THE EFFECT OF DIETARY INSULINOGENIC AMINO ACID RESTRICTION ON GLUCOSE METABOLISM IN NEONATAL PIGS

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Title of Study: THE EFFECT OF DIETARY INSULINOGENIC AMINO ACID RESTRICTION ON GLUCOSE METABOLISM IN NEONATAL PIGS

Major Field: ANIMAL SCIENCE PRE-VET

Abstract:

Many commercial human infant formulas contain a protein content greater than what is naturally found in human breast milk. Prolonged consumption of protein greater than nutritional requirements in early infancy has been linked to increased risk of obesity and type 2 diabetes, which is characterized by decreased insulin sensitivity and impaired glucose homeostasis. Due to the negative impacts of low-protein diets on infant growth, their use in commercial formula is limited. Alternatively, dietary restriction of insulinogenic amino acids (IAA, i.e., Leu, Ile, Val, Thr, Phe, Arg, and Ala) may be considered. This study aimed to determine the impact of IAA restriction in formula on glucose and lipid metabolism in a neonatal piglet model for human infants. 32 seven-day-old Yorkshire barrows were randomly assigned to one of three dietary treatment groups for 21 days: 1) NR: 0% IAA restriction; 2) R50: 50% IAA restriction; and 3) R75: 75% IAA restriction; with each diet being isonitrogenous and isocaloric. After 21 days, all animals were sacrificed, and liver, skeletal muscle, and white adipose tissue (WAT) samples were collected. Using RT-qPCR the expression of glucose and lipid metabolism and insulin signaling genes in target tissues was determined. Data were analyzed with Univariate GLM with Dunnett's post-hoc (SPSS®). Relative to NR, the R75 treatment group increased the mRNA abundance of key rate-limiting glycolytic enzymes and glucose transporters including hepatic glucose transport 1 (GLUT 1), hepatic pyruvate kinase (PFKL), hepatic pyruvate kinase liver type (PKLR), and WAT glucokinase (GCK). R50 increased the mRNA expression of hepatic and muscular PKLR. Insulin signaling markers significantly improved via increased mRNA of muscular serine/threonine kinase 1 (AKT) and insulin receptor substrate 1 (IRS1) when R75 was compared to NR. IAA restriction by 75% increased the mRNA expression of hepatic fibroblast growth factor 21 (FGF-21), with R50 increasing the mRNA expression of hydroxyacyl-CoA dehydrogenase (HADH). In conclusion, restriction of dietary IAA improved glucose and lipid metabolism in a neonatal piglet model likely through increasing the rates of glycolysis via upregulation of key glycolytic pathway enzymes, and gene expression of key enzymes involved in lipid metabolism.

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CHAPTER I

INTRODUCTION

Background Information on Insulinogenic Amino Acids

Amino acids are the biological building blocks for all proteins consumed by humans. However, this becomes a major health issue because several common amino acids are also considered to be insulinogenic, and they are related to greater insulin resistance and the presence of type 2 diabetes (4). Some of the most common insulinogenic amino acids (IAA) include leucine, isoleucine, valine, lysine, threonine, phenylalanine, arginine, and alanine (4). The following study is one of many that aimed to determine which of the twenty main amino acids were associated with heightened insulin tolerance and insulin sensitivity.

American Diabetes Association Finland Study

In a study involving about 10,000 randomly chosen Finnish men aged 45 to 73 years of age, their glucose tolerance was evaluated over a two-hour oral glucose tolerance test following the ingestion of a concoction of 75g glucose diluted in sterile saline (4). This study also aimed to determine if and to what level any of the twenty main amino acids were associated with insulin secretion and sensitivity (4). The glucose tolerance of the participants was recorded at zero, 30, and 120 minutes after ingestion based on the research criteria determined by the American Diabetes Association (4). Out of the initial test group, 5,181 men participated in the subsequent study after about 7 years, and 522 men of this subset exhibited clinical and laboratory characteristics of type 2 diabetes (4). In a related cross-sectional study, researchers found that phenylalanine, tryptophan, tyrosine, alanine, isoleucine, leucine, valine, aspartate, and glutamate were all consistently associated with reduced insulin secretion, which is a major contributor in

the conversion to type 2 diabetes (4). When cross-examined with the results of the Finnish study, researchers found that phenylalanine, tyrosine, alanine, aspartate, and glutamate were all linked to reduced insulin secretion and insulin sensitivity (4). Tyrosine, alanine, isoleucine, aspartate, and glutamate were also associated with incident type 2 diabetes and increased insulin resistance (4). This study, among others, helped to determine which of the main twenty amino acids were insulinogenic and increased insulin sensitivity in humans.

Correlation Between IAA Consumption and Type 2 Diabetes

The intake of insulinogenic amino acids has been shown to have a correlation with the growing rate of type 2 diabetes among infants, children, and young adults in the United States. In many cases, patients undergo prolonged periods of prediabetes, which later converts into type 2 diabetes when the pancreas ultimately fails to compensate for the heightened insulin resistance and impaired insulin secretion (2). Insulin resistance mainly refers to the body's ability to resist the metabolic effects of insulin, including glucose uptake and changes to metabolic rates (9). It is often associated with type 2 diabetes and obesity when the body's ability to transport glucose and properly metabolize is significantly decreased (9).

Insulin resistance becomes a major issue because the body is designed to respond to insulin when the blood glucose levels are becoming too elevated. However, if a patient is suffering from insulin resistance, the necessary insulin receptors fail to work, and the pancreas ultimately fails to produce additional insulin since the body no longer responds to the hormone. Furthermore, those who experience prediabetes typically experience higher cholesterol levels, elevated blood pressure, greater obesity rates, and lower insulin sensitivity (1). The Centers for Disease Control and Prevention (CDC) has reported that one in five children and one in four young adults under the age of 20 suffer from prediabetes (2).

Center for Disease Control 2020 Type 2 Diabetes Overview

In 2018, 210,000 children and young adults under 20 years of age were diagnosed with type 2 diabetes out of roughly 30 million people total (2). Unfortunately, nearly 7.3 million young adults were unaware they met the national criteria for type 2 diabetes, thus increasing the prolonged health issues caused by undiagnosed cases (2). Those who suffer from type 2 diabetes are also predisposed to "chronic kidney disease and cardiovascular disease," further exacerbating the growing health risks (1). Concerningly, it has been estimated that the prevalence of type 2 diabetes will increase by about four times within the following decades (3). Due to the severe health ramifications, the aim to reduce the prevalence of type 2 diabetes, especially in infants, children, and young adults, has become crucial.

The protein content of various foods has increased over the last several years, especially in commercially produced baby formulas (4). It has been recently documented that most baby formulas that contain dairy protein have a significantly higher protein content compared to human breast milk (5). However, the recommended protein requirements for children and young adults have decreased by 8-25% (6) due to increases in the prevalence of type 2 diabetes, childhood obesity, and insulin resistance (7,8). Constant exposure to high insulin levels due to the increased levels of proteins ultimately decreases the uptake of glucose and is often linked with late-stage metabolic diseases, including type 2 diabetes and severe insulin resistance (9,10).

American Journal of Nutrition 2008 Childhood Obesity Study

A study conducted in 2008 aimed to determine that higher protein intake increased the secretion of insulin-like growth hormone (IGF-I) and extensive cell proliferation, thus leading to rapid weight gain in infants and predisposing them to childhood obesity (12). The study looked

at 1,138 healthy, formula-fed infants up to two years of age, and they were split into two experimental groups based on a lower or higher protein content of cow milk-based formula (12). Birth weight, body length, body width, and BMI measurements were recorded each time the children were present at the research center up until they reached the age of two (12). In addition, the food intake of all the participants was recorded on a three-day basis (12).

At the conclusion of the 2008 study, nearly 572 children were excluded from the study (12). The reasons for exclusion varied among the excluded children, ranging from illness, medication use, failure to follow-up, or lack of compliance (12). The children in the higher protein formula group were significantly larger in weight and BMI scores up until six months of age (12). The children in the lower protein formula group, by comparison, were reportedly smaller in both height, length, and weight throughout the duration of the study (12). Ultimately, it was concluded that limiting the dietary protein content of infant and follow-up formula may prove to be a beneficial approach in reducing the prevalence of childhood obesity and childhood type 2 diabetes (12).

Neonatal Pigs as Agromedical Models for Human Infants

Besides the previously mentioned study, there is limited data available regarding low-protein formulas, which is why most pediatricians are hesitant to recommend their usage. In addition, these same issues related to insulin tolerance are being raised with the feed content of swine because if swine consumed higher protein diets, the subsequent food products derived from these swine will only exacerbate the above issues. Due to the similar anatomy, physiology, and genomes, swine populations are often used as models in research studies regarding human health and safety. This is why neonatal and nursery pigs are then used as models for human

infants and children when studying the effects of amino acid restriction on glucose tolerance, insulin sensitivity, and subsequent growth rates.

CHAPTER II

OBJECTIVE AND HYPOTHESIS

Type 2 diabetes has become a rising concern amongst the public, especially due to its prevalence in infants, children, and young adults. This disease is linked to prolonged periods of insulin resistance, which affects the body's ability to control its glucose levels and negatively impacts its metabolic rates (9). In patients suffering from increased insulin sensitivity, the receptors responsible for maintaining the body's insulin control begin to fail, and the pancreas will eventually exhaust itself because any additional insulin produced will no longer have any effect on the body's blood glucose levels.

The main objective of this research project is to determine how the restriction of dietary insulinogenic amino acids impacts glucose tolerance and insulin sensitivity in nursery piglets. In addition, we aim to determine the prevalence of different genes related to glucose metabolism, thus creating new potential therapies for treating obesity and diabetes. By reducing the intake of insulinogenic amino acids in swine, consumers of swine-derived products should face a lower risk of developing insulin resistance and type 2 diabetes.

CHAPTER III

MATERIALS AND METHODS

Animals and Housing

The baseline project conducted by Dr. Adel Pezeshki and his graduate students studied the effects of restricting insulinogenic amino acids at two different doses and how each dose affected the glucose tolerance and growth rate of neonatal pigs (11). Thirty-two seven-day-old Yorkshire barrows were housed in individual pens, and all were fed with a milk replacer feeder for three weeks. Each pen consisted of a heated plastic floor mat and lighting protocols of sixteen-hour light and eight-hour dark cycles were followed throughout the preliminary study.

Experimental Design

In this study, the piglets were split into three experimental groups based on three milk-based diets. These diets included the control group with no amino acid restriction (NR), the 50% amino acid restriction group (R50), and the 75% amino acid restriction group (R75) (11). After two weeks and one overnight fast, an oral glucose tolerance test was performed in all the piglets using a 50% dextrose solution diluted with sterile saline (11). After an eight-hour fast on day 21, the piglets were permitted access to their respective diet for 30 minutes, and then baseline blood samples were collected from the jugular vein. Then, subsequent blood samples were taken in 30-minute increments up until 120 minutes post-meal. Blood glucose levels were recorded using a handheld glucometer immediately after each round of blood sampling, and the blood plasma and serum were stored at -80°C for future analysis via RNA isolation and RT-qPCR. The piglets were humanely euthanized, then tissue samples of the spleen, liver, hypothalamus, pancreas, and muscle and colon contents were collected, frozen in liquid nitrogen, and stored at -80°C.

RNA Isolation and RT-qPCR

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is the main focus of the undergraduates involved in this experiment. We have seen improvements in glucose tolerance, but it is currently unknown to the level of correlation between insulin sensitivity and gene expression, which is why we are looking at the metabolism markers in muscle, liver, and adipose tissue. The RNA was initially transcribed into the complementary DNA form, which is then used as the template for real-time polymerase chain reactions. Then, the amount of amplification product and RNA abundance was quantified for different genes of interest using a standardized melt curve and the respective primers for each gene of interest. Each test plate was completed using a standardized procedure developed by Dr. Pezeshki, then the plates were spun down using a microplate spinner to remove all bubbles. Once all bubbles were removed, the plates were placed into a plate reader that followed said standardized melt curve, and each run occurred for two hours. RT-qPCR was completed for different genes of interest including, but not limited to, glucokinase (GCK), glucose transporter 2 (GLUT 2), phosphofructokinase liver type (PFKL), insulin receptor (IR), fibroblast growth factor (FGF 21), glucose synthase kinase 3 alpha (Gsk-3α), glucose synthase kinase 3 beta (Gsk-3β), and glucose transporter 4 (Glut 4).

Statistical Analysis

Specific genes and RT-qPCR data were analyzed using the GLM univariate procedure (IBM SPSS Statistical Version 26, Armonk, NY, USA), followed by running the data through Dunnett's post hoc test (IBM SPSS Statistical Version 26, Armonk, NY, USA). Hepatic FGF-21, IR, GLUT4, S6K, LPL, skeletal muscle HK2, PKLR, SREBP1, and WAT GCK, PKLR, S6K, FGF-21, and ACC expression distribution was abnormal, so the data set was then normalized via an inverse distribution function (IDF-normal). An outlier test based on the Interquartile rule was

performed in SPSS (IBM SPSS Statistical Version 26, Armonk, NY, USA), followed by a normality test before statistical analysis of all data. Data with *P*-values ≤ 0.05 were considered significant, whereas data with P-values between $0.05 \leq 0.10$ were considered trends. All significant data was then placed into Prism to create high-quality graphs highlighting any definitive differences between NR, R50, and R75 across genes of interest within liver, muscle, and white adipose tissue.

CHAPTER IV

RESULTS AND DISCUSSION

Glucose Metabolism

Significant improvements to glucose metabolism through increased mRNA expression occurred in hepatic PFKL, PKLR, and adipocyte GCK (Figure 1A - C). These improvements were noted in the R75 treatment group across hepatic PKLR, PFKL, and adipocyte GCK. Improved glucose metabolism allows the body's metabolism to efficiently process glucose and store it as an energy source. Furthermore, increased glucose metabolism leads to better insulin resistance and sensitivity, thereby reducing the risk of type 2 diabetes because blood glucose levels are being properly maintained.

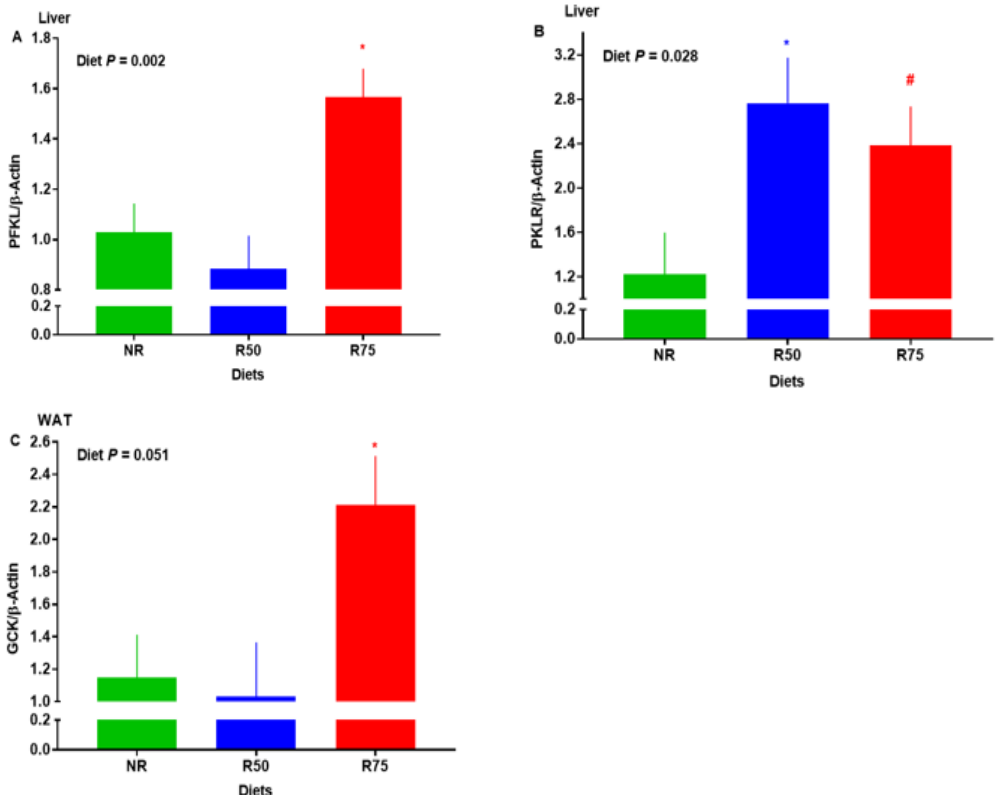

Figure 1. mRNA expression related to glucose metabolism markers of hepatic PFKL (A), hepatic PKLR (B), and adipocyte GCK (C).

Glycogen and Lipid Metabolism

Glycogen metabolism increased in the R50 treatment group compared to the NR treatment group via elevated mRNA expression of hepatic GSK-3β and muscle Gys2 (Figure 2D - E). In addition, lipid metabolism was significantly increased in the R50 treatment group through increased mRNA expression of adipocyte FAS and hepatic HADH compared to the NR treatment group (Figure 2F – G). Hepatic LPL expression significantly decreased in the R75 treatment group compared to the NR treatment group (Figure 2H). Improved glycogen and lipid metabolism prevent the accumulation of excess glycogen and low-density lipoprotein, thus reducing the risk of impairing pancreatic functions and developing type 2 diabetes (15).

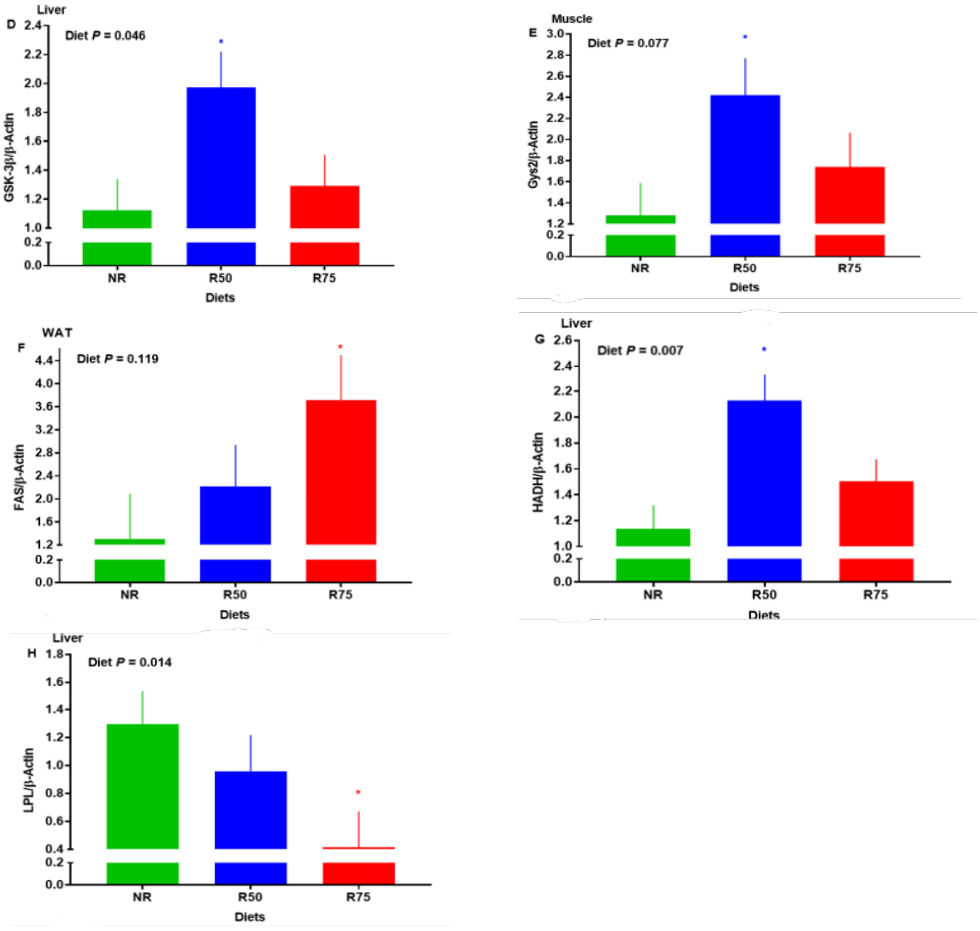

Figure 2. mRNA expression concerning glycogen metabolism markers of hepatic GSK-3 β (D) and muscle Gys2 (E); mRNA expression related to lipid metabolism markers of adipocyte FAS (F), hepatic HADH (G), and hepatic LPL (H).

Insulin Signaling Pathway

Finally, it was determined that the mRNA expression of IRS1 and AKT in skeletal muscle was significantly elevated in R75 compared to NR (Figure 3I – J). Therefore, the R75 treatment group improved the insulin signaling pathway in IRS1 and AKT in skeletal muscle in comparison to NR. Type 2 diabetes blocks the signal released by insulin receptors, which prevents the necessary signals from registering in the cells designed to regulate blood glucose and lipid metabolism, thereby causing hyperglycemia (16). Furthermore, the lack of signal recognition causes the body to overproduce insulin, which eventually causes cells to become more insulin resistant. However, by improving the effectiveness of insulin receptors, the body becomes more sensitive to insulin, allowing the body to successfully regulate blood glucose and lipid concentrations.

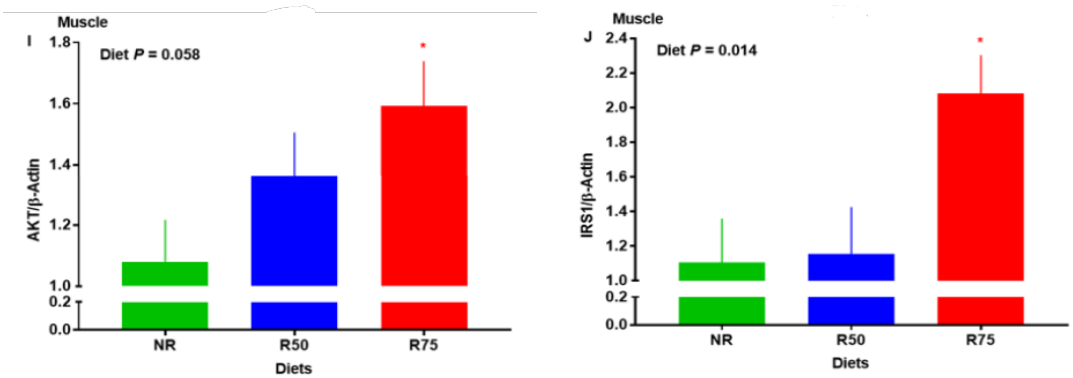

Figure 3. mRNA expression correlating to insulin signaling pathway markers of muscle IRS1 and AKT (I, J).

CHAPTER V

CONCLUSION

Both R50 and R75 altered the lipid and glycogen metabolism in the liver, muscle, and white adipose tissue samples compared to NR. In addition, R75 improved glycolysis in the liver and white adipose tissue samples and enhanced the insulin signaling pathway in skeletal muscle samples compared to NR. Restricting the insulinogenic content did exhibit improvements in increasing insulin sensitivity and insulin resistance, but additional tests are likely needed to determine the full range of restriction without causing additional health problems. Future experiments may observe more long-term effects in these experimental groups to determine if the amino acid restriction has any inhibitory functions during growth development.

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I am from Frisco, TX and I was born on July 16th, 2002. I currently work part-time during semester breaks at Frisco Emergency Pet Care in Frisco, TX. I graduated from high school with a 3.9 GPA. Since being in college, I have achieved Early Admission into OSU-CVHS and I am pursuing a bachelor's degree before entering veterinary school in Fall 2024. I have also made it through Organic Chemistry I and II, Organic Chemistry Lab, and Survey of Biochemistry with a 3.97 GPA while balancing my other animal science classes and Honors courses.

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