens that are located in facilities compatible with newborn pigs and which we have used previously for such studies. The pens are 0.62 m x 1.2 m, have a slatted floor to facilitate cleaning, and have a suspended infrared heating lamp.

All the pigs were fed a commercial milk replacer that meets the known energy and nutrient requirements of suckling pigs (Table 1). This milk replacer does not induce necrotizing enterocolitis and promotes good growth and health of newborn term pigs. After the pigs were trained to drink from bottles with nipples they were be allowed to drink from a bottle feeder, designed specifically for raising suckling pigs. The volume of formula voluntarily consumed by each group was recorded. At each feeding, pigs were observed for activity and responsiveness, general health status, and the cage was cleaned. The pigs were weighed twice each day: 12:00 and 18:00. The dietary intervention period was 20 days.

Table 1. Composition of milk replacer

Guaranteed analysis		
Crude protein	Minimum	30.0 %
Crude fat	Minimum	26.0 %
Calcium	Minimum	0.8 %
	Maximum	1.3 %
Phosphorus	Minimum	0.9 %
Selenium	Minimum	1 PPM
Vitamin A	Minimum	12.500 IU/LB
Other: Dried whey, Lactose, Dicalcium Phosphate, Potassium Chloride, Lecithin, Mineral oil, Magnesium Sulfate, Ferrous Sulfate, Zinc Proteinate, Selenium Yeast, Vitamin E Supplement, Ascorbic acid, Zinc Sulfate, L- Carnitine, Copper Proteinate, Biotin, Vitamin A Acetate, Niacineamide, d-Calcium Pantonthenate, Vitamin D3 Supplement, Manganese Sulfate, Vitamin B12 Supplement, Thiamine, Calcium Iodate, Folic acid		

Dietary supplement composition

The treatment supplement added to the milk replacer contained functional nutrients, that were hypothesized would promote growth, primarily as lean body mass and encourage growth and functional development of vital organs. With the supplement added, each liter of the formula contained 3.3 ml of krill oil (7.7 g/100 g DHA, 13.2 g/100 g EPA) 10 g of medium chain triglyceride (MCT) oil (Caprylic acid 73.72%, Capric acid 26.16%, water 0.0048%, free fatty acid 0.0066, other 0.1086 %), and 10 g of the amino acid leucine (76 mM). A preliminary study using initial dosages of MCT and vegetable oil at 20 ml/L of prepared formula resulted in the pigs developing steatorrhea, which was considered as a sign of lipid intolerance. The dosage of MCT and vegetable oils was reduced to 10 ml/L of prepared formula, the symptoms of intolerance were suppressed. The krill oil remained at 3.3 ml/L.

The placebo supplement was isonitrogenous by combining the same amounts of vegetable oil blend (Caprylic acid 1.4%, Lauric acid 8.9%, Palmitic acid 20.8%, Stearic acid 3.7%, Vaccenic acid 37.1%, γ-Linolenic acid 1.5%, else 0.1 %) and alanine (6.8 g/L; 76 mM) (Figure 2), (Table 2).

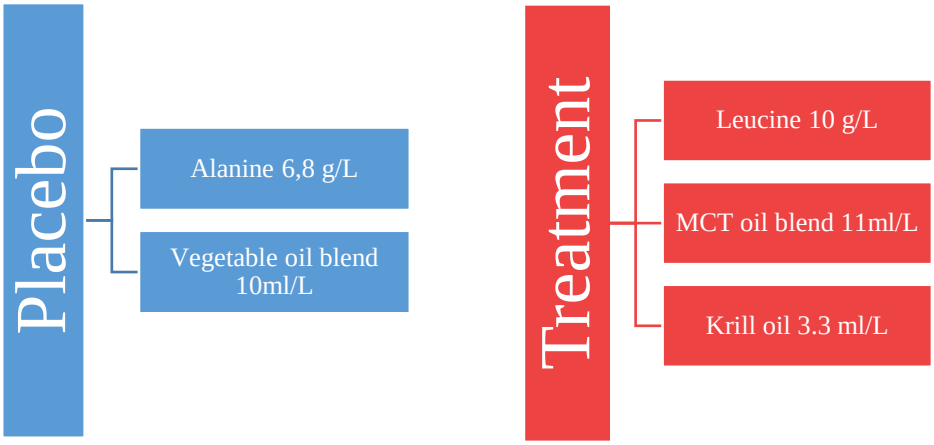

Figure 2. Composition of experimental diets. Placebo and treatment diets

Table 2. Composition of krill oil as reported by supplier

ω -3 (usually < 30g/100g)	
Eicosapentanoic acid (EPA)	13.2
Docosahexanoic acid (DHA)	7.7
α - Linolenic acid	1.2
Stearidonic acid	3
ω -6	
γ-Linoleic acid	0.2
Arachidonic	0.3
ω -9	
Oleic acid	10.7
Other	
Palmitic acid	13.5
Palmitoleic acid	2.5
Stearic acid	0.6

Sampling

Blood samples were collected prior to the necropsy on day 21 of study and distributed to tubes containing either ethylenediaminetetraacetic acid (EDTA) for isolation of plasma or in serum separator tubes. Plasma and serum samples were aliquoted into 1.5 ml microtubes and stored at -80°C until analysis.

Necropsy protocol

At conclusion of the feeding trial the pigs were sedated with telazol 0.2 ml/kg, imaged using dual x-ray absorptiometry (DEXA) to assess the percentage of lean and fat body mass, then anesthetized using isoflurane for intracardiac collection of blood before euthanasia (Euthasol; 1 ml per 4 kg)

After death, each pig was necropsied and internal organs were removed, observed, weighed, and representative tissues fixed using 10% neutral buffered formaldehyde.

Serum and plasma were collected for general metabolic parameters such as: albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), calcium, cholesterol, creatine kinase (CK), creatinine, glucose, lactate dehydrogenase (LDH), triglycerides. The measurements were made using an Alfa Wasserman Vet Axcel blood chemistry analyzer (Diagnostic Technologies, LLC)

Outcome measures

Dual energy x-ray absorptiometry (DEXA)

DEXA provides reliable measurements of bone density, fat and lean body mass, and has been used to evaluate body composition of neonatal pigs and human (Picaud et al., 1999; Picaud, Rigo, Nyamugabo, Milet, & Senterre, 1996). Hologic discovery Dual Energy X-ray Absorptiometer were used to determine if the treatment supplement preferentially promotes growth of lean body mass rather than adipose tissue.

Statistical analysis

SAS 9.4 was used to perform all statistical analyses of body weight, DEXA parameters, and weights of brain, cerebellum, heart, lungs, liver, spleen, and pancreas.

Aim 1: Determine whether supplementation improves growth and body composition. Statistical analysis of obtained dataset was performed using the Wilcoxon signed-rank test, two-tailed paired Student's t-tests to evaluate if daily weight gain and daily supplement consumption differed between the two groups (placebo and treatment).

Aim 2: Determine if SGA pigs fed formula with the supplement experience have accelerated organ growth. The data were analyzed by Wilcoxon signed-rank test, two-tailed paired Student's t-tests.

For all comparisons, P-values less than 0.05 were considered as significant.

RESULTS

Analysis of initial groups of SGA and AGA pigs studies the first day of life

The initial groups of newborn SGA and AGA pigs (n=6 per group) were delivered to the University of Memphis for immediate organ and tissue collection to determine if initial organ weights and baseline differences existed between SGA and AGA piglets for weights of the kidneys, liver, spleen, pancreas, lungs, heart.

Body weight analysis

As seen from figure 3, body weight of AGA group was significantly larger assessed via Wilcoxon Signed-Rank test. Summary of weight statistics is presented in table 3.

Table 3. Summary of weight (grams) statistics in initial SGA and AGA groups

Group	Mean	Std. Deviation	P-value		Extreme Observations	
			t-test	Wilcoxon score	Min	Max
AGA	1640	267.8	<0.0001	0.0039	1240	1998
SGA	790	71.88			695	914

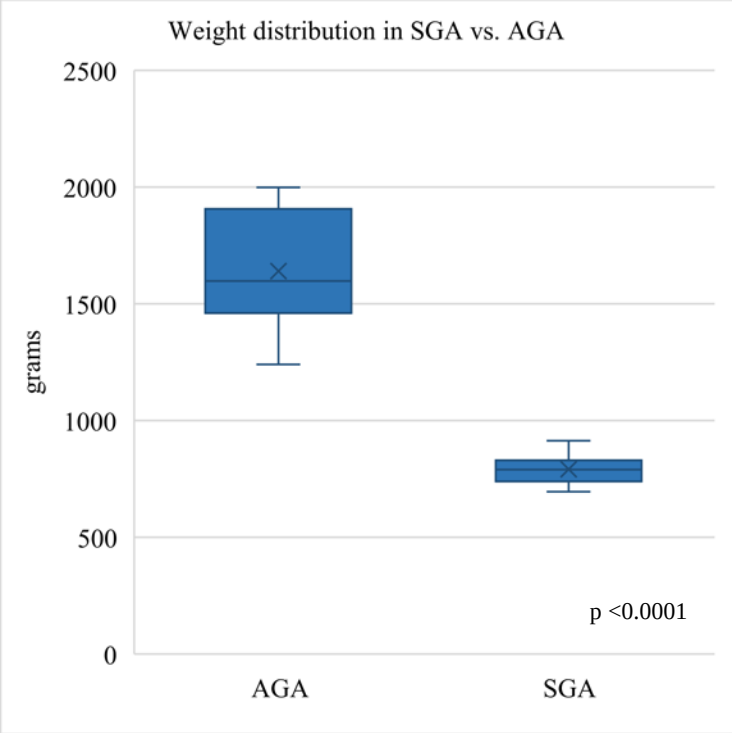

Figure 3. Distribution of body weights for the initial groups of SGA and AGA groups

Organ weights

The liver and spleen were significantly larger in newborn AGA pigs. All organ weights were normalized to body mass to account for differences in birth weights (g/kg body weight).

a) Spleen weight

Analysis of relative spleen weight (g/kg) showed that SGA pigs had significantly smaller spleens [(AGA 1.6 ± 0.15 vs. SGA 1.23 ± 0.08, n=12; p=0.043)]. (Figure 4)

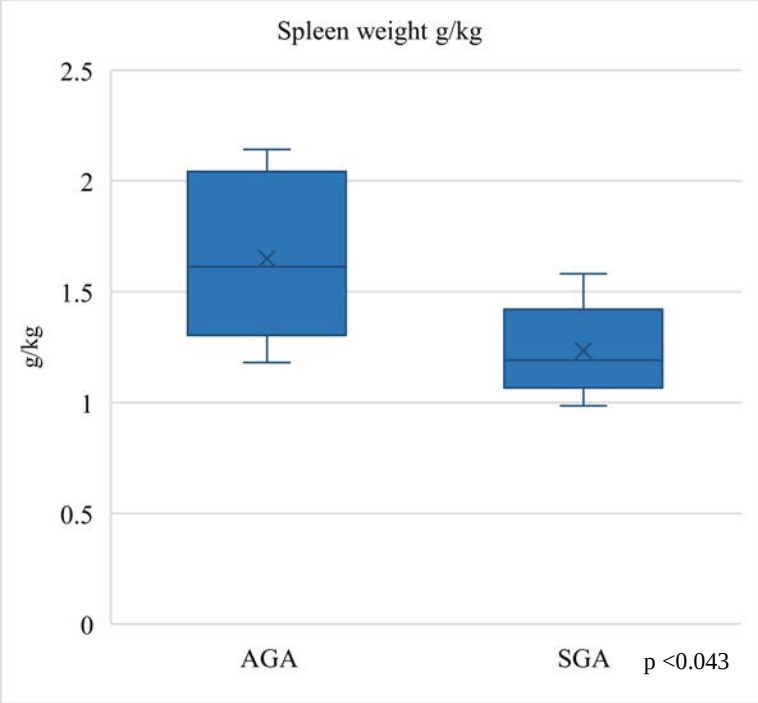

Figure 4. Spleen weight g/kg of body weight for initial groups of SGA and AGA pigs

b) Liver weight

Analysis of liver weights determined that SGA group has significantly lower relative liver weights (Figure 5) [(AGA 27.7 ± 8.7 vs. SGA 21.6 ± 1.92, n=12; p=0.03)].

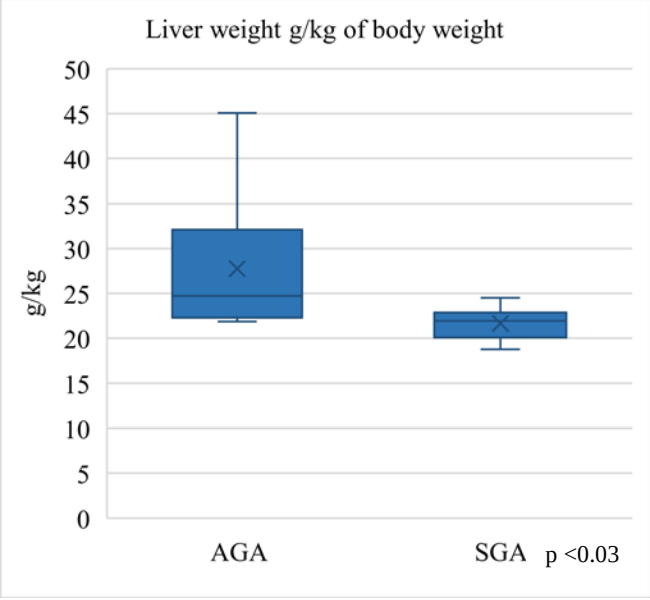

Figure 5. Liver weight g/kg of body weight for initial groups of SGA and AGA

c) Kidney weight. Kidney weight description is presented in table 4.

Table 4. Comparison of kidney weight (g/kg) between initial groups of SGA and AGA pigs

Group	Mean		Std. Deviation		P-value			
	kidney		kidney		t-test		Wilcoxon score	
	right	left	right	left	right	left	right	left
AGA	0.004	0.005	0.002	0.003	0.58	0.5	0.63	0.63
SGA	0.0043	0.0044	0.001	0.003				

d) Lung weight. Comparison of lung weights is presented in table 5.

Table 5. Comparison of lung weight (g/kg) between groups.

Group	Mean	Std. Deviation	P-value	
			t-test	Wilcoxon score
AGA	0.018	0.004	0.36	0.336
SGA	0.021	0.0058		

e) Heart weight. Comparison of heart weights is presented in table 6.

Table 6. Comparison of heart weight (g/kg) between initial groups of SGA and AGA pigs

Group	Mean	Std. Deviation	P-value	
			t-test	Wilcoxon score
AGA	0.0097	0.00251	0.27	0.42
SGA	0.00843	0.0011		

f) Table 7 reflects basic metabolic parameters in initial groups of SGA and AGA. There are significant differences in LDH, ALT and CK.

Table 7. Basic metabolic parameters AGA vs. SGA

Group	ALB g/dL	ALP U/L	ALT U/L	AST U/L	Ca mg/dL	Chol. mg/dL	CK U/L	Creat. mg/dL	LDH U/L	TP g/dL	TG mg/dL
AGA	0.71	1318	18	57.6	8.3	44	192	0.7	430	4.2	33
SGA	0.6	1451	26	94.3	9.1	48	773	1	865	3.5	30
p-value	0.1	0.69	0.023	0.1	0.1	0.6	0.01	0.1	0.0036	0.34	0.58

Analysis of experimental groups of SGA pigs after 20 days of feeding

A total of 16 newborn SGA piglets were delivered to the University of Memphis, were randomly assigned to placebo or treatment groups, and were fed for 20 days milk replacer either with the treatment supplement with leucine, MCT oil and krill oil or placebo supplement with the vegetable oil blend and alanine.

On day 21 of the study the pigs were sedated, DEXA scans were made and blood was collected before euthanasia and the collection of organs for measuring weight.

Body weight and weight gain analysis

Average body weight of placebo group was larger [(placebo 3417± 780.89 vs. treatment 2665± 725.9, n=16; p=0.06)], despite the fact that both groups were provided the same amount of formula per day (150 ml/kg/day).

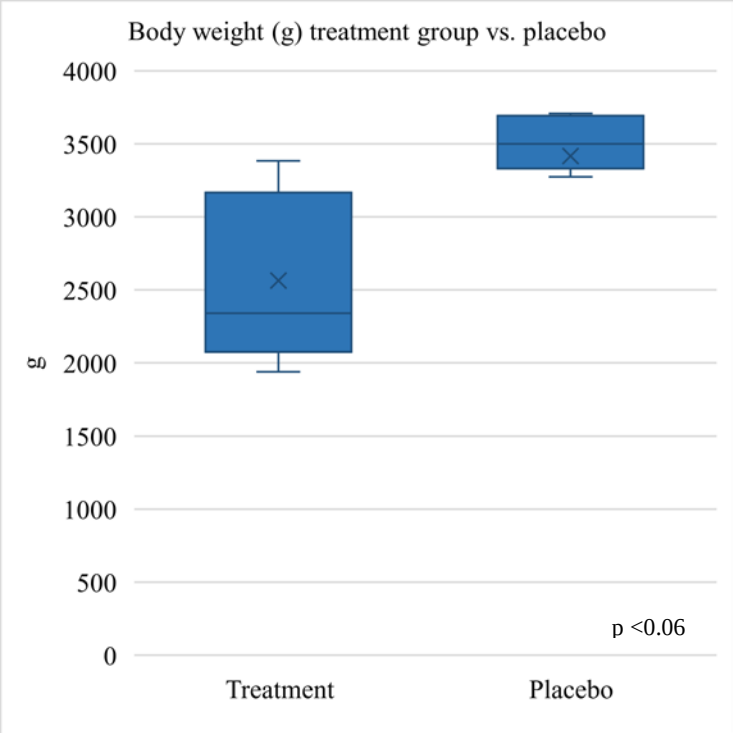

Figure 6. Body weight (g) treatment group vs. placebo

Average daily weight gain for the placebo group was 98 g per day compared with 63 g per day for the treatment group. These values corresponded with formula consumption per day of 708 ml per day in placebo group vs. 618 in treatment group. However, accurate assessments of consumption were difficult because of spillage of formula by the pigs. Throughout the study, treatment group pigs were more active and playful. The higher level of activity may have contributed to the differences in weight gain.

Figures 6 and 7 display daily weight gain percentages between groups. Our findings suggest that treatment group pigs gained less weight than placebo group pigs respectively to birth weight: Placebo 7% vs. 4.99% in treatment group. Daily weight gain percentage over period between groups is reflected in figure 7. As shown in figure 8 both groups had comparable weight gains for the first week of feeding. Thereafter, the placebo group started to gain weight faster

than treatment group and this increased weight gain persisted throughout the third week of the feeding period.

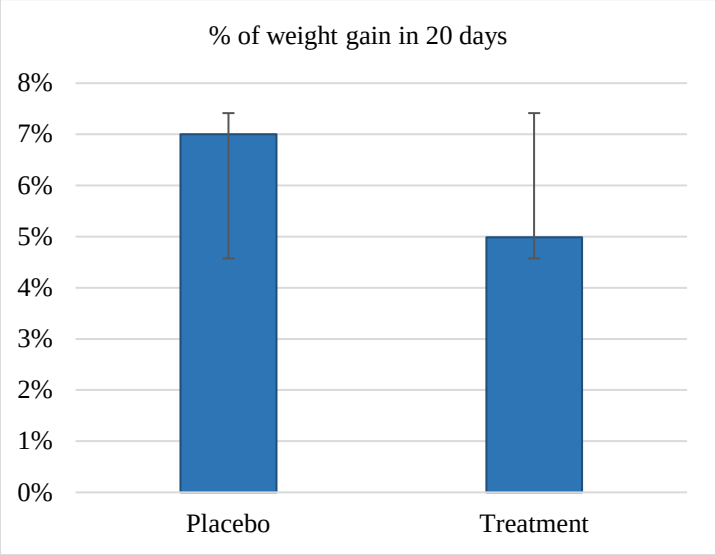

Figure 7. Average weight gain percentage between groups. Data is represented as means ± std. deviation.

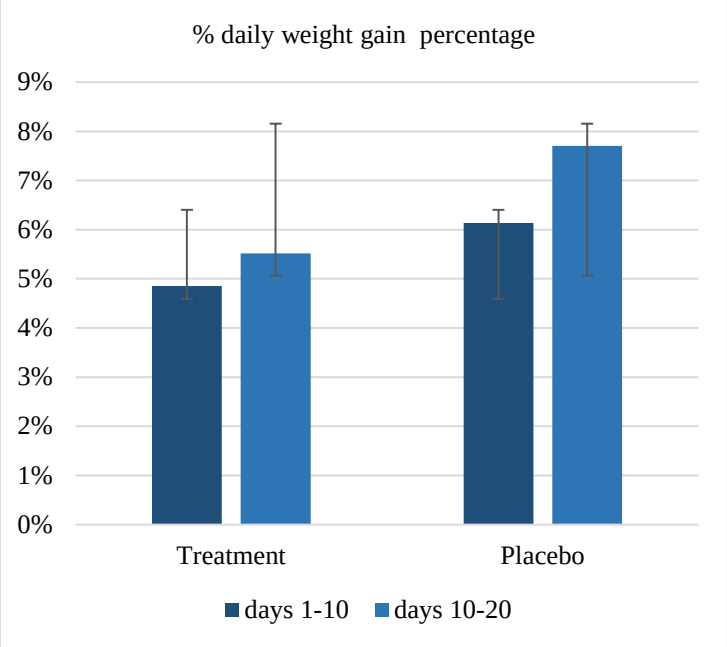

Figure 8. Comparison of weight gain during dietary intervention. Data is represented as means ± std. deviation.

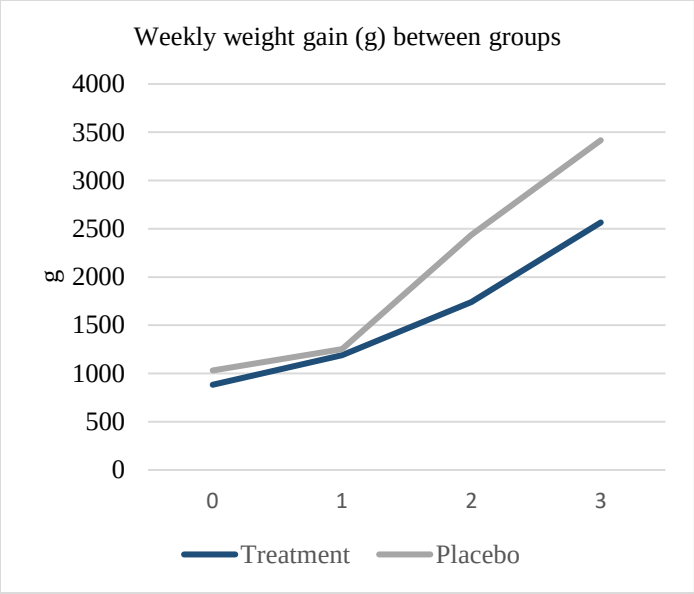

Figure 9. Weight gain (g) per week between groups

Organ weight analysis between groups.

a) Liver weight. The absolute and relative weight of the livers of placebo pigs were significantly larger [(placebo 145.4 ± 21.36 g vs. treatment 89.4 ± 34.02 g, n=16; p=0.0015)], [(placebo 44.2 ± 3.4 g/kg vs. treatment 33.0 ± 1.5 g/kg, n=16; p=0.001)]

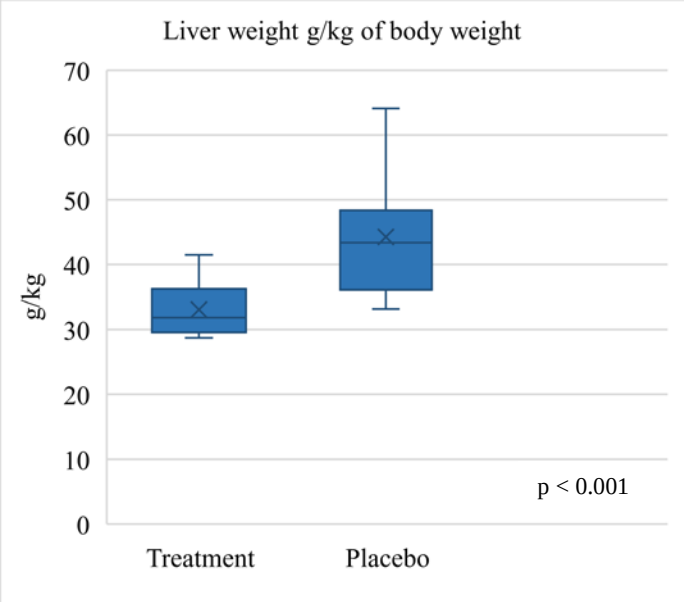

Figure 10. Liver weight g/kg of body weight

b) Whole brain weight comparison is presented in Table 8.

Table 8. Brain weight (g) comparison in both groups

Group	Mean	Std. Deviation	P-value	
			t-test	Wilcoxon score
Placebo	39.1450	4.3852	0.81	0.07
Treatment	38.7263	2.2		

Wilcoxon score-rank test revealed a p -value of 0.07, which indicates possible significance if repeated with more subjects.

c) Cerebellar weight (g) comparison is illustrated in Table 9.

Table 9. Cerebellar weight (g) comparison in both groups

Group	Mean	Std. Deviation	P-value	
			Student's t-test	Wilcoxon score
Placebo	4.92	0.39	0.8	1
Treatment	4.98	0.76		

d) Heart weight (g/kg) are reflected in table 10.

Table 10. Heart weight (g/kg) in both groups

Group	Mean	Std. Deviation	P-value	
			Student's t-test	Wilcoxon score
Placebo	9.28	1.53	0.4	0.1
Treatment	9.83	1.0		

e) Pancreas weight (g/kg) (Table 11).

Table 11. Pancreas weight (g/kg) in both groups

Group	Mean	Std. Deviation	P-value	
			Student's t-test	Wilcoxon score
Placebo	1.15	0.61	0.2	0.5
Treatment	1.24	0.34		

f) Spleen weight (g/kg) (Table 12).

Table 12. Spleen weight (g/kg) in both groups

Group	Mean	Std. Deviation	P-value	
			Student's t-test	Wilcoxon score
Placebo	4.385	0.63	0.5	0.2
Treatment	5.11	1.59		

g) Comparison of lung weight (g/kg) between groups (Table 13).

Table 13. Lung weight (g/kg) in both groups

Group	Mean	Std. Deviation	P-value	
			Student's t-test	Wilcoxon score
Placebo	16	2.9	0.56	0.4
Treatment	17.8	4.8		

h) Kidney weight g/kg (Table 14).

Table 14. Comparison of kidney weight (g/kg) between groups

Group	Mean		Std. Deviation		P-value			
	kidney		kidney		t-test		Wilcoxon score	
	right	left	right	left	right	left	right	left
Placebo	4.48	4.6	2	2.1	0.2	0.2	0.1	0.1
Treatment	3.44	3.7	0.49	0.26				

Body composition analysis

a) Body fat percentage

The percentage of body fat was significantly lower in treatment pigs [(placebo 10.8 ± 1.29 vs. treatment 9.7 ± 0.89, n=16; p=0.05)].

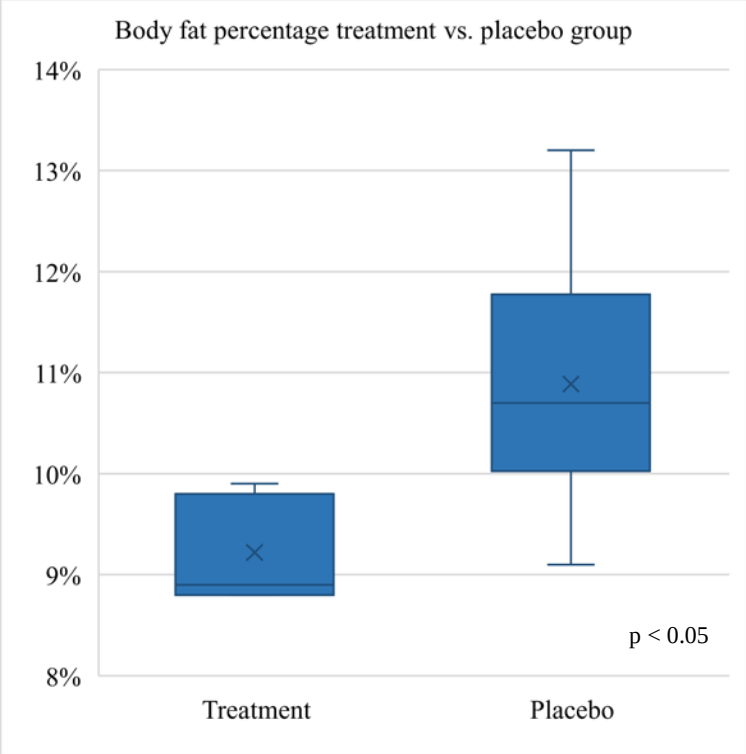

Figure 11. Body fat percentage treatment vs. placebo group

b) Body fat mass (g)

The DEXA assessment reveled a significantly larger fat mass (g) in placebo pigs [(placebo 401 ± 69.5 vs. treatment 268.2 ± 91.7, n=16; p=0.0157)], which was also significant when normalized to body mass (g/kg) [(placebo 120.6 ± 18.79 vs. treatment 99.49 ± 10.1, n=16; p=0.0157)].

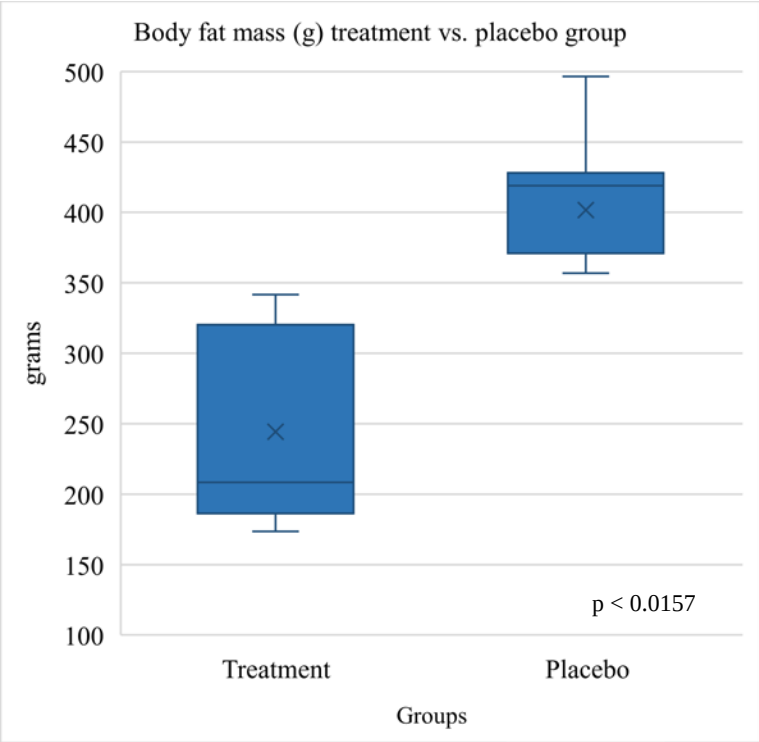

Figure 12. Body fat mass (g) treatment vs. placebo group

c) Lean mass percentage between groups

Statistical analysis revealed a trend for a greater percentage of lean mass in treatment pigs [(placebo 0.878 ± 0.014 vs. treatment 0.889 ± 0.0079, n=16)]. However, the difference did not achieve significance (p< 0.07).

Table 15. Summary of lean mass percentage statistics in treatment vs. placebo

Group	Mean	Median	Std. Deviation
Placebo	0.878	0.880	0.014
Treatment	0.889	0.888	0.0079

d) Lean mass between groups

Lean body mass grams [(placebo 3230.2 ± 385.7 g vs. treatment 2411.6 ± 616.4 g, n=16 p< 0.7)] and lean body mass g/kg [(placebo 983.7 ± 209.3 vs. treatment 908.6 ± 38.2, n=16 p< 0.7)]

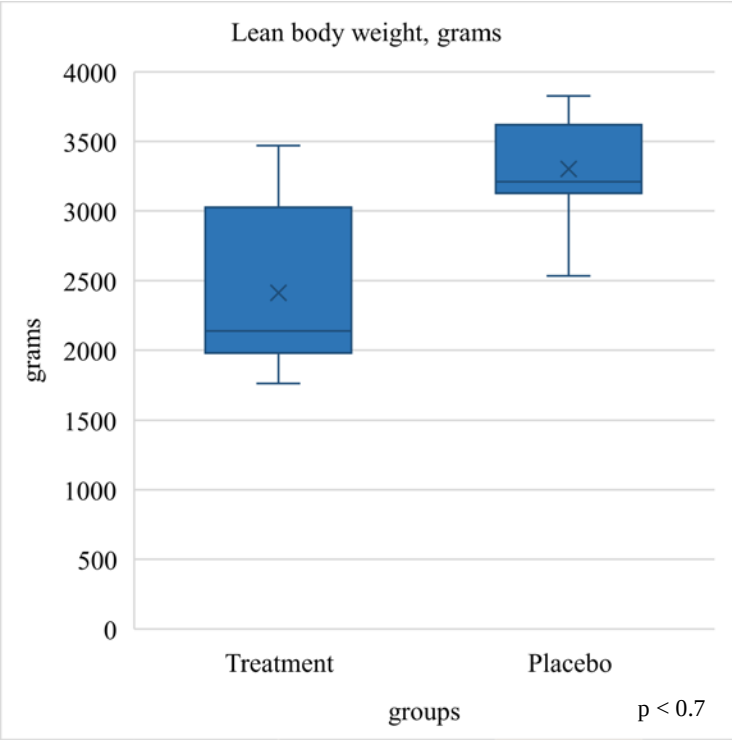

Figure 13. Distribution of lean body weight (g) between groups

e) Bone mineral content (BMC) (g) and bone mineral density (BMD) (g/cm²) analysis

Results of statistical analysis determined greater BMC and BMD in placebo group. It is associated with greater body weight. BMC: [(placebo 47.67 ± 6.5 g vs. treatment 34.0 ± 5.9 g, n=16; p<0.0007)], BMD: [(placebo 0.13 ± 0.00659 g/cm² vs. treatment 0.1258 ± 0.129 g/cm², n=16; p<0.0007)]. Pearson correlation analysis in Fisher's z transformation determined that there is correlation between weight (g) and BMC (g) (p-value < 0.002), therefore we can conclude that higher BMC can be explained simply by larger size and it is not associated with dietary intervention.

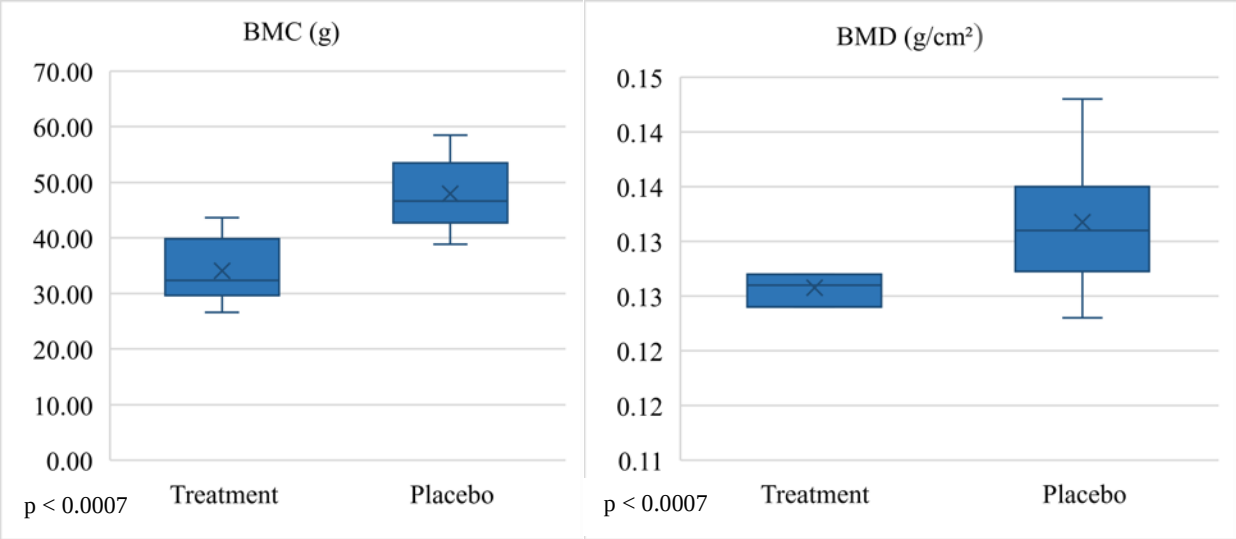

Figure 14. BMC (g) and BMD (g/cm²) in treatment vs. placebo group

Blood biochemistry analysis

Basic metabolic parameters were compared using blood samples collected immediately before death for the placebo group (n=8) and treatment group (n=8). The placebo pigs had higher serum albumin, alanine aminotransferase, lactate dehydrogenase, total protein, and triglycerides.

Table 16. Summary of blood analysis

Group	ALB g/dL	ALP U/L	ALT U/L	AST U/L	Ca mg/dL	Chol. mg/dL	CK U/L	Creat. mg/dL	LDH U/L	TP g/dL	TG mg/dL
Treatment	2.2	431	11	27.6	9.2	79	324	0.69	375.8	3.7	52.12
Placebo	2.77	589	20.1	27.6	9.4	76.8	469	0.62	574.6	4.03	38.6
P-value	0.0077	0.1	0.049	1	0.855	0.68	0.09	0.266	0.002	0.03	0.07

DISCUSSION

There is increasing evidence that SGA neonates are prone to metabolic abnormalities and experience chronic disorders. Currently, there is no successful nutritional strategy that can prevent health-related issues in SGA infants. This research is novel by determining if a combination of functionally active nutrients can influence body composition and potentially improve growth of SGA infants.

Consequence of being SGA at birth. Baseline data collection included organ weights and blood of SGA and AGA newborn term pigs. The analysis of organ weights revealed significant differences for liver and spleen weights, with SGA pigs having significantly smaller livers and spleens even after normalizing to body mass (g/kg). The growth and maturation of the brain and cerebellum are of concern for SGA infants, but did not differ between newborn SGA and AGA pigs. The differences in serum chemistries are suggestive of differences in liver functions, but the specific issues were not determined.

Responses of SGA pigs to the supplement. Evaluating experimental dietary interventions using SGA neonates is very difficult due to ethical considerations and may carry safety issues. For this reason, newborn SGA pigs were used as a translational model for SGA neonates. This model was previously validated as a credible model of a neonate in nutritional research (Lennon, Zanganeh, & Borum, 2011; Svendsen & Koch, 2013).

During the dietary intervention, the treatment group was visually leaner, more active and playful. In contrast, the placebo pigs after day 10 were less active, spent more time inactive, and interacted less with the ball placed in the pen for enrichment.

Despite the fact that during the intervention both groups were provided similar amounts of formula (~150 ml/kg/day), daily weight gains differed between the two groups. There are several possible reasons. First, the treatment group was more active. Second, placebo pigs had a greater amount of fat body mass. On the other hand, enteral leucine enhances muscle protein synthesis in neonatal pigs by activating the mTOR pathway (Columbus, Fiorotto, et al., 2015; Suryawan et al., 2008).

DEXA results confirmed that there were significant differences in body composition. Placebo pigs were significantly larger, mostly because of greater accumulation of body fat.

Treatment pigs had significantly smaller amounts of fat mass. This result fully corresponds with SGA pediatric research studies (Ibáñez, Ong, Dunger, & de Zegher, 2006; Monteiro & Victora, 2005; Ong et al., 2000).

Organ development in SGA neonates is delayed compared to AGA infants. Moreover, similar to premature infants, SGA infants experience catch-up growth period (Cho & Suh, 2016). Analysis of collected organ weights during the study, revealed significantly larger livers of placebo pigs. Recent studies have reported that being born SGA *per se* is an important risk factor for non-alcoholic fatty liver disease (NAFDL) (Alterio, Alisi, Liccardo, & Nobili, 2014; Nobili et al., 2007). Rapid weight gain is a major risk factor for later development of metabolic complications, and has been implicated in the presence and severity of NAFLD in both children and adults (Nobili et al., 2013). The larger livers of the placebo pigs had areas of discoloration with that were not seen in the livers of treatment pigs. Moreover, ALT levels in placebo group were two-fold higher than for treatment pigs (20.1 ± 10.7 vs. 11 ± 2.39; $P=0.049$). The larger livers and higher liver enzyme activities in the serum may be indicative of early liver damage.

Although SGA infants can have differences in brain structure, compared to AGA neonates and there is also catch-up growth period of cranial structures (Radlowski et al., 2014). However, collected brain weights after 20 days of feeding did not differ between groups.

Summarizing all the results of the study, we can conclude that current supplement composition is safe for use and improves body composition in SGA piglets by decreasing fat deposition.

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

Considering metabolic risks of individuals who were formerly SGA infants, efficient dietary supplementation during early postnatal life is essential for growth and development that

promotes quality of life. The data reported herein formula with a combination of MCT oil, krill oil and leucine could improve body composition and growth of newborn piglets. Treatment group was significantly leaner, more active than placebo group.

Although, brain weights were not significantly different between groups, the lack of difference may be related to the relatively small sample size. Corresponding with this some organ weights, such as the spleen and kidneys were trending to be significant levels between groups. Therefore, for the future research, larger sample sizes will be needed.

Future studies should include immunohistochemistry analyses and labeling of proliferating cells with 5-bromo-2'-deoxyuridine (BrdU). A longer period of study may be recommended. Finally, the responses of AGA pigs to the same placebo and supplement should be assessed.

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IACUC PROTOCOL ACTION FORM

To:	Randal Buddington
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	February 10, 2017

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No.

0798 Nutrition of pigs that are small for gestational age

☒ Your protocol is approved for the following period: From: To:

February 9, 2017 | February 8, 2020

Your protocol is not approved for the following reasons (see attached memo).

February 9, 2017 | February 8, 2020

☐

Your protocol is renewed without changes for the following period: From: To:

February 9, 2017 | February 8, 2020

☐

Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated for the following period:

From: To:

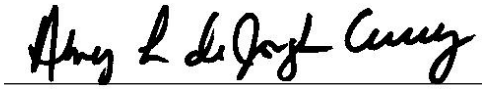
February 9, 2017 | February 8, 2020

☐

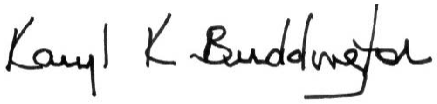
Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated

February 9, 2017	February 8, 2020

☐



Amy L. de Jongh Curry, PhD, Chair of the IACUC



Dr. Karyl Buddington, University Veterinarian and Director of the Animal Care Facilities

IACUC PROTOCOL
FOR USE OF LIVE VERTEBRATES FOR RESEARCH, TEACHING OR DEMONSTRATION
UNIVERSITY OF MEMPHIS

Date submitted to Attending Veterinarian for pre-review: 26 November 2016

IACUC Protocol # 0798 Date Submitted to IACUC February 9, 2017

Dates Protocol will be in effect: from February 9, 2017 to February 8, 2020
(not to exceed three years including two yearly renewals)

Is this protocol related to an external grant or contract application? Yes ☐ No ☒

If yes, complete the following:

Agency: _____ Date Submitted _____

Grant # _____

University account for Animal Care Facility per diem charge: Development account

If the protocol is not related to an external grant or contract application, complete the following:

University account for Animal Care Facility per diem charge: To be established

Project Title: (If project relates to a grant or contract application, give that title; if multiple protocols relate to one grant, give unique titles for each protocol; if the project is related to a class, give the course name and number):

Nutrition of pigs that are small for gestational age

I. Personnel

Investigator/Instructor: Randy Buddington

Department: School of Health Studies

Academic Rank: Professor

Campus phone: X4743 Emergency phone: 662 418 2666 / 417 719 2372

Attending Veterinarian: Karyl Buddington

Phone: X2359; 258-1232 Emergency phone: 901 258 1232

List all individuals that will handle animals using this protocol, their affiliation, and their level of expertise (e.g. relevant qualifications). If the protocol applies to a class then so specify.

1) Randy Buddington, Marie van der Merwe, Samantha Davis, Lauren Wells, Kristy Levin, Thomas Wong, Mitchel Penninger, Taisiya Yakimkova. All of these individuals have taken the animal training course prior to participating. All of the personnel are actively participating in our studies using small for gestational age pigs.
2) Karyl Buddington and Donny Ray: veterinarian and facility manager, both with extensive experience in swine research
3) It is likely additional individuals will be added to the protocol to assist with the care and observations of the pigs. They will attend the training session offered by Dr. K. Buddington, receive additional and specialized training in the care and handling of small for gestational age pigs, and will be added to the protocol via an amendment prior to participating.

If additional personnel become involved in handling animals used in this protocol, it is the responsibility of the principal investigator to notify the Animal Care Facility in writing before they start.

Has the investigator/instructor and all personnel listed above received the appropriate vaccinations (tetanus, rabies)? Yes ☐ No ☐ Not Applicable ☒

Is it necessary for personnel listed on this protocol to be tested for TB?
Yes ☐ No ☒

If you have questions about the kind of vaccination or about TB, call the Animal Care Facility at 678 2359.

All U of M personnel involved in this protocol must complete the animal care and use training program before animals can be procured or before experiments/teaching or demonstration. In submitting this protocol, I, as Principal Investigator/Instructor accept the responsibility for compliance with this requirement.

In addition, the Principal Investigator/Instructor must be willing to provide appropriate supervision for all persons working on this protocol. In the case of a class, the Instructor must be responsible for training any students in classes involved prior to using animals.

II. Project Description

A. Summary **(Enter a brief description below of your project, using lay terminology)**:

Each year, more than 20 million infants worldwide are born with low birth weight (LBW). These babies are often called being small for gestational age (SGA), which by definition means that they are born in the lower 10% of birth weights. For term infants this is ~1.5 kg or babies born at ~3.5 pounds and less. These SGA babies have an increased risk of death and disability.

There is increasing information about the differences and long term consequences between infants born SGA and those born of appropriate weight for gestational age (AGA). This includes compromised brain growth and development of SGA infants. Other organs are impacted by an infant being SGA. These differences are associated with lifelong effects.

Studies with SGA human infants are extremely difficult due to obvious ethical considerations. In the light of this fact, there is a need for animal models to understand the impact of being SGA and to evaluate possible interventions. The neonatal piglet is a highly tractable translational animal model that has been successfully used for SGA studies.

What is critical for the SGA infant is to provide nutrition support that will promote growth and normal development. Currently, providing breast milk is considered the best approach. However, breast milk is not available for all SGA infants and in reality may not be good enough. The breast milk fed to many SGA infants is fortified with protein to encourage better growth. Although this does improve gain in weight, most of the gain is fat, not lean body mass, and this increases the risk of obesity, metabolic syndrome, cardiovascular disease, and other health issues later in life. There is a need to improve nutrition support for the SGA infant to improve outcomes.

Our previous studies with preterm SGA pigs showed how supplementing formula increased weight gain dramatically, and without an apparent increase in the amount of fat. We also know that SGA infants have a need for essential fatty acids, and particularly long chain omega 3 fatty acids, such as those in fish oil. These findings led us to the concept of a supplement consisting of different nutrients that can be added to breast milk or existing infant formula that will improve growth by increasing gain in lean body mass and will promote normal development.

We will determine if newborn term pigs that are SGA will benefit when they are fed formula with the supplement by comparing them with those that don't receive the supplement. This will be accomplished by measuring growth, evaluating how much fat and lean body mass they have using dual x-ray absorptiometry (known as DXA), performing serum chemistries, and evaluating the growth and development of critical organs, including but not limited to the brain, gut, kidneys, lungs, and immune system.

Our objective is to learn how to improve nutrition support for the SGA infant and then work with neonatology colleagues and industry sponsors to translate our findings into improving outcomes for babies that are SGA.

B. Describe IN DETAIL the procedures you will follow. Include accompanying documentation and reference to previously published work in the box below. Provide a complete bibliographic citation and describe any variations from the published technique. The bibliography may be included in the box below or appended to this protocol.

We will obtain newborn term pigs at <24 h of age with body weights of <800 g and are considered as SGA. Additional pigs of average weight at term (1,200 – 1,600) will be used as controls. All of the pigs will originate from the same commercial producer that supplies the sows we use for our preterm pig studies. The newborn pigs will be allowed to suckle the sow until they are brought to UM. Once at UM the pigs will be fed a commercial milk replacer (formula) for pigs that meets or exceeds known nutrient requirements for suckling pigs.

The pigs will be housed in facilities specifically designed for care and handling preterm and newborn term pigs, and which we have used previously for such studies. We request approval to house the pigs in incubators with square footage that is smaller than what is listed for housing pigs, but large enough to allow for free movement and activity. The incubators have dimensions of 2' x 4' and have a slatted floor which will facilitate cleaning. The incubators will have heating lamps to keep the pigs warm, like the heating lamps used to keep preterm infants warm. Housing the pigs in the incubators is like keeping infants in cribs, and will enhance our ability to feed and monitor the pigs individually. From our previous studies we know that the pigs will huddle together for most of the time. The pigs will be observed and fed throughout the day (minimum of 7 times. At each time the pigs will be fed, the caging cleaned, and health status recorded. The pigs will be weighed a minimum of two times each day which will be an important indicator of health and well-being. Additional observations will be made of general activity and responsiveness to touch.

The SGA pigs will be fed the milk replacer for up to 20 days. Some pigs will be studied earlier than 20 days to determine the responses at earlier time points. The supplement (or placebo) will be added to the milk replacer. The supplement will include nutritional ingredients that previous studies (our own and from the literature) have shown are likely to improve growth and development. Our studies will use different combinations rather than single ingredients to obtain synergistic benefits. During the project period we will try different combinations. The ingredients to be evaluated as components of the supplement will include fatty acids, lipids, amino acids, proteins, carbohydrates, and vitamins and minerals. The placebo will include other forms of the same nutrients that are not considered to provide benefit. For examples, we will use alanine rather than leucine, vegetable oil rather than fish or krill oil, maltodextrin rather than fermentable fiber.

Blood will be collected from each pig one or two times during the period of feeding. This will be accomplished by first anesthetizing the pig with isoflurane and while they are anesthetized place them on their back to access the subclavian vein. We have done this previously with newborn and juvenile pigs. If our findings suggest that more frequent blood sampling is desirable, we will have jugular catheters surgically placed using the same surgical approach that we use for our other studies using pigs with jugular catheters. The catheters will be used to collect blood samples for analysis during the feeding period, to inject bromydeoxyuridine and puromycin (below), and to administer euthanasia (euthasol). Pigs with jugular catheters will be housed individually in the plastic tubs we used to house the pigs for our studies of short-bowel syndrome that also required individual housing.

We will inject pigs with a combination of BrdU (50 mg/kg) and puromycin (18.8 mg/kg; 0.04 mmol/kg) 30 – 60 min prior to euthanasia. The BrdU is used to label cells that are proliferating as an indicator of growth. The puromycin labels proteins that are being synthesized as a secondary indicator of growth, with the emphasis on the increase in protein synthesis and lean body mass. The BrdU and puromycin will be administered via the jugular catheter, if one is in place. Pigs without jugular catheters will be sedated with telazol then placed under gas anesthesia so the BrdU and puromycin can be administered via the subclavian vein. While these pigs are anesthetized they will be imaged using dual x-ray absorptiometry (DXA) to assess the percentage of lean and fat body mass. These pigs will remain under anesthesia for 30 min after the administration of BrdU and puromycin until blood is collected for analysis followed immediately by the administration of euthasol. This approach will avoid the need to use anesthesia twice.

After death each pig will be necropsied and organs will be removed, observed, weighed and tissues collected for analysis.

C. Rationale for Involving Animals and the Appropriateness of Species and Number Used.

Indicate (**here**) briefly the short and/or long-term benefits (to humans and/or other animals) of this use of animals for research, teaching or demonstration. Provide rational for and the number of animals to be used. In addition, state briefly why living animals are required for this study, rather than some alternative model.

There are no non-animal models that allow us to study if and how SGA pigs respond to nutrition support protocols that could be used for preterm and term infants that are also SGA.

The pig is considered as a clinically relevant animal model for research focused on the preterm and term infant. Alternative animal models (neonatal rats and mice) are available, but because of small size and characteristics that differ from human infants, rodent models have limited use for the proposed research because they are not considered translational. Although non-human primates would be suitable, they do not provide benefits beyond those available from preterm pigs. Lambs are not appropriate because of differences with humans for organ structure and development and feeding habits, they are seasonal breeders, and SGA lambs are in limited supply, are not available locally,

D. Do the procedures described in B above, have the potential to inflict more than momentary pain or distress (this does not include pain caused by injections or other minor procedures)? Yes ☐ No ☒

I have considered alternatives to procedures that might cause more than momentary or slight pain/distress, and I have not found such alternatives. As such, I have used one or more of the following methods and sources to search for such alternatives: **(check below each method used)**

☐ Agricola Data Base	☒ Medline Data Base	☐ CAB Abstracts
☐ TOXLINE	☐ BIOSIS	☐ Lab. Animal Sci. Journal
☐ Lab. Animals Journal	☐ Lab Animal	☐ Animal Welfare Info Center
☐ ATLA (Alternatives to Laboratory Animal Journal)		☐ Quick Biblio. Series
☐ Lab Animal Welfare Bibliography (QL55L27311988)		☐ "Benchmarks"

☐ "Alternatives to Animal Use in Research, Testing and Education"

☐ Current Contents

☐ CARL

☐ Direct contact with colleagues **(if selected, you MUST document this below)**

List search words for the literature search:

SGA, small for gestational age, pig, nutrition, preterm, neonate, infant, nutrition, formula

What is the length of time that the literature search covers?

1990 – 2016 (present)

III. Animal Use

A. List all animal species to be used (example below).

Species	Number[1]	Age[2]	Sex[2]	Weight[2]	Where Housed (Bldg./Rm#)
pig	80 per year	neonate	both	500-800 g	Fieldhouse 135
Pig	20 per year	neonate	Both	1,200 – 1,800	Fieldhouse 135

[1]Individuals using ectotherms need to only approximate numbers.
[2]Individuals using fish or other ectotherms need not answer this question.

Is any species threatened or endangered? Yes ☐ No ☒

B. Source of animals

☒ Commercial vendor (Source Prestage Farms, MS)
☐ Bred at The University of Memphis
☐ Captured from wild. Identify method of capture: _____
☐ Transferred from another study (IACUC Protocol Number _____)
☐ Donated (Source _____)
☐ Tennessee Wildlife Resources Agency

Is the supplier a USDA approved source? Yes ☐ No ☒

If not, explain why: The pigs will be obtained from Prestage Farms. This is a closed production facility that maintains strict biocontainment to maintain specific pathogen free conditions. The sows that produce the pigs are of the same genetic lineage.

☐ Animals are already in residence at U of M

C. Will surgery be conducted on animals? Yes ☒ No ☐

If yes, complete this section:

☐ Non Recovery Surgery ☒ Recovery Surgery
☐ Multiple Survival Surgery (**if the latter is checked, complete section F)**

Surgeon(s) (Name/Job/Title/Academic Rank)	Location of Surgery (Bldg. & Room #)
Karyl Buddington or her designee	422 Psychology

Will Anesthetic(s), Analgesic(s), or
Tranquilizing agents be administered? Yes ☒ No ☐

If yes, complete this section (example below).

Species & Sex	Agent	Dose	Route	Performed by (Name/Title/Academic Rank)
Pig	Isoflurane	1-2%	Inhal.	K. Buddington or designee
Pig	Telazol	0.04 mg/kg	IM	K. Buddington or designee
Pig	Rimadyl	0.5 ml/pig	IM	K. Buddington or designee
Pig	Meloxicam	0.5 ml/pig	oral	K. Buddington or designee

E. Will euthanasia be carried out? Yes ☒ No ☐

If yes, complete this section (example below).

Species & Sex	Agent	Dose	Route	Performed by (Name/Title/Academic Rank)
Pig	Euthasol or equivalent	1 ml/4.5 kg (10 lb)	IV	K. Buddington or designee

If no, describe disposition of animal(s) at conclusion of this study in box below.

F. Will special housing, conditioning, diets or other conditions be required? Yes ☒ No ☐

If yes, please explain in box below.

Most of the pigs will be group housed in plastic pens that are 2' x 4' sq. ft. that we have used successfully for housing of 4-6 newborn term pigs. Pigs that have jugular catheters will need to be housed individually in plastic pens of 1.5' x 2.5'. The pens will be provided with heating lamps.

The pigs will be trained to feed from the bottles with nipples. This will allow the pigs to feed themselves and for the research team to monitor consumption.

We are requesting and exemption from the square footage recommended in 8th Edition of the Guide for the Care and Use of Laboratory Animal, which suggests 6 sq ft per pig when pigs <15 kg are housed in groups of 2-5 pigs. We seek an exemption for using housing with smaller dimensions since the pigs are not expected to reach more than 5-6 kg. Based on our previous studies using the same pens, the amount of available space will does not impair movement and provided enough space for the pigs to freely move and play. When groups housed in the larger pens, we request permission for a maximum of 6 pigs in each of the pens. Pigs to be housed individually will be kept in the smaller pens.

G. Will animals be removed from the U of M campus at any time? Yes ☐ No ☒
If yes, please indicate to where and for how long in box below.

H. If they are to be housed for more than 24 hours outside approved facilities at U of M, provide a scientific justification in box below.

IV. Toxic and Hazardous Substances

A. Check off any of the following below that will be used in these experiments?

☐ Infectious agents (Fill out a, b)
☐ Radioisotopes (Fill out a, b, e)
☒ Toxic chemicals or carcinogens (Fill out a, b)
☐ Recombinant DNA (Fill out a)
☐ Experimental drugs (Fill out a)
☐ Malignant cells or hybridomas (Fill out a, c)
☐ Adjuvants (Fill out a)
☒ Controlled substances (Fill out a, d, e)

For each checked off category, answer the questions indicated below:

a. Identify the substance(s) and completely describe their use, including how will be injected or given to the animal(s):

We will use Euthasol (or equivalent) to euthanize the experimental pigs (1 ml/4 kg; IV). The bromydeoxyuracil (BrdU) and puromycin will be administered IV via subclavian access and used to label proliferating cells as an indicator of growth responses. The BrdU will be prepared in a fume hood located in room 231 of Smith Chemistry. This will be done by adding BrdU to sterile 0.9% saline at 10 mg/ml and using a sonicator to put the BrdU in solution. The BrdU solution will be drawn up into a syringe just prior to administration. Preparation of the BrdU will be done by R. Buddington or his trained designee.

The puromycin will be purchased as a powder and added to 0.9% sterile saline for IV administration via the subclavian vein.

The euthasol solution will administered via the jugular catheter, if present, or via cardiac puncture or IV via the subclavian vein.

The Telazol and Rimadyl will be administered IM. The meloxicam will be given orally.

b. Describe all procedures necessary for personnel and animal safety including biohazardous waste, carcass disposal and cage decontamination:

The Euthasol, bromydeoxyuracil, puromycin, Rimadyl, and Telazol will be administered using a syringe to avoid contact with project personnel. The resulting carcasses will be disposed of by incineration. There will be no cage contamination.

c. If transplantable tumors or hybridoma cells are to be injected into the animals, have the tissues/cells been tested for inadvertent contamination by viruses or mycoplasma? Yes ☐ No ☒

If yes, what was the result (indicate in box below).

d. In the box below, provide a complete list of these substances, and if their use is not explicitly explained in the materials already provided, explain their use and role in the research.

Provide DEA license # covering the use of these substances: BB9988847

To whom (or what entity) is the license issued? Karyl K. Buddington

e. Provide Radioisotope License Number: _____

To whom is the license issued? _____

V. Categories of Animal Experimentation Based Upon Level of Manipulation and Pain: (check off each category that is applicable to this application)

Animals will be involved in teaching, research, experiments or tests involving no pain, distress, or use of pain-relieving drugs.

☐ B. Animals will be subject to mild stress only (e.g., food or water deprivation of less than 24 hours for use in behavioral studies such as operant conditioning; physical restraint for less than 30 minutes), and will not be subject to surgery, painful stimuli, or any of the other conditions described below. Procedures described in this protocol have the potential to inflict no more than momentary or slight pain or distress on the animal(s)----that is, no pain in excess of that caused by injections or other minor procedures such as blood sampling.

☒ C. Animals will have minor procedures performed, blood sampling, etc. while anesthetized.

The majority of pigs will not have surgeries for placement of jugular catheters and are appropriate for this category.

☐ D. Live animals will be humanely killed without any treatments, manipulations, etc. but will be used to obtain tissue, cells, sera, etc.

☐ E. Live animals will have significant manipulations, surgery, etc. performed while anesthetized. The animals will be humanely killed at experiment termination without regaining consciousness.

☐ F. Live animals will receive a painful stimulus of short duration without anesthesia (behavior experiments with flight or avoidance reactions--e.g., shock/reward) resulting in a short-term traumatic response. Other examples in this category are, blood sampling, injections of adjuvants, or drugs, etc.

☒ G. Live animals will have significant manipulations performed, such as surgery, while anesthetized and allowed to recover. Such procedures cause post-anesthetic pain/discomfort resulting from the experiment protocol (e.g., chronic catheters. surgical wounds, implants) which cause a minimum of pain and/or distress. Also included are mild toxic drugs or chemicals, tumor implants (including hybridomas). tethered animals, short-termed physically restrained animals (up to 1 hour), mother/infant separations.

This applies to the pigs that will have surgery to place jugular catheters.

☐ H. Live animals will have significant manipulations or severe discomfort, etc. without benefit of anesthesia, analgesics or tranquilizers. Examples to be included in this category are: toxicity testing, radiation sickness, irritants, burns, trauma, biologic toxins, virulence challenge, prolonged: restrictions of food or water intake, cold exposure, physical restraint or drug addiction. All use of paralytic agents (curare-like drugs) must be included in this category. Describe any abnormal environmental conditions that may be imposed. Describe and justify the use of any physical restrain devices employed longer then 1 hour.

VI. Justifications for Category G Studies and Deviations from Standard Techniques

Describe in the box below any steps to be taken to monitor potential or overt pain and/or distress during the course of this study and how such pain or distress will be alleviated. Be as detailed as necessary to justify your procedure.

Pigs that require surgery for placement of a jugular catheter will be fully anesthetized for the surgery. Rimadyl will be administered before the pigs recover from surgery to provide analgesia. Meloxicam will be provided the day after surgery for analgesia.

VII. Certifications
(By submitting this protocol, I am acknowledging that I comply with the certifications included in Section VII.) *(check one)*

☒ Animal Use for Research. I certify that the above statements are true and the protocol stands as the original or is essentially the same as found in the grant application or program/project. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not purchase animals nor proceed with animal experimentation until approval by the IACUC is granted.

☐ Animal Use for Teaching/Demonstration. I certify that the information in this application is essentially the same as contained in the course outline and a copy of the laboratory exercises using animals is on file in the IACUC office. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not proceed with animal experimentation until approval by the IACUC is granted.

Estimate the cost of maintaining animals used in this protocol based on current per diem charge at University of Memphis.

Please specify cost per unit of time: $ 1,000 per month

Specify anticipated total costs for project duration: $ 3,000

As supervisor of this project it is required that you inform your department chair concerning any animal per diem costs related to this project that are to be paid by the department.

By submitting this protocol, the Principal Investigator/Course Director indicates that the following have been considered:

1. Alternatives to use of animals.
2. Reduction of pain and stress in animals to the lowest level possible.
3. The proper needs of the animals with respect to housing and care.
4. The lowest number of animals used that will give the appropriate experimental results.
5. Use of the most primitive species that will give the appropriate experimental results.
6. Proper training of all personnel in the care and handling of the species used and in the procedures called for in this protocol before beginning the experiment/teaching or demonstration.
7. That this protocol is not an unnecessary repeat of results already in the literature or in the case of teaching/demonstrations, results that can be demonstrated using models or video material.

Principal Investigator/Course Director (Type Name) Randal K. Buddington

e-mail address rbddngtn@memphis.edu

Date 12-1-2016

Federal Law requires that members of the IACUC be given adequate time to read and review protocols including any changes or revisions in them.

Pre-review of protocols by the Attending Veterinarian is required before submission to the IACUC. New protocols or modifications or renewals to protocols must be submitted to the IACUC Chair by the 1st business day of the month to be considered for review during that month. Incomplete protocols will be returned to the principal investigator.

E-mail the completed protocol to the IACUC Chair, Dr. Amy de Jongh Curry, adejongh@memphis.edu

February, 2015