5. Discussion

Type 2 diabetes mellitus is a metabolic disorder of fuel homeostasis characterized by hyperglycemia and altered lipid metabolism caused by islet β cells being unable to secrete adequate insulin in response to varying degrees of over nutrition, inactivity, consequential overweight or obesity, and insulin resistance. The estimated worldwide prevalence of diabetes among adults was 285 million (6.4%) in 2010, and this value is predicted to rise to around 439 million (7.7%) by 2030 [43]. Surprisingly, these estimates of the global prevalence of diabetes indicate that, Egypt will be ranked as the 10th country worldwide which have the highest number of people with diabetes[44]. The pandemic of type 2 diabetes, along with its high human and economic costs, is showing no signs of abatement and, therefore, new approaches are urgently needed to predict, prevent, slow the progression, and limit the consequences of this disease [47].

The elevated prevalence of this disease results in part from an increased rate of obesity in individuals with genetic predisposition for T2DM. Genetic studies have demonstrated that known variants account for less than 10% of the estimated overall genetic contribution to T2DM predisposition, suggesting that additional unidentified factors contribute to susceptibility of this disease [183]. The fetal nutrient environment has been proposed as another component that might modify the risk for developing diabetes later in life [184]

The mammalian fetus is completely dependent on the nutrients supplied by its mother. Disturbances in this supply can induce structural and functional changes during fetal development, with long-lasting consequences for growth and metabolism of the offspring throughout life. The intra-uterine milieu, therefore, programs to a certain extent the health of an individual throughout life. This effect has been called "fetal origin of adult disease" [47]. The altered maternal/fetal metabolism appears to be associated with a diabetogenic effect in the adult offspring even in the absence of genetic predisposition. This fetal programming of type-2 diabetes might considerably contribute to the global burden of diabetes [48, 49].

Extensive epidemiological and laboratory evidence indicates that a suboptimal environment during fetal and neonatal development in both humans and experimental animals impacts on offspring susceptibility to later development of altered carbohydrate metabolism [185]. Previous studies in rats have shown that altered maternal carbohydrate and protein metabolism during pregnancy and/or lactation due to maternal low protein diets can result in altered carbohydrate metabolism in offspring. Multiple animal models, including maternal protein restriction, caloric restriction, and intrauterine placental ligation models, have identified common metabolic phenotypes in offspring and have identified the pancreatic β cell as particularly sensitive to nutrients very early in life [186].

The molecular mechanism(s) of intra-uterine programming for T2DM in the offspring of malnourished mothers is (are) unclear. Also, the offspring gender difference in diabetogenic susceptibility requires detailed study of the different pathways involved in glucose sensing and metabolism. The present study aimed to explore the alteration of peripheral glucose sensing and mitochondrial biogenesis and function in the offspring of low-protein (LP) nourished mothers. Also, role of post-natal feeding with high caloric diet (HCD) was investigated.

In the present study the maternal protein malnutrition was induced pre-gestational just after weaning. Most studies dealing with the fetal programming induce the maternal insult during late (last week) or mid (2nd week) gestation [187,188]. But, such designs for studying the effect of nutrient restriction are complicated by the fact that short-term deficiencies are buffered to varying degrees by intracellular reserves of nutrient. Also, pre-gestational challenges are important to cover the pre-implantation period which may be particularly sensitive. Also, our model resembles to large extent the human situation.

5- Discussion

In the present study, maternal protein malnutrition appears to affect pregnancy outcome as it caused significant decrease in the number of pups per litter. The decreased number of pups may be resulted from increased fetal deaths and resorption [(189)] or decreased quality of the maternal ova. It was documented that, maternal metabolic disturbances, like a negative energy balance or obesity and type II diabetes are associated with ovarian dysfunction. Changes in the growth pattern of the ovarian follicle, due to suboptimal metabolic conditions, can affect oocyte quality. Furthermore, maternal metabolic disorders (nutritionally induced or caused by obesity) may alter the endocrine and biochemical composition of the microenvironment of the growing and maturing female gamete (follicular fluid). Any perturbation in oocyte's direct environment has the potential to reduce the oocyte's developmental competence. Also embryo quality is significantly reduced due to maturation in adverse conditions [(190)]. The results indicated that, pups of LP mothers have lower weight than control pups. In agreement with these results, Snoeck et al., demonstrated a link between the maternal low protein diet and lower birth weights in the offspring as well as decreased beta-cell proliferation and altered adipocyte properties [(191)]. Also, using intrauterine artery ligation (as a model of intrauterine impaired fetal nutrition) similar results were obtained [(65)].

It was reported that the maternal health even during pre-gestation alone can result in diabetogenic and obesogenic tendency in the offspring [(192)]. This could create different developmental windows of diabetogenic programming namely; pre-gestational, gestational and post-natal. In this study the maternal malnutrition was induced pre-gestational and continued through gestation and lactation which resembles to large extent the human situations, however we manipulated the post-natal feeding using normal balanced control diet (CD) and high-caloric diet (HCD) to examine the role of the type of nutrition on the intra-uterine programming.

Follow up of F1 offspring indicated that, the male and female offspring of LP mothers became over weight from the 10th and 15th week of age; respectively, and post-natal feeding with HCD further increases weight gain. This indicated obesogenic behavior of the offspring which is considered a risk factor for the development of insulin resistance and T2DM .

The offspring of malnourished mothers maintained under control diet showed normal glucose tolerance and insulin sensitivity while those maintained under high caloric diet showed age- dependant decline in glucose tolerance and insulin sensitivity which became significant from the 15th week of age especially in male. With age 30 week, mild hyperglycemia was detected in the male offspring of LP mothers (under HCD only) while in females no hyperglycemia was detected and IGT was aggravated. These results imply that maternal malnutrition alone is not sufficient to induce T2DM at this young age while post-natal HCD plays important role in accelerating the development of insulin resistance and T2DM phenotype in the F1 offspring of maternal malnutrition.

Many studies previously demonstrated IGT and insulin resistance in the offspring of maternal protein malnutrition. The maternal malnutrition is a known inducer of offspring metabolic syndrome and diabetes risk. It was documented that gestational caloric and protein restriction resulted in metabolic alterations, reduced pancreatic cell and insulin and diabetogenic tendency in the offspring [(191)]. In line with the present data, Isganaitis et al., developed a mouse model of low birth weight (LBW) by maternal food restriction by 50% from gestational days 12.5– 18.5 [(193)]. In this model, LBW mice with accelerated post- natal growth have increased adiposity in early life and increased risk of diabetes and of obesity in adulthood. Also, they confirm the role of early postnatal feeding in this programming. In rats, it was documented that, female fed a LP Diet during gestation give birth to pups that exhibit a lower weight at birth but that catch up quickly during the early life. In their adulthood, the offspring were prone to develop glucose intolerance, especially when fed a high fat diet after weaning [(194)]. These results in rodents are also replicated in other animal models including

sheep [195], swine and piglet [196] where an isoenergetic low-protein diet during pregnancy affects not only the birth weight but also has long-term consequences for the offspring .

Previous reports on rat offspring derived from mothers fed a low protein diet during pregnancy indicated that the offspring did not always show reduced adolescent and adult body weight as compared to controls which is a consequence of catch-up growth [197]. Catch-up growth is a compensatory growth. It may occur at any stage of growth but is most commonly observed in the early life [68]. This overgrowth may occur as a result of the mis-match between the undernutrition environment in early life (pre-natal) and overnutrition in later life (Post-natal) [65]. Studies have found that catch-up growth often results in overcompensation, whereby the organism exceeds normal weight and often has excessive fat deposition. This rapid and excessive growth has been associated with the development of adult obesity, insulin resistance, metabolic syndrome, and type 2 diabetes[68,69]. These adverse adult health outcomes are associated with permanent changes in the expression of genes involved in glucose homeostasis [198].

The immunohistological data indicated that, the pancreas of LP mothers under HCD (LPF1-HCD) showed normal acinar cells and the islets are present with a large proportion of islet cells though with a large volume as compared to control but with inflammatory cell infiltration. The offspring of LP mother showed decreased total β-cell mass while the individual β-cell area and mass are significantly increased especially in the male rats under HCD. These pattern of changes; decreased total β-cell mass and area together with increased individual β-cell area may indicate a state of inflammation in β-cells. This suggestion is confirmed in the histological examination of pancreatic tissues which indicated that, the pancreas of LP mothers under HCD (LPF1-HCD) showed mild dilatation of acinar cells and islets, increase with a large proportion of islet cells volume as compared with control. Also, the examination indicated the infiltration of pancreas with inflammatory cells.

The histological examination also confirmed the results of increased plasma insulin level in offspring of LP mothers under HCD (especially males). The examination indicated strong reaction of insulin expression in all β-cell of those offspring which indicated enhanced insulin expression and excretion in these cells that may be a response to insulin resistance in those offspring.

Animal studies in rodents demonstrate that the protein supply during pregnancy plays a key role in the development of β cells [191,199,200]. It is known that most of the β-cell mass of adult rats forms after birth and that maturation of insulin secretion occurs in early postnatal life [201]. Therefore, any environmental factor in this critical window of development could have long-term consequences on pancreatic function.

Offspring exposed to LP in gestation followed by a normal post-natal diet underwent slow catch-up growth and showed normal glucose homeostasis while those feed high caloric diet post-natal showed rapid catch-up growth and increased serum insulin maintaining relatively normal glucose homeostasis during early ages. Increased plasma insulin suggests that insulin resistance is already present in these offspring by 15th week of age. During the later-life challenges to pancreatic function may well precipitate overt diabetes. Previous studies with animals experiencing gestational diabetes indicate that increased insulin concentrations neonatal at postnatal day 21 [187] and these high levels are still observed in adulthood and that nutritional challenges lead to glucose intolerance and insulin resistance [202]. In line with our data, these studies documented that these alterations in insulin secretion are accompanied by changes in β-cell structure and functions [187]. Also, in accordance with the present study other studies indicated that, offspring of rats fed a low-protein diet during gestation, a model of intrauterine growth restriction (IUGR); exhibit reduced neonatal β cell proliferation, islet size, and vasculature [191] as well as impaired glucose tolerance in adulthood [200].

5- Discussion

The observed abnormalities in glucose homeostasis in the offspring of LP mothers are associated with lipid profile disturbances. The total cholesterol and triglycerides are significantly elevated from the 25th week of age, while high density lipoprotein-cholesterol (HDL-C) significantly declined from 20th week of age in the offspring of LP mothers maintained under HCD compared to control offspring. Also, the level of non-esterified fatty acids (NEFA) is highly elevated in the offspring of LP mothers at earlier age (10th week) than others lipid abnormalities. These disturbances are more apparent in the male than female offspring; this may be cause or consequence of gender difference in the insulin resistance and glucose homeostasis observed in the present study.

Disturbance of lipid metabolism appears to be an early event in the development of type 2 diabetes, potentially preceding the disease by several years in human. The observed derangements of lipids may be a cause or a consequence of the diabetic state and insulin resistance. The dyslipidemia associated with insulin resistance (also referred to as atherogenic dyslipidemia) is characterized by moderately increased triglyceride (TG) levels carried in very-low-density lipoprotein (VLDL) particles and reduced HDL-C levels carried in small HDL particles [(203)]. It was documented that Insulin resistance is associated with increased levels of serum insulin and results in impaired regulation of circulating lipoprotein and glucose levels [(204)]. Data suggest impairment in the ability of insulin to suppress hepatic production of large TG-rich VLDL (VLDL-TGs) in patients with type 2 diabetes results in a subsequent elevation in plasma TG levels [(204)].

The significant elevation of serum NEFA levels may be explained on the basis of impaired insulin action at the level of the adipocyte which may results in defective suppression of intracellular hydrolysis of TGs with the release of NEFA into the circulation [(205)]. The increased influx of NEFAs to the liver promotes triglycerides synthesis and the assembly and secretion of large VLDL; this results in elevated plasma VLDL levels and triglycerides. Moreover, the increased flux of NEFA directly affects insulin signaling, diminishes glucose uptake in muscle, exaggerates triglyceride synthesis, induces gluconeogenesis in the liver, and contributes to β-cell failure [(206)]. The correlation between increased FA availability and reduced insulin-stimulated glucose metabolism is well established. Despite this clear relationship, to date, there has been no unifying mechanism that explains lipid-induced reductions in insulin action under all circumstances. The most described mechanisms are that toxic lipid intermediates and/or activation of inflammatory and stress signaling pathways act to decrease the phosphorylation and function of proteins in the insulin signaling pathway, and this explains the decreased insulin stimulated glucose uptake observed with lipid accumulation [(207)].

The observed shifts in glucose homeostasis were associated with changes in the peripheral sensing of glucose in muscle and adipose tissues which start at insulin receptor and ended by translocation of Glut4 to the plasma membrane of the cell. The offspring of LP mothers maintained under control diet showed similar or even higher level of IR in muscle tissues especially in females while in adipose tissue the IR was lower during the first 15 week of age after which its level become insignificant from control offspring. Those offspring under HCD showed the lowest level of IR in muscle and adipose tissues especially in males which showed the least level and at earlier age than females.

The regulation of IR gene expression occurs at different levels of control; transcriptional, post-transcriptional and/or post-translational. Insulin is known to down regulate IR protein by inducing its degradation [(208)] and by inhibiting its gene transcription [(209)]. So, the observed down regulation of IR in the offspring of protein malnourished mothers under HCD may explained by the increased insulin level in those offspring. Also, the lower level of insulin in the offspring under control diet may explain the increased muscle and adipose tissues content of insulin receptor .

5- Discussion

Insulin activation of IR leads to tyrosine (Tyr) autophosphorylation of both β-subunits with the major 3 sites at Tyr-1158, Tyr-1162 and Tyr-1163[210]. In the present study we used a kit for assessment of Tyr-1162/1163 phosphorylated β-subunit (Phospho-IR) after 30 minutes of insulin injection and the result indicated down regulation of adipose tissues content of Phospho-IR from 25th week age in male offspring of LP mothers maintained under HCD compared to control. The muscle content of Phospho-IR showed no significant change with age or sex. Also, no significant difference was observed between different groups of offspring, however the lowest levels were detected in the offspring of LP mothers under HCD

It was reported that, prenatally at gestational day 17 , up regulation of fetal adipose tissues content of phospho-IR in the fetuses of malnourished mothers [189]. This prenatal effect may activate negative mechanisms to down regulate this up regulation by inducing expression of protein tyrosine phosphatases 1B and this epigenetic change may persist post-natal while even though no hyperglycemia present, but this assumption needs further experimental proof.

Another important observation is the absence of significant changes in the phospho-IR level in the muscle and adipose tissues in the male offspring of LP mothers under HCD earlier than 25th week of age while insulin resistance (detected by HOMA) and IGT (detected by OGTT) were observed at earlier age (15 week) which may indicate that resistance to insulin action at the early age may occur downstream the insulin receptor activation especially in the muscle. In adipose tissue the insulin resistance occurs early downstream insulin receptor activation while at later age (from 25 week) the resistance starts from the activation of insulin receptors as indicated by lower phosphor-IR contents. These results point out the early role played by adipose tissues for the programming for the development of T2DM in the offspring of LP mothers.

The liver plays an essential role in the control of glucose and lipid homeostasis, and insulin is a central regulator of multiple hepatic metabolic functions. By modifying the expression and enzymatic activity of key players, insulin promotes glycogen synthesis, lipogenesis and lipoprotein synthesis, and inhibits gluconeogenesis, glycogenolysis, and VLDL secretion in the liver also plays a pivotal role in the control of cholesterol homeostasis[211]. Therefore, hepatic insulin resistance contributes substantially to glucose intolerance and dyslipidemia [212]. While the offspring of LP mothers under CD showed higher hepatic content of IR and Phospho-IR compared to control offspring, those offspring feed HCD showed significant lower hepatic contents of IR and Phospho-IR at earlier age than that observed in adipose tissues .

These results indicated the offspring of LP mother maintained post-natal under control healthy balanced diet showed no worse or even better changes in the insulin signaling parameters (IR and Phospho-IR) than control offspring and displayed a more insulin sensitive state compared to control offspring, at least until the 30th week age (follow up period). On the other hand, those offspring of LP mothers maintained under high caloric diet showed early and great derangements in the insulin signaling parameters; especially in liver and adipose tissues, and a high insulin resistance states compared to control offspring even under HCD. In line with these observations it was reported that during early life, the low protein offspring show a significantly improved glucose tolerance when compared to controls. Then low protein offspring undergo an age-dependent loss of glucose tolerance and develop impaired glucose tolerance which may results in type 2 diabetes [213].

The important mediators in the glucose sensing and metabolism are mitochondria [214]. Mitochondria play an important role in maintaining energy homeostasis by balancing ATP generation and expenditure at the cellular level. Most of the ATP required to maintain the normal functions of the cell is produced through oxidative phosphorylation (OXPHOS) in mitochondria. A key adaptation enabling the fetus to survive in an unbalanced energy

environment may be the reprogramming of mitochondrial function. Deregulation of energy homeostasis is a common feature of metabolic syndrome and a number of studies have shown that maternal nutrition programs offspring mitochondria OXPHOS activity in various types of tissues, including liver, pancreatic islet and skeletal muscle in rats [215-217].

Many studies relate the reduced mitochondria in the muscles and adipose tissue to the pathogenesis of T2DM. Mitochondria in adipocytes play pivotal role in the regulation of lipolysis as fatty acids resulting from lipolysis can be oxidized by the fatty acid β-oxidation. This process of removal of fatty acids within white adipocytes would protect against fatty acid leakage out of adipocytes [214]. So, the reduced mitochondrial biogenesis and function would result in enhanced efflux of fatty acid into circulation that contributes to the insulin resistance, since fatty acids impair muscle and liver insulin sensitivity [218].

Since mitochondrial number and function require both nuclear and mitochondrial-encoded genes, coordinated mechanisms exist to regulate the two genomes and determine the overall oxidative capacity. The mitochondrial function and biogenesis is under the control of nuclear encoded proteins; mainly mitochondrial transcription factor A (mTFA) and uncoupling proteins (UCPs). The mTFA is nuclear encoded transcription factor which acts as a key regulator of mitochondrial transcription and mtDNA replication [116]. The relative gene expression of mTFA in muscle tissues is up regulated in nearly all offspring (CF1-HCD, LPF1-CD and LPF1-HCD) compared to control offspring from the 15th week of age in males and females. In adipose tissues, the control offspring respond to HCD by up regulating the expression of mTFA, this up regulation may be a compensatory mechanism to overcome the increased flux of fatty acids (as a result of increased caloric intake) through induction of mitochondrial biogenesis and mtDNA transcription. On the other hand, the offspring of LP mothers showed down regulation of mTFA in the adipose tissues which may decrease the OXPHOS capacity of the adipocytes that favors the lipogenesis instead of lipid degradation. Moreover, the offspring of LP mothers subjected to HCD failed to up regulate mTFA expression in the adipose tissues like control offspring, instead the expression level declined further and earlier than those offspring under CD. These effects may cause insulin resistance and increased adipogenesity of the adipose tissues. Also in the hepatic tissues, all the offspring even control offspring under HCD showed down regulation in mTFA with the lowest activities observed in the male offspring of LP mothers under HCD. This mean that the OXPHOS capacity of the liver is decreased in those offspring which may responsible for the insulin resistance and accumulation of lipids and non-esterified fatty acids that may leads to susceptibility for the development of non-alcoholic fatty liver (NAFLD). In line with these results Park el al demonstrated that low protein diet during gestation significantly decrease mtDNA number in liver and muscles tissues of the offspring [219].

The expression of UCP2 appears to depend on the type of diets used especially in adipose tissues and liver, the control offspring under HCD showed up regulation of UCP2 expression at mRNA level. On the other hand, while the male and female offspring of LP mothers under CD showed down regulation of hepatic UCP2 expression, those offspring under HCD showed up regulation compared to both control offspring groups. This up regulation may be considered an adaptive mechanism to eliminate the excess fuel intake and inhibit production of reactive oxygen species.

The abnormalities in the expression of mTFA and UCP2 in the peripheral tissues of the offspring of LP mothers may imply defective mitochondrial biogenesis and function. It was documented that defects in mitochondrial functions have been suggested to lead to inadequate substrate oxidation, precipitating a build-up of intracellular lipid metabolites, impaired insulin signalling and the subsequent development of insulin resistance [220]. Also, it has been shown that mitochondrial dysfunction is associated with insulin resistance in skeletal muscle, as well as in other tissues, including liver, fat, heart, vessels, and pancreas [221]. Thus, insulin resistance

caused in part by mitochondrial dysfunction may contribute to a common pathophysiologic etiology for many chronic diseases like T2DM, NAFLD and other components of the metabolic syndrome [(222)]

From the above results and discussion we can suggest that pre-gestational and gestational maternal protein malnutrition affect the offspring insulin sensitivity and glucose sensing parameters in the different peripheral tissues. The post-natal diets differentially affect the studied parameters; the control healthy balanced post-natal diets has no effect or even ameliorate the glucose sensing and insulin sensitivity in the offspring while high caloric post-natal feeding reverses the situation completely; as the offspring showed derangements in the glucose sensing (down regulation of insulin receptor and phosphor-insulin receptor) and lipid metabolism, expression of genes controlling mitochondrial biogenesis and functions, and impaired glucose tolerance and even hyperglycemia. There are many hypotheses that could explain this contradictory figure observed in offspring of maternal malnutrition. The first one is "thrifty phenotype" which suggests that poor intra-uterine environment causes the development of fetal organs (brain) at the expense of other organs like pancreas and cardiomyocytes which results in survival of fetuses under this poor condition [(49)]. But this -hypothesis can't explain the development of insulin resistance observed in the offspring. It was also postulated that fetus develop insulin resistance to ensure proper supply of glucose and amino acids to the brain [(65)]. Catch-up growth is another hypothesis which assumes that the mismatch between nutritionally poor intra-uterine environment and post-natal nutrient rich environment results in early post-natal catch-up growth associated with long-term adverse metabolic effects including insulin resistance and IGT [(65)]. The male F1 offspring appear to be more sensitive for fetal diabetogenic programming than female offspring. Also, the study points out to the role of adipose tissues in triggering the programming for development of T2DM.