Université de Montréal

Impact of Neonatal Total Parenteral Nutrition and Early Glucose-enriched Diet on Glucose Metabolism and Physical Phenotypes in Guinea Pig

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Ce mémoire intitulé:

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RÉSUMÉ

Les oxydants infusés avec la nutrition parentéral (NP) néonatale induisent une modification du métabolisme des lipides et du glucose, donnant lieu à l'âge adulte à un phénotype de carence énergétique (faible poids, baisse de l'activité physique). L'hypothèse qu'une diète précoce riche en glucose prévient ces symptômes plus tard dans la vie, fut évalué chez le cobaye par un ANOVA en plan factoriel complet à deux facteurs ($p < 0.05$) : NP du jour 3 à 7, suivit d'une nourriture régulière (chow) (NP+) *vs.* chow à partir du $3^{\text{ième}}$ jour (NP-), combiné avec une eau de consommation enrichie en glucose (G+) ou non (G-) à partir de la $3^{\text{ième}}$ semaine. Les paramètres suivant ont été mesurés à l'âge de 9 semaine: taux de croissance, activité physique, activité de phosphofructokinase-1 et glucokinase (GK), niveau hépatique de glucose-6-phosphate (G6P), glycogène, pyruvate et potentiel redox du glutathion, poids du foie, glycémie, tolérance au glucose, concentrations hépatiques et plasmatiques en triacylglycérides (TG) et cholestérol. Le groupe G+ (*vs.* G-) avait un taux de croissance plus bas, une activité de GK et une concentration en G6P plus élevée, et un potentiel redox plus bas (moins oxydé). Le niveau plasmatique de TG était moins élevé dans le groupe NP+ (*vs.* NP-). Les traitements n'eurent aucun effet sur les autres paramètres. Ces résultats suggèrent qu'indépendamment de la NP, une alimentation riche en glucose stimule la glycolyse et déplace l'état redox vers un statut plus réduit, mais ne surmonte pas les effets de la NP sur le phénotype physique de carence énergétique.

Mots-clés: Nouveau-né, nutrition parentérale, métabolisme du glucose, potentiel redox, programmation métabolique, cobaye, impact à long terme.

ABSTRACT

Neonatal exposure to oxidant molecules from total parenteral nutrition (TPN) alters future lipid and glucose metabolism, resulting in an energy deficient phenotype characterized by lower body weight and physical activity. Using a guinea-pig model, the hypothesis that early diet supplementation with glucose could overcome such symptoms at week 9 of age was tested in a two-factor full-factorial ANOVA design ($p < 0.05$): TPN day 3-7, chow thereafter (TPN+) *vs*. chow from day 3 (TPN-), combined with glucose-enriched diet from week 3 (G+) *vs*. plain chow throughout (G-). The growth rate, physical activity, phosphofructose kinase-1 and glucose kinase (GK) activities, glucose-6-phosphate (G6P), glycogen and pyruvate concentrations, relative liver weight, fasting blood glucose, glucose tolerance, hepatic and plasma triacylglyceride and cholesterol levels, individual glutathione levels and GSH/GSSG-based redox potential were determined at 9 weeks. Glucose supplementation (*vs*. the lack thereof) resulted in a lower growth rate, higher GK activity, and higher G6P concentration at week 9. Plasma triacylglycerides at week 9 were lower in TPN+ (*vs*. TPN-) subjects. Hepatic GSH/GSSG-derived redox potential shifted to a more reduced state in G+ (*vs*. G-) subjects. No other parameters showed significant differences. Independently of TPN, an early glucose-rich diet stimulated the glycolysis pathway, shifted the redox potential towards a more reduced status ; however, it did not overcome the effects of TPN on future physical and metabolic phenotype.

Keywords: Neonates, total parenteral nutrition, glucose metabolism, redox potential, metabolic programming, guinea pig, long-term impact.

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LIST OF ABBREVIATIONS

ATP	Adenosine Tri-Phosphate
BMI	Body Mass Index
BSA	Bovine Serum Albumin
DNA	Deoxyribo Nucleic Acid
DTT	1, 4-Dithiothreitol
EGTA	Ethylene Glycol Tetraacetic Acid
GI	Gastro Intestinal
GK	Glucokinase
GSSG	Glutathione Disulfide
GSH	Glutathione
G6P	Glucose 6-Phosphate
G6PH	Glucose-6-Phosphate Dehydrogenase
HNE	4-Hydroxy-2-Nonenal
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NICU	Neonatal Intensive Care Unit
OS	Oxidative Stress
PFK-1	Phosphofructokinase-1
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
TCA	Trichloroacetic Acid
TPN	Total Parenteral Nutrition

I would like to dedicate my thesis to

my beloved parents, brother, and dear husband

for all their faith, support and love.

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CHAPITRE 1

LITERATURE REVIEW

Premature birth occurs in 12.5% of deliveries in the United States and 8% in Canada (2006-2007) [1]. Many of these infants do not develop any prematurity problems since they are born only a few weeks prior to full term delivery; however, others are at risk of serious complications throughout their lifetime. For instance, infants born before 28 weeks of gestation are at high risk for chronic diseases such as diabetes and cardiovascular disorders [2–9]. Epidemiological evidence demonstrates that a suboptimal gestational and postnatal environment, including nutrition, plays a key role in the associated increase of metabolic diseases observed in adults [10–13].

Infants born before 28 weeks of gestation cannot be fed enterally [3, 6, 7] due to the immaturity of their gastrointestinal tract [10–17]. Intravenous nutritive support - or total parenteral nutrition (TPN) - is therefore essential for their growth and development. Unfortunately, TPN formulations are contaminated with oxidant molecules [18–21], mainly hydrogen peroxide (H_2O_2) [22]. Such peroxides are the end products of reactions between oxygen dissolved in the TPN solution and various electron donors, such as ascorbate, amino acids, and lipids [18, 19, 22–25]. Given that such reactions are catalyzed by photo-excited riboflavin, a vitamin present in TPN formulations [26, 27], protection against ambient light can halve the peroxide concentrations in TPN solutions [28]. Laborie S *et* al. observed that the use of TPN not afforded full protection from light was correlated with an increase in peroxide concentrations in the urine of premature infants [29]. Their observation suggests that this population is unable to quench this oxidant load, possibly as a result of their immature antioxidant defense system [29–34]. In the short-term, infusion of TPN or a solution bearing a similar quantity of peroxides during the neonatal period is associated with a perturbation of lipid and glucose metabolism [35]; however, the long-term impacts of delivering such oxidant molecules through TPN infusions remain unclear.

"The developmental origin of adult health and disease hypothesis" affirms that the origin of several adult metabolic diseases (*e.g.* cardiovascular diseases, type II diabetes, and hypertension) can be traced back to fetal and postnatal life [36–38]. In an animal model of neonatal parenteral nutrition, Kleiber N *et* al. demonstrated that early intravenous exposure to peroxides, as generated in this nutritive solution, induces permanent modifications in the metabolism of lipids and glucose later in life [35]. They suggested that peroxides contaminating TPN solutions permanently modify the metabolism of energy, thus perturbing growth rate, physical activity, and metabolic responses later in life [35]. The phenotype of animals thus afflicted was one of energy deficiency [35].

Several studies support the notion that one mechanism by which postnatal nutrition affects the long-term outcome is developmental programming [9, 39]. Using a guinea pig model of neonatal parenteral nutrition, the present study was designed to investigate the long-term (eight weeks after ending TPN) effects of a 4-day neonatal exposure (Day 3 to 7 of life) to TPN, combined with an early diet enriched in glucose (Week 3 to 9 of life) on their glucose metabolism 8 weeks after stopping TPN (*i.e.*, 9 weeks from birth). The introduction of glucose to the diet was intended to assess if high caloric nutrient enrichment of the diet could overcome the energy deficiency previously observed by Kleiber N *et* al. [35].The following sections briefly explain the notion of developmental programming and oxidative stress observed in neonates in relation to parenteral nutrition.

1.1 Concept of Developmental Programming

The association between early life events and the development of adult diseases has recently become widely recognized [9]. This association is mainly attributed to the programming concept stemming from Barker's hypothesis of "developmental origin of adult health and disease" [9]. Programming, or the modulation of the gene expression, represents the ability to adapt to environments encountered during development [9]. Although

the short-term gains are adaptation to the environment, the long-term costs are permanent changes in the character of tissues [40] and in gene expression profiles [9].

1.2 Risk Factors in Postnatal Programming

Several risk factors hinder postnatal programming. One such factor is the infants' size at birth. Barker has shown that there exists a causality between low birth weight and diseases that may generate adult coronary heart disease as well as metabolic disorders such as hypertension, type II diabetes, and hyperlipidemia [2, 4, 5, 8]. It is well established that the correlation between small birth size in neonates kept in the NICU (Neonatal Intensive Care Unit) and impaired glucose regulation later in life is much greater than that for individuals born at term [41].

The second factor is postnatal nutrition and dietary composition. Preterm infants are at risk of postnatal over-nutrition and rapid catch-up growth [39, 42]. Kashyap S *et* al. observed that high non-protein energy feeding of low birth weight infants resulted in a weight increase due to body fat rather than lean body mass [43]. However, high-protein diets may induce the proliferation of adipose formation and obesity later in life [44]. The Dortmund Nutritional and Anthropometric Longitudinally Designed Study (DONALD), a longitudinal study of 216 individuals, evaluated the associations between added sugar intake during early childhood and body mass index (BMI) and body fat at 7 years of age [45]. The study showed that while a higher total added sugar intake during the first year of age was correlated with a lower BMI at 7 years of age, the same added sugar intake during the second year of life was associated with a higher BMI at 7 years of age. However, no associations were observed with the percentage of body fat [45]. Kleiber N *et* al. demonstrated in guinea pigs the relation between early TPN and a decrease in level of plasma triglyceride later in life [35]. Various dietary regimens may likewise have an effect on organ development. Rat pups with high protein intake showed greater growth rate in the short term, as well as insulin resistance [46]. In the rat pups-in-the cup model with high carbohydrate intake, alterations in adult metabolism, along with obesity,

were observed [47–54]. In this population, modification in individuals' insulin secretory capacity increased hexokinase activity and insulin biosynthesis [47–54]. In this model, a high carbohydrate diet in early life augments the quantity of pancreatic islets [47–54]. Furthermore, evidence suggests that breastfeeding during the postnatal period prevents the development of metabolic diseases in full term infants. Later in life, breastfed infants have lower blood pressure, total cholesterol, and prevalence of overweightness and obesity [55–57].

The third risk factor is postnatal growth rate. It has been observed that in low birth weight infants, rapid postnatal growth rate over the first 4 months leads to a greater body weight later in life [58]. Animal studies have demonstrated the effect of postnatal growth rate in metabolic diseases. In the rat model, accelerated growth altered the metabolic parameters in response to a high-fat diet, as well as increased weight and percentage of body fat [59]. These observations confirm the Lucas hypothesis of "accelerated growth", which illustrates the connection between faster early postnatal growth in preterm infants and an increased risk of the metabolic syndrome indicators [60, 61].

Another risk factor in programming is oxidative stress (OS). Oxidative stress programming occurs directly through the modulation of gene expression or indirectly through the oxidation of lipids, proteins, and DNA [62]. In preterm infants, oxidative stress may increase the prevalence of the metabolic syndrome, type II diabetes, and several other metabolic disorders later in life [35–38]. Kleiber N *et* al. have shown in the guinea pig model that exposure to oxidative stress early in life effects a permanent modification in the programming of lipid and glucose metabolism, resulting in energy deficiency observed later in life [35]. Such modifications in the guinea pig animal model had long-term effects on the animals' growth and physical activity [35].

1.3 Oxidative Stress

Reactive oxygen species (ROS) are byproducts of mitochondrial electron transport in the cellular respiratory chain. Moreover, they are inevitable byproducts of several cellular and extracellular redox reactions [62–64]; hence, they are constantly produced within the organism. In moderate concentrations, ROS regulates enzymes and redox-sensitive gene expression [63, 65, 66]. The antioxidant system sustains the homeostasis of ROS inside the cells.

Conversely, an imbalance between ROS load or its generation and antioxidant defenses within the cell, known as oxidative stress, can cause serious cellular damage. Superfluous amounts of oxidants may react with cellular macromolecules, thereby inducing lipid peroxidation and modifications of proteins and DNA [67, 68]. Biological activities generally decrease with the attack of oxidized products of ROS, resulting in metabolic dysregulation and an alteration in cellular signaling functions [43, 53]. Studies have demonstrated that premature infants are more prone to oxidative stress due to environmental, biological, and nutritional conditions, such as early postnatal use of parenteral nutrition [18, 19, 22–24].

1.4 Total Parenteral Nutrition

Parenteral nutrition was first introduced in 1967 to human infants suffering from extreme short bowel syndrome [69]. Today, parenteral nutrition refers to the administration of supplemental intravenous nutrients via a continuous infusion through a peripheral or central vein [68]. Since it contains all the essential nutrients for metabolic requirements and growth, it was named total parenteral nutrition (TPN) [11, 70]. This intravenous solution includes glucose, amino acids, lipids, vitamins, and trace elements [11, 70]. In North America, TPN is administered without adequate light protection [71, 72].

1.5 Total Parenteral Nutrition as a Source of Oxidant Molecules

Silvers KM *et* al. showed that exposure to light decreases the levels of antioxidant vitamins (*i.e.* vitamins A, C, and E) in the TPN solution [25]. The absence of full photo protection also increases or catalyzes the generation of byproducts of nutrient oxidation such as hydrogen peroxide (H_2O_2) [19], lipid peroxides [18], as well as aldehydes, especially 4-hydroxy-2-nonenal [24], and ascorbylperoxide [23]. These products are generated by a reaction between oxygen dissolved in the solution and different electron donors such as ascorbate, amino acids, or lipids [20, 21, 73]. Lavoie JC *et* al. demonstrated that the H_2O_2 represents approximately 80% of the total peroxides generated in TPN solutions [22]. Given that ascorbate is the prime electron donor, the main source of peroxides is the multivitamin preparation [22, 26, 27]. Furthermore, multivitamin preparations used in parenteral nutrition also contains riboflavin, a photo-sensitive component which acts as a catalyst in the photo-oxidation of nutrients [26, 27]. Lavoie JC *et* al. showed a rise in urinary peroxide concentration in newborn premature infants who received TPN exposed to light [29]. The study confirms premature infants' incomplete capacity to scavenge peroxides [29]. This fact may be related to the immaturity of the antioxidant defenses of preterm newborns. Indeed, several studies have demonstrated that newborn antioxidant defenses increased in function of the age of gestation. Frank L *et* al. [74, 75] showed that activities of antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase increase in the last trimester of gestation. Lavoie JC *et* al. [76] reported that the level of glutathione, the key antioxidant molecule, is directly proportional to the gestational age. Therefore, this population is more prone to oxidative stress due to the high oxidant load from TPN and their low antioxidant levels.

The impact of oxidative load from TPN cannot be adequately studied in premature infants since appropriate control group, premature infants (<28 weeks of gestation) without TPN, does not exist. Therefore, only a reduction of peroxides in TPN by light protection can be done. However, light protection is difficult in clinical situations. The few scientific teams who have studied the impact of oxidants infused with TPN have

reported that the absence of light protection is associated with higher oxidative stress in premature infants [27, 28], lower plasma triacylglycerol and blood glucose [35], lower arterial blood pressure [77], and higher incidence of chronic lung diseases [27] in the short term. No data is available on the long-term impact of light protection of TPN in humans.

The guinea pig model of neonatal TPN has been developed in our laboratory to study the impact of TPN compared to a control group without TPN. Several studies with this animal model reported that TPN is related to oxidative stress in the lungs, blood and liver [27, 28, 78, 79], hepatic steatosis [28], and higher plasma triacylglycerol levels [80]. One study demonstrated that the administration, in the first week of life, of either TPN or a solution of H_2O_2 at a concentration equivalent to the total peroxides measured in the TPN, both induced a perturbation of energy metabolism 12 to 13 weeks after cessation of the infusion [35]. Compared to control groups, without TPN or peroxides, animals infused with TPN or peroxides had a lower plasma triacylglycerol level, a lower glucose tolerance, a lower body weight, and a lower propensity to spontaneous activity. We can conclude from this study that TPN induced an energy deficiency later in life [35].

Following these observations in the guinea pig system, the present research with this animal model was designed to explore the impact of a diet enriched in glucose, as a supplemental source of calories, on glucose metabolism and physical phenotypes 8 weeks after that the administration, or not, of TPN during their first week of life. The next section provides an overview of glucose metabolism.

1.6 Glucose Metabolism Pathways

Carbohydrates such as glucose are important parts of our diet. Glucose is an important source of energy for body cells. In animals and humans, glucose has three main fates (figure 1.1) [81]:

– It may be oxidized into pyruvate via the glycolysis pathway to provide ATP and metabolic intermediates if the body and cells need energy [81].
– It may be stored as glycogen via the glycogenesis pathway if the body has excess blood glucose [81].
– It may be oxidized via pentose phosphate pathway into ribose 5-phosphate for synthesis of nucleic acids and NADPH for reductive reactions, especially glutathione reduction [81].

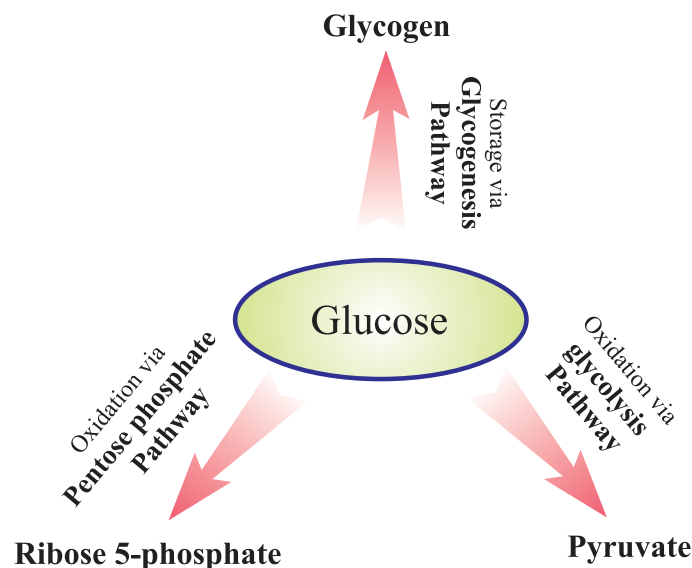

FIGURE 1.1 – Major pathways of glucose in the body (adapted from Lehninger C [81]).

1.6.1 Overview of Glycolysis Pathway

Glycolysis, the first and central pathway of glucose catabolism, was discovered in 1897 by Eduard Buchner [81]. In glycolysis, a molecule of glucose is degraded in a series of enzyme-catalyzed reactions to yield two molecules of the three-carbon compound pyruvate. During these sequential reactions of glycolysis, some of the free energy released from glucose is conserved in the form of ATP and NADH. The breakdown of the

six-carbon glucose into two molecules of the three-carbon pyruvate occurs in ten steps, which can be categorized in two phases as illustrated in figure 1.2;

- The preparatory phase, in which glucose is phosphorylated and converted into glyceraldehyde 3-phosphate.
- The pay off phase, in which glyceraldehyde 3-phosphate is oxidized into pyruvate, while generating ATP and NADH.

To summarize, in the preparatory phase of glycolysis, the energy of ATP is invested, raising the free-energy content of the intermediates, and the carbon chains of all the metabolized hexoses are converted into a common product, glyceraldehyde 3-phosphate, while in the payoff phase the desired energy is released [81].

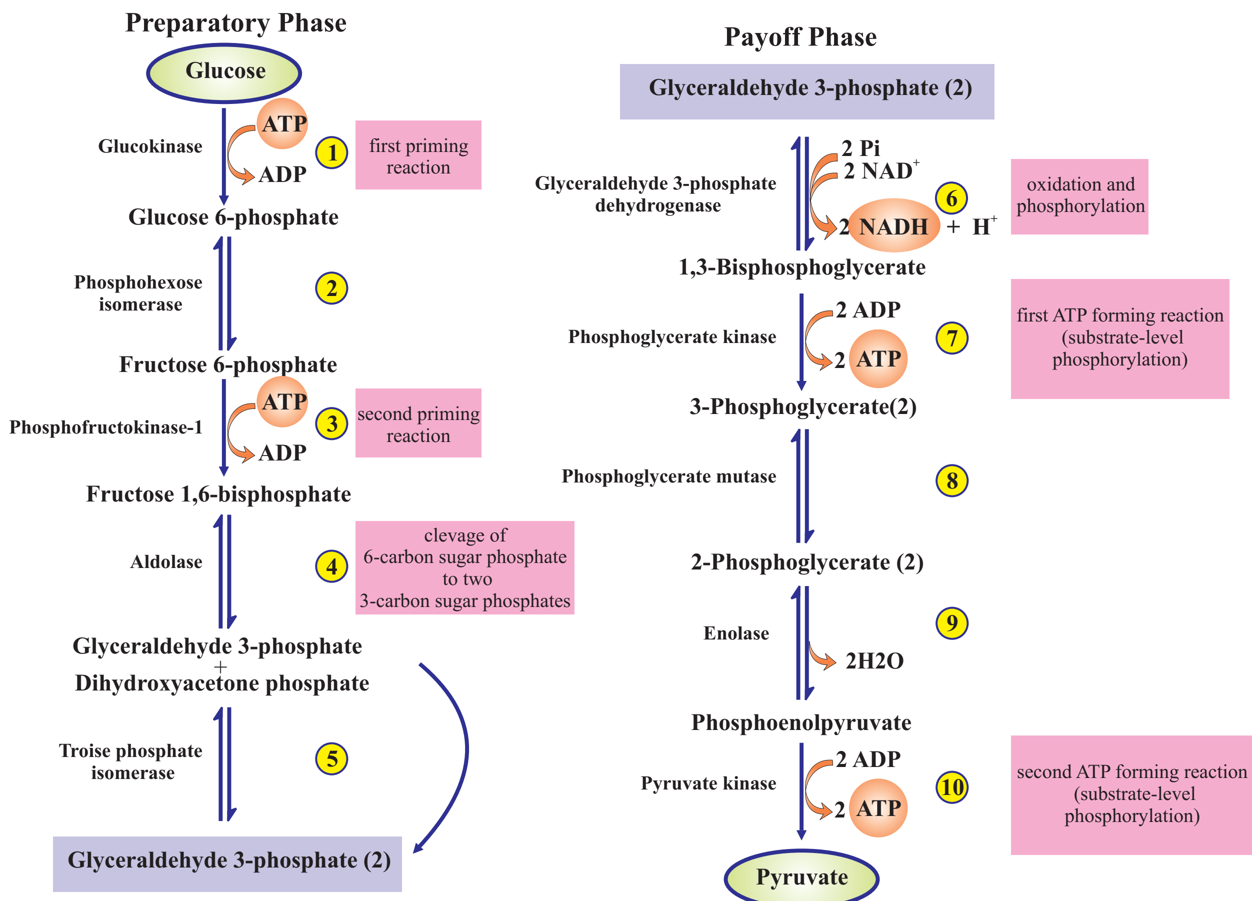

FIGURE 1.2 – Glycolysis pathway (adapted from Lehninger C [81]).

The rate of glucose metabolism is adjusted by a complex interplay between ATP consumption, NADH regeneration, and allosteric regulation of several glycolytic enzymes, including hexokinase, phosphofructokinase-1 (PFK-1), and pyruvate kinase, and by second-to-second fluctuations in the concentration of key metabolites that reflect the cellular balance between ATP production and consumption [81]. Neonatal exposure to oxidant molecules has been noted to lead to permanent modification on the programming of glycolysis, resulting in metabolic diseases later in life [35, 81]. The most important modifications are lower phosphofructokinase activity, higher glucokinase activity, lower blood glucose, lower glucose tolerance and lower hepatic and plasma triacylglycerol concentrations [35]. Considering the origin of the "adult health and disease" hypothesis, such a metabolic modification early in life holds several consequences for metabolic disorders such as cardiovascular disease, type II diabetes and lower overall energy for growth and development later in life [36–38]. In the present study, the effects of neonatal exposure to oxidant molecules, along with an early glucose supplementation, on the programming of glycolysis was investigated. To ascertain if glucose has entered the glycolysis (*vs.* the glycogenesis or pentose phosphate) pathway, glucose 6-phosphate (G6P) and pyruvate levels, as well as glucokinase (GK) and phosphofructokinase-1 (PFK-1) activities, were measured. These glycolytic pathway catalysts are briefly discussed in the next few sections.

1.6.1.1 Glucokinase (Hexokinase IV))

GK is the first key enzyme in glycolysis. It catalyzes the phosphorylation of glucose substrate to glucose 6-phosphate (section 1.6.1.2). Glucokinase or hexokinase IV is the hepatocyte form of hexokinase, which differs from other forms of hexokinase in its kinetic and regulatory properties [82]. Such differences have the potential to fine-tune metabolic rates. For example, the sensitivity of glucokinase, an allosteric enzyme [82], to be inhibited by glucose 6-phosphate is different from that of other types of hexokinase present in other tissues [73, 81]. Glucokinase has a lower affinity for glucose than the other hexokinases. This enzyme plays a key role in the liver to maintain blood glucose homeostasis by removing blood glucose for its transformation into glycogen (glycoge-

nesis pathway) or pyruvate (glycolysis pathway), depending on the prevailing glucose concentration [73]. Kleiber N *et* al. demonstrated that GK activity during guinea pigs' first 13 weeks of life were greater when these were exposed to neonatal oxidative stress caused by the oxidant load of TPN [35]. The present study focuses on the effects of neonatal exposure to oxidant molecules on GK activity at guinea pigs' ninth week of life, and the effects of early glucose supplementation on this activity. In fact, one of the aims of the present study is to demonstrate the probable relation between early glucose supplementation and alteration of GK activity in animals exposed or not to TPN in their first week of life.

1.6.1.2 Glucose 6- phosphate

Glucose 6-phosphate is glucose molecule phosphorylated on carbon 6 [83]. This compound is very common in cells since almost the entire amount of glucose entering a cell becomes phosphorylated in this way [83]. Glucose 6-phosphate initiates all of the glucose metabolism pathways; glycolysis, glycogenesis, and the pentose phosphate pathway [81, 84]. The phosphorylation of glucose is catalyzed by the enzyme hexokinase in most cells and by glucokinase in liver cells.

1.6.1.3 Phosphofructokinase-1

PFK-1 is the next key enzyme in glycolysis. It phosphorylates fructose 6-phosphate to produce fructose 1,6-bisphosphate [81, 85]. It has an allosteric regulatory role in the body, thereby regulating the rate of glycolysis [81, 86–88]. Its activity increases when ATP for cell requirements is depleted, and is inhibited under high levels of ATP or fatty acids in the organ [81].

1.6.1.4 Pyruvate

Pyruvate is the final product of the glycolysis pathway, where one molecule of glucose breaks down into two molecules of pyruvate. Through glycolysis, energy is released ; however, further energy may be obtained upon the pyruvate entering one of two cycles :

(i) its conversion into acetyl-coenzyme A through the Krebs cycle or (ii) its conversion into oxaloacetate through the citric acid cycle. Measurement of pyruvate as the final product of the glycolysis pathway reveals the probability of the entrance of glucose into this pathway [81].

1.6.2 Overview of Glycogenesis Pathway

The storage of glucose in the form of glycogen in the liver and other body tissues occurs via a metabolic activity known as glycogenesis. In theory, it is defined as a process in which glucose molecules are added to glycogen chains. Glycogenesis is activated when the body is in a state of rest or during instances of high glucose levels in the blood. Insulin activates this process to reduce blood sugar levels [81].

Glycogenesis contains two main steps. However, similar to the other glucose metabolism pathways, initially glucose is converted into glucose 6-phosphate by the action of glucokinase. Then, glucose 6-phosphate is converted into glucose 1,6-bisphosphate by phosphoglucomutase and later into glucose 1-phosphate.

– The first step involves synthesis of UDP-glucose from glucose 1-phosphate in the presence of UDP-glucose pyrophosphorylase. A hydrolytic process converts pyrophosphate to orthophosphate, making this step a non-reversible reaction [81, 89].
– Glycogen synthase catalyzes the linkage of the previously generated UDP-glucose through a hydroxyl group in the glycogen molecule, forming α-1,4-glycosidic link. This binding requires a protein like glycogenin which contains the glycogen branching enzyme [81, 89].

In this study, the possibility of the entrance of glucose into the glycogenesis pathway is investigated through the measurement of glycogen levels.

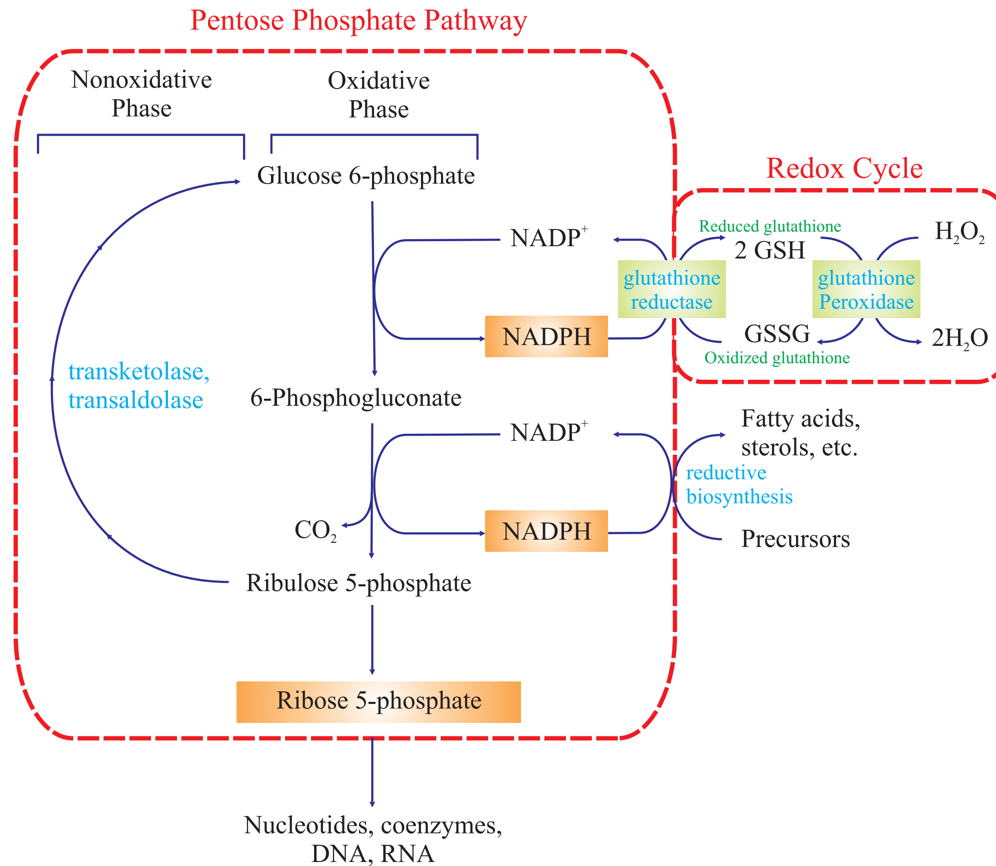

FIGURE 1.3 – Pentose phosphate pathway in the body in connection with redox cycle (adapted from Lehninger C [81]).

1.6.3 Overview of Pentose Phosphate Pathway

Althought glucose 6-phosphate is mostly converted into pyruvate through glycolysis, G6P may enter an alternative catabolic pathway known as the pentose phosphate pathway. This pathway (figure 1.3) contains two distinct phases: (i) the oxidative phase, where NADPH is generated, and (ii) the non-oxidative synthesis of pentose [81]. In the oxidative phase, two molecules of $NADP^+$ are reduced to NADPH, using the energy from the conversion of G6P into ribose 5-phosphate (figure 1.3). The ribose 5-phosphate produced can be used in the synthesis of nucleotides and nucleic acids. Within cells that are not using ribose 5-phosphate for biosynthesis, the nonoxidative phase recycles six molecules of the pentose into five molecules of the hexose glucose 6-phosphate,

allowing continued production of NADPH and converting glucose 6-phosphate (in six cycles) to CO_2 [81, 90].

One of the most important uses of NADPH in cells is to prevent oxidative stress. It reduces glutathione (GSSG $\rightarrow$ 2GSH) via glutathione reductase, which converts reactive H_2O_2 into H_2O through the action of glutathione peroxidase. This cycle is known as the redox cycle and is illustrated in figure 1.3 in connection with the pentose phosphate pathway. In the absence of this cycle, H_2O_2 would be converted into hydroxyl free radicals, which can attack the cells. By maintaining a reducing atmosphere (a high ratio of NADPH to $NADP^+$ and a high ratio of reduced (GSH) to oxidized (GSSG) glutathione), this cycle could prevent or undo oxidative damage to proteins, lipids, and other sensitive molecules [81, 90].

It was previously mentioned that TPN contains peroxides which may cause oxidative stress within the cells. Through the pentose phosphate pathway and NADPH production, the oxidative stress can be decreased by the reduction of glutathione. Glutathione is the main antioxidant within the cells, especially in the liver, and plays a key role in balancing the cellular redox potential. Therefore, given the importance of investigating the possibility of the entrance of glucose into the pentose phosphate pathway, glutathione levels were measured in the present study.

1.6.3.1 Glutathione

Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) and is found widely distributed and at high levels (0.1-10 mM) in body tissues, especially the liver (5-10 mM). It is one of the key antioxidant that prevents intracellular damage caused by reactive oxygen species such as peroxides and free radicals [91, 92]. Glutathione exists in the thiol-reduced (GSH) and disulfide-oxidized (GSSG) forms. In a healthy situation, more than 95% of total glutathione is in the reduced form, and less than 5% exists in the disulfide form. The ratio of GSSG/GSH increases during oxidative stress, and is therefore usually considered as its indicator. The concentrations of both forms (GSH and GSSG)

are important in the balancing of the redox potential within the cells. In fact, glutathione is considered as the buffer of the intracellular redox potential [90–92].

In the presence of glutathione peroxidase, GSH serves as an electron donor and is oxidized to its disulfide form (GSSG). Once oxidized, glutathione can be reduced back in the presence of glutathione reductase, using NADPH as an electron donor (figure 1.3) [90–92].

CHAPITRE 2

HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Previous studies on guinea pigs have shown that neonatal exposure to TPN induced a perturbation of glucose metabolism, leading to a phenotype of energy deficiency characterized by a lower body weight and a more lazy state. We hypothesize that supplementation of diet, early in life, with a high energetic component such as glucose, will prevent modifications in body weight and spontaneous physical activity related to the neonatal infusion of TPN.

2.2 Objectives

2.2.1 General Objective

Assess the mechanisms by which oxidants from TPN contribute to modifications in the programming of glucose metabolism and how they affect health later in life.

2.2.2 Specific Objective

Assess the impact of neonatal exposure to TPN (first week of life) and early glucose supplementation (introduced in the third week of life) on the physical (growth rate and physical activity), biochemical (enzymatic activity of PFK-1 and GK, along with glucose 6-phosphate, glycogen and pyruvate concentrations), and clinical (relative liver weight, fasting blood glucose, glucose tolerance, triacylglyceride and cholesterol concentrations in the liver and plasma) phenotypes as well as on the glutathione defenses (levels of reduced glutathione (GSH), glutathione disulfide (GSSG), and calculation of the redox potential) in guinea pigs up to their ninth week of life.

CHAPITRE 3

MATERIALS AND METHODS

3.1 Materials

The chemicals that were used in this research study and their sources are presented in this section.

Tris-hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide phosphate (NADP), glucose 6-phosphate dehydrogenase (G6PD), ethylene glycol tetraacetic acid (EGTA), and 1,4-dithiothreitol (DTT) were provided by the Roche Diagnostics GmbH Co., Mannheim, Germany. Potassium chloride (KCl), magnesium chloride ($MgCl_2$), glucose, D-fructose 6-phosphate (F6P), adenosine 5′-triphosphate disodium (ATP), triethanolamine, potassium fluoride (KF), and aldolase were purchased from SIGMA Life Science Co., Saint Louis, Missouri, USA. Isoflurane and saline were obtained from Baxter Corporation, Deerfield, Illinois, USA. 2-Mercaptoethanol was provided by BioRad, Hercules, California, USA and xylazine by Bayer Inc., Calgary, Alberta, Canada. Bovine serum albumin (BSA), ammonium sulfate ($(NH_4)_2SO_4$), imidazole, methanol, sulfuric acid, and bis-tris were purchased from Fisher Chemical, New Jersey, USA. Potassium hydroxide (KOH) and boric acid were provided by J.T. Barker Analyzed Reagent Chemical, Austin, Texas, USA. Glyceraldehyde 3-phosphate dehydrogenase and trichloroacetic acid (TCA) were purchased from MP Biomedical LLC, Solon, Ohio, USA. Sodium hydroxide (NaOH) was provided by A&C American Chemicals Ltd., Saint Laurent, Quebec, Canada, and silver sulfate (Ag_2SO_4) by Laboratoire Mat Inc., Quebec, Canada. Pyruvate was obtained from Dehringer Mennhein GmbH, Germany and ketamine from Wyeth Canada, Inc. Markham, Ontario, Canada.

3.2 Animal Model

In the present study, three-day-old guinea pigs were employed as a newborn animal model due to their adequate similarities to the human being. Guinea pigs have striking similarities to humans in terms of hepatic cholesterol and lipoprotein metabolism [93]. They responses to dietary factors, exercise, and oxidative stress undoubtedly mimic the human situation [93]. In addition, many of the mechanisms by which guinea pigs regulate cholesterol and lipoprotein metabolism as a response to diet are analogous to those reported in clinical experiments [93]. These trends make them suitable for metabolic studies [93]. Moreover, guinea pigs experience rapid growth during the third trimester, can be weaned at the second day of age, and can also develop fatty livers during fetal life [93–95]. Therefore, they are appropriate models for developmental studies. Finally, an important corresponding characteristic of newborn guinea pigs and human neonates is their immature glutathione antioxidant defense systems [94], which makes guinea pig a suitable model for investigation of the effects of oxidative stress.

3.3 Experimental Set up and Design

The three-day-old male guinea pigs (n=13) were provided by the Charles River Laboratory, St. Constant, Quebec, Canada. The protocol of this study was approved by the "Institutional Committee for Good Practice in Animal Research of CHU Sainte-Justine Research Center, Montreal, Canada" in accordance with the guidelines of the "Canadian Council on Animal Care". Neonatal guinea pig catheter (1 Fr Silicone Tip, 74 cm 3 Fr Silicone, 2 Fixed Beads, SA1 Infusion Technologies) was fixed in the jugular vein of all guinea pig pups on their third day of life. The surgery to fix the catheters was done under anesthesia with 125 mg/kg-(body weight) ketamine and 10 mg/kg xylazine. The catheter was exteriorized in the scapular region. The circadian rhythm was adjusted to 12 hours light and 12 hours darkness. The room temperature was kept at 20°C.

Animals were randomly assigned to two groups: Control and TPN.

– Control Group: The catheter was closed by a node. Animals had free access to

regular solid chow (High Fiber Guinea Pig Diet 2041, Harlan Teklad Global, Wilmington, DE) with 2.5 kcal/g (table 3.I) from the third day of life until the end of the study.

TABLE 3.I – 2041 Teklad Global High Fiber Guinea Pig Diet

Macronutrient Information	
Nutrient	Chow Diet
Energy [1] (kcal/kg diet)	2500
Carbohydrates [2] (g/kg diet)	369 (56% of energy)
Fiber (g/kg diet)	150
Lipids (g/kg diet)	45 (16% of energy)
Amino acids (g/kg diet)	183 (28% of energy)
Methionine (g/kg diet)	2.9
Cyst(e)ine (g/kg diet)	2.7
Zinc (mg/kg diet)	86
Manganese (mg/kg diet)	134
Copper (mg/kg diet)	18
Iron (mg/kg diet)	265
Selenium (mg/kg diet)	0.21
Riboflavin (mg/kg diet)	94
Vitamin E (mg/kg diet)	10.50
Vitamin C (mg/kg diet)	13.8
β-Carotene (mg/kg diet)	46

1) Energy was calculated via the carbohydrates (3.8 kcal/g), amino acids (4 kcal/g), and lipids (9 kcal/g) from the diets. 2) Carbohydrates exclude fiber.

– TPN Group: For 4 days (3rd to 7th day of life), the guinea pig pups were fed exclusively with TPN via the catheter. Through our experience, we have concluded that 4 days of exposure to the TPN would be sufficient to investigate the effect of peroxide. Our lab team tried to increase the number of days ; however, consequently, an increase in the incidence of infiltration was observed. Moreover, the death rate of animals has increased. The TPN solution was infused at a constant rate of 220 mL/kg/day and contained 4.8 g/kg/day amino acids + 3.8 g/kg/day lipids + 8.7% (w/v) dextrose + 1% multivitamin preparation (MVP) + 1 unit/mL heparin (table 3.II). The composition of this nutritive solution was close to what

newborn human infants receive. Heparin is added to the TPN solution to prevent blood coagulation during fixing the catheter and for its maintenance. At seventh day of life, TPN was stopped and the animals were fed ad libitum enteral with the same diet as control group (High Fiber Guinea Pig Diet 2041).

TABLE 3.II – TPN

Macronutrient Information	
Nutrient (cal/22ml solution/day)	TPN
Energy	9.75
Dextrose	4.26
Lipid	3.66
Amino acids	1.83

Moreover, in order to assess the effect of an early diet enriched in glucose on the programming of glycolysis later in life, each group was divided into two subgroups from the 3rd week of life onward. The second week served as a transition period for the animals to learn how to eat by themselves. Since, Guinea pigs are refractory to a cafeteria-like diet [96, 97], in order to improve the caloric intake, animals received tap water with glucose (100 g/L ; similar to sucrose concentration of Coca Cola) whereas the control subgroup drank regular tap water without any glucose. The animals daily water consumption was 10 mL per 100 g of body weight. The high concentration of glucose in the glucose water had some side effects on the guinea pigs, such as painful oral ulcers. Therefore, the animal facility staff rinsed the mouth of animals with water to prevent this side effect. Four animals were assigned to each cage due to the space limitation in the animal facility as well as ensuring an appropriate level of social life for them.

At the 4th week of life, the growth rate, physical activity, blood glucose, plasma triacylglycerol (TG) and cholesterol were measured. The glucose tolerance curve was measured at the 8th week of life. We hypothesized that supplementation of diet with a high energetic component such as glucose would prevent modifications in body weight. Since, the growth rate is an important characteristic indicating the weight gain rate, al-

lowing animals to reach their maximum growth rate is important. Based on unpublished research study of our laboratory (Dr. Lavoie JC), guinea pigs reach their maximum growth rate between 6th to 9th week of age (figure 3.1). Therefore, animals were sacrificed at the 9th week of life, after 18 hours of fasting, under anesthesia with isoflurane and oxygen. Isoflurane was chosen to provide the accurate length and depth of anesthesia. Isoflurane is usually used with oxygen to prevent hyperventilation. Livers were removed, weighed, rinsed with 0.9% NaCl, and rapidly minced on the ice bath. Samples were conserved at −80°C for further assays. Plasma samples were collected and stored until determinations.

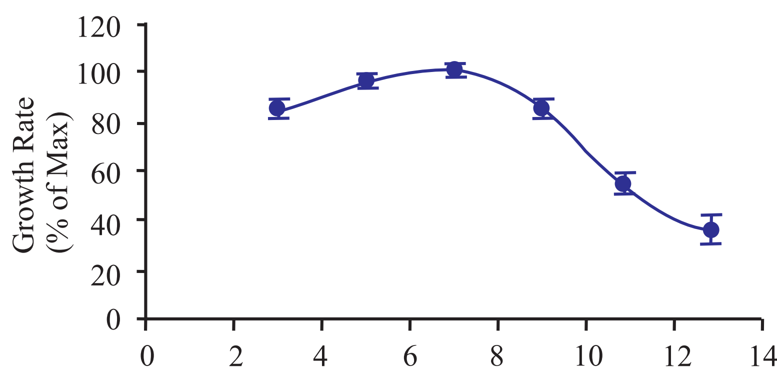

FIGURE 3.1 – Guinea pig growth rate versus time (unpublished data from Dr. Lavoie JC's laboratory).

Figure 3.2 schematically presents the different stages of the present study as well as the timeline for the physical, clinical, and biochemical determinations.

3.4 Analytical Measurements

3.4.1 Physical Phenotype Determinations

3.4.1.1 Growth Rate

Body weight was monitored at the beginning of each week. The purpose of this evaluation was to document the effect of the neonatal TPN and glucose diet on the growth rate of animals and its impact on their body weight later in life.

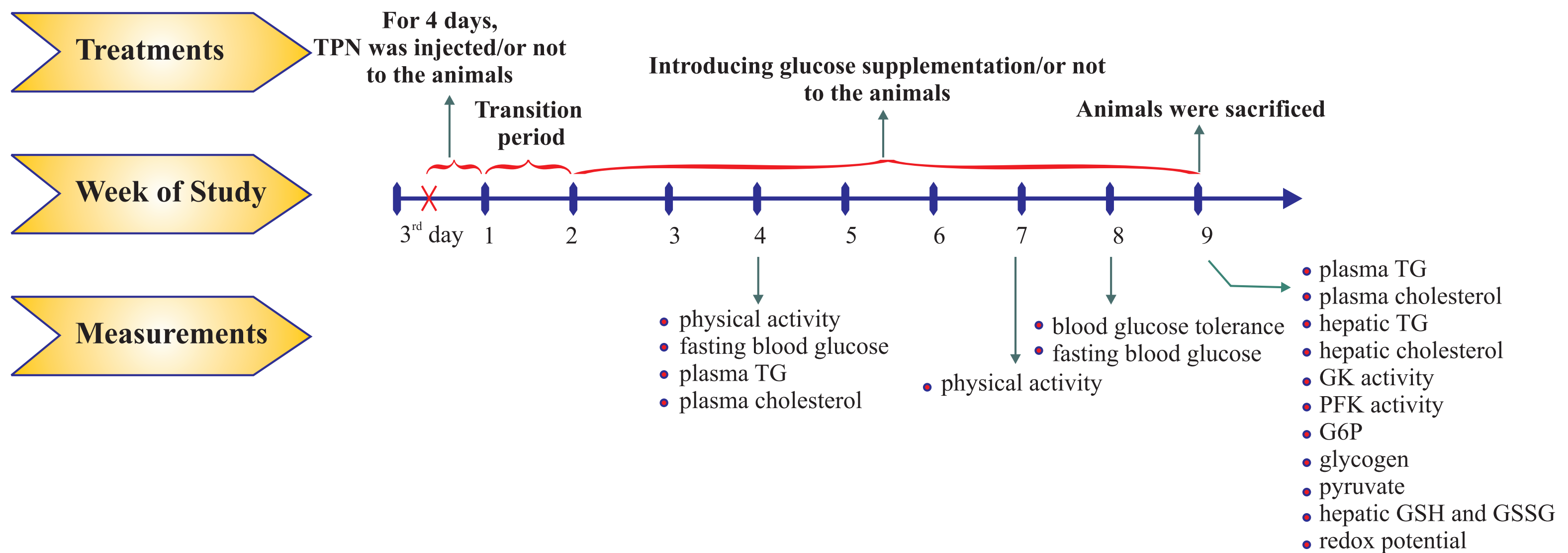

FIGURE 3.2 – The study timeline.

3.4.1.2 Physical Activity

Physical activity was measured at the 4th and 7th week of the animals' lives as counts of spontaneous ambulatory activity using the AccuScan infrared instrument (AccuScan Instruments, Inc., Fusion 2.1, Columbus, Ohio, USA) [98]. Animals did not have access to chow during the monitoring but water was supplied. Measurements were taken over a period of 4 hours; however, the results included in this study correspond to the last 2 hours, as the first 2 hours were considered as the adaptation period. The instrument was equipped with 8 light beams as horizontal lines. Infrared detectors collected the animals' movements and transferred the information to a computer software which processed the input signals [98].

3.4.2 Clinical Phenotype Determinations

3.4.2.1 Fasting Blood Glucose

For this assay, animals had no access to chow for 6 hours. Blood samples were obtained from the saphenous vein. Blood glucose was measured through the administration of one peck of blood samples on the strip of a digital glucometer (One Touch ultra Meter, LifeScan Europe, Division of Cilag GmbH International, Switzerland). All recorded results were displayed in mM values [99]. The remaining blood samples were kept in K_3EDTA-coated tubes and were centrifuged (Beckman Co, Fullerton, CA, USA) at 10 000 RPM for 4 minutes. The plasma (supernatant) was decanted and stored at −80°C for triglycerides and cholesterol assays.

3.4.2.2 Glucose Tolerance Test

An intraperitoneal injection of glucose solution (1g/kg-(body weight)) was performed at the 8th week, after a fast of 18 hours. Measurement of blood glucose of each guinea pig was performed from the saphenous vein every 30 minutes up to 150 minutes. Blood glucose was measured using a digital glucometer [99].

3.4.2.3 Plasma Triglycerides and Cholesterol Concentrations

Plasma triglycerides (TG) and cholesterol concentrations were measured at the 4th and 9th week of guinea pigs' lives using an enzymatic colorimetric method. For the plasma TG measurement, a colorimetric commercial kit (Triglycerides GPO-PAP #12016648, Roche diagnostics, Indianapolis, IN, USA) was used. The kit method is based on the work by Wahlefeld AW *et* al. [100] using a lipoprotein lipase from microorganisms for the rapid and complete hydrolysis of triglycerides to glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The resultant hydrogen peroxide reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase, forms a red dyestuff. The color intensity of the red dyestuff is directly proportional to the triglyceride concentration and can be measured photometrically. In this study, glycerol (0-2 mM) was used as a standard. For the assay, 1 mL of the kit reagent was added

to 10 μL of plasma and incubated for 10 minutes at room temperature resulting the red color solution. The color intensity measurement was performed at a wavelength of 500 nm using a spectrophotometer (DU 640, Beckman Co, Fullerton, CA, USA).

For the plasma cholesterol measurement, another colorimetric commercial kit (Cholesterol CHOD-PAP # 11489437, Roche diagnostics, Indianapolis, IN, USA) was used. The kit method is base on the work by Allain CC *et* al. [101] and Roeschlau *et* al. [102]. Cholesterol is determined enzymatically using cholesterol esterase and cholesterol oxidase. First, cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. Then, cholesterol is converted by oxygen with the aid of cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide. The resulted hydrogen peroxide forms a red dyestuff by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The color intensity is directly proportional to the concentration of cholesterol and can be determined photometrically. In our study, cholesterol (0-4.2 mM) was used as the standard curve. For the assay, the same steps as for TG measurement were employed. For both assays, the results were expressed in μM.

3.4.2.4 Liver Triglycerides and Cholesterol Concentrations

Hepatic triglycerides (TG) and cholesterol concentrations were measured at the 9th week of guinea pigs' lives using the same methods and kits as described for the plasma in the previous section. However, an additional step for the extraction of the liver lipid was required to prepare the initial solution for the assay. In our study, the Folch method was used for the liver lipid extraction [103]. Initially, liver samples (500 mg wet wt.) were homogenized in two volumes of saline (0.9% NaCl solution) by three passes (10 seconds per pass) in a homogenizer (Polytran Teader, Biospec products Inc, Dermel-Racine, WI, USA). 100 μL of the homogenate was mixed with 2 mL of Folch solution (chloroform: methanol 2 :1 v/v) and were agitated for 1 hour, at 4°C. Subsequently, 400 μL of saline (0.9% NaCl solution) was added to each homogenate sample. Samples were centrifuged at 2500 $\times$ g for 10 minutes at room temperature (25°C), which resulted in two separate phases. The upper liquid phase of samples, containing small organic polar

molecules, were removed by siphoning and the lower solid phase, containing lipids, were evaporated under a nitrogen stream and re-suspended in 100 μL of Folch and 125 μL of thesit 20% (v :v), diluted in chloroform. The resultant mixtures were evaporated again under nitrogen stream, which yields oily residues. The residues were suspended in 1.25 mL distilled water and finally, 50 μL of suspensions were used for the assay. The protein assay was performed using the Bradford method (see below). The results were presented as nmol/mg protein.

3.4.3 Biochemical Phenotype Determinations

3.4.3.1 Dosage of protein

Determination of the protein contents of liver fractions and blood cells were performed by a modification of the Bradford MM *et* al. method [104], using Bio-Rad reagent containing 100 mg Coomassie brilliant blue G-250 in 50 mL 95% ethanol and 100 mL 85% (w/v) phosphoric acid. The method is based on the binding of protein with Coomassie brilliant blue in acid solution. Hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change, which is detected photometrically.

Initially, proteins from liver and blood pellets were solubilized with 1N NaOH (500 mg of liver pellets in 1100 μL of 1N NaOH; 200 μL of blood pellets in 600 μL of 1N NaOH). The solubilizing of samples was performed by incubating at 4°C for 12 hours. Subsequently, 50 μL of each solution was transferred to the spectrophotometer cuvette containing 1 mL of Bio-Rad reagent. Cuvettes were incubated for 10 minutes at room temperature (25°C). The resulting color intensity was directly proportional to the concentration of protein and was determined spectrophotometrically at 595 nm. The protein concentrations were compared to a standard curve of bovine serum albumin (0 - 120 pg/μL).

3.4.3.2 Glucose 6-Phosphate

The approach used for this assay was the adapted enzymatic method described by Bergmeyer HU *et* al. [105]. This method is based on the conversion of G6P to 6-phosphogluconate and NADPH in the presence of NADP, with the aid of glucose 6-phosphate dehydrogenase (G6PD). The formation of NADPH is proportional to the amount of G6P. Using the spectrophotometer, at 340 nm and knowing the molar extinction coefficient of NADPH at this wavelength to be 6.22 $mM^{-1}cm^{-1}$, the concentration of G6P was determined.

For the assay, liver samples (200 mg wet wt.) were pulverized on dry ice with mortar and pestle, resulting in a powder. Each powdered sample was transferred to a 50 mL tube containing 300 μL of 3 M cold perchloric acid and 1 mL cold distilled water. Samples were homogenized by three passes (10 seconds per pass) in the homogenizer. The homogenates were centrifuged at 5000 $\times$ g for 10 minutes at 4°C. A sample (1 mL) of the resultant supernatant was mixed with 290 μL of a solution of cold 2M KOH and 0.4 M imidazole. The supernatants were again centrifuged at 5000 $\times$ g for 10 minutes at 4°C. The protein assay was performed on the resultant pellets using the modified Bradford method (section 3.4.3.1). Afterward, 50 μL of the resultant supernatants was transferred to the spectrophotometer cuvette containing 20 μL of 20 mM NADP, 10 μL of 0.5M $MgCl_2$, 20 μL of 150 mM ATP, and 950 μL of 0.4 M triethanolamine buffer. The absorption of samples was recorded at 340 nm, 0, 1, and 2 minutes after mixing, and the mean value calculated (mean absorbance #1). At 2 min, 5 μL of 2.5 μM G6PD was added to the each supernatant, which enhanced the reaction rate. The absorption of samples was determined at 340 nm at 6, 7, and 8 minutes, and averaged (mean absorbance #2). To measure the G6P concentration, the difference between absorbance 2 and 1 was divided by the molar extinction coefficient of NADPH (6.22 $mM^{-1}cm^{-1}$) and was reported in the pmol/mg of protein.

3.4.3.3 Liver Enzyme Activity Assays

- **Liver Glucokinase Activity**

Although both hexokinase and glucokinase are present in the liver, glucokinase is predominant. These enzymes catalyze the production of glucose 6-phosphate from glucose. In the present study, liver glucokinase activity was measured using the method of Ming-zhi Xu *et* al. [106] and Ferre T *et* al. [107]. Based on this method, the extent of glucokinase activity can be measured through the reduction of NADP in the presence of G6PD during the conversion of glucose 6-phosphate to 6-phosphogluconate. Since, the amount of NADP can be measured through a colorimetric method; knowing the molar extinction coefficient of NADPH at a specified wavelength, the glucokinase activity could be determined.

For the assay, liver samples (200 mg wet wt.) were homogenized in an ice bath by three passes (10 seconds per pass) in the homogenizer, in the presence of 2 volumes of homogenate buffer containing 100 mM tris-HCl, pH 7.5, 5 mM EDTA, 5mM $MgCl_2$, 150 mM KCl and 0.7 μL of 2-mercaptoethanol per mL. Homogenates were centrifuged at 1400 $\times$ g at 4°C for 20 minutes. Samples of resultant supernatants were centrifuged again at 12 000 $\times$ g for 10 minutes at 4°C. Supernatants were kept in an ice bath and resultant pellets were suspended in 500 μL of homogenate buffer. The resuspended pellets were recentrifuged (12 000 $\times$ g for 10 minutes at 4°C) and the resultant supernatants were combined with the supernatants from the first centrifugation.

The calculation of total enzyme activity of GK was based on the difference between the glucose phosphorylation capacity in the presence of 100 mM *vs* 0.5 mM glucose. Tubes containing 3 mL of reaction buffer (100 mM Tris-HCl, pH 7.5, 0.2 mM NADP, 5 mM ATP, 5 mM $MgCl_2$, 0.2 unit G6PD), containing either 100 mM glucose (high-glucose) or 0.5 mM glucose (low-glucose) were warmed at 30°C for 5 minutes. Clear liver supernatant (20 μL), warmed to room temperature (25°C) was added into each tube to start the reaction, which was allowed to proceed for 60 minutes. The rate of formation of NADPH from NADP was monitored spectrophotometrically from the rate of change in absorbance at 340 nm. The rate of increase in absorbance achieved in the low-glucose tube was subtracted from that achieved in the high-glucose tube. Protein content was

determined by a modified Bradford method (section 3.4.3.1). Liver glucokinase activity was reported as μmol/min/mg protein.

– **Liver Phosphofructokinase-1 Activity**

The assay was based on the method of Karadsheh NS *et* al. [108] and Hamer MJ *et* al. [109]. The PFK-1 activity was measured by determining the rate of NADH oxidation and fructose1,6-bisphosphate formation at room temperature (25°C). Similar to previously mentioned determinations, the amount of NADH could be measured through a colorimetric method knowing its molar extinction coefficient at a particular wavelength (at $\lambda = 340$ nm, the molar extinction coefficient of NADH is 6.22 $mM^{-1}cm^{-1}$). The PFK-1 activity could be determined based on the rate of NADH oxidation.

Liver samples (300 mg wet wt.) were homogenized by three passes (10 seconds per pass) in a homogenizer, in 9 volumes of 50 mM triethanolamine, pH 7.35 (20°C), containing 1 mM EDTA, 15 mM KF, 2mM $MgCl_2$, and 3 mM EGTA. Homogenates were centrifuged at 12 000 $\times$ g for 2 minutes at 4°C. Supernatants were then centrifuged at 100 000 $\times$ g for 10 minutes at 4°C to produce a clear extract for analysis of PFK-1 activity.

The total Phosphofructokinase-1 activity was measured at pH 8.1 in an assay system (1 mL total volume) which contained 50 mM Tris-HCl pH 8.14, 1 mM fructose-6-phosphate, 2 mM $MgCl_2$, 1 mM ATP, 0.16 mM NADH, 2.5 mM dithiothreitol (DTT), 5mM $(NH_4)_2S0_4$, 1 mM EDTA, 0.4 unit of aldolase, 2.4 units of triosephosphate isomerase and 0.4 unit of glycerol-3-phosphate dehydrogenase. Assay was performed at 25°C by adding an 0.01 mL aliquot of Phosphofructokinase-containing extract to the assay system. Absorbance of each sample was read at a wavelength of 340 nm over a period of 10 minutes. One unit of phosphofructokinase activity corresponded to the oxidation of 2 μmol of NADH, which is equivalent to the production of 1 μmol of fructose1,6-bisphosphate/min under these conditions. Protein content was determined using the modified Bradford method (section 3.4.3.1). The results were expressed in

terms of μmol/min/mg protein.

3.4.3.4 Pyruvate

A pyruvate assay kit (Bio Vision #K607-100) was used for the pyruvate determination. The assay is based on the oxidizing of pyruvate by pyruvate oxidase to generate color. Since the color intensity is proportional to pyruvate content, the pyruvate concentration can be accurately measured.

Liver samples (100 mg wet wt.) were homogenized in an ice bath by three passes (10 seconds per pass) in a homogenizer, initially containing 400 μL pyruvate assay buffer. The resultant homogenates were centrifuged at 7200 $\times$ g for 2 minutes to obtain a clear pyruvate-bearing supernatant. Using a 96 well plate, individual aliquots of supernatant (1 μL) were mixed with 50 μL of pyruvate assay buffer (46 μL pyruvate assay buffer, 2 μL pyruvate probe, and 2 μL pyruvate enzyme) in an individual well and incubated for 30 minute at room temperature, protected from light. The absorbance of each sample was determined at 570 nm by spectrophotometer. The absorbance values were compared to those of a series of pyruvate standards (0-8 nmol/well). Protein content was measured according to a modified Bradford method (section 3.4.3.1). Pyruvate concentrations were expressed as nmol/mg protein.

3.4.3.5 Glycogen

Determination of the liver glycogen followed the basis Kemp A *et* al. method [110], based on the color reaction that occurs by heating a diluted glycogen solution with concentrated sulphuric acid.

Liver samples (25-75 mg wet wt.) were homogenized in an ice bath by three passes (10 seconds per pass) in a homogenizer containing 5 mL methanol 80% (v/v). The homogenates were centrifuged at 3000 RPM for 5 minutes. The resultant supernatants were

removed. Pellets were ground with 5 mL of deproteinizing solution (5% trichloroacetic acid and 3.21 mM silver sulphate). The fluids levels were marked on the tubes and the tubes were covered with glass caps. Although pure glycogen dissolved in the deproteinizing solution, only part of it can be extracted from the liver tissue with a cold solution. To extract the full complement of liver glycogen, supernatant tubes were heated in a boiling-water bath for 15 minutes. The tubes were then cooled under running tap water and were filled up to the mark with the deproteinizing solution to compensate for evaporation. Samples were then centrifuged at 3000 RPM for 5 minutes. One mL of 36 N sulphuric acid was added to 333 μL of each clear supernatant and heated for 6 minutes. Subsequently tubes were cooled in running tap water. The intensity of the resulting pink color was proportional to the amount of glycogen and was measured in the spectrophotometer at 520 nm. The glycogen concentration of each sample was compared to a standard curve for glucose (0-800 μM). Protein content of the homogenate dosage was measured based on a modified Bradford method (section 3.4.3.1). The values were reported in nmol/mg protein.

3.4.4 Determination of Redox Status

The overall tissue redox status was quantified by the redox status of the couplet of oxidized glutathione (GSSG) and reduced glutathione (GSH), as described by Schafer FQ *et* al. [111]. In our laboratory, the assay of GSH and GSSG levels was based on the capillary electrophoresis method developed by Lavoie JC *et* al. [112]. Immediately after sacrificing animals, liver samples (500 mg wet wt.) were acidified in 5 volumes of iced and freshly prepared 5% (w/v) metaphosphoric acid and homogenized by 2 passes (10 seconds per pass) in the homogenizer. Metaphosphoric acid reduces the rate of oxidative processes. Homogenates were centrifuged at 7200 $\times$ g for 3 minutes. The resultant pellets and supernatants were frozen at $-80°$C until protein and glutathione determination assays. The assay was performed after the frozen supernatants were thawed and diluted 5-fold in water. The GSH and GSSG from 50 μL supernatant aliquots were separated by capillary electrophoresis (P/ACE^{TM} MDQ Beckman Coulter). The silica capillary had

an inner diameter of 75 μm and was 50 cm in length. The electrophoresis buffer consisted of 75 mM boric acid and 25 mM Bis-Tris, pH 8.4. Electrophoresis was performed at a voltage of 18 kV for 10 minutes and samples GHS/GSSG levels were detected at 200 nm. The glutatione-based redox potential was calculated by using the Nernst equation: $E_{hc} = -240 - (59.1/2)\log([GSH]^2/[GSSG])$ mV at 25°C, pH =7 [111].

3.5 Statistical Analysis

Results of the present study were expressed as mean ± standard error of the mean (sem). Main effects and interaction effects were tested by ANOVA ([TPN vs. Control] × [± Glucose diet]) or ([age] × [TPN vs. Control] × [±Glucose diet]). The Bartlett's Chi square test was used to assess homoscedasticity. Each group contained at least 3 samples. If a significant interaction occurred between parameters, data was analyzed according to the parenteral nutrition and control group, glucose-rich-diet or age. A significant difference between the TPN and control group would suggest that neonatal peroxide exposure, in the form of exposure to TPN, induces some form of metabolic programming. A significant effect of age would suggest that the stage of development modulates the clinical phenotype. A significant effect of glucose supplementation would suggest that the nutritional environment is important for the clinical manifestation of the metabolic phenotype. The threshold of significance was set at $p < 0.05$.

CHAPITRE 4

RESULTS

According to Barker's hypothesis of "developmental origin of adult health and disease", several adult disorders can be traced back to the neonatal period. In the present study, several physical, clinical, and biochemical determinations were made to assess the impact of neonatal exposure to oxidant molecules and early glucose supplementation on the modulation of glucose metabolism programming later in life.

Four test groups were compared: 1) "Control" animals were fed regular chow from the 3rd day to the 9th week of life. 2) "Control + G" were fed regular chow from the 3rd day and supplemented with a diet enriched in glucose from the 3rd week of life. 3) "TPN" animals received total parenteral nutrition from the 3rd to the 7th day of life and were fed with regular chow thereafter. 4) "TPN + G" were treated as the "TPN" animals, but their diet was enriched in glucose from the 3rd to the 9th week. All the animals were nourished with mother's milk until their 3rd day of life.

4.1 Physical Phenotypes

The growth rate of animals that had received glucose from the 3rd to 9th week of life was lower ($p < 0.05$) than the rate measured in animals that had received water devoid of glucose (Figure 4.1). The TPN treatment had no significant effect on the growth rate and there was no interaction between parameters.

The physical activitiy, characterized by the number of movements per hour, measured at the 4th and 7th week of age, are presented in figure 4.2. All treatments were without significant effect. Similarly, relative liver weight (figure 4.3) was not affected by the treatments.

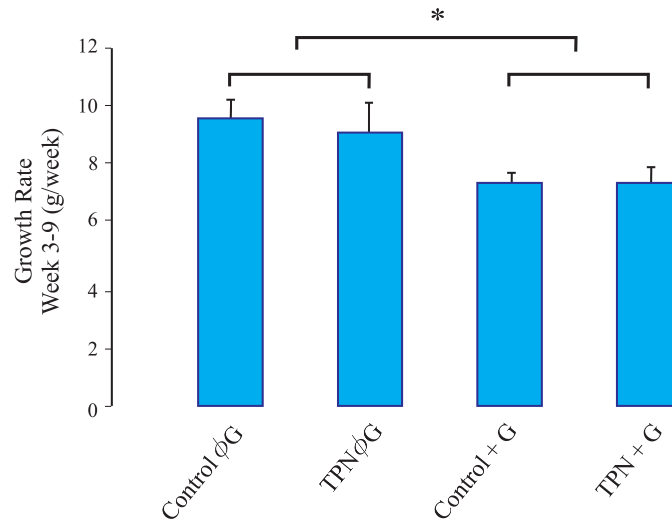

FIGURE 4.1 – Growth rate during the 3rd to 9th weeks of age. Control: animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. The growth rate was lower ($p < 0.05$) in animals receiving glucose than those not receiving glucose. Mean±sem, n=3-4 per treatment combination.* $p < 0.05$

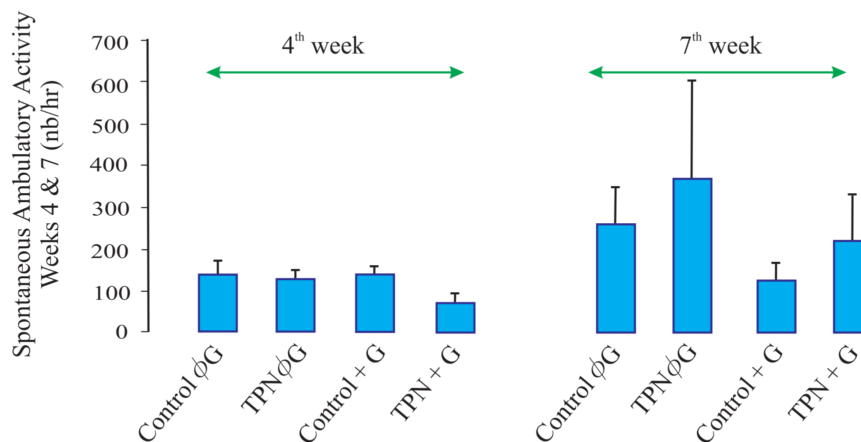

FIGURE 4.2 – Physical activity at the 4th and 7th week of age. Control: animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. There was no significant difference between any treatments at either age. Mean±sem, n=3-4 per treatment combination.

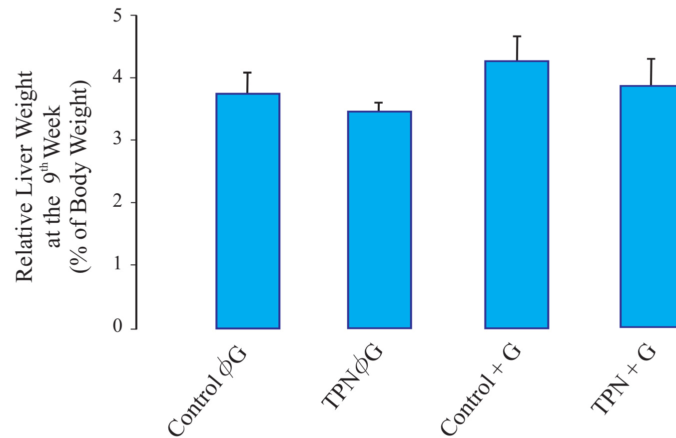

FIGURE 4.3 – Relative liver weight at the 9th week of age. Control: animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. There was no significant difference between any treatments. Mean±sem, n=3-4 per treatment combination.

4.2 Clinical Phenotypes

Glucose tolerance results are presented in figure 4.4. None of the treatments affected blood glucose levels over time after intraperitoneal injection of sucrose. The fasting blood glucose observed at the 4th and 8th week of life did not differ significantly between treatments (figure 4.5).

Plasma triacylglycerol (TG) and total cholesterol at the 4th and 9th week are shown in figure 4.6 (a to d). Cholesterol levels were not significantly different for any treatment factor. At week 4 there was no effect of any treatment on TG levels (figure 4.6(a)); however, at week 9, within the no-glucose treatment TG was higher for the control than the TPN treatment (figure 4.6(a)); however, this difference was not apparent in the glucose-treated animals. This long-term impact of TPN ($p < 0.05$) has been previously reported by our team [35]. Levels of hepatic cholesterol (figure 4.7) and TG (figure 4.8) showed no significant differences for any treatment factor.

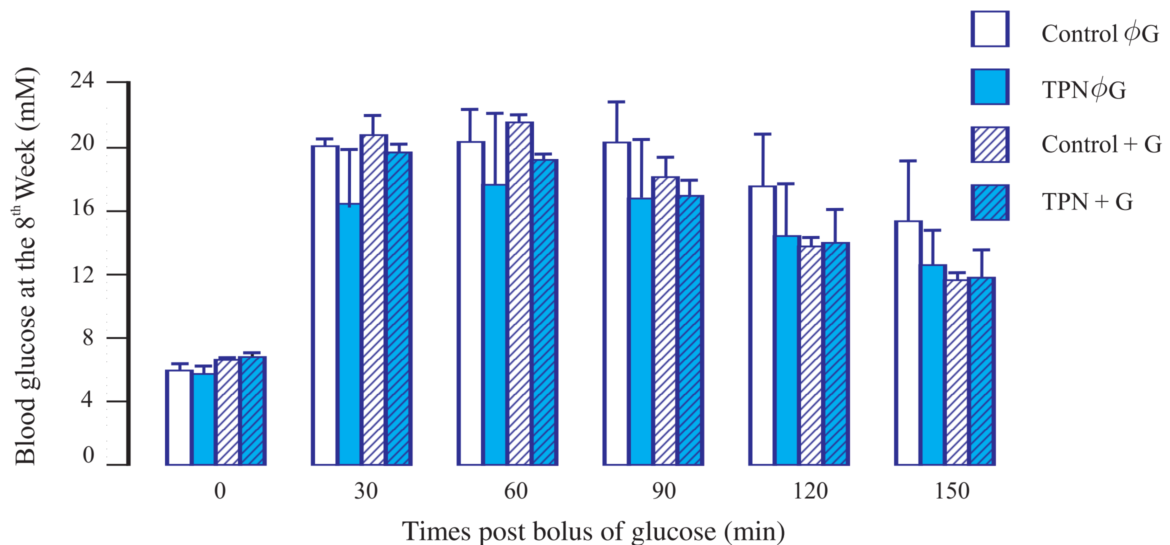

FIGURE 4.4 – Blood glucose tolerance at the 8th week of age. Control : animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. There was no significant difference between any treatments at any time. Mean±sem, n=3-4 per treatment combination.

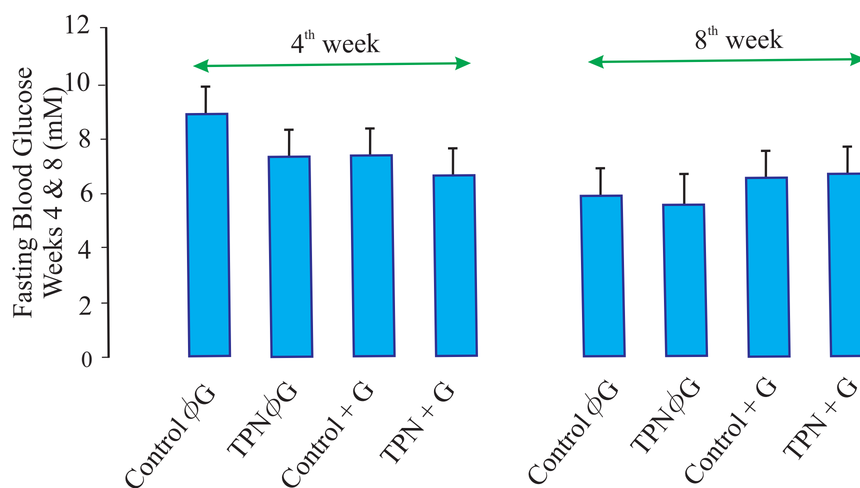

FIGURE 4.5 – Fasting blood glucose at the 4th and 8th week of age. Control: animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. There was no significant difference between any treatments at either age. Mean±sem, n=3-4 per treatment combination.

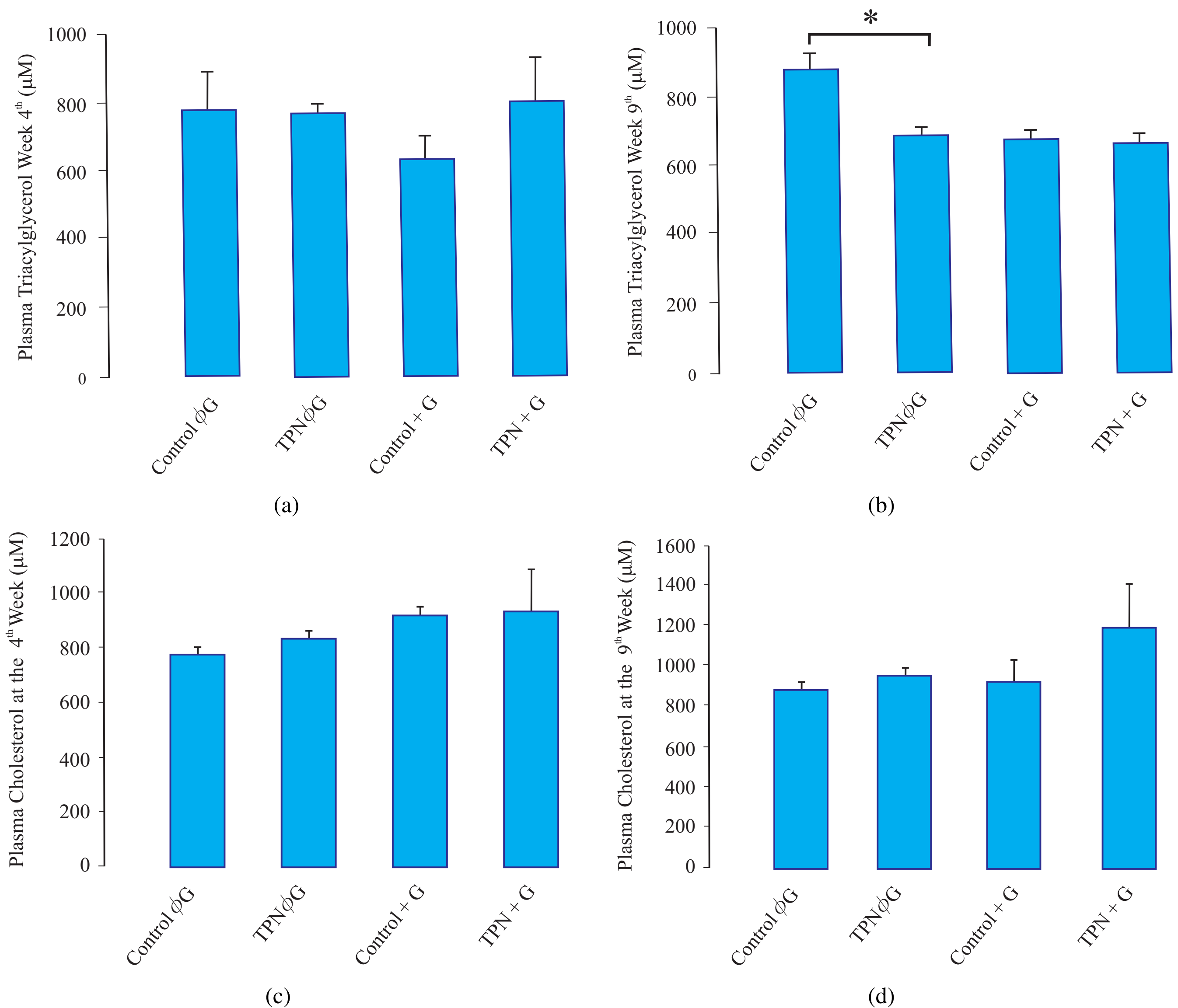

FIGURE 4.6 – a) Plasma triacylglycerol at the 4th week, b) Plasma triacylglycerol at the 9th week, c) Plasma cholesterol at the 4th week, and d) Plasma cholesterol at the 9th week of age. Control: animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. The only significant difference between treatments was observed in panel (b), where a TPN effect was observed for glucose-free control treatments ($p < 0.05$), but disappeared when animals received glucose in their drinking water. Mean±sem, n=3-4 per treatment combination. *: $p < 0.05$.

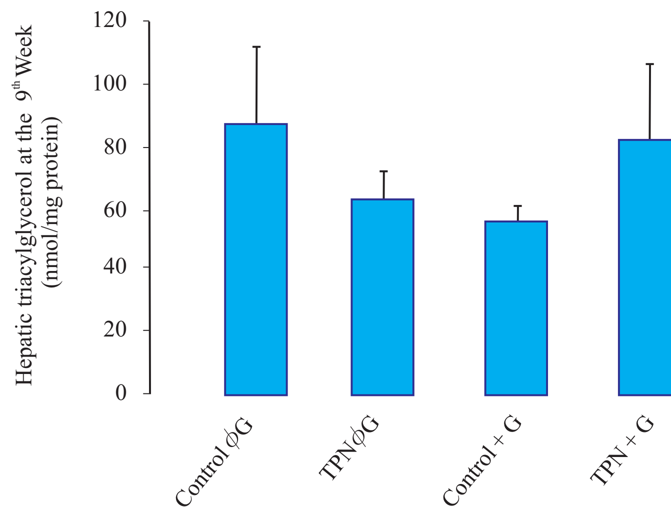

FIGURE 4.7 – Liver triacylglycerol at the 9th week of age. Control: animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. There was no significant difference between any treatments. Mean±sem, n=3-4 per treatment combination.

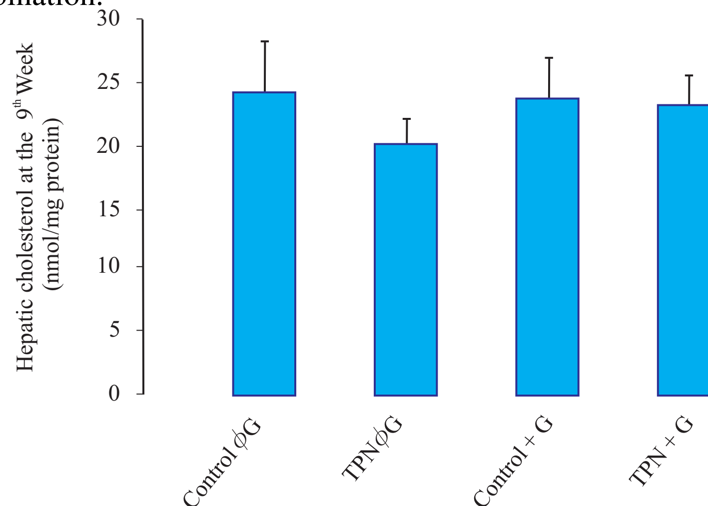

FIGURE 4.8 – Liver cholesterol at the 9th week of age. Control: animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. There was no significant difference between any treatments. Mean±sem, n=3-4 per treatment combination.

4.3 Biochemical phenotypes

At 9 weeks of age, animals which had received glucose supplementation from their 3rd week of life onwards showed significantly greater GK activity than those receiving just water ($p < 0.05$; figure 4.9(a)). However, TPN had no effect on the GK activity.

For PFK activity, a significant interaction ($p < 0.05$) between (±TPN) and (±glucose), illustrated in figure 4.9(b), led us to analyze data according to whether animals were fed glucose-water or not. Data shows that the PFK activity increased in the TPN (*vs.* control) treatments when no glucose supplementation occurred ($p < 0.05$), but no difference existed between TPN and control treatments in glucose-treated animals, suggesting a different effect of glucose for GK and PFK.

The hepatic level of G6P at the 9th week of the guinea pigs' lives are shown in figure 4.10. The significant interaction between (±TPN) and (±glucose) for this parameter led us to analyze data according to whether animals drank glucose water or not. The level of G6P was significantly higher ($p < 0.01$) in animals exposed to TPN compared to control groups, but only if the animals received glucose in their diet.

At 9 weeks of age, no significant difference in the level of glycogen (figure 4.11) or pyruvate (figure 4.12) was apparent between any treatment factors.

4.4 Glutathione status

There was no significant difference between treatments with respect to hepatic concentrations of glutathione (GSH) and glutathione disulfide (GSSG) (figures 4.13(a) and 4.13(b), respectively). However, the GSH/GSSG-derived redox potential (figure 4.14) was more reduced (less oxidized) ($p < 0.05$) in the liver of the animals which received (*vs.* did not receive) the early glucose supplement in their diet. There was no statistical effect of TPN. It should be noted that as a marker of oxidative stress, redox potential is more precise than glutathione. Redox potential is a strong function of GSH level since accor-

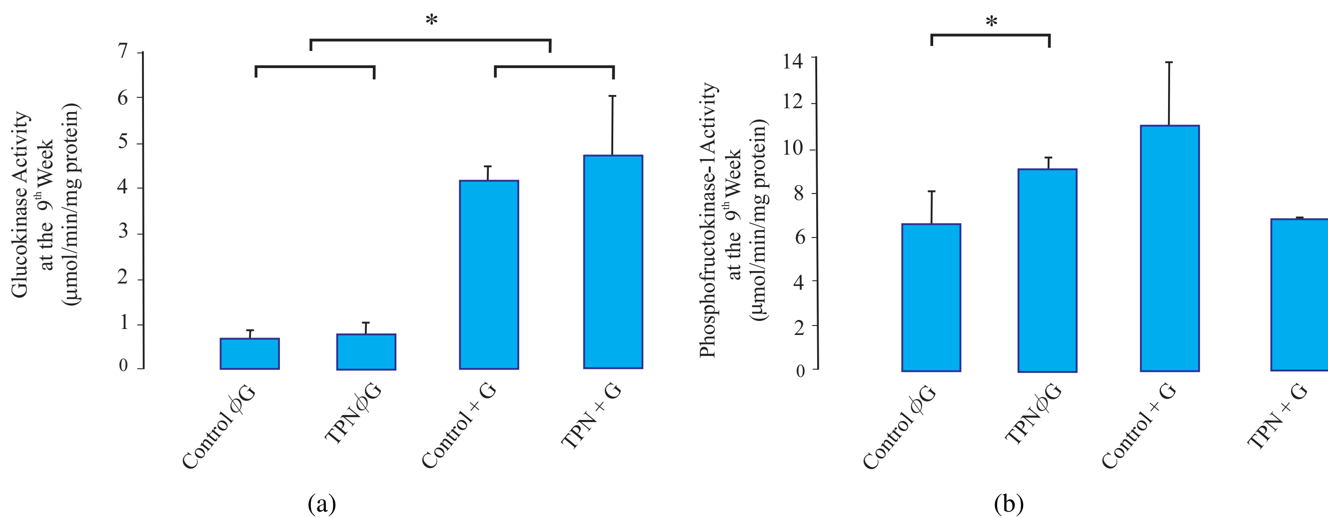

FIGURE 4.9 – a) Glucokinase activity, and b) Phosphofructokinase-1 activity at the 9th week of age. Control: animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. Glucokinase activity was higher ($p < 0.05$) in glucose-fed animals than those receiving plain water. Phosphofructokinase-1 activity was increased ($p < 0.05$) in the TPN group without any early glucose supplementation. Mean$\pm$sem, n=3-4 per treatment combination.*: $p < 0.05$.

ding to the Nernst equation, $E_{hc} = -240 - (59.1/2)\log([GSH]^2/[GSSG])$ mV at 25°C, pH =7 [111], the square value of GSH influences the redox potential. Therefore, a small change in GSH, not statistically significant, may cause a significant deviation in the redox potential.

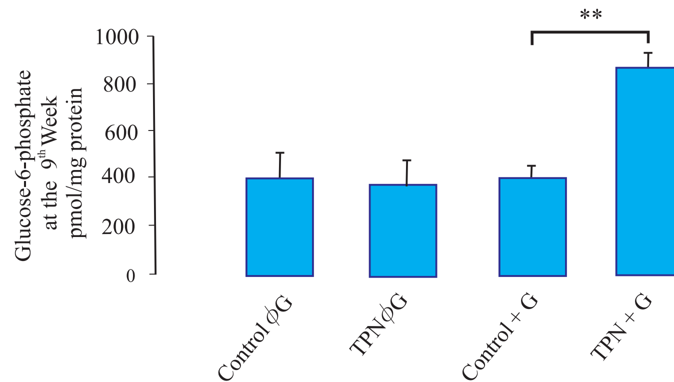

FIGURE 4.10 – Glucose 6-phosphate at the 9th week of age. Control : animals fed regular food; TPN : animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G : animals drank sugared water from their 3rd week of life. Glucose 6-phosphate was higher ($p < 0.01$) in TPN group with early glucose supplementation compared to the other groups. Mean±sem, n=3-4 per treatment combination.** : $p < 0.01$.

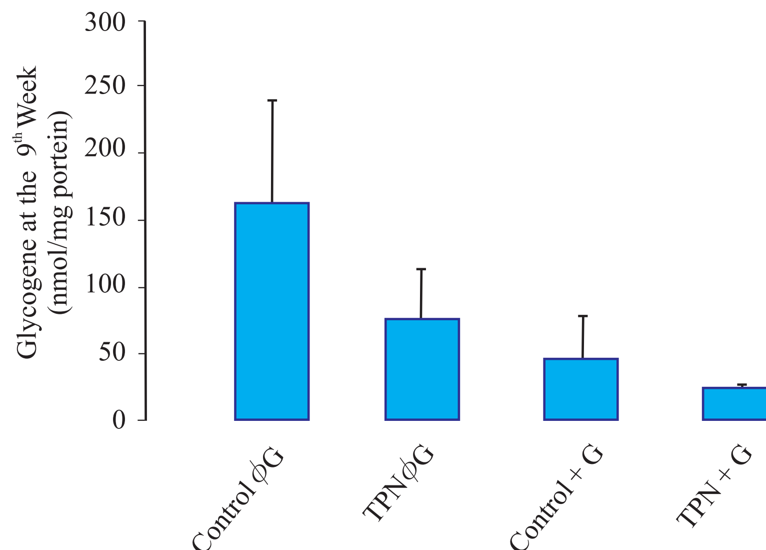

FIGURE 4.11 – Glycogen at the 9th week of age. Control : animals fed regular food ; TPN : animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. There was no significant difference between any treatments. Mean±sem, n=3-4 per treatment combination.

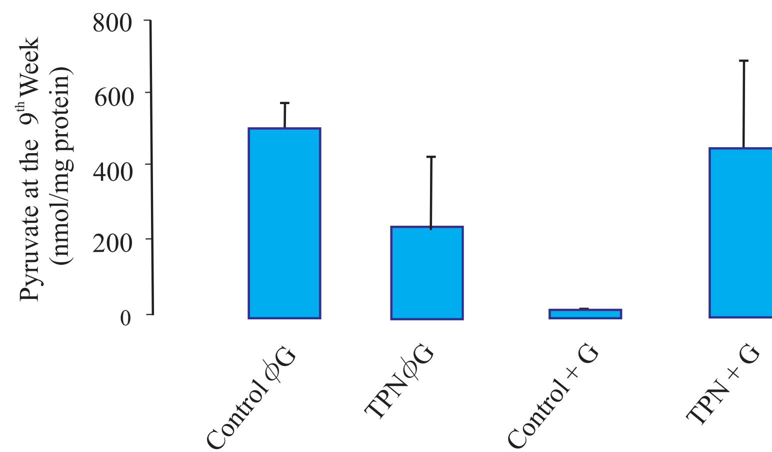

FIGURE 4.12 – Pyruvate content at the 9th week of age. Control: animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. There was no significant difference between any treatments. Mean±sem, n=3-4 per treatment combination.

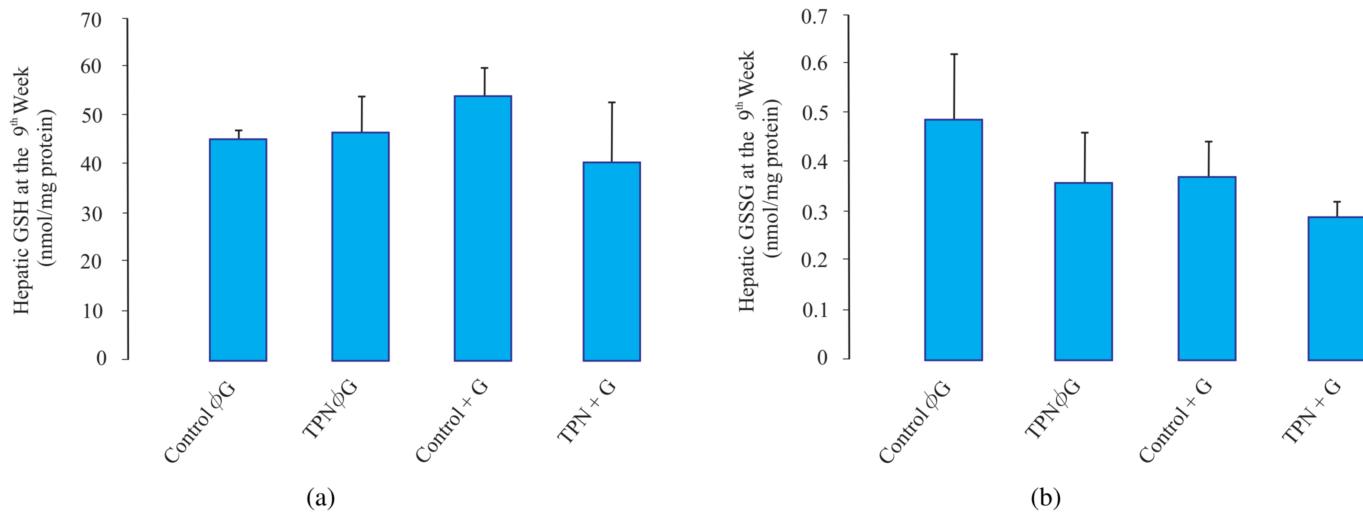

FIGURE 4.13 – a) Glutathione (GSH), and b) Glutathione disulfide (GSSG) at the 9th week of age. Control: animals fed regular food; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. There was no significant difference between any treatments. Mean±sem, n=3-4 per treatment combination.

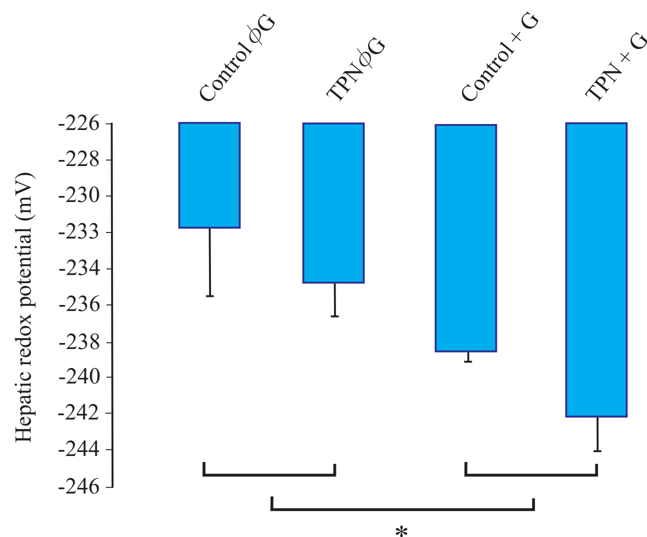

FIGURE 4.14 – Redox potential at the 9th week of age. Control: animals fed regular food ; TPN: animals fed exclusively by parenteral nutrition between day 3 and 7 of their lives followed by regular food; G: animals drank sugared water from their 3rd week of life. The redox potential in animals receiving glucose in their water was more reduced (less oxidized) ($p < 0.05$) than in animals drinking glucose-free water. Mean±sem, n=3-4 per treatment combination. * : $p < 0.05$.

CHAPITRE 5

DISCUSSION

To our knowledge, the present study is a pioneer work investigating the interaction between neonatal exposure to TPN and early glucose supplementation on the programming of glucose metabolism. Our research study illustrates several alterations of physical, biochemical, and clinical phenotypes during the developmental stage of guinea pigs before reaching their maximum growth rate (9 weeks of age ; figure 3.1). Kleiber N *et* al. explored the connection between neonatal exposure to TPN and the modulation of lipid and glucose metabolism in guinea pigs at 13 weeks of age [35]. However, it is unclear whether the observed alterations stem from the period prior to or following their maximum growth rate. The present study follows in the footsteps of the findings of Kleiber N *et* al. [35] who demonstrated the impact of neonatal TPN on energy metabolism later in life, up to 13 week of age. We were interested to know if these manifestations occurred earlier in life. Since Kleiber concluded that neonatal TPN induces a modification of glucose metabolism later in life, leading to a phenotype of energy deficiency, a second objective was to know if introduction of an early glucose-enriched diet could alter this phenotype.

In our experimental design, no modification of parameters was observed at the 4th week of life, whereas by the 9th week, modification in several components of glycolysis were documented, as well as modification of plasma TG and redox potential. For some of these modifications, there were significant interactions with glucose diet.

To understand the mechanisms by which glycolysis may be perturbed, the activity of two key enzymes of this pathway (glucokinase and phosphofructokinase-1) was investigated. There were no significant differences in GK activity (figure 4.9(a)) between animals receiving TPN or standard chow for either glucose-supplemented or glucose-free water treatments ; however, glucose supplementation gave raise to significantly higher

GK activity than it's absence. In contrast, Kleiber N *et* al. observed greater GK activity in the TPN (*vs.* standard chow) group at the animals'13 week of life [35]. These observations could suggest a probable effect of TPN on the modulation of GK activity later in life after reaching the maximum growth rate level, *i.e* (after 9 days). Several studies have shown glucokinase is sequestered in the nucleus of the hepatocyte [81, 113, 114], and released when the cytoplasmic glucose concentration increases [113–120]. In the present study, showing the same behavior, the GK activity of groups that received a glucose-enriched (*vs.* glucose-free) diet, increased at the 9th week. This illustrates the modulation of GK activity by postnatal glucose supplementation, thereby initiating the normal steps of glycolysis.

As discussed previously, GK is the hepatic enzyme that allows hepatocytes to continue phosphorylating of glucose into G6P during the rise in glucose concentration [121]. In this study, a greater production of G6P is expected considering the higher GK activity in groups that received a glucose-enriched diet [121]. Statistical analysis confirms this, but only for the group exposed to TPN. Accordingly, one may wonder about this high level of G6P accumulation. In fact, several studies have observed that G6P is at the crossroads of carbohydrate metabolism in the liver [95]. It may have several major fates and enter different metabolic routes depending on the metabolic demands of the organism [81]. The liver directs the flow of G6P into different pathways through the action of several allosteric regulatory enzymes and the hormonal regulation of enzyme synthesis and activity [81, 121–123]. G6P can be dephosphorylated via the enzyme glucose 6-phosphatase [81, 113]. This reaction converts G6P to free glucose, which is exported to replenish blood glucose and provide adequate energy for the brain and other tissues [81, 113]. In the present study, in order to verify if G6P entered this pathway, the blood glucose level of animals was measured, but no significant differences were observed.

Another possible fate of glucose 6-phosphate is its conversion to hepatic glycogen, assuming that the body does not require blood glucose immediately [81, 114, 117, 118].

In order to determine if G6P in animals that have a glucose-enriched diet has entered into glycogenesis pathway one can measure the amount of liver glycogen, as was done in the present study. Measurements showed that no treatment had a significant effect on the amount of glycogen.

On the other hand, several investigations have confirmed that glucose-6-phosphate can also be broken down through glycolysis and decarboxylation of the pyruvate via the pyruvate dehydrogenase reaction [35, 120]. However, the level of pyruvate measured in the present study showed no significant differences between groups. In addition, many studies have indicated that pyruvate can be converted to acetyl coenzyme A [81, 113, 120]. This component could be oxidized through the citric acid cycle to produce energy, with ensuing electron transfer and oxidative phosphorylation yielding ATP [81, 113, 120]. Normally fatty acids are the preferred fuel for energy production in the liver [81]. Acetyl coenzyme A may also serve as the precursor of fatty acids or cholesterol [81, 113, 120]. Such fatty acids are incorporated into triacylglycerol and phospholipids [81]. Much of the lipid synthesized in the liver is transported to other tissues through the mechanism of blood lipoproteins. In our investigation, the concentration of plasma and hepatic cholesterol, as well as that of hepatic TG was measured ; however, no significant differences were apparent between diets with or without TPN for animals receiving early glucose supplementation. The monitoring acetyl coenzyme A will be the domain of future studies.

The final fate of glucose 6-phosphate may be its entrance into the pentose phosphate pathway [81]. A NADPH-powered reduction is required for the biosynthesis of fatty acids and cholesterol [122]. The plasma and hepatic cholesterol measurement in our study showed no significant differences between any treatments. NADPH is also an essential cofactor in the detoxification and elimination of many metabolites in the liver [122]. Cells require NADPH for reductive biosynthesis or to counter the damaging effects of oxygen radicals [81, 122]. The detoxification of peroxides depends directly on the availability and the regeneration of NADPH. Within cells and tissues, the pen-

tose phosphate pathway appears to be the predominant deriver of the regeneration of NADPH [124–127]. Kussmaul L *et* al. illustrated that detoxification of peroxide, which is a metabolic process, is linked to the availability of glucose as a metabolic substrate [145] and the hydride donor for the regeneration of NADPH needed in the reduction of glutathione (GSSG $\rightarrow$ 2GSH) by glutathione reductase [127].

Our results showed a reduction of the redox potential at the 9th week of age in groups that received glucose, independently of whether they received neonatal TPN or not. Redox potential is essentially associated with the biological status of the cell and could influence several metabolic pathways. For instance, in several pathways, the thiol status is important for its function. This status is strongly influenced by the redox potential of the cell. In general, pathways regulated by phosphorylation are susceptible to be influenced by the redox potential. These results support the hypothesis of the entrance of G6P into the pentose phosphate pathway. By reducing GSSG to GSH, via glutathione reductase, NADPH contributes greatly to a shift in the redox potential toward a more reduced status. Therefore, it may prevent or undo the oxidative damage of proteins, lipids, and other sensitive molecules [81, 122]. NADPH likewise protects cells from oxidative damage from hydrogen peroxide (H_2O_2) and superoxide free radicals [81, 122] through the normal detoxification process previously described in section 1.6.3.

In the present study, no significant difference was observed in glucose tolerance at the 4th or 8th week of life for any treatment. These results indicate that there was no resistance to insulin, a major energy metabolism modulator [35]. We did not measure the concentration of insulin given the unavailability of a commercial antibody for guinea pig insulin [35]. However, higher PFK activity and lower plasma TG were observed in animals that received TPN without any glucose supplementation, although it could not confirm insulin dysregulation [35]. In the Kleiber N *et* al. study, a significant difference existed in glucose tolerance in 13-week-old guinea pigs [35]. These findings suggest that the modulation of glucose tolerance might be linked with the age and stage of development. However, it may occur after reaching the maximum level of growth rate, at or

around the 9th week. At this time PFK activity increased in those animals which received TPN only, whereas in the Kleiber N *et* al. study its activity decreased. This reveals a perturbation of PFK activity caused by an early exposure to oxidant molecules and its direct correlation with the developmental stage of the animals' life. The high activity of PFK observed in our experimental study might have been expected to alter the amount of pyruvate ; however, this was not the case. The activity of enzymes involved in lipid metabolism and related metabolic pathways remains to be investigated.

In the present study, several physical determinations were examined in order to observe any correlation between a neonatal exposure to oxidant molecules alone or in combination with an early diet enriched in glucose (as a source of energy) and physical phenotype alterations later in life. One of these physical phenotypes was the animals' growth rate. The growth rate of guinea pigs having received a glucose-enriched diet was lower than that of guinea pigs having received glucose-free water. This runs counter to the study's hypothesis. One of the probable reasons is that a high concentration of glucose in water caused mouth ulcers in this experimental population, thus disabling or reducing normal food intake. However, this complication was quickly corrected by the action of the personnel of the animal facility that regularly rinsed the mouth of animals with sugar-free water. After the TPN/no TPN treatment period, all animals had free access to the chow food. Other studies have demonstrated suppression of food intake when the level of glucose entering the brain rose [128]. Therefore, the groups that received glucose supplementation likely experienced a decreased appetite, and therefore did not take in sufficient calories for growth.

Nevertheless, according to the Lucas hypothesis of "accelerated growth," increasing the growth rate could cause adult obesity. Lucas explains the association between faster early postnatal growth in infants who have a delay in their growth rate during their intrauterine life and an increased risk of metabolic syndrome indicators later in life [9, 60, 61]. According to Barker and Lucas, the origin of several adult diseases can be traced back to the neonatal and postnatal stage of life. One of the risk factors in programming of adult

disease is the postnatal growth rate [9]. Pylipow M *et* al. studied 463 seven-year old adult human born with low birth weight. They observed that an early rapid postnatal growth rate led to a greater body weight later in life [58], while a lower growth rate did not trigger obesity later in life in the same population. Isganaitis E *et* al., performed a low-birth weight mouse study design demonstrating the effects of postnatal growth rate in metabolic diseases later in life [129]. They observed that high postnatal growth rate affected the adipose tissue's lipogenic gene expression, resulting in larger adipocytes [129]. In addition, in their rat model, Desai M *et* al. observed that accelerated growth altered the metabolic parameters in response to a high-fat diet, as well as increased weight and percentage of body fat [59] later in life. Therefore, the result of a lower growth rate due to the early addition of glucose to the diet of animals that received neonatal oxidant molecules may modify metabolic disorders later in life.

The body weight of guinea pigs exposed to TPN in the Kleiber N *et* al. study decreased by the 13th week of life [35]. In the present study, this phenotype showed no significant difference at the 9th week in the TPN group without glucose supplementation. These observations might suggest the programming that leads to alterations in physical phenotypes later in life may occur after the 9th week. We measured physical activity as a physiologic marker of energy expenditure [130]. There was no significant difference in this physical phenotype at either 4th or 7th week of life. In the Kleiber N *et* al. study the decrease in physical activity was observed in the 13th week of life in animals that received early TPN. These results suggest that modulation of this phenotype may depend on the stage of the animals' development. Likewise, in the present study, the physical activity of the groups that received a glucose-enriched diet did not change. These results suggest that decreasing the level of plasma TG may affect the physical activity phenotype modulation and the perturbation of lipid and glucose metabolisms, thus inducing energy production later in life [35].

5.1 Limitations

This study was subject to several limitations, including :

(i) the sample size of the study was small mainly due to the limited funding of the welcome laboratory. Statistically, small sample sizes increase the standard deviation and prevent obtaining a greater statistical power to demonstrate a difference when it exists [131].

(ii) several studies demonstrated that when the level of glucose entering the brain rises, food intake is suppressed [128]. Therefore, the high concentration of glucose in the present study might prevent the animals' normal and proper food intake. Moreover, the free accessing of animals to the chow might limit the precise body growth rate observed.

(iii) the present study was performed on male guinea pigs. Choi *et* al. in their rat study design demonstrated that metabolic programming alterations depend on the sex and can increase metabolic risk factors more in adult males than females [132]. They showed that cholesterol levels and liver TG content were lower in females compared to males. The Chessex P *et* al. study on preterm infants (< 1000 g) receiving TPN containing different amounts of peroxides, demonstrated that among infants who received higher levels of peroxide, only females had a higher blood pressure and cardiovascular effects [77]. These observations support the notion that the metabolic programming might depend on the gender. Therefore, the results of the present study may not be generalized to females.

(iv) Due to the space limitation of the animal facility, four animals were assigned to each cage. Therefore, it was not possible to measure the food consumption of guinea pigs individually. Besides, since within the cages, four animals are from different groups, averaging of the food consumption per cage would not be useful. For the future studies, we suggest that animals from the same group be assigned to each cage.

5.2 Future Studies

Regan FM *et* al. suggested that neonates fed with a high carbohydrate diet might suffer from greater growth rate and a greater reduction in insulin sensitivity later in life [133]. Insulin resistance was observed early in the pathogenesis of adult metabolic diseases, and an isolated reduction in insulin sensitivity had been reported in low birth-weight neonates and children [133]. An explanation for the association between low birth-weight infants and later metabolic disease in adulthood is early impairment of insulin sensitivity. It may increase obesity and induce insulin resistance in adulthood [133]. Furthermore, they demonstrated that TPN with high amounts of carbohydrate may impair insulin sensitivity in later life of infants and may lead to obesity as well as obesity-related disorders such as high blood pressure [133]. In the prospective studies, the proper concentration of glucose diet should be measured to prevent the probable alterations of several physical and biochemical phenotypes, such as BMI and body fat, later in life [45].

In future studies, experimental designs investigating the impacts of high caloric diet on clinical phenotype in animals exposed to oxidative stress in their early life should be improved. The activity of several enzymes that contribute to the pentose phosphate pathway and lipid metabolism pathway as well as the amount of acetyl coenzyme A should be determined. Finally, in order to generalize the results, the sample size needs to be increased in the prospective studies.

5.3 Conclusion

Diet strategies should be investigated in order to prevent the programming of adult metabolic disorders associated with neonatal oxidative stress. Infants, who are born prematurely (<28 weeks of gestation) need TPN for growth and development [80]. The complete photo protection of this solution against ambient light, which catalyzes the peroxide generation, is clinically impossible [71]. As a result, several oxidant molecules [18, 19, 22, 24, 133] are generated, thus leading to neonatal oxidative stress [134].

This early mode of nutrition has several short and long-term effects on the clinical, physical, and biochemical phenotypes in this fragile population [27–29, 35, 77–80, 134–136]. These modifications all depend on the stage of development.

The neonatal and postnatal period may be a starting point of adult diseases [46]. Present study suggests that the quality of TPN and nutrition have an impact on metabolism later in life. Parenteral nutrition has several long-term effects on the programming of lipid and carbohydrate metabolism. For example, the level of plasma triglyceride is lower among animals that received neonatal TPN and may lead to the alteration of energy metabolism later in life [35]. The activity of enzymes involved in the glycolysis pathway shifts at various stages of development. The present study indicates that the quality of neonatal and postnatal diets may also have an effect on the development of an oxidative defense system [46, 136]. A glucose-enriched diet may improve the immature antioxidant defense system of this fragile population by increasing NADPH regeneration [124–127, 137]. This has beneficial effects on cell detoxification, especially with respect to oxidative stress, and can thus maintain cell integrity [127]. The early glucose-enriched diet may improve the glucose metabolism pathway, thereby preventing the reduction of plasma triglyceride later in life. Moreover, this early supplementation has positive effects on the physical phenotypes, thus decreasing growth rate acceleration. This reduction may also decrease the risk of metabolic syndrome indicators [39, 42, 60, 61] and adult coronary heart diseases, such as hypertension, and type II diabetes [2, 4, 8].

In summary, results suggest that the diet supplementation of glucose in guinea pigs exposed to oxidative stress may stimulate the glycolysis pathway, shift the redox potential towards more a reduced status, as well as induce several alterations in physical, biochemical, and clinical phenotypes later in life.

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