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DOES PRECONCEPTION ALCOHOL ABUSE MAKE THE OFFSPRING
VULNERABLE TO DEVELOPING TYPE II DIABETES?

by

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ABSTRACT OF THE DISSERTATION

Does Preconception Alcohol Abuse Make the Offspring Vulnerable to Developing Type
II Diabetes?

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Recently, it was reported in our laboratory that binge-like alcohol drinking for three weeks prior to conception by female rats produces poor birth outcome and defects in the offspring's body stress response. These effects occur even after alcohol abstinence prior to conception, during pregnancy and postnatal care. Offspring born after preconception alcohol exposures (PCAE) show abnormalities in expression of the stress regulatory gene Pro-opiomelanocortin (Pomc) in the hypothalamus, resulting in higher stress hormone responses to a stress challenge and increased anxiety-like behavior. Since stress is often connected with increased body risk for metabolic diseases and since POMC-expressing neurons are known to control glucose homeostasis, I hypothesized that PCAE disrupts POMC neuronal function to increase susceptibility to developing hyperglycemia in animals. To test this hypothesis, I determined if PCAE had the ability to alter glucose homeostasis and increase the susceptibility to develop high fat diet (HFD)-streptozotocin (STZ)-induced diabetes in the offspring. The aims of my study were to determine the effects of PCAE on pancreatic, hepatic, and hypothalamic POMC/BEP neuronal abnormalities, and how the BEP replacement ameliorates these

effects. In this study, 21 female adult Sprague Dawley rats were divided body weight-matched and randomly into three groups; the control group receiving rat chow and water ad libitum (AD; N=7), the alcohol-fed group (AF; N=7) receiving a liquid diet containing ethanol (6.7%), or the calorie-matched pair-fed (PF; N=7) group receiving an isocaloric liquid control diet. After 30 days of feeding, all rats were given normal chow ad libitum for three weeks; the estrus cycle was monitored and bred them with normal adult males to produce offspring. The pups were weaned at the age of postnatal day (PND) 25 and were randomly selected one animal from each litter to have three body weight-matched different groups (AD, PF, and AF, N=7). At the age of two months, offspring of AF exposed animals showed significantly higher fasting blood glucose and leptin and lower insulin and glucagon levels as compared with AD and PF offspring. Oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (IPITT) data showed higher blood glucose and lower insulin production in AF in comparison with AD and PF offspring. Further, glucose-stimulated insulin secretion (GSIS) results demonstrated that AF group showed lowest insulin secretion as a response to an oral glucose solution in comparison with AD and PF groups. To evaluate the mechanistic link between PCAE effects on pancreatic homeostasis, I measured inflammatory markers such as; COX-2, IFN- γ , IL-6, and CD3 in whole (endocrine & exocrine) pancreatic tissue, insulin and glucagon in pancreatic islets by immunohistochemistry (IHC). Compared to AD and PF control groups, offspring from AF animals displayed higher levels of inflammatory markers accompanied with low pancreatic insulin and high glucagon expression, indicating the metabolic alterations at pancreas. However, there was no significant

change in the expression of Ki-67 and caspase-3 as markers of cell proliferation and cell death, respectively in AF group compared with AD and PF.

Further, gene expression analysis at liver showed that PCAE markedly increased several genes related to glucose homeostasis such as forkhead box O1 (Foxo1) and glucose-6-phosphatase (G6pc) and decreased insulin receptor (Insr) and protein kinase b (Akt) levels, which reflected an increase in glycogenolysis. However, Western blot results showed no significant changes in insulin receptors levels at muscular and adipose tissues among all of AD, PF, and AF groups. To evaluate the effect of PCAE on diabetes susceptibility, I induced type 2 diabetes in AD, PF, and AF groups by feeding them on 40% HFD for two weeks followed by single injection of STZ 40mg/kg. My results demonstrated that AF-HFD-STZ group showed the highest blood glucose and glucagon levels and lowest insulin in comparison with AD-HFD-STZ and PF-HFD-STZ groups. TNF- α , IL-6, and IL-1 β as markers of inflammation were increased significantly in AF-HFD-STZ in comparison with AD-HFD-STZ and PF-HFD-STZ animals which can reflect the high susceptibility to diabetes in AF group. In a previous study, our lab found that PCAE has the ability to disturb hypothalamic POMC/BEP neurons in the offspring and that might be the central regulation of initiating inflammatory processes in peripheral tissue, such as the pancreas. In this study, I measured the number of beta-endorphin expressing neurons in arcuate nucleus of the hypothalamus and found that AF group showed the lowest BEP secreting neurons in comparison with AD and PF. I therefore proposed that upregulating hypothalamic POMC/BEP would suppress inflammatory processes and reverse PCAE effect on glucose homeostasis by promoting pancreatic tissue functions and having a better glucose homeostasis. At age of two months, I did the

intracerebroventricular (ICV) injection of dibutiryl cyclic adenosine monophosphate (db-cAMP)-delivering nanospheres and considered as a BEP subgroup or plain nanosphere (Sham subgroup) and left one subgroup of animals without surgery for all of AD, PF, and AF groups. I showed that AF-BEP animals demonstrated lower blood glucose and higher blood insulin in comparison with AF-no surgery and AF-Sham subgroups after overnight fasting. There were no changes in basal glucagon and leptin levels. OGTT and GSIS were improved in BEP subgroups of all of AD, PF, and AF. For type 2 diabetes model, I induced a non-insulin dependent diabetic-like state in all of control (no surgery), Sham, and BEP subgroups of AD, PF, and AF subgroups by feeding them on 40% HFD for two weeks followed by single injection of STZ 40mg/kg. BEP sub groups of AD, PF and AF showed lower blood glucose and highest blood insulin in comparison with control-no surgery and Sham surgery sub groups and did not find any change in blood glucagon and leptin level as well.

Taken together, PCAE could significantly induce abnormal glucose homeostasis and increased the susceptibility to non-insulin dependent or type 2 diabetes state in the offspring by disturbing hypothalamic POMC/BEP neurons. One mechanism for this dysregulation of glucose homeostasis is a reduction of hypothalamic beta-endorphin (BEP) neuronal numbers and production in the PCAE offspring. The data from this study indicate that, improving BEP neuronal numbers/ expression could attenuate the abnormal glucose homeostasis and lower the risk to diabetes in AF offspring.

DEDICATION

I would like to dedicate this work to my fabulous family, my dad Dr. Mosa, my mom, my brothers and sisters.

I also dedicate this dissertation to my wife, Dalya, my daughters Baneen, Sadan & Zainab and my son Basheer.

For all of them, I dedicate this work for their passion, support, and guidance.

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LIST OF ABBREVIATIONS

ACTH Adrenocorticotrophic hormone

AD Ad libitum

AF Alcohol fed

AgRP Agouti-related Peptide

AH Anterior hypothalamus

Akt Serine/threonine kinase

α -MSH alpha-melanocyte stimulating hormone

ARBD Alcohol related birth defects

ARC Arcuate Nucleus

ARND Alcohol related neurobehavioral disorders

AUC Area under the curve

BBB Blood Brain Barrier

BCGS Brain-centric glucose control system

BEP B-endorphin

CAT Catalase

CCK Cholecystokinin

CDC Center for Disease Control and Prevention

CLIP Corticotropin-like intermediate lobe peptide

CNS Central nervous system

CORT Corticosterone

COX-2 Cyclooxygenase-2

CPEE Chronic prenatal ethanol exposure

CREB c-AMP response elements binding protein

CRH Corticotropin-releasing hormone

CSF Cerebrospinal fluid

dbcAMP dibutyl-cAMP

DMH Dorsal Medial Hypothalamus

ELISA Enzyme-linked immunosorbent assay

FAS Fetal alcohol syndrome

FASD Fetal alcohol spectrum disorder

FGF19 Fibroblast growth factor 19

FOXO1 Forkhead box O1

G-6-Pase Glucose-6 phosphatase

GIT Gastrointestinal tract

GK Glucose kinase

GLP-1 Glucagon-like peptide 1

GLUT2 Glucose transporter 2

GLUT-4 Glucose transporter-4

GSIS Glucose-Stimulated Insulin Secretion

HCV hepatitis C virus

HFD High fat diet

HMGB1 High motility group box 1

HPA Hypothalamic-pituitary-adrenal

IFN- γ Interferon-gamma

IL Interleukin

iNOS induction of nitric oxide synthase

InsR Insulin receptor

ITT Insulin tolerance test

LH Lateral hypothalamus

LPS Lipopolysaccharide

MBH Mediobasal hypothalamus

ME Median eminence

MTII Melanotan II

NAFLD non-alcoholic fatty liver disease alcoholism

Nhlh2 Nescient helix-loop-helix transcription factor

NK Natural Killer

NO Nitric oxide

NPY Neuropeptide Y

NSC Neuronal stem cells

OGTT Oral glucose tolerance test

PACAP Pituitary adenylate cyclase-activating peptide

PAMP Pathogen associated molecular pattern

PBMC Pro-inflammatory peripheral blood mononuclear cells

PCAE Preconception alcohol exposure

PND Postnatal day

PDK1 Pyruvate dehydrogenase kinase

PF Pair fed

PH Posterior Hypothalamus

PI3K Phosphoinositide 3-kinase

POA Preoptic Area

POMC Proopiomelanocortin

PSNS Parasympathetic nervous system

PTEN Phosphatase and tensin homolog

PVN Paraventricular nucleus

ROS Reactive oxygen species

S6K1 S6 protein kinase-1

SCN Suprachiasmatic Nucleus

SEM Standard Error of Measurement

SNS Sympathetic nervous system

SON Supraoptic Nucleus

STZ Streptozotocin

T1D Type 1 diabetes

TCR T cell receptor

TLR Toll-like receptor

TNF- α Tumor necrosis factor alpha

US United States

VMN Ventromedial Nucleus

WAT White adipose tissue

WHO World Health Organization

CHAPTER 1: BACKGROUND OVERVIEW

1.1 ALCOHOLISM NEGATIVE IMPACT:

Alcohol consumption and its harming effects have been observed since earliest times and alcohol drinking was prohibited, especially in women of reproductive age. For instance, wine consumption was limited in people under 40 years old as Plato proposed (Abel, 1984). In some ancient cultures, such as the Carthaginian Empire, Biblical and Talmudic, and ancient Vedic writings list prohibitions for alcohol drinking, suggesting acknowledgement of some negative effects of alcohol drinking, though it is not clear whether an impact on offspring was known (Mead & Sarkar, 2014).

Alcohol drinking abuse can be considered as one of the most serious health issues. There are many health complications related to alcoholism that result in diseases, disability and deaths, especially in developing countries. It is estimated by the National Institute of Alcohol Abuse and Alcoholism (NIAAA) that about 80,000 people around the world die each year from alcohol-related reasons and it is also predicted that fourteen million Americans (7.4%) have alcohol consumption problems that can be classified as either alcoholism or alcohol abuse (Rachdaoui & Sarkar, 2013). Many people worldwide drink alcohol socially on special occasions such as weddings or at parties and they have the ability to consume alcohol without facing severe health consequences. However, some people drink alcohol heavily and they are classified as “problem drinkers” and may suffer from the negative impact of alcohol consumption that can lead to alcohol dependence (Siglow, 2013).

Alcohol drinking is associated with some health problems that can be classified and categorized as; alcohol dependence, alcohol abuse, harmful drinking, hazardous drinking, and heavy drinking. Alcohol dependence is characterized by at least 3 of these

signs; tolerance, serious withdrawal symptoms, diminished control, anxiety, recreational disability, and alcohol drinking despite adverse consequences. Alcohol abuse has at least one of these symptoms; abnormal social and occupational obligation, alcohol use can occur with some legal problems, alcohol use persists even though there are social and interpersonal problems (Reid et al., 1999). Heavy drinking is defined as an alcohol consumption that exceeds the threshold level of alcohol consumption. According to the NIAAA, the threshold is about 14 drinks/week or 4 drinks/occasion for men, 13 drinks/week or 3 drinks/occasion for women, and more than 7 drinks/week in people who are older than 65 years old (Adams et al., 1996 and Reid et al., 1999). Alcohol hazardous drinking is one of alcohol consumption patterns and it causes serious health problems to its consumers. Hazardous drinking is estimated to reach 21 or more drinks/week or more than 7/occasion in men, and it is more than 14 drinks/week or 5 drinks/occasion in women (Saunders et al., 1993). Alcohol harmful drinking is the alcohol drinking pattern in which, alcohol consumption can lead to physical or psychological harming effects (Reid et al., 1999).

Globally, it is probable that close to 16% of drinkers are heavy drinkers, who consume 60g (i.e., 4.3 drinks) or more of alcohol per occasion, and the percentage of alcohol dependence is greater than 5% in several countries (WHO, 2014). There are some epidemiological data (2012-2013) showed that the incidence of alcohol use disorders, which are very serious mortal disorders can sometimes reduce the expected lifespans more than ten years as a result of alcohol consumption, was 29% in the United States (Schuckit, 2006 and Witkiewitz et al., 2017).

Excessive alcohol drinking such as more than 4 drinks for men or 3 for women per a day, leads to serious health problems accompanied with psychological, physical, and social issues. In addition to that, alcohol abuse is a risk factor for many other diseases including pancreatitis, liver cirrhosis, gastrointestinal tract and liver cancers, cardiovascular diseases, breast cancer, and fetal alcohol syndrome. According to the World Health Organization (WHO), alcohol can be considered as the third principal leading factor for disability and death. It is also estimated by WHO that binge drinking causes approximately two million and half deaths every year and that is close to 4% of total deaths worldwide (Edenberg & Foroud, 2006).

Francis Bacon writings in 1627 described his concern about the effect of maternal alcohol consumption on offspring, and in the early 18th century in England, the “gin epidemic” gave an explanation that drinking during pregnancy can harm the fetus (Abel, 1984). A century old study in guinea pigs was the first definitive evidence that paternal chronic alcohol exposure before conception can cause significant rise in the mortality rate even in the third generation offspring (i.e., grandchildren) and demonstrated the trans-generational effects of alcohol consumption (Stockard, 1913).

Alcohol drinking during pregnancy (fetal alcohol) can cause teratogenic effects on the developing fetus. The term Fetal Alcohol Spectrum Disorder (FASD) refers to wide range of negative impact caused by ethanol exposure during pregnancy. Among them, fetal alcohol syndrome (FAS) is the most severe, characterized by physical and developmental defects such as facial dysmorphology, pre- and postnatal growth deficiencies and central nervous system (CNS) dysfunction. Sometimes, alcohol drinking during pregnancy can also result in alcohol-related birth defects (ARBD), alcohol-related

neurobehavioral disorders (ARND) and other alcohol-related effects. It is estimated that in the United States the incidence of FAS is as high as 1 in 500 live births (Franklin, 2013).

A recent study by Jabbar et al., 2016 showed that alcohol consumption in rat dams three weeks before conception led to abnormal hypothalamic-pituitary-adrenal (HPA) stress axis in rat offspring and the effects were linked with the changes in expression and methylation profiles of several stress-related genes across many brain regions. The reported effects of alcohol in the offspring suggest that preconception exposure to alcohol can affect normal physiological development in the next generation, but other physiological effects were not investigated in that study.

1.2 TYPE 2 DIABETES MELLITUS:

Diabetes mellitus (DM) can be considered as one of the oldest disease that is known by human. Diabetes was originally documented in 1552 B.C. when an Egyptian physician by the name of Hesy-Ra documented his symptoms of frequent urination for an unknown disease that he had (Nwaneri, 2015). The word “diabetes” was first used by Araetus of Cappodocia (81-133AD) and followed by adding “mellitus” which means honey sweet by Thomas Willis, British scientist, when he confirmed that urine of diabetic patients has sweet taste. In 1776, it is completely confirmed by Dobson that the cause of sweetness is the presence of sugar in urine (Ahmed, 2002). The differentiation and characterization between type one (i.e., insulin-dependent or juvenile) and type two DM was completed in 1936. Type 2 DM was described as a part of metabolic syndrome in 1988 and known as non-insulin dependent diabetes mellitus (NIDDM). Type 2 DM

resulted from alteration in genetic, environmental, and behavioral causes and it is characterized by many signs such as; high blood glucose, insulin resistance, and consequently hypoinsulinemia (Ahmed, 2002 and Olokoba et al., 2012). The incidence of type 2 diabetes is about 90-95% of total diabetic patients and those people have insulin resistance and relative hypoinsulinemia at initial stage, but usually throughout their life they do not require any exogenous insulin. Most people of type 2 DM have obesity, which can play a role in initiating insulin tolerance. Even though those type 2 DM patients that have no obesity, the percentage of body fat in the abdominal region is higher than normal (American Diabetes Association, 2014).

It is predicted that 439 million people will be diagnosed with type 2 DM by 2030 worldwide. This incidence is different according to the geographical region and it is related to the life style (Chamnan et al., 2011 and Zimmet et al., 2001). According to Center for Disease Control and Prevention (CDC) report, the total number of people diagnosed with diabetes in the U.S was 25.8 million in 2011 and that is about 7.8% of the population with 90-95% of them were diagnosed with type 2 DM (Olokoba et al., 2012).

Type 2 DM is developed as a result of contribution of lifestyle or genetics or both (Ripsin et al., 2009). The risk factors related to lifestyle can include low level of activity, smoking, and alcohol drinking (Hu et al., 2001). Obesity is also considered as a one important causative agent of type 2 diabetes and it is estimated that obesity is found in about 55% of type 2 diabetes cases. The high prevalence of type 2 DM from 1960 to 2000 is assumed to be related to a higher incidence of obesity in children and youths (Spear et al., 2007).

1.3 PATHOPHYSIOLOGY OF TYPE 2 DIABETES:

The development of type 2 DM is related to the imbalance and abnormal relationship between insulin production and insulin sensitivity, which is indicated by gradual drop in pancreatic beta cells functions as a result of insulin insensitivity (Bacha et al. 2010). Many organs such as; pancreas, liver, skeletal muscle, adipose tissue, and nervous systems interplay a vital role in the pathophysiology and can affect the normal glucose homeostasis and consequently lead to type 2 DM (DeFronzo, 1988).

It is well demonstrated that a high percent of type 2 DM patients are obese or overweight, and the role of fat in type 2 DM was not highly respected until 1990s. Insulin resistance is one of the first related signs to type 2 DM, is initiated and promoted by obesity. The major effect of obesity on glucose homeostasis is depends on the distribution of fat in muscular, visceral, subcutaneous, and liver organs rather than the degree of obesity itself (D'Adamo & Caprio, 2011). Hence, white adipose tissue is a very crucial player in the progression of type 2 DM especially, when it is accompanied with the ectopic fat storage syndrome that is defined as a presence of high level of triglycerides in hepatic, muscular, and pancreatic tissues (Fujioka, 2007).

Histologically, adipose tissue is composed of adipocytes, immune cells, pre-adipocytes, and endothelium. Adipocytes hypertrophy and hyperplasia related with obesity development and progression lead to reduction in blood supply which might cause hypoxia, and consequently, can lead to necrosis and infiltration of macrophages in adipose tissue. As a result, adipocytokines such as; glycerol, free fatty acids, TNF- α , IL-6, plasminogen activator inhibitor-1 (PAI-1) and C-reactive protein (CRP) will be over

produced as an indicator of local white adipose tissue inflammation which can be spread out and cause systemic inflammation and obesity development (Lau et al., 2005 and Trayhurn & Wood, 2004).

Presence of these adipocytokines affects the endocrine system, insulin sensitivity, energy balance, and inflammatory responses. That gives an idea that white adipose tissue is not only functioning as a site of lipid storage, but it also works as an endocrine organ by producing many cytokines (Saleem et al., 2009 and Kaur, 2014). There are many methods to diagnose type 2 DM. First, a patient can receive 75 g of glucose and conducting oral glucose tolerance (OGT) for two hours glucose concentration of 200mg/dL or more. Second, a blood glucose reading of 200 mg/dL or more for random blood glucose. Third, multiple measurements of 126 mg/dL or more of fasting blood glucose (Wingard & Barrett-Connor, 1995). People who are suffering from impaired glucose tolerance (IGT) are predicted to develop type 2 DM in the future (Mahler & Adler, 1999), but improving diet and regular exercise can significantly stabilize IGT and reduce the risk of type 2 DM in pre-diabetic states (Alberti, 1996).

1.4 BETA CELLS DYSFUNCTION IN TYPE 2 DIABETES:

Before developing of IGT, pancreatic beta cells get dysfunctional changes at first and that is reflected by abnormal glucose-stimulated insulin secretion (GSIS) (Ward et al., 1986). Normally, GSIS is a response to glucose molecules in the blood and glucose sensors (receptors) in the beta cells to induce glucokinase (GK), a rate-limiting enzyme of hepatic glucose metabolism. GK in turn, stimulates insulin secretion and acts as glucose sensor a connecting insulin secretion and body metabolic status. Compared with normal,

in type 2 DM patients altered GSIS-GK link and reduced and/or affected beta cells glucose transport has been seen (Mahler & Adler, 1999).

The relationship between beta cells dysfunction and insulin resistance and how it is linked to the progress of type 2 DM was controversial and referring to insulin resistance as an initial stage of type 2 DM compared to insulin secretion inability which is seen at the late stages (Kahn et al., 2014). Earlier, insulin resistance, which is resulted by obesity, makes pancreatic beta cells produce more insulin to normalize GT, but later, when they become unable to normalize it, hyperglycemia will be seen (Kahn et al., 2014). The function of beta cells in type 2 diabetes was studied and investigated for many years and both of beta cells insufficiency and apoptosis are found in human type 2 DM (Butler et al., 2003).

The three main mechanisms that can cause beta cells dysfunction in diabetics are; First, is the presence of genetic problem as it seen in maturity-onset diabetes of youth (MODY) due to GK gene mutation (Polonsky, 1995). Second is the “thrifty phenotype hypothesis” that is related with nutrient deficiency during in-utero fetal development and excessive body fat accretion during later development (Hales & Ozanne, 2003). Third, is the bad metabolic environment such as; presence of hyperglycemia which can lead to glucotoxicity (Yki-Jarvinen, 1992), increase of non-esterified fatty acids might lead to lipotoxicity (Unger, 2002), and accumulation of triglycerides in pancreatic tissue may increase nitric oxide and consequently, induces oxidative defect and apoptosis of beta cells (Unger, 2002).

In conclusion, insulin production by pancreatic beta cells can be disrupted by abnormal insulin signaling that is impaired by insulin resistance in peripheral organs, giving an idea that insulin resistance plays an important role in induction of beta cells dysfunction and accordingly, can be one of the causative agents of type 2 DM development (Greenberg & McDaniel, 2002 and Scheen, 2003).

1.5 HIGH FAT DIET (HFD) AND STZ AS A MODEL OF TYPE 2 DIABETES:

In 1959, the term of high fat diet (HFD) was first described to be related to the induction of obesity and abnormal nutritional status (Masek & Fabry, 1959). There are many following studies showed that the HFD has the ability to induce hyperglycemia and insulin resistance in the whole body in addition to other studies have shown the effect of HFD on muscular and hepatic glucose homeostasis and insulin signaling pathway. Accordingly, the idea of using HFD as a model to generate metabolic disorder, insulin resistance, and abnormal beta cells function was highly accepted (Oakes et al., 1997, Ahren et al., 1999, and Lingohr et al., 2002).

Streptozotocin (STZ) (2-deoxy-2-(3-methyl-3-nitrosourea)-l-d-glucopyranose) (α and β) is an antibiotic that is isolated from *Streptomyces achromogenes* var. *streptozoticus* in 1959 when it was found at Blue Rapids, Kansas during collecting soil specimens (Agarwal, 1980).

STZ has the ability to inhibit insulin secretion and subsequently induce diabetes through selective pancreatic beta cell toxicity (necrosis) (Lenzen, 2008). There are several mechanism has been postulated for STZ mediated toxicity in animals. STZ is a less lipophilic molecule, selectively accumulates in pancreatic beta cells through Glucose

transporter 2 (GLUT2) (Tjalve et al., 1976 and Karunanayake et al., 1976). Whereas, beta cells lack of GLUT2 have the ability to resist STZ mediated toxicity (Ledoux et al., 1984 and Schnedl et al., 1994). STZ causes beta cells toxicity through DNA alkylating effect by transferring its methyl group to DNA molecule to cause DNA damage. Further, to neutralize the STZ effect, induction of poly (ADP-ribose) polymerase (PARP), an DNA repair enzyme will results in the utilization of cellular NAD⁺ and ATP and the drop in total cellular energy leads to necrosis of beta cells (Lenzen, 2008).

The HFD/STZ induced model of type 2 diabetes was first proposed by Reed et al., in the year 2000. In this study it is shown that feeding Sprague–Dawley rats with 40% HFD for 2 weeks followed by single STZ injection of 50 mg/kg led to the development of insulin resistance in rats (Reed et al., 2000). Subsequently, another study shows that multiple low doses of STZ can induce type 2 DM in rats. (Srinivasan et al., 2005). Zhang et al., in 2009 showed that HFD and STZ injection could initiate inflammation and beta cells destruction and cause obesity, insulin resistance, and abnormal glucose tolerance in animal models. HFD/STZ model mimics human type 2 diabetes because the consequence of type 2 diabetes events start with excessive weight gain to promote glucose tolerance and insulin resistance conditions, and are followed by beta cells impairment cause by STZ (Zhang et al., 2009). Beta cells destruction in type 1 diabetes is caused by autoimmune disease is not typically seen in HFD/STZ model (Di Gialleonardo et al., 2012). In HFD/STZ model, destruction followed by regeneration of beta cells was commonly seen in addition to lipotoxicity (gluco-lipotoxicity), insulin resistance, endoplasmic reticulum (ER) stress, and inflammation (Prentki & Nolan, 2006). In conclusion, this model of HFD/STZ has face validity for type 2 DM, and has construct

validity since it causes impairments of glucose homeostasis by promoting insulin resistance and beta cell dysfunction.

1.6 ALCOHOLISM AND GLUCOSE HOMEOSTASIS:

In humans, heavy alcohol consumption can be considered as a diabetogenic factor when it relates to additional caloric intake and obesity, initiation of pancreatitis, disturbance of carbohydrate and glucose metabolism, and impairment of liver function (Holbrook et al., 1990).

Chronic alcohol consumption can affect the normal glucose tolerance, a good indicator of glucose homeostasis, is resulted by abnormal insulin secretion and resistance. Thus, glucose intolerance indicates there is a transition from normal glucose homeostasis toward diabetes in a state known as “prediabetic” (Kim & Kim, 2012).

In alcoholics, it is found that the cause of diabetes was related to low insulin resistance and chronic pancreatitis (Nealon et al., 1988) or due to decreased insulin production that is concomitant with alcohol-induced liver changes (Ikai et al., 1995). It has been also found that reversible insulin resistance, abnormal insulin binding, inhibition of intracellular insulin signaling can be related to chronic high doses of alcohol (Umeki et al., 1989). In addition, OGTT results showed that alcohol dependency was related to impaired glucose tolerance which suggests that heavy alcohol consumption might negatively affect fasting and postprandial glycemic controls and therefore a risk factor for type 2 DM (Umeki et al., 1989). Another study has shown that heavy alcohol drinking can be considered as a risk factor of metabolic and cardiovascular diseases through its effect in elevating blood glucose levels (Leggio et al., 2009).

Whereas in animals models, it has been shown that exposure of mothers with alcohol during their pregnancy could lead to abnormal glucose tolerance with diminished glucose tolerance index to their offspring (Chen & Nyomba, 2004). This might advocate that alcohol exposure during prenatal stage could have the ability to impair glucose tolerance. The rats that were nursed by alcohol drinking dams, without previous exposure to alcohol during pregnancy, also had an affected glucose tolerance in spite of normal body weight and growth pattern (Chen & Nyomba, 2004). Another study by Lindtner et al., 2013 using Sprague-Dawley rats showed that the daily alcohol consumption of 3g/kg for three days, to mimic the alcohol binge drinking in humans, showed that these rats developed insulin resistance, and the resistance was existed for 54 hours even though the blood alcohol concentration was not detectable. In the same study, these rats showed impaired hypothalamic insulin response to suppress liver glucose production, and lipolysis. Accordingly, phosphorylation of hepatic insulin receptors and Akt were decreased as a result of alcohol binge drinking (Lindtner et al., 2013). Moreover, it is also found that diabetic rats consumed chronic alcohol showed higher postprandial plasma glucose level than the non-drinking diabetic rats (Jung et al., 2011).

1.7 FETAL ALCOHOL SPECTRUM DISORDER (FASD) AFFECTS NORMAL GLUCOSE HOMEOSTASIS:

Fetal alcohol spectrum disorders (FASDs) are a group of health problems associated with alcohol consumption during pregnancy (Chudley et al., 2005). In addition to nervous system injuries that can be caused by alcohol consumption during pregnancy, alcohol drinking during pregnancy has the ability to damage other organs such as; heart, lung, and kidney in addition to some findings suggest that alcohol drinking during pregnancy

could disturb the normal metabolism in the offspring (Chen & Nyomba, 2003; Yao & Nyomba, 2008).

In United States it is estimated that FASD affects 2% of people every year and are considered an important medical and social burden on society (Tan et al., 2015). It is also observed that chronic heavy alcohol consumption by adult humans can be considered as substantial risk factor for symptoms of metabolic syndrome, including impaired glucose homeostasis, diabetes mellitus, hypertriglyceridemia, abdominal obesity and high blood pressure. However, the negative impact of chronic alcohol consumption during pregnancy on those metabolic diseases in the offspring is still not well known. It is also found that metabolic pathways such as; insulin signaling, glucose metabolism, gluconeogenesis, insulin resistance were impaired after prenatal alcohol exposure (Chen & Nyomba, 2003). Some findings from human studies showed that the children with fetal alcohol syndrome had high blood insulin hyperinsulinemia and high blood glucose hyperglycemia in oral glucose tolerance test (OGTT) in comparison with normal healthy children. In other words, these results suggest the children with fetal alcohol syndrome have an insulin resistance; nevertheless, the relationship between prenatal alcohol consumption and the risk of metabolic syndrome is still not confirmed in the offspring (Dobson et al., 2012).

The offspring of rats exposed to alcohol showed decreased glucose uptake by muscular tissue (Elton et al., 2002). In another study by Yao and Nyomba, 2007 showed that fetal alcohol-exposure to rats decreased GLUT-4, PDK1, and Akt that are very important in mediating insulin signaling in the muscular tissue, in comparison with control rats. In the same study, it is found that female fetal alcohol rats expressed higher

phosphatase and tensin homolog deleted on chromosome 10 (PTEN). In conclusion, fetal alcohol consumption could disturb the normal insulin signaling and that might give an acceptable mechanism of the affected glucose tolerance (Yao and Nyomba, 2007).

1.8 ROLE OF BRAIN IN GLUCOSE HOMEOSTASIS AND IMMUNE SYSTEM:

In addition to brainstem, several hypothalamic areas such as; arcuate, ventromedial and paraventricular nuclei regulates blood glucose, and it is found that injection of glucose or insulin in these areas can lead to increase insulin sensitivity by hepatic tissue and has blood glucose lowering effect (Obici et al., 2002 and Lam et al., 2005). Similarly, reestablishing leptin receptor in the hypothalamus in the animals lack them can lower blood glucose and increase insulin sensitivity (Coppari et al., 2005 and Morton et al., 2005). On the other hand, deletion of leptin receptors in hypothalamic areas can significantly cause hyperglycemia and insulin resistance, which highlights the importance of hypothalamus in regulating glucose homeostasis (Jordan et al., 2010 and Hill et al., 2010).

The role of brain in controlling glucose homeostasis has been described as “Brain-centric glucose control system” (BCGS), which explains the relationship between the brain and glucose homeostasis. When the body has high blood insulin and glucose during a meal, many signals will be initiated to stimulate BCGS and that will increase glucose disposal by stimulation of insulin-dependent glucose disposal and insulin-independent mechanism as well, and as a result of that, blood glucose will be decreased back to normal (Schwartz et al., 2013). The brain has an important role in regulating blood glucose through several mechanisms, including insulin-independent mechanisms. For

example, there are some studies showed that injection of low doses of leptin into brain ventricles of insulin-deficient diabetic rats and mice has the ability to normalize blood hyperglycemia even though the plasma insulin level is still low (German et al., 2010 and Morton & Schwartz, 2011). In other insulin-deficient diabetic rodent model, it was reported that systemic administration of leptin has shown similar effects of central leptin injection (Yu et al., 2008 and Kruger et al., 2011). Even though it is believed that glucose tolerance reflects the ability of body to lower blood glucose level by many ways that are controlled by insulin action on different body organs, intracerebroventricular injection of leptin could normalize the glucose tolerance in diabetic animals through lowering blood glucose and increasing glucose uptake by different body organs (German et al., 2010) (Figure 1).

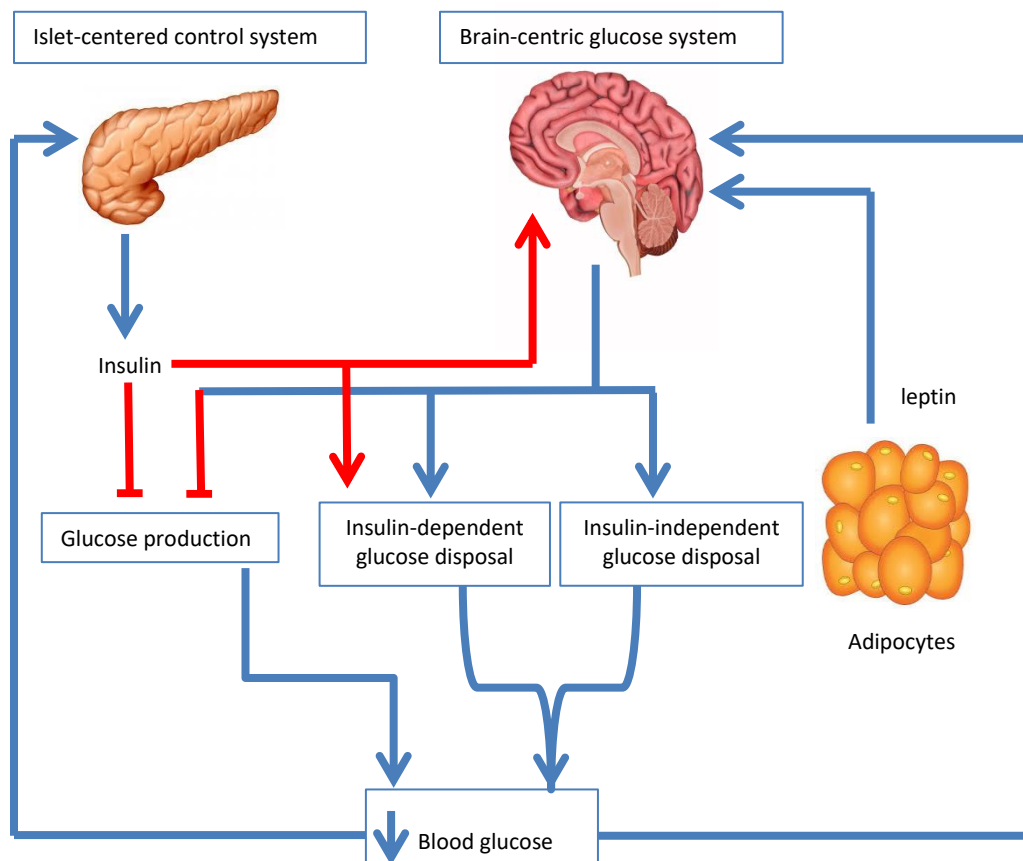


FIGURE 1: BLOOD GLUCOSE HOMEOSTASIS IS CONTROLLED BY “BCGS”: when the blood glucose is high as a result of nutrients ingestion there is a glucose-stimulated insulin secretion. High blood insulin will affect the blood glucose levels by many ways, such as; direct inhibition of glucose production by liver, stimulating insulin-dependent glucose disposal, and stimulates the brain to play its role lowering blood glucose. All of those factors, in addition to leptin’s role on the hypothalamus, will decrease peripheral blood glucose level. If the blood glucose is lower than normal that will stimulate islet-centered control system and BCGS to elevate it back to normal. Adapted from (Schwartz et al., 2013).

Accordingly, any disruption in BCGS such as; deletion of hypothalamic leptin receptors in mice (Jordan et al., 2010 and Hill et al., 2010) is able to induce reduction in insulin sensitivity in hepatic tissue with less effect on blood glucose due to insulin compensatory effect as a response to high blood glucose. Leptin deficiency is related to state called lipodystrophy which is characterized by increased blood glucose and hepatic glucose production regardless of the hyperinsulinaemia (Petersen et al., 2002). In conclusion, the disruption of both BCGS and normal pancreatic islet functions will lead to wide range of health problems that are related to glucose homeostasis. Even though there is still a capacity to compensate the damage happened in BCGS by islets, that capacity will be limited. Therefore, BCGS is working together with islet function to promote glucose homeostasis, and when it affected, islets will compensate if the damage is mild, but when it is advanced, there is no capacity for the islets to compensate or reverse (Schwartz et al., 2013).

Medial orexigenic Neuropeptide Y (NPY)/Agouti-related Peptide (AgRP) neurons and lateral anorexigenic proopiomelanocortin (POMC) neurons are the primary neuronal populations which occupy the ARC. These two sets of cell types play opposing roles to modulate food intake and energy expenditure and are sensitive to changes in lipidemia or glycemia. The neurons of the ARC are appropriately situated proximal to the

fenestrated vessels of the median eminence (ME) and the cerebrospinal fluid (CSF) that bathes the third ventricle where they may aptly respond to circulating signals from the periphery. Molecular communicators like insulin, ghrelin, glucose, leptin glucagon-like peptide 1 (GLP-1), and free fatty acids can act in concert at the hypothalamus to address issues of energy expenditure and regulation. In POMC and AgRP neurons, p70 ribosomal S6 protein kinase-1 (S6K1) is critical for appropriate glucose-sensing and hepatic glucose production as well as insulin sensitivity (Berglund et al., 2014; Hill et al., 2008; Hill et al., 2010).

Glucose levels are converted to neuronal signals by ATP-sensitive K⁺ ion channels which modulate excitability of hypothalamic POMC/AgRP neurons and in turn induce down-stream signaling cascades to bring the system back to its set point in the periphery (Levin et al., 2004). Accordingly, insulin release is also mechanistically linked to central nervous function by sympathetic and parasympathetic innervations. In fact, it has been well-characterized that insulin can be secreted even before glucose enters the bloodstream, in what is known as the cephalic phase of digestion. The visual stimulus of food triggers neuronal excitability to parasympathetic connections which terminate in the pancreas to release insulin (Teff et al., 1993) in a feed-forward mechanism.

POMC is cleaved into several smaller peptides including alpha-melanocyte stimulating hormone (α -MSH), Adrenocorticotrophic hormone (ACTH), Beta-Lipotropin, gamma lipotropin, corticotropin-like intermediate lobe peptide (CLIP), and β -endorphin (POMC/BEP) (Smith & Funder, 1988).

Alpha MSH is a potent appetite-suppressant and binds MCR4 on AgRP Neurons in the ARC. Treatment of melanocortin agonist melanotan II (MTII) centrally in rats

resulted in increased insulin sensitivity and decreased blood glucose as measured by insulin tolerance test (ITT).

Beta endorphin (BEP), another cleavage product of POMC, is an endogenous opioid shown to modulate immune function, glucose homeostasis, analgesia, and stress (Logan et al., 2015). BEP's most appreciated function is to act as an opioid and its ability to diminish the stress response and anxiety. In response to acute stress, the HPA axis and sympathetic nervous system are activated, glucose and fat stores are mobilized, corticosteroids down regulate immune function, and secretion of catecholamines promotes vasodilation for increased oxygen delivery to muscles and hormone-sensitive lipase activity. Under normal conditions, the stress response is controlled by predominating inhibitory GABAergic synapses at PVN of the hypothalamus. Counter-intuitively, GABAergic signals can become excitatory during the stress response via GABA(A) receptor activation, anion transporter KCCS phosphorylation, and subsequent depletion of the chloride current (Hewitt et al., 2009; Sarkar et al., 2011).

POMC/BEP secreted from the ARC or the pituitary can bind presynaptic μ -opioid receptors with high efficiency, engaging trimeric G-protein $G_{o\alpha}$, phospholipase C, and IP3/DAG/RyR-induced intracellular Ca^{2+} release (Velazquez-Marrero et al., 2014) to reduce neurotransmitter release to parvocellular neurosecretory cells in the PVN, thus attenuating sympathetic responses to the body (Iremonger and Bains, 2009).

Primary and secondary lymphoid tissues like the bone marrow, spleen, and lymph nodes are among tissues heavily innervated by sympathetic fibers. The nerves terminating near maturing lymphocytes at the spleen can release norepinephrine, the primary catecholamine of the sympathetic division of the autonomic nervous system and are aptly

poised to regulate immune function, cell growth, survival, and lineage. Immune signals like cytokines released from immune cells into the blood can also modulate central nervous system activity. Stress challenges and inflammation increase pro-inflammatory cytokines, interleukin-1 (IL-1), IL-6, and Tumor Necrosis Factor (TNF) which act at different levels of the HPA axis. IL-1-mediated CRH release can induce secretion of POMC/BEP that inhibits further release of CRH from the hypothalamus. These findings are consistent with the fact that removing the POMC/BEP mediated inhibition on CRH neurons can result in chronic stress and inflammation. Like alpha-MSH, POMC/BEP also has numerous roles in the periphery (Millington, 2007).

The vital role of BEP in the hypothalamic-sympathetic-adrenal response in mediating stress, inflammation, and glucose homeostasis was highlighted. For instance, μ -receptors are localized to peripheral blood mononuclear cells (PBMC) such as monocytes or lymphocytes (T cells, B cells, NK cells) and studies from our lab have shown POMC/BEP neuronal transplants increase PBMC and NK cell activity (Boyadjieva et al., 2009). The ability for β -endorphin to bind μ -receptors with an extremely high affinity led us to investigate its role in natural killer cell function in rodent immune systems. Using *in vitro* differentiated β -endorphin cells transplanted into the hypothalami of experimental animals, we were able to show these cells produce POMC and POMC/BEP. The immune-protective effect of POMC/BEP is thought to be mediated through the suppression of sympathetic neurons that results in an increased peripheral natural killer (NK) cell activity (Boyadjieva et al., 2009).

1.9 ALCOHOLISM AND INFLAMMATION:

Chronic alcoholism mediates inflammation and considered as one of the important etiological factor for chronic inflammation at various organs. It is observed that individuals with alcoholic liver diseases have a high numbers of proinflammatory cytokines circulating in their blood (McClain et al., 1999). Tumor necrosis factor (TNF- α), a pro-inflammatory cytokine, is found at higher level in alcohol-exposed mouse brain and thought to be related to neuro-degeneration. To confirm this relationship, it is found that mice that have knocked out TNF- α receptor did not show any alcohol-induced injuries (Yin et al., 1999).

In general, alcohol-related inflammation, is mainly mediated by the direct effect of alcohol to induce cell damage or derived from gut microflora, specifically, lipopolysaccharide (LPS). Several studies have shown that alcohol or acetaldehyde, a metabolite of alcohol, can change the permeability of the gut and increase the LPS leak from the gut lumen by induction of nitric oxide synthase (iNOS) and NF- κ B signaling, which will control the expression of tight junction proteins including miRNA-122 direct action (Ferrier et al., 2006 and Tang et al., 2008). It is also reported that alcohol can also alter gut integrity and permeability indirectly by different ways such as; first, Zn²⁺ deficiency, known to be detected in alcoholics, second, increased circulating inflammatory cytokines and LPS may promote further increases in gut leakiness. Third, alcohol, by itself, has the ability to modify the mucosal immune system, and sequentially, affect the structure or function of the gut barrier. Moreover, alcohol consumption leads to the elevation of reactive oxygen species (ROS) at cellular and tissue level, and there by it activate the nuclear factor- κ B (NF- κ B), a major transcription factor that regulates genes

responsible for transcription of DNA, cytokine production and cell survival. Alcohol metabolism also induces hypoxia which is also known to induce inflammation as a response (Oliver et al., 2009). Heavy alcohol consumption is a well-known risk factor for the developing chronic pancreatitis (AP) and chronic pancreatitis (CP), an inflammatory disorder of the pancreas (Herrerros-Villanueva et al., 2013).

Alcohol mediated systemic inflammatory signals are sensed rapidly by neural circuits of the CNS by activating the HPA axis and SNS, which in turn, exerts anti-inflammatory function to control the inflammatory process. Glucocorticoids produced by HPA axis exert their effect by binding to their receptors on leukocytes and other immune cells which lead to inhibition of the production of pro-inflammatory cytokines and stimulate the release of anti-inflammatory cytokines including IL-10 (Ogawa et al., 2005 and Richards et al., 2000). Separately, SNS can also be stimulated by peripheral inflammation and that will lead to peripheral release of catecholamines (epinephrine and norepinephrine), that also have anti-inflammatory effects at peripheral level. These catecholamines can promote anti-inflammatory cytokines by interacting with specific receptors on innate immune cells. The net effect of catecholamines is to suppress pro-inflammatory signals. In conclusion, the glucocorticoids and catecholamines (epinephrine and norepinephrine) that made by the HPA axis and SNS will all work together with the anti-inflammatory cytokines produced by immune cells to recover inflammation. This pro-inflammatory response will cause tissue damage because of high toxicity level and in this case, the counter regulatory will be needed to protect body tissue (Wang et al., 2010).

When HPA is activated, the release of CRH stimulates the cleavage of ACTH from POMC expressing cells in anterior pituitary gland. ACTH produced by this

cleavage is then circulated in the blood and stimulates production of CORT. POMC neurons that are located at ARC of the hypothalamus are cleaved by CRH, and as a result of that cleavage, BEP will be produced in the PVN. BEP can exert its function and modulate HPA axis by inhibiting CRH release from CRH-producing neurons in the PVN (Franklin, 2013).

By using different alcohol exposure models such as prenatal and postnatal models, it is found that the exposure to alcohol led to increase the apoptotic rate of hypothalamic cells, including BEP-expressing POMC neurons in the ARC (Sarkar et al., 2007 and Sarkar et al., 2008). Furthermore, it is also demonstrated the BEP neurons expression was decreased after the exposure to prenatal ethanol and as a result it can lead to HPA hyper response to immune challenge in adulthood (Boyadjieva et al., 2009). There are many mechanisms explain how alcohol abuse has the ability to induce neurotoxicity including the role of oxidative stress and inflammatory cytokines or they both together can initiate apoptosis process (Alfonso-Loeches and Guerri, 2011).

Type 2 DM is an important medical problem worldwide which is characterized by abnormal insulin secretion/response or both as it termed insulin resistance. The outcomes of that is hyperglycemia is mainly due to the impairment in glucose uptake by adipose and muscular tissue while there is high glucose production by liver and it is commonly associated with obesity (Hotamisligil, 2006 and Shoelson & Goldfine, 2006). Type 2 DM is also characterized by chronic low grade inflammation induced by oxidative stress, endoplasmic reticulum stress, hypoxia, glucotoxicity, and lipotoxicity (Shu et al., 2012). The persistence of activated HPA axis might activate catecholamine and inflammatory

cytokines production (Henry, 1992). Since BEP has the ability to suppress CRH and HPA axis, it is predicted that treatment with BEP will be effective in type 2 diabetes.

HYPOTHESIS

To determine whether preconception alcohol exposure PCAE has the ability to increase the susceptibility to develop hyperglycemia in the rat offspring through disruption of POMC/BEP neuronal function.

AIMS OF THE STUDY

Aim1: To determine if preconception binge alcohol exposure in rat dams alters glucose homeostasis in rat progeny at fasting and tolerance states

Aim2: To determine if preconception ethanol exposure increases inflammatory milieu in pancreas, glucose production by liver, and the susceptibility to diabetes in PCAE rat progeny

Aim3: To determine if decreased hypothalamic POMC/ β -endorphin neuronal function plays a role in the enhanced hyperglycemic condition observed in PCAE rat progeny

CHAPTER 2

2.1 INTRODUCTION

The pancreas as an endocrine organ has a vital role in glucose homeostasis (Khorami et al., 2015). Insulin, the main regulator produced by beta-pancreatic cells, exerts its physiological effect by stimulating body cells to uptake blood glucose by stimulation of glucose transporter 4 (GLUT4) transcription and translocation, in addition to inhibition of glucose production by the liver (Khorami et al., 2015).

Similar to beta-pancreatic cells, α -cells have an essential role in glucose homeostasis. For instance, in the case of hypoglycemia, pancreatic α -cells will be stimulated to release glucagon to elevate blood glucose to normal by activating liver glycogenolysis and gluconeogenesis, which in turn, leads to elevated blood glucose level (Nadal et al., 1999 and Quesada et al., 2006). In other words, insulin acts as an anabolic factor by stimulating glucose uptake by tissues to form proteins and lipids while glucagon exerts a catabolic effect, by activating liver glycogenolysis and gluconeogenesis (Dunning et al. 2005).

Glucose homeostasis is also regulated by another axis named as an adipo-insular axis, which shows the relationship between insulin and leptin, demonstrates that insulin promotes leptin secretion and, in turn, leptin inhibits insulin secretion when it is presented in high concentration. The net effect of leptin on insulin levels is concluded by its inhibitory effect on proinsulin synthesis and insulin secretion as well (Kieffer, & Habener, 2000 and Covey et al., 2006). Here, I tested the effect of drinking alcohol before pregnancy on the glucose homeostasis in the offspring by measuring blood

glucose, insulin, glucagon, and leptin in fasting and/or during glucose and insulin tolerance tests.

2.2 MATERIALS AND METHODS

Animal Use

Twenty one two months old adult female Sprague Dawley female rats were purchased from Charles River Laboratories, Wilmington, MA. The rats were housed in 12 hours light/ 12 hours dark cycles (lights on at 7:00 am and off 7:00 pm) on a constant temperature (22°C) throughout the study. Animal care was performed in accordance with institutional guidelines and complied with National Institutes of Health policy.

Preconception Alcohol Exposure

Female rats were divided randomly into three groups; (1) the control group receiving rat chow and water ad libitum (AD; N=7), (2) the alcohol fed group (AF; N=7) receiving a liquid diet (BioServ, Flemington, NJ) containing ethanol (6.7%), or (3) the pair fed (PF; N=7) group receiving an isocaloric liquid control diet (BioServ, Flemington, NJ) to match the daily intake of the rats receiving the alcohol-containing diet by matching the volume of food consumed by AF animals with the volume given to PF animals since both of AF and PF diets are prepared in a way to contain the same calories/unit of volume. Adult cyclic female rats were given free access to 6.7% alcohol containing liquid diet and drinking water, frequently drank alcohol to achieve a blood alcohol level between 0.08 -0.13 gm/dl.

Breeding and Conducting Glucose Homeostasis Monitoring in the Offspring

After 30 days of feeding, all rats were given normal chow ad libitum. Three weeks later, the estrus cycle was monitored to breed them with control adult males to produce males and female offspring. Litters were normalized to have four females and four males by selecting them randomly since I did not have significant difference in the body weight within or between groups. The new pups kept with their mothers at the same cage until age of weaning (PND25). At this age, they were weaned and I randomly selected one female from each litter to have three different groups (AD, PF, and AF, N=7) (Figure 2). I monitored glucose status at fasting glucose, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT), and measured blood levels of insulin, glucagon, and leptin in female offspring. At age of 90 days, female animals were sacrificed and blood, pancreatic tissues were collected and used for hormone and histological studies. Male offspring are not included in this study.

Basal and During Glucose and Insulin Tolerance Glucose Level

Basal blood glucose level was measured by using AlphaTrak blood glucose monitoring system and AlphaTrak blood glucose test strips.

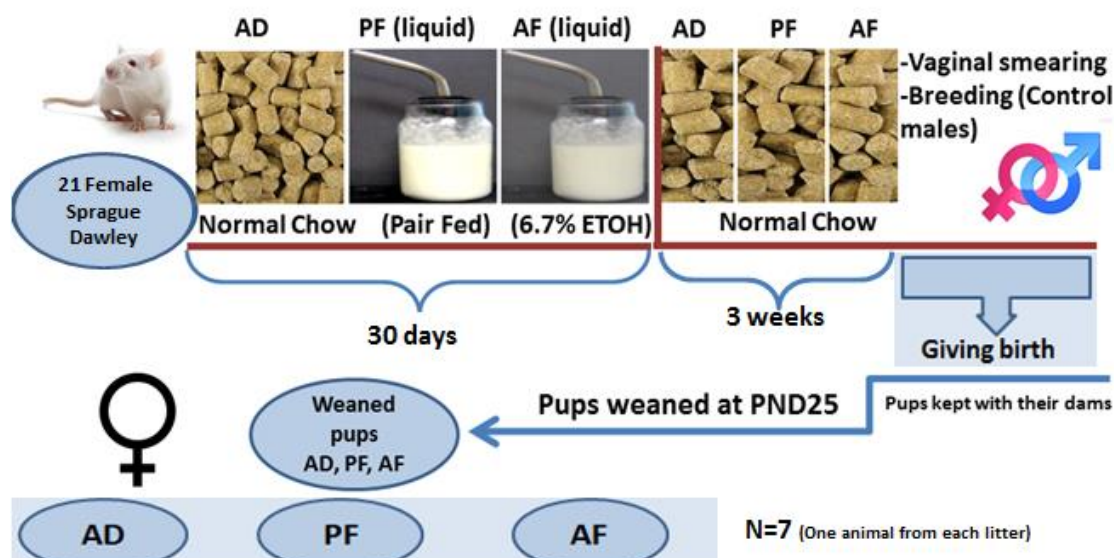


FIGURE 2: THE STUDY DESIGN OF PCAE. Twenty one adult female Sprague Dawley female rats were used as dams. Female rats were divided randomly into three groups; (1) the control group receiving rat chow and water ad libitum (AD; n=7), (2) the alcohol fed group (AF; n=7) receiving a liquid diet containing ethanol (6.7%), or (3) the pair fed (PF; n=7) group receiving an isocaloric liquid control diet to match the daily intake of the rats receiving the alcohol-containing diet. After 30 days of feeding, all rats from different groups returned to be fed by normal chow. Three weeks later, the estrus cycle was monitored to breed them with control adult males to produce males and females offspring. The new pups kept with their mothers at the same cage until age of weaning (PND25). At this age, they were weaned and we randomly selected one animal from each litter to have three different groups (AD, PF, and AF, n=7).

Oral Glucose Tolerance Test (OGTT)

Glucose tolerance was tested in the offspring and conducted by overnight fasting followed by oral administration of 2 g/kg of glucose solution (Sigma Aldrich; Cat# G8270). Blood samples were collected at 0 time before gavaging glucose solution, 30 minutes, 60 minutes, 90 minutes, and 120 minutes after glucose solution gavage to measure glucose level by using AlphaTrak glucometer and glucose strips.

Glucose-Stimulated Insulin Secretion (GSIS)

Glucose solution (2g/kg) was administered orally and blood samples were collected from tail vein. Blood plasma was separated by centrifugation at 4500 rpm for 15 minutes. Plasma samples were used to measure insulin concentration by using rat insulin ELISA kit to evaluate the body GSIS.

Intra-Peritoneal Insulin Tolerance Test (IPITT)

After one hour fasting, insulin (Humulin R U-100) was injected intraperitoneally 1 IU/kg. Blood glucose level was measured at 0, 30, 60, 90, and 120 minutes after injection.

Blood Insulin, Glucagon, and Leptin

Ultra Sensitive Rat Insulin ELISA Kit, Rat Glucagon ELISA Kit and Rat Leptin ELISA Kit (Crystal Chem, Catalog #:90060, Catalog# 81519, and Catalog# 90040, respectively) were used to detect blood insulin, glucagon, and leptin by following the instructions provided by the company.

Immunohistochemistry

Pancreatic tissues were harvested and kept in neutral formalin for fixation. Pancreatic tissues were dehydrated, cleared, and embedded and completely processed by the Histopathology Core of the Environmental Occupational Health Sciences Institute at Rutgers University. Pancreatic paraffin samples were sectioned at 6 μ m. To do immunohistochemistry IHC, slides were incubated overnight at 60°C, followed by deparafinization in three series of xylene (20 minutes, 15 minutes and, 10 minutes)

followed by tissue rehydration by using different concentrations of ethanol (from 100% to 50%). Antigen retrieval was performed by boiling the slides in 10 mM sodium citrate (pH 6.0) at 95°C for 20 min followed by cooling them at room temperature. Pancreatic sections from different groups were incubated with 2% bovine serum albumin in PBS and then with primary antibodies anti-insulin and anti-glucagon (Santa Cruz Biotechnology: anti-insulin (H-86), and anti-glucagon (FL-180) rabbit monoclonal) for overnight at 4 °C. The following day the tissues were washed with PBS and incubated with secondary antibody (Vector, Burlingame, CA) for 60 minutes at room temperature. Slides were counterstained with DAB (Vector Laboratories SK-4100 DAB Substrate Kit, Brown) followed by mounting with per mount prolong media. By using 20X magnification, ten pictures were taken randomly per section and the staining intensity was measured by using ImageJ software.

Statistical Analysis

Graph Pad Prism version 4 software was used for statistical analysis of data. Quantitative results are indicated as mean \pm SEM. The significance between treatment and controls was assessed using one way and two-way ANOVA between treatment and days of exposure. $P < 0.05$ was considered significant.

2.3 RESULTS

To understand the effect of PCAE on normal glucose homeostasis, fasting blood glucose, insulin, glucagon, and leptin were measured at age of PND60. Preconception alcohol exposure significantly increased basal blood glucose level in AF animals in comparison with AD and PF control animals (Figure 2-a). This high blood glucose was accompanied with low basal blood insulin level in AF group as compared with AD and PF groups (Figure 2-b). Interestingly, blood glucagon was lower in AF group when it compared with control groups (Figure 2-c). Blood leptin level, as a glucose homeostasis modulator, was measured and the results showed that PCAE could significantly increase leptin level (Figure 2-d).

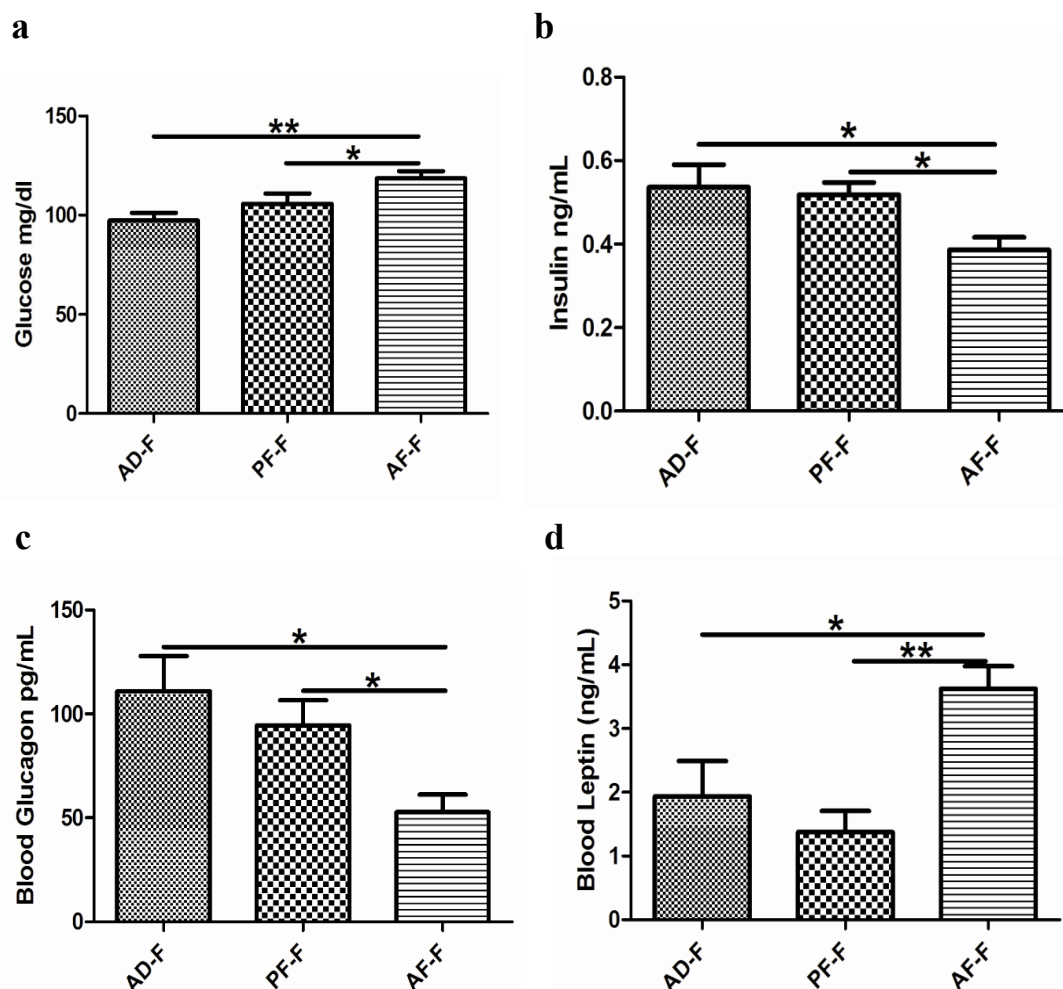


FIGURE 3: PRECONCEPTION ALCOHOL EXPOSURE ALTERS BASAL LEVELS OF GLUCOSE, INSULIN, GLUCAGON AND LEPTIN IN FEMALE OFFSPRING OF AF DAMS

Blood glucose homeostasis was evaluated by measuring glucose, insulin, glucagon, and leptin after overnight fasting. **a.** Histograms showing blood basal levels of glucose (using Alphatrak glucometer and strips) in AD, PF, and AF groups, “F” letter refers to the gender “female”. **b.** Blood basal level of insulin was measured by insulin ELISA kit. Histograms showing the representative blood insulin level in AD, PF, and AF female rats. **c.** Glucagon blood level was measured by using glucagon ELISA kit. **d.** Histograms showing blood leptin level in AD, PF, and AF female offspring by using ELISA kit. Data are means \pm SEM (One-way ANOVA) and Newman-Keuls Multiple Comparison Test. N=7, *P<0.05 and **P<0.01.

Since I have found a significant effect of PCAE at basal level, I then decided to conduct other experiments that related with glucose homeostasis such as; oral glucose tolerance, glucose-stimulated insulin secretion, and insulin tolerance test. My data showed that PCAE could significantly change and disturb the normal glucose homeostasis. OGTT data revealed that the PCAE increases blood glucose level in AF group (* $P < 0.01$ and $P < 0.05$) as compared with AD and PF control groups, respectively, shown in curves and areas under the curve (Figure 3-a & b). To evaluate the body response to sudden high blood glucose after gavaging of glucose solution, I measured GSIS. GSIS data showed that AF group had the highest blood glucose ($P < 0.05$) in comparison with AD and PF as it clearly shown in the area under the curve (Figure 3-c & d). Baseline data showed that PCAE decreases the production of insulin by measuring the basal level of insulin in blood. Here, I decided to provide insulin and evaluate the body response to that insulin by measuring the blood glucose that resulted from IPITT. My findings showed that AF group has the highest blood glucose ($P < 0.05$) when it compared with both of AD and PF control group (Figure 3-e & f).

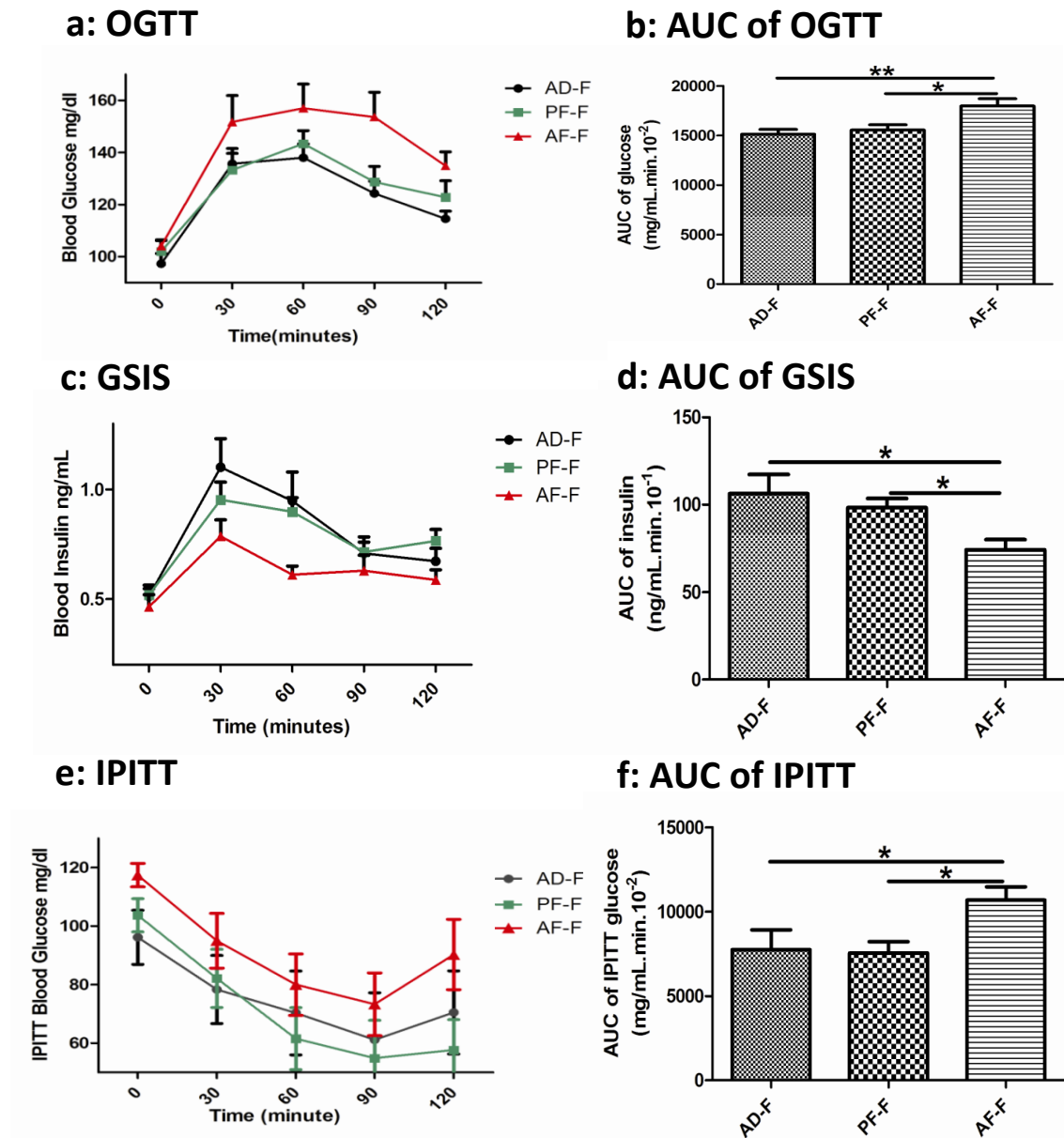


FIGURE 4: PCAE REDUCES GLUCOSE TOLERANCE AND INSULIN SENSITIVITY IN FEMALE OFFSPRING OF AF DAMS

PCAE disturbs the normal glucose homeostasis by different ways. All of OGTT, GSIS, and IPITT have shown abnormal hyperglycemia in AF female animals. **a, c, and e:** Curves represent blood glucose level after oral glucose tolerance test (OGTT) Interaction: $F(8,76)=1.218$, $P>0.05$, Time: $F(4,76)=44.04$, $P<0.0001$, and Treatment: $F(2,76)=3.923$, $P<0.05$, glucose-stimulated insulin secretion (GSIS) Interaction: $F(8,76)=2.552$, $P<0.05$, Time: $F(4,76)=35.60$, $P<0.0001$, and Treatment: $F(2,76)=2.885$, $P>0.05$, and intraperitoneal insulin tolerance test (IPITT) Interaction: $F(8,68)=0.5282$, $P>0.05$, Time: $F(4,68)=16.23$, $P<0.0001$, and Treatment: $F(2,68)=1.379$, $P>0.05$, respectively

(two-way ANOVA) and Bonferroni posttests. **b, d, and f:** Histograms represent the means and SEM area under the curve of blood glucose during OGTT, GSIS, and IPITT. N=7, *P<0.05 and**P<0.01.

To evaluate whether PCAE has the ability to exert its effect at tissue level, paraffin pancreatic sections were used for IHC to detect insulin and glucagon production in pancreatic tissues obtained from PCAE animals and compare them with other animals from AD and PF control groups. IHC data showed that the intensity of insulin staining was lower in AF as it shown in pictures of (Figure 4-a) and in the histogram (P<0.05) (Figure 3-c). Glucagon intensity was also altered in AF animals. AF animals showed significant increase (P<0.01) of glucagon expression in pancreatic tissues in comparison with other animals of AD and PF control groups (Figure 4-b & d).

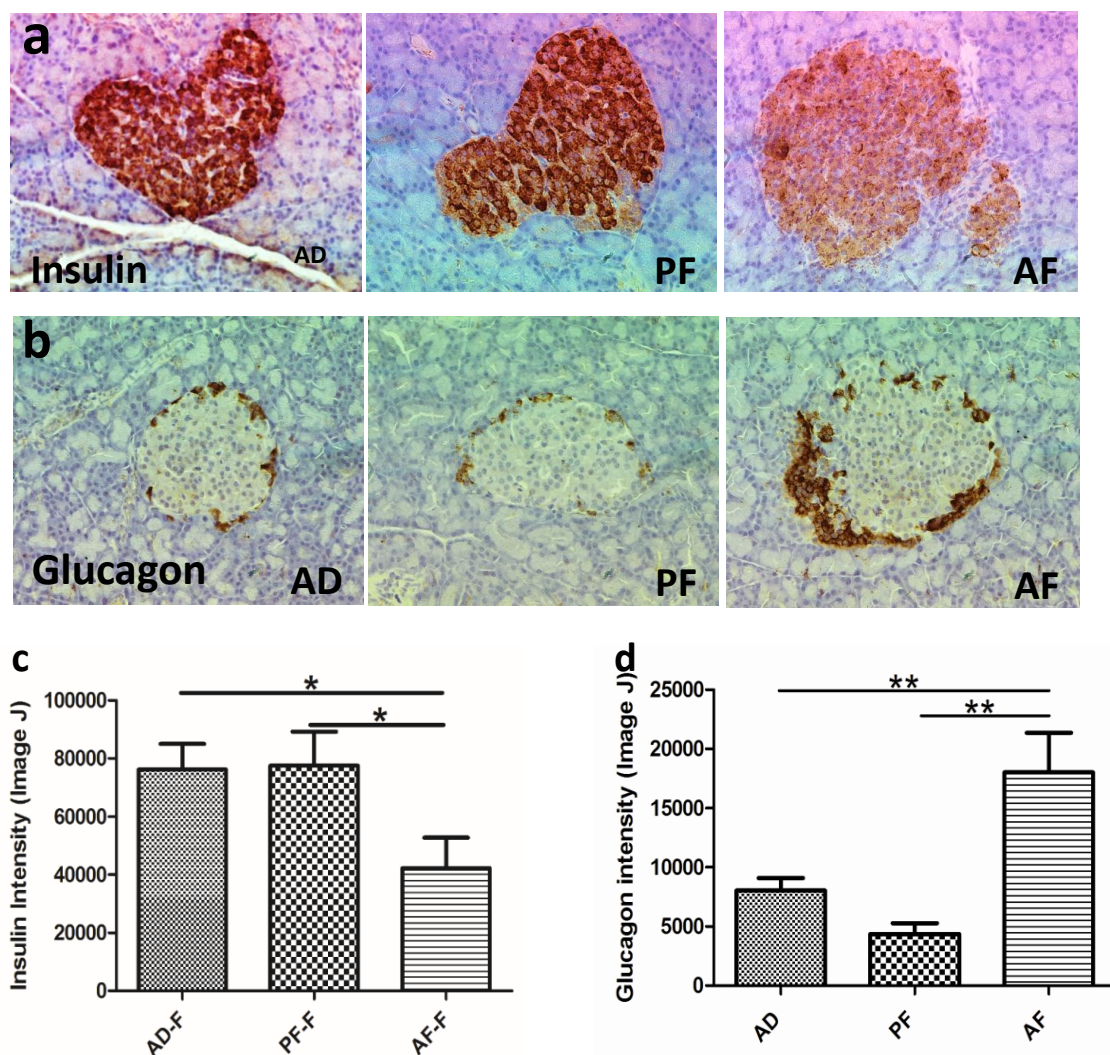


FIGURE 5: PRECONCEPTION ALCOHOL EXPOSURE ALTERS THE INTENSITY OF PANCREATIC INSULIN AND GLUCAGON IN THE OFFSPRING

PCAE has the ability to exert some histological changes in the offspring pancreatic tissue. **a:** IHC of pancreatic tissues showed that the intensity of insulin production was lower in AF animals in comparison with AD and PF (20X magnification). **b.** Immunostaining of pancreatic tissues showing that the intensity of glucagon was much higher in pancreatic tissue of AF animals. **c & d:** Histograms showing means and SEM of AD, PF, and AF for insulin and glucagon, respectively. Insulin intensity decreased significantly ($P < 0.05$) in PCAE group while the same group showed high significant intensity ($P < 0.05$) in comparison with other AD and PF groups. One way ANOVA and Newman-Keuls Multiple Comparison Test, $N=7$ * $P < 0.05$ and ** $P < 0.01$.

2.4 DISCUSSION

I show here for the first time that PCAE has the ability to alter the normal glucose homeostasis in the offspring. At first of all, I provide that the heavy alcohol drinking before pregnancy could significantly disturb the basal level of glucose and other essential hormones that can be considered as keys of normal glucose homeostasis. I found that alcohol consumption before pregnancy has elevated basal blood glucose level. This increment can be resulted by the effect of PCAE on pancreatic function as a direct effect as I found in regards of insulin and glucagon alteration or by the effect of leptin on normal insulin production and secretion.

It is already reported that heavy alcohol drinking can be considered as a risk factor for diabetes development (Holbrook et al., 1990). It is also confirmed by many studies that heavy alcoholics have higher risk than light/moderate drinkers for developing diabetes. In the Paris Prospective Study, diabetic patients had a higher risk of death by liver cirrhosis, which was caused by heavy alcohol drinking (Balkau et al., 1991). It has been suggested that the mechanism by which, alcohol can make the body susceptible to diabetes, is through its direct toxic effect on the pancreatic islet cells (Wannamethee et al., 2002). The impaired insulin-mediated glucose uptake has been seen in many previous studies that showed a negative correlation between alcohol consumption and blood insulin level (Lazarus et al., 1997).

The direct effect of alcohol consumption on pancreatic function was well-studied to understand the mechanism behind its action. Alcohol can be one of the risk factors for pancreatic necrosis. Some findings showed that the pancreas that already exposed to

alcohol was more sensitive to LPS-induced cells damage because of the high sensitivity to necrotic cell death rather than apoptotic cell death and that would suggest this mechanism may occur in acute alcoholic pancreatitis patients (Fortunato et al., 2006). As known, alcohol metabolites such as acetaldehyde and FAEEs have the ability to harm body cells and can make metabolic alterations by inhibition of stimulated secretion as a response to stimuli (Chowdhury & Gupta, 2006).

Here in this study I am focusing on the intragenic effect of alcohol. It is found that F0 alcohol consumption can make changes in behavior, neurochemical signaling, and brain morphology in F1 offspring. Those changes include reduced alcohol intake and increased behavioral response to alcohol, Decreased fear and increased aggression, decreased attention and increased impulsivity, deficits in passive avoidance learning, and decreased POMC expression in arcuate nucleus and many other behavioral, neurochemical, and cellular structure abnormalities can be seen in F1 due to F0 exposure to alcohol (Yohn et al., 2015). However, to date, there is no work or study has described the effect of preconception alcohol exposure on glucose homeostasis in the offspring and here I suggest that the PCAE may have some intragenerational effects can be detected in the offspring.

Those intragenic effects of PCAE could give an explanation for the abnormal glucose, insulin, and glucagon assuming that the negative impact of alcohol on alcoholics can be transferred to the offspring. In addition, PCAE increased the blood level of leptin, which is known to suppress insulin production and secretion by its negative on proinsulin synthesis and insulin production as well, and that can also give us a good etiology of the low blood insulin level (Covey et al., 2006).

CHAPTER THREE

3.1 INTRODUCTION

Pancreatitis caused by alcoholism can be considered as one of the common causes of death (DALGAARD, 1956). In the U.S, there is about one third of acute pancreatitis are alcohol related and it is about 60%-90% of pancreatitis patients had a history of chronic alcoholism (Strate et al., 2002). The risk of pancreatitis developing increases with increases both amount and duration of alcohol consumption (Chowdhury & Gupta, 2006). In addition to alcoholism, it is thought that other factors can also play a role in developing pancreatitis such as; environmental, genetic, and race (Chowdhury & Gupta, 2006). Nowadays, it is become accepted that alcoholic acute and chronic pancreatitis, that caused by alcohol consumption, same disease but at different stages. Repetitive pancreatic inflammation and death leads to periductular obstructive scarring and protein plug formation and eventually extensive fibrosis. This repeated inflammation makes the patients with acute pancreatitis more susceptible to develop chronic pancreatitis and that what is seen in heavy alcohol drinking (Ammann, & Muellhaupt, 1994).

Likewise, the liver is very important organ and has a considerable in carbohydrate metabolism because it is responsible for the balance of blood glucose levels by regulating glycogenesis and glycogenolysis (Holstein et al., 2002). If the liver has any disease, hepatic glucose homeostasis will be impaired as it related to insulin resistance, abnormal glucose tolerance, and diabetes. Additionally, the prevalence of diabetes is really related with liver disease such as; non-alcoholic fatty liver disease (NAFLD), alcoholism, hepatitis C virus (HCV) and hemochromatosis (Garcia-Compean et al., 2009).

It is reported that gluconeogenesis and abnormal glucose tolerance develop in adult offspring after exposure to ethanol during pregnancy in rats. The high gluconeogenesis rate was elucidated by increased expression of major genes PEPCK and G6Pase in the liver. These data are also found when the rats were exposed to prenatal ethanol during the entire duration of pregnancy. In addition, there is another study showed that exposing rats to prenatal alcohol for only one week could exert high gluconeogenesis due to increased expression of histone deacetylases (Yao et al., 2013). Since the negative effects of alcohol are seen in alcoholics themselves, here in this study, I examine that the PCAE can exert similar effects in the offspring. Since healthy functioning pancreatic and hepatic tissues are required for normal glucose homeostasis, I evaluated the inflammatory status in pancreatic tissue in addition to estimating the glucose production by liver by collecting pancreatic and hepatic tissues.

3.2 MATERIALS AND METHODS

Animal Use, Study Design, and Tissue Collection

As described before in chapter 2, I used the same study design. At age of 90 days, animals were sacrificed and I collected pancreatic and hepatic tissues and I kept them in neutral formalin and -80, respectively.

Susceptibility to Diabetes

To study the effect of PCAE on diabetes susceptibility, rats were fed on 40% HFD (Research Diet, INC, New Brunswick, NJ) for two weeks followed by intraperitoneal single injection of STZ 40mg/kg. At 90 days. Animals were sacrificed and blood,

pancreas, and liver tissues were collected and used for hormone, gene measurements, and histological studies.

Immunohistochemistry

Pancreatic tissues were harvested and kept in neutral formalin for fixation and I followed the same protocol that mentioned in chapter two. Primary antibodies for immunohistochemistry were used to detect specific proteins in pancreatic paraffin sections as the following: Anti Ki-67 (rabbit monoclonal), anti-COX2 / Cyclooxygenase 2 antibody, anti-CD3 antibody and IL-6 (mouse monoclonal)(Abcam, Cambridge, MA), cleaved caspase-3 (Asp175) (5A1E) Rabbit mAb (Cell Signaling Technology, Inc., Danvers, MA), and anti-insulin (H-86), anti IFN- γ (J-7), and anti-glucagon (FL-180) rabbit monoclonal; Santa Cruz Biotechnology, Inc. Dallas, Texas followed by incubation with secondary antibodies (anti-mouse or anti-rabbit, Immpress Reagent Kit, Vectorlabs, Burlingame, CA). ImageJ software was used to detect the intensity of protein expression in tissue or counting the positive cells per microscopic fields (10 microscopic fields/section).

Real time PCR for gene expression:

Gene expression levels of *Akt*, *Glucokinase*, *Glut2*, *Pepck*, *Foxo1*, *glucose-6-phosphatase*, and *InsR2* (Table 1) in the hepatic tissue measured by quantitative real time PCR (SYBR green assay). Total RNA from pituitary gland was extracted using the All in One Purification Kit (Norgen Biotek, Ontario, Canada). Total RNA (1 μ g) was converted to first strand complementary DNA (cDNA) using a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). All the primer sequences used for

the study are given in Table 2. Real time quantitative PCR was performed at 95°C for 5 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 40 sec using Applied Biosystems 7500 Real time PCR system. The quantity of target gene expression was measured using standard curve method. GAPDH was used as internal control. Gene expression levels as GAPDH ratios are presented in the figures.

Primers		Sequence 5' to 3'
INSR2	Forward	ATTCCCTGGTATTTGGGCGG
INSR2	Reverse	AGGGAAAGGCAGTGGGTCTA
Akt	Forward	ACCATGAAGACGTTCTGCGG
Akt	Reverse	CTCCGTTCACTGTCCACACAC
GLUT2	Forward	GCAACATGTCAGAAGACAAGATCAC
GLUT2	Reverse	TGGTAGCTCTTCGGTCATCC
PEPCK	Forward	GGATGTGGCCAGGATCGAAA
PEPCK	Reverse	ATACATGGTGCGGCCTTTCA
G-6-Pase	Forward	AACTCCAGCATGTACCGCAA
G-6-Pase	Reverse	TCCAGCATTACAGCTTTCCT
FOXO1	Forward	TACACGTACGGCCAATCCAG
FOXO1	Reverse	GACTGTTGGGTTGAGCCACT
GK	Forward	TGGCCTAATGAAAGCTGGGG
GK	Reverse	ACACACTTATGGCCCGTGTT

TABLE 1: FORWARD AND REVERSE SEQUENCING OF PRIMERS THAT THEIR EXPRESSION WAS MEASURED IN HEPATIC TISSUE

Western Blotting

Proteins levels of PDK1, PTEN, IL1-b, IL6, TNF- α , InsR and β -actin were determined by western blot. Pancreatic, tissue samples were collected and protein was extracted and estimated and 50 μ g of protein was run in 4-20% SDS PAGE. The primary antibodies used were: PDK1, PTEN (Cell Signaling Technology, Inc., Danvers, MA), IL-1b, IL-6, and TNF- α (Abcam, Cambridge, MA), and mouse anti-actin antibody (EMD

Millipore, Billerica, MA). The protein band intensities were determined by Image Studio Lite software (LI-COR Biotechnology, Lincoln, NE).

Statistical Analysis

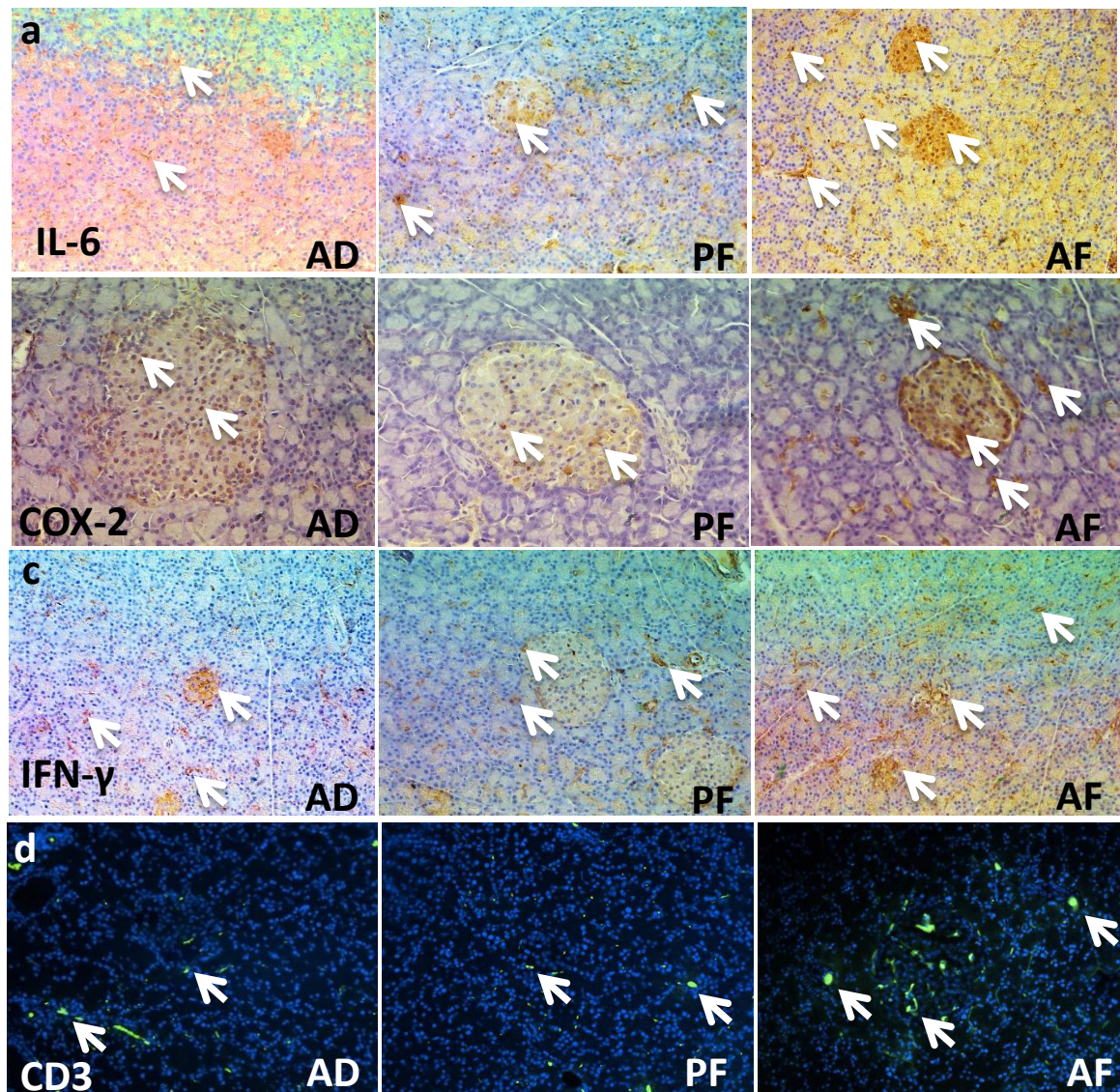
Graph Pad Prism version 4 software was used for statistical analysis of data. Quantitative results are indicated as mean \pm SEM. The significance between treatment and controls was assessed using one-way ANOVA between treatment and days of exposure. $P < 0.05$ was considered significant. Newman Keuls post-hoc testing was performed when justified.

3.3 RESULTS

It is already found and mentioned above that alcohol is one of the risk factors for fatal pancreatitis in alcoholics. Inflammation of pancreas will directly affect pancreatic function negatively. As a result of that, glucose homeostasis will be disturbed since the normal pancreatic endocrine function is impaired. To study the intragenic alcoholic harming effects, I measured the expression of some important inflammatory markers such as IL-6, COX-2, IFN- γ , and CD3 as indicators of pancreatic inflammation and to confirm that alcohol drinking by mothers before pregnancy can lead to abnormal pancreatic function due to pancreatitis. As it shown in (Figure 5:a-c) pancreatic tissues of PCAE showed significant ($P<0.05$ and $P<0.01$) increase of IL-6, known pro-inflammatory marker, as compared with AD and PF control groups, respectively, (Figure and pictures 5-a & a1).

Cyclooxygenase COX-2, as another pro-inflammatory marker, was increased significantly in PCAE. In comparison with AD and PF control groups, PCAE increased the tissues expression of COX-2 significantly, $P<0.05$ and $P<0.01$, respectively (Figure and pictures 5-b & b1). IFN- γ data revealed that PCAE led to increase IFN- γ significantly $P<0.05$ in pancreatic tissue of AF group animals when it compared with AD and PF groups (Figure and pictures 5-c & c1). CD3, cluster of differentiation protein, part of the T cell receptor (TCR) complex on a mature T lymphocyte, is also increased incredibly and significantly in AF animals as compared with AD and PF control groups (Figure and pictures 5-d & d1).

In conclusion, I found here that PCAE could significantly increase the expression of inflammatory markers in pancreatic tissue suggesting a mechanism to explain the low insulin intensity in beta cells of AF group which is related to high blood glucose level that I have seen in AF group.



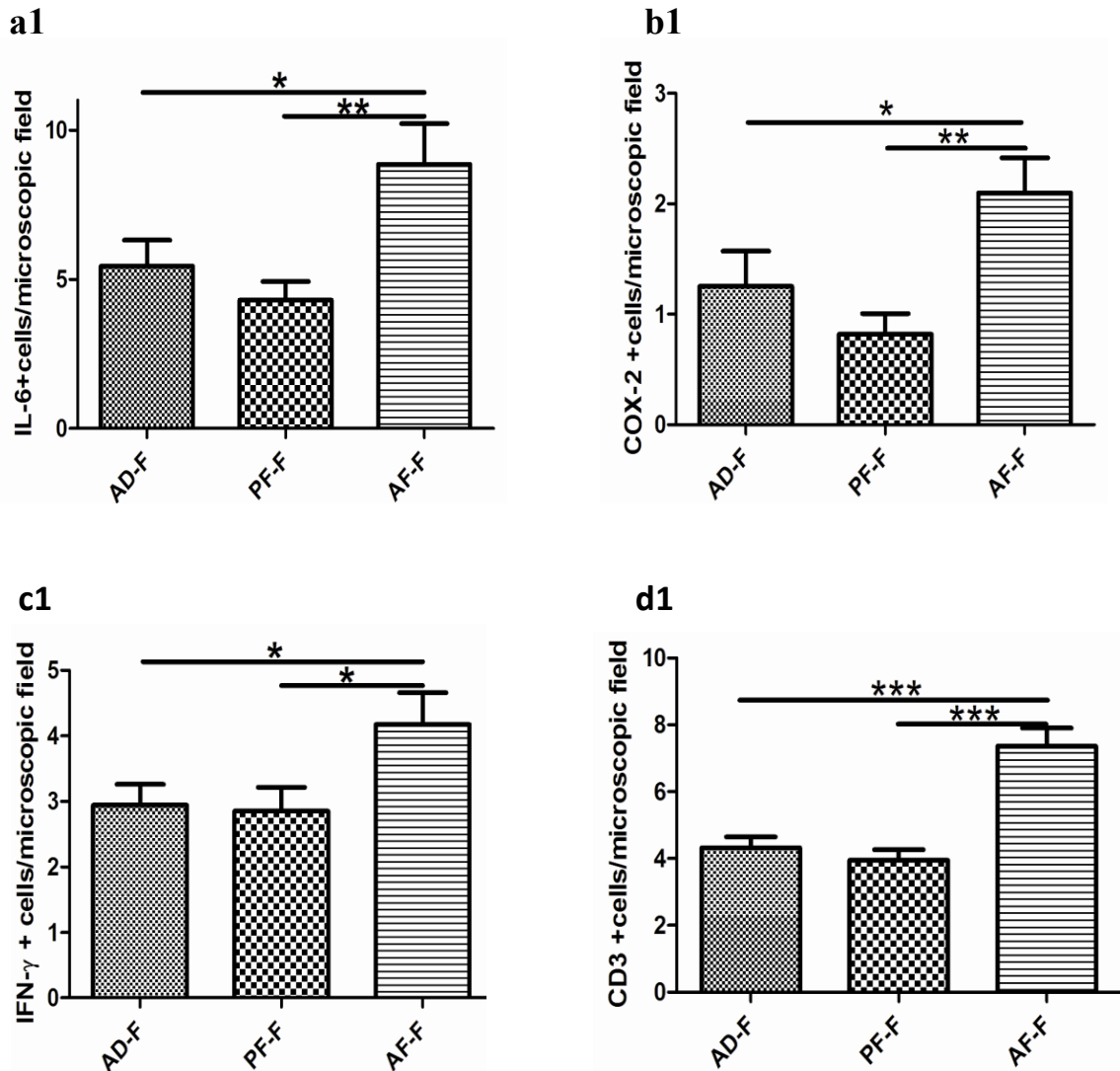


FIGURE 6: PRECONCEPTION ALCOHOL EXPOSURE INCREASES THE EXPRESSION OF PANCREATIC INFLAMMATORY MARKERS IN THE OFFSPRING

PCAE increased the expression of inflammatory markers in the offspring pancreatic tissue. **a:** IHC of pancreatic tissues shows that the number of IL-6 positive cells is higher in AF animals in comparison with AD and PF (20X magnification). **b.** Immunostaining of pancreatic tissues showing that COX-2 was much higher in pancreatic tissue of AF animals. **c & d:** IFN- γ and CD3 IHC staining pictures show the increment of both inflammatory markers in AF pancreatic tissue in comparison with AD and PF control groups. **a1, b1, c1, and d1:** Histograms showing means and SEM of AD, PF, and AF for IL-6, COX-2, IFN- γ and CD3, respectively. One way ANOVA and Newman-Keuls Multiple Comparison Test, N=7 *P<0.05, **P<0.01, and ***P<0.001. Using positive cells counting/10 microscopic fields.

Since it found the pancreatic injury leads to pancreatitis and as a sequence of that will lead to cell death, here in this experiment I evaluated the cell proliferation and cell death in pancreatic tissues by detecting the tissue expression of Ki67 as a cell proliferation marker, and caspase-3 as an apoptotic factor in pancreatic tissue collected from animals of AD, PF, and AF groups. IHC data showed that the PCAE did not affect the normal cell proliferation or cell death and the P value was $P>0.05$ (Figure 7).

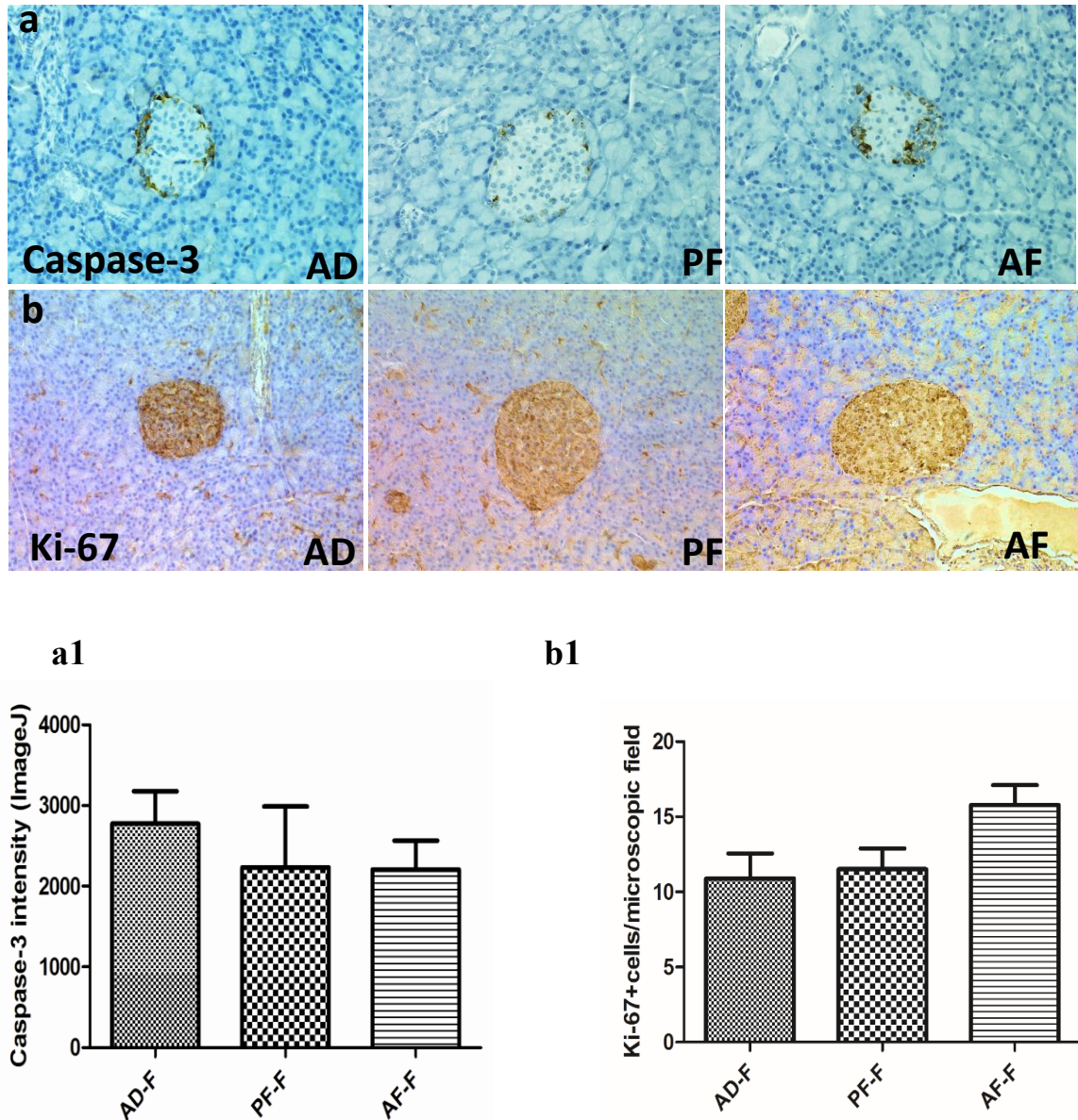
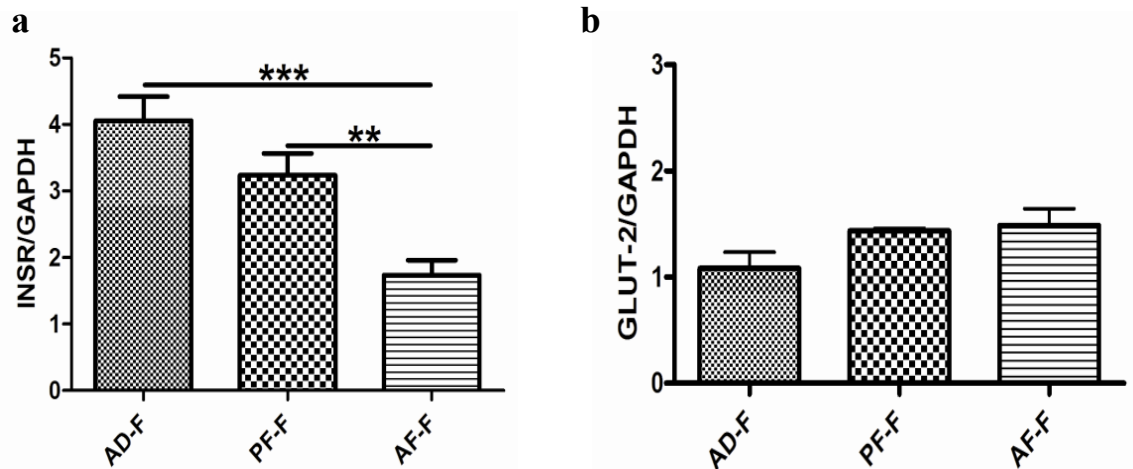


FIGURE 7: PRECONCEPTION ALCOHOL EXPOSURE DID NOT AFFECT APOPTOSIS OR CELL PROLIFERATION IN PANCREATIC ISLETS OF THE OFFSPRING

PCAe did not change the cell death or proliferation in the offspring pancreatic tissue. **a & b:** IHC of pancreatic tissues showing the intensity of caspase-3 and ki-67, respectively in AD, PF, and AF animals (20X magnification). **a1 & b1:** Histograms showing means and SEM of AD, PF, and AF for caspase3 and ki-67, respectively. There is no significant change among different groups. One way ANOVA and Newman-Keuls Multiple Comparison Test, N=7.

Glucose homeostasis is a coordinated process involving many body organs such as pancreas, brain, liver, fat tissue, and muscles. As a result of this process, blood glucose will be maintained within the normal range. Since I have found abnormal insulin, glucagon, and leptin levels in PCAE animals' blood in addition to the high expression of inflammatory cytokines, here I evaluated the glucose homeostasis in regards of the liver's role. Gene expression data showed that PCAE could significantly; decrease InsR and Akt, and increased FOXO1 and G-6-Pase. The data did not show any significant change in GLUT2, GK, and PEPCK (Figure 8).



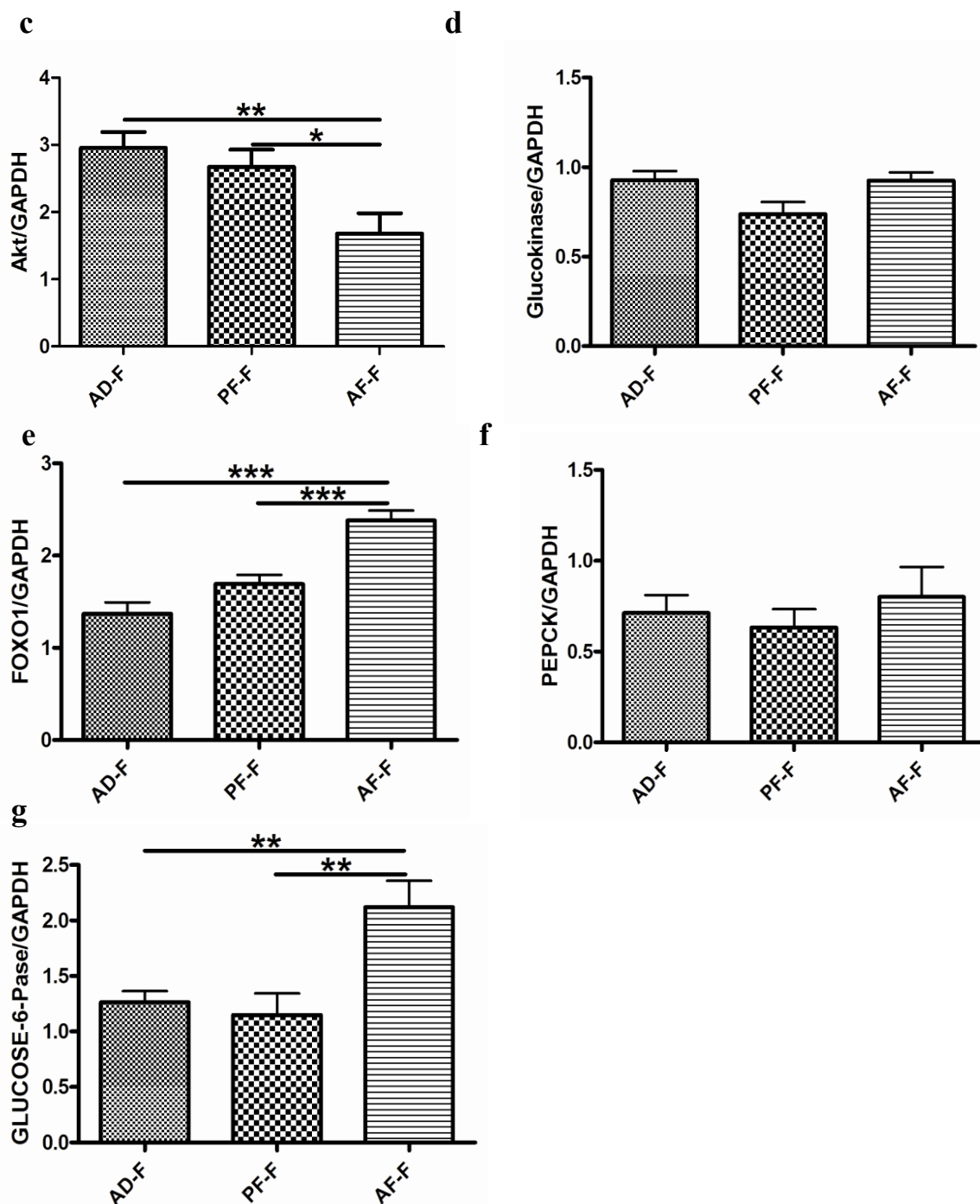


FIGURE 8: PRECONCEPTION ALCOHOL EXPOSURE REDUCES THE EXPRESSION LEVEL OF INSULIN RECEPTOR AND ALTERS THE EXPRESSION LEVEL OF GENES CONTROLLING LIVER GLYCOGENOLYSIS AND GLUCONEOGENESIS

PCAE is able to change hepatic expression of genes that are related to liver glucose homeostasis. **a-g:** Histograms showing the data of RT-PCR of InsR, GLUT2, Akt, GK,

FOXO1, PEPCK, and G-6-Pase for AD, PF, and AF groups using hepatic tissues. Among those genes, InsR was decreased significantly (** $P < 0.01$ and *** $P < 0.001$) in comparison with AD and PF, respectively. Akt is also decreased significantly in AF group when it compared with AD (** $P < 0.01$) and PF (* $P < 0.05$). FOXO1 gene expression shows the PCAE increased significantly (*** $P < 0.001$) in comparison with AD and PF control groups. G-6-Pase increment was induced by PCAE and led to significant difference (** $P < 0.01$) as compared with AD and PF female offspring. One-way ANOVA and Newman-Keuls Multiple Comparison Test. $N=6$.

The role of insulin signaling in hepatic tissue is very important to control the entire glucose homeostasis. In addition to the negative PCAE effects that I have seen at gene level, I conduct Western Blotting to understand the entire signaling at proteins level. To do that, I measured the protein expression of PDK1 and PTEN. PDK1, pyruvate dehydrogenase kinase 1, is an important protein facilitates insulin signaling. On the other hand, phosphate and tensin homolog (PTEN), suppresses insulin signaling. Detecting those two proteins showed that PCAE increased PTEN (* $P < 0.05$) in comparison with AD group. PDK1 was decreased (* $P < 0.05$) in AF animals in comparison with AD and PF groups (Figure 9).

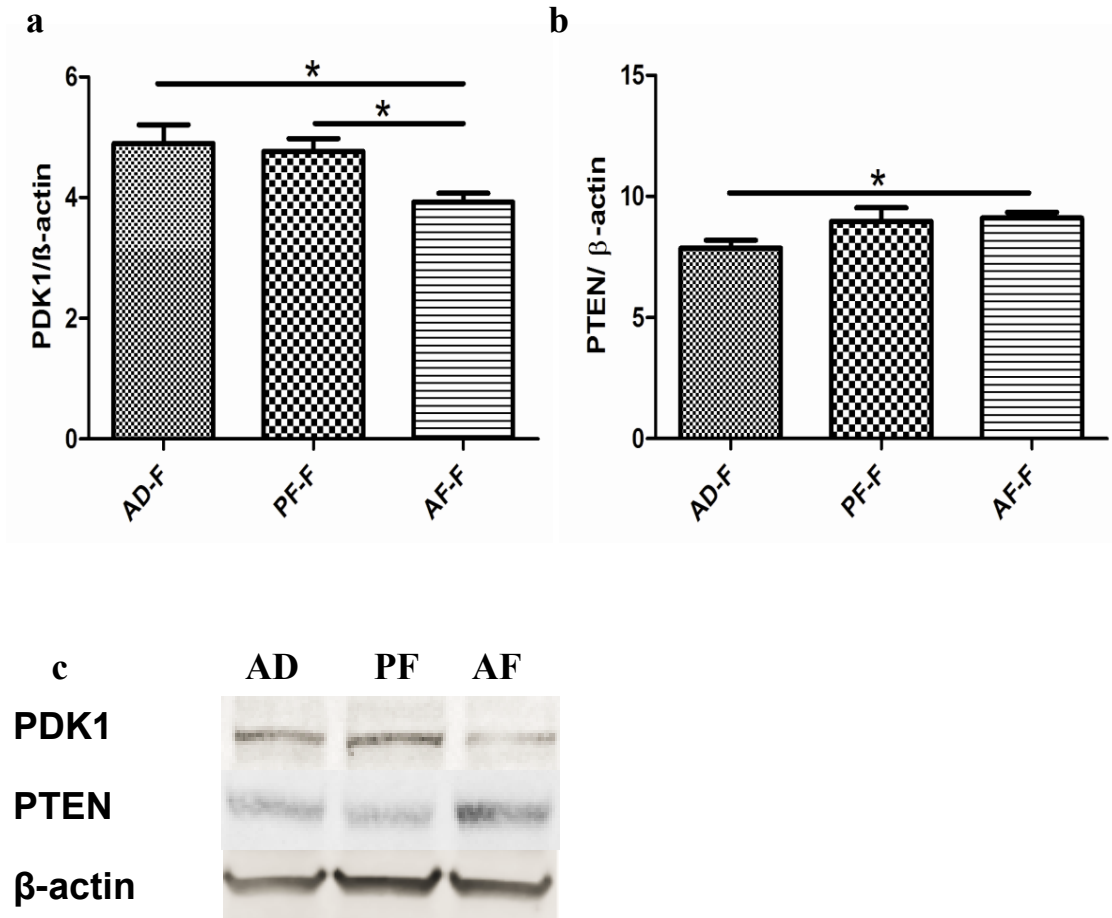


FIGURE 9: PCAE ALTERS THE NORMAL STATUS OF HEPATIC PROTEINS THAT RELATED TO INSULIN SIGNALING

PCAE significantly change the expression of PDK1 and PTEN proteins in liver. **a & b:** Histograms showing the protein expression as a ratio to β -actin for PDK1 and PTEN in hepatic tissue of AD, PF, and AF. One-way ANOVA and Newman-Keuls Multiple Comparison Test, $*P < 0.05$. **c:** Western Blotting protein bands for PDK1, PTEN, and β -actin. The protein band intensities were determined by Image Studio Lite software (LI-COR Biotechnology). N=6.

Since insulin action required insulin receptors at liver, muscular, and adipose tissue, insulin receptors at muscular and adipose tissues of AD, PF, and AF offspring. Data represent that insulin receptor protein expression did not show any significant

changes in InsR protein expression neither in muscular nor adipose tissues (Figure 10-a & b).

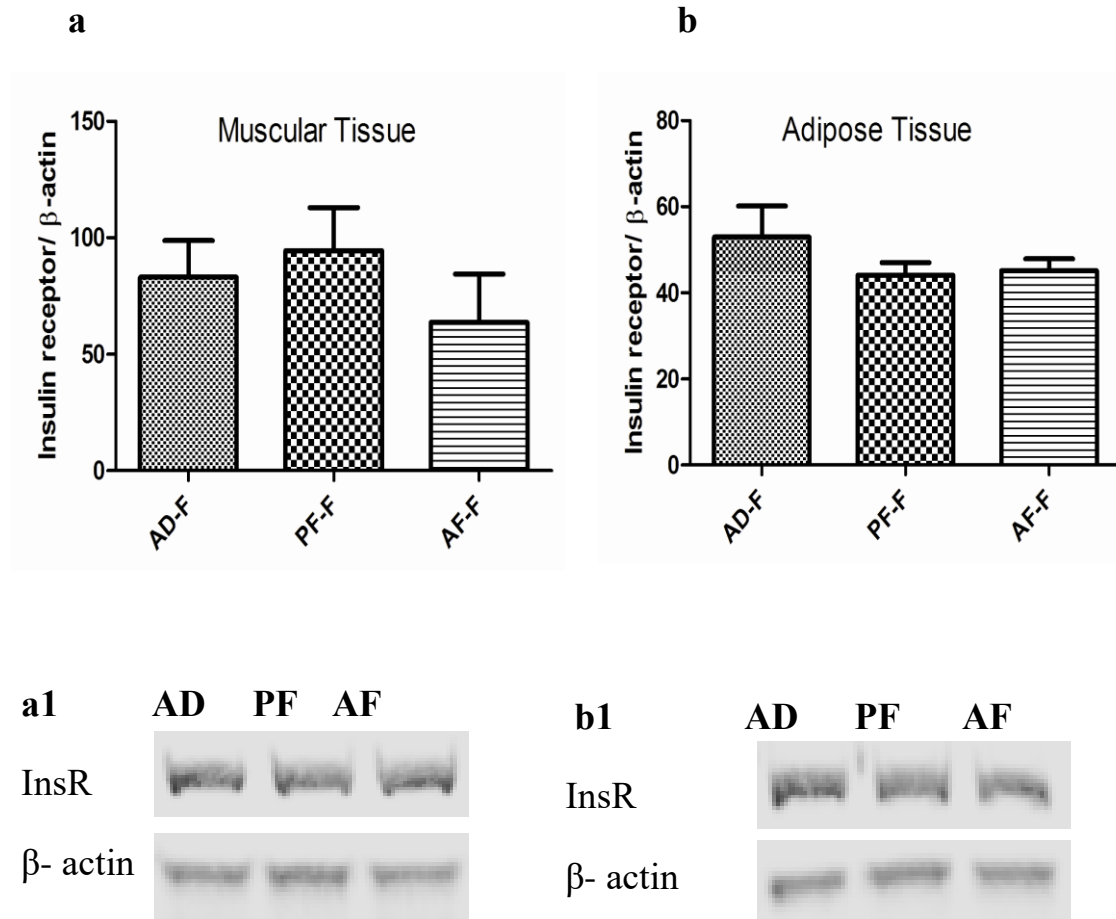


FIGURE 10: PCAE DID NOT AFFECT INSULIN RECEPTOR PROTEIN EXPRESSION IN MUSCULAR AND ADIPOSE TISSUE. Insulin receptors' expression in muscular and adipose tissue was not changed in PCAE offspring. **a & b.** Histograms show the protein expression by Western Blotting of InsR in muscular and adipose tissue, respectively. $P > 0.05$. $N = 4-5$. One-way ANOVA and Newman-Keuls Multiple Comparison Test. **a1 & b1.** Western Blotting of insulin receptors in muscular and adipose tissues, respectively by using Image Studio Lite software (LI-COR Biotechnology).

The negative impact I have found in pre-diabetic model encouraged us to study the effect of PCAE on the susceptibility to diabetes in the offspring. There are many studies have shown and established a model to simulate type two diabetes. This model is concluded as using high fat diet HFD 40-60% for short period of time such as two weeks followed by single dose injection of streptozotocin STZ 30-40 mg/kg (Reed et al., 2000; Srinivasan et al., 2005; and Sugano et al., 2006). At age of PND90, female animals of AD, PF, and AF groups were fed on 40% HFD for two weeks followed by single dose of STZ 40 mg/kg. Blood samples for glucose and insulin evaluation, pancreatic tissues for Western Blotting were all collected to evaluate the general susceptibility to diabetes.

My data revealed that PCAE showed the highest susceptibility to type II diabetes model in comparison with other AD and PF control groups. The susceptibility to diabetes was estimated by measuring blood glucose and insulin levels in addition to measuring the expression of some inflammatory markers such as; IL-6, IL-1b, and TNF- α . My result show that PCAE group has the highest blood glucose level among other AD and PF diabetic groups ($P < 0.05$). Blood insulin level was also decreased significantly ($P < 0.05$) in AF-HFD-STZ group in comparison with AD-HFD-STZ and PF-HFD-STZ groups (Figure 11: a & b). The blood levels of glucagon and leptin in diabetic animals were detected by using ELISA kits to estimate the effect of PCAE on diabetes susceptibility in the offspring since glucagon and leptin are considerable regulators of glucose homeostasis. My data revealed that diabetic PCAE animals showed the highest blood glucagon among other AD and PF control animals (Figure 11-c). The high blood glucagon level accompanied with low insulin level in AF animals could explain and

demonstrate the high blood glucose level I have seen in AF diabetic animals. I did not find any significant change among all of AD, PF, and AF diabetic groups (Figure 11-d).

At tissue level after induced diabetes, I have seen that AF-HFD-STZ expressed the highest level of pro-inflammatory cytokines such as; IL-6, IL1-b, and TNF- α . PCAE increased significantly ($P<0.05$ and $P<0.01$) as compared with other control diabetic groups (Figure 12: a-d).

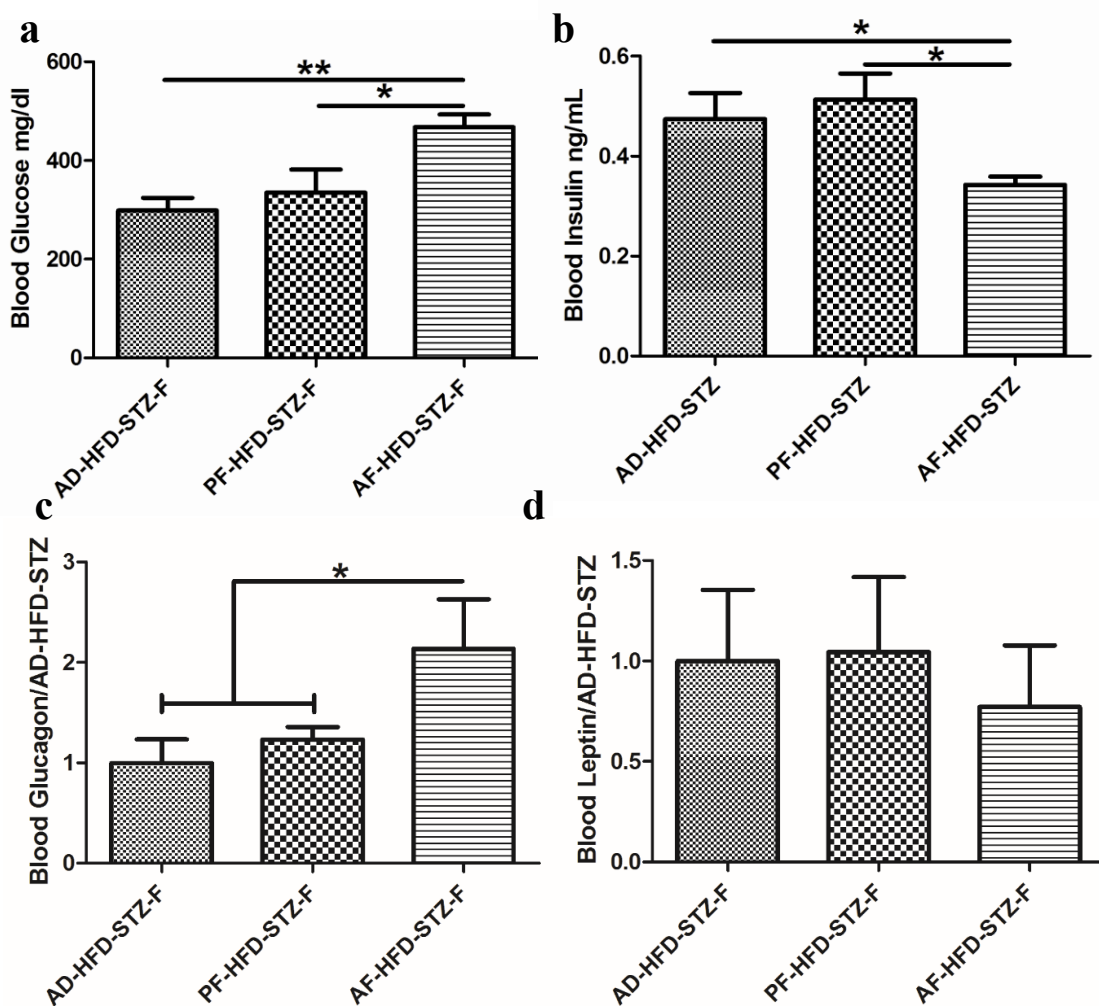
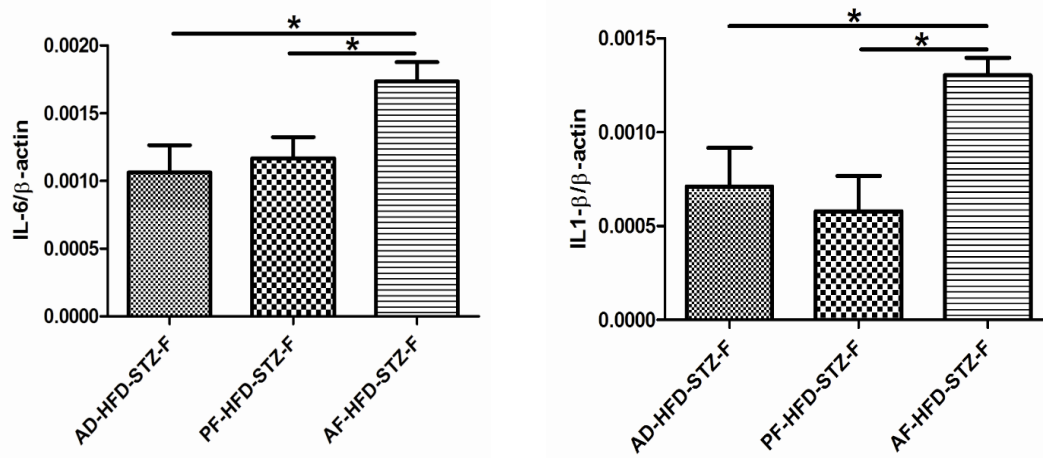


FIGURE 11: PRECONCEPTION ALCOHOL EXPOSURE INCREASES THE SUSCEPTIBILITY TO DIABETES IN THE OFFSPRING

PCAE significantly increased blood glucose level and lower blood insulin in the female offspring. **a.** Histogram shows the effect of PCAE on the blood glucose level after two weeks of 40% HFD followed by single injection of 40 mg/kg of STZ measured by AlphaTrak glucometer and strips. **b-d.** Histogram represents the plasma insulin, glucagon, and levels in diabetic AD, PF, and AF female offspring, respectively, by using ELISA kits. One-way ANOVA, N=5-7, *P<0.05 and **P<0.01.

a



c

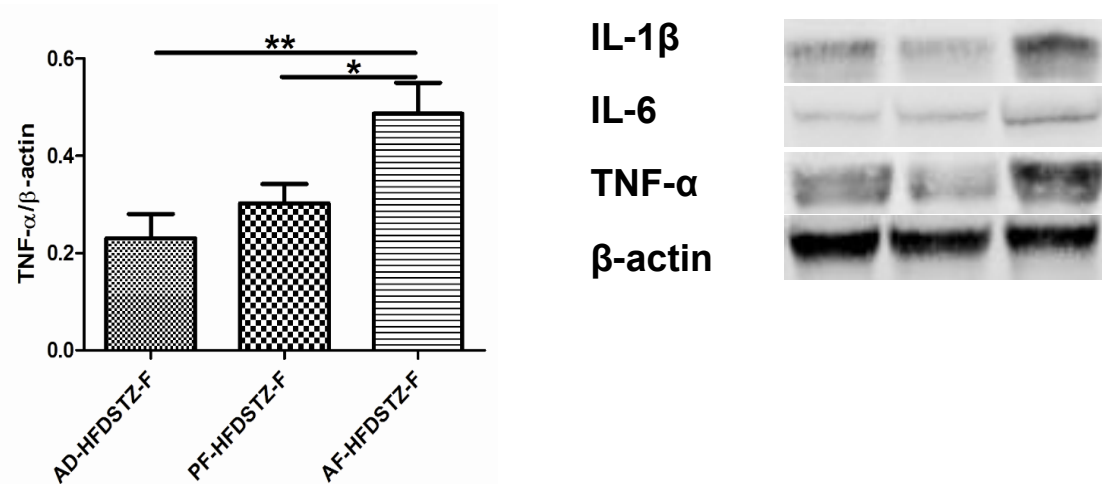


FIGURE 12: PRECONCEPTION ALCOHOL EXPOSURE INCREASED DIABETES SUSCEPTIBILITY IN THE OFFSPRING

PCAE increase the expression of inflammatory cytokines in diabetic pancreatic tissue of the offspring. **a, b, and c:** Histogram showing Western Blotting of the pancreatic inflammatory cytokines/β-actin ratio such as; IL-6, IL-1b, and TNF-α, respectively. One-way ANOVA and Newman-Keuls Multiple Comparison Test. N=6. *P<0.05 and

****P<0.01. d.** Western Blotting bands measured by Image Studio Lite software (LI-COR Biotechnology).

3.4 DISCUSSION

My data show for the first time that the PCAE has the ability to change the normal status of pancreatic tissue. I show here that alcohol drinking before pregnancy led to increase the inflammatory milieu in the offspring pancreatic tissue. To understand the relationship between alcoholism and inflammation, alcohol drinking facilitates LPS leakage from gut to the blood through portal vein or lymphatic vessels. The liver is a very important organ in detoxification of LPS that present in blood circulation and as a result of that, limited balanced cytokine production will be initiated and the inflammation will be contained. Those circulating LPS will cause damage to other organs when the hepatic detoxification is impaired. The presence of LPS and pro-inflammatory cytokines will stimulate HPA axis to produce cortisol, which in turn, inhibits the inflammatory response. If the body exposed to chronic alcohol drinking, this mechanism will be disrupted and the liver detoxification and generation of balanced inflammation will be impaired in addition to affect brain regulatory response to inflammation (Wang et al., 2010).

The effect of alcoholism on pancreatic function is characterized by induction of pancreatitis, which in turn, leads to high expression of pro-inflammatory cytokines. The effect of alcohol drinking on pancreas can be concluded by its effect in inhibition of pancreatic secretion from acini, which causes microtubular dysfunction, and finally induces oxidative stress. The other mechanism by which alcohol can induce pancreatitis

is through increase transcription of NF- κ B and TNF- α , IL-6, iNOS and other pro-inflammatory cytokines (Chowdhury & Gupta, 2006).

The high level of leptin I have seen in chapter two can be related to the data that represent the PCAE-induced inflammatory status. There are many evidences concluded by Jaworek & Konturek (2014) showed that leptin can be considered as a factor can initiate non-specific immune defense and inflammation. It is also found that leptin blood level increased significantly in pancreatitis. Leptin has the ability to initiate innate immune response and as a result of that, inflammation process will be started. However, in intensive inflammation, leptin keeps stimulating the immune system to produce pro-inflammatory cytokines. Continues stimulation of immune system by leptin will support the progression of inflammation (Jaworek & Konturek, 2014).

Since the glucose homeostasis is also controlled by liver, here I examined the PCAE effect on hepatic function that is related to glucose metabolism. In this study, I conducted gene expression for many genes that play a role in insulin signaling. Insulin receptors considered as a key of insulin signaling that controls the glycolysis and glycogenolysis. My data revealed that PCAE reduced the gene expression of insulin receptors in hepatic tissue which an indicator of abnormal insulin tolerance that I have seen in IPITT. Akt role in Phosphatidylinositol 3-kinase activation can be considered as a compliment of insulin receptor substrate proteins to play a critical role in insulin-induced metabolic effect in liver (Ono et al., 2003).

My data show that PCAE could significantly decrease Akt in hepatic tissue in comparison with AD and PF control groups. This decrement in Akt could be caused by the chronic hyperglycemia that was seen in PCAE animals. The data I have could be

similar to the findings of Ono et al., (2003) that Akt is directly related to glucose level. Hypoglycemia is induced by Akt associated with upregulation of glycogen synthesis, downregulations of PEPCK and G6Pase.

According to another study by Oku et al., in 2001, in skeletal muscle, chronic high blood glucose level can affect the efficiency of the insulin-signaling by affecting Akt, and that will play a role in hyperglycemia-induced insulin resistance (Oku et al., 2001). The net effect of insulin in liver is to suppress the glucose production by liver and that is very important to maintain the normal glucose homeostasis and insulin resistance in liver. FOXO1 is one of the insulin targets and it plays a role in gluconeogenesis and promotes glucose production both in isolated hepatocytes and in transgenic mouse models. Activated Akt by the interaction between insulin and insulin receptors, suppresses FOXO1 (InSug et al., 2015).

FOXO1, in turn, stimulates the expression of G6Pase gene which in turn, plays a role in pathological status such as diabetes and obesity. Chronic hyperglycemia can also promote O-glycosylation of FoxO1 in obese or diabetic patients, which will lead to over-expression of G6Pase. The final result of low blood insulin and low Akt is accompanied with increased hepatic glucose production and abnormal glucose intolerance (Kuo et al., 2008).

Taken together, in my study I found the PCAE reduces blood insulin and insulin receptors in hepatic tissue, which in turn, resulted in low Akt. As a result of low Akt, FOXO1 got highly expressed in liver and led to high G-6-Pase and that explains the high blood glucose I have seen in AF group (Figure 13).

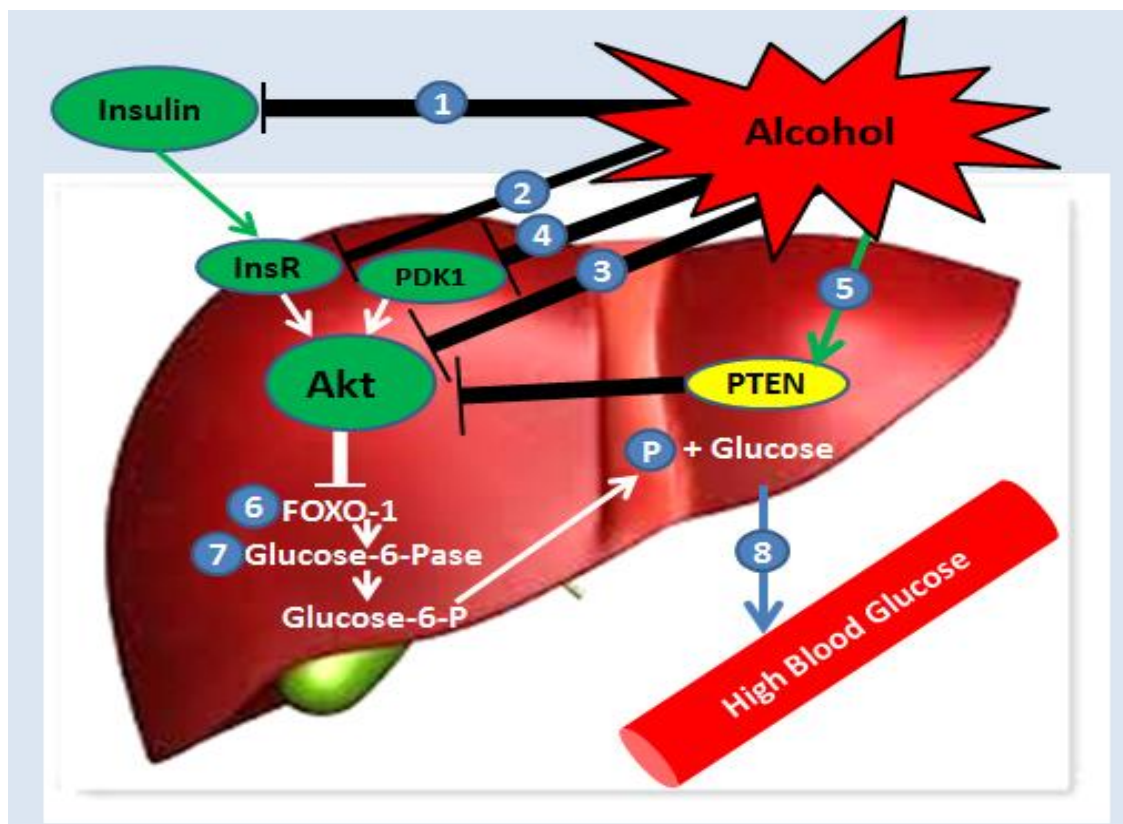


FIGURE 13: PCAE IMPAIRS LIVER GLUCOSE HOMEOSTASIS: 1. PCAE reduces blood insulin which is the key regulator of hepatic glucose homeostasis. 2. InsR is also reduced in hepatic tissue as a result of PCAE and that affects insulin pathway signaling directly. 3. Serine/threonine-protein kinase (Akt) gene is very important in conducting insulin signaling. Total Akt was significantly reduced in AF animals. 4. PCAE reduces pyruvate dehydrogenase kinas (PDK1) in the offspring and that will affect liver glucose homeostasis negatively through its effect on Akt. 5. Phosphatase and tensin homolog (PTEN) was increased in PCAE animals and that will play a role in inhibition of Akt and impairs the normal homeostasis. 6 & 7 As a result of 1-5, the inhibitory action of Akt will be abolished and FOXO1 & G-6-Pase will be expressed highly and that will lead to increase glucose production by liver and will play a role in high blood glucose.

All of the data I found such as the high expression of inflammatory markers in pancreatic tissue in addition to high glucose production by liver in AF group, led to increase the susceptibility to HFD-STZ diabetic model by showing the highest blood glucose level, lowest blood insulin, and highest expression of inflammatory cytokines in comparison with AD and PF control groups.

CHAPTER 4

4.1 INTRODUCTION

As a toxic substance, alcohol can exert a variety of noticeable harming effects on different body systems such as central nervous system (Zahr et al., 2011) and that can particularly affect POMC/BEP neurons especially if the exposure happened during the fetal developmental period (Rachdaoui and Sarkar, 2013).

In fetal alcohol animal model, it has been found that alcohol exposure decreases Pomc gene expression and lowers BEP neurons number (Del Arbol et al, 2007; Boyadjiva et al, 2009). It is also found by Chen et al., in 2006 that alcohol induces BEP neuronal apoptosis (Chen et al., 2006). The same alcohol-induced BEP apoptosis was found *in vivo* animal model (Kuhn and Sarkar, 2008).

In preconception alcohol exposure model, Jabbar et al., in 2016 showed that drinking alcohol three weeks before pregnancy could have the ability to alter the stress axis in the rat offspring. In the same study, it is found that alteration in stress response was partially mediated by the abnormalities in POMC/BEP neurons through the effect in regulating HPA stress axis (Jabbar et al., 2016). Here in this chapter, I evaluate whether the effect of PCAE on POMC/BEP is related to the abnormal glucose homeostasis and how BEP replacement is effective to ameliorate that abnormality.

4.2 MATERIALS AND METHODS

Animal Use

I followed the same preconception study design which is already mentioned above to have three groups of offspring AD, PF, and AF. Here in this chapter, I am studying the effect of PCAE on neuronal BEP and how that relates to the abnormal glucose homeostasis that I have seen in AF offspring. To conduct that, I have two more groups of AF offspring (N=7) to use them as negative and positive BEP treatment groups. By using of cyclic adenosine monophosphate, dbcAMP, and PACAP (pituitary adenylate cyclase-activating peptide) our lab established a method to differentiate *in vitro* BEP neurons from hypothalamic neuronal stem cells (NSCs) (Sarkar et al., 2008). It is also found that BEP can be differentiated *in vivo* by using 13-20 nanometers (nm) in diameter nanospheres have the ability to carry cAMP-activating agents. If those nanospheres injected in brain third ventricle, they will stay there and start releasing the attached agents to stimulate BEP differentiation.

Nanosphere Injection to Animals

Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, Butler Schein, Columbus, OH). By using stereotactic device, animals were either injected with 10 μ L plain nanospheres to be considered as control sham surgery group just to verify if the surgical pinch itself can alter the function, or nanospheres that deliver 70 nmol dbcAMP (cAMP) in the third ventricle to be considered as BEP group. The surgery can be concluded as I shaved rats' head hair and I sterilized it using iodide solution followed by ethanol. Incision was made by using surgical scalpel and blade. By using

brain atlas, I injected the nanospheres in coordination of 2.0 mm behind the bregma, midline, and 8.0 mm below the cortex using a 10 μ L Hamilton syringe. After injection is done, skin was closed with wound clips.

BEP immunofluorescent staining

Brains were collected and kept at -80 °C to be cut later using the Cryostat Microtome (LEICA CM1850 Cryostat Microtome). Here I used 30 μ m as a thickness of brain sections and I collected the entire hypothalamic arcuate by using brain atlas and collecting the sections from plate 18 to plate 23 of the stereotaxic atlas (Paxinos and Watson, 1989) to cover the whole hypothalamic arcuate nucleus. Brain slides were fixed with 4% paraformaldehyde for 15 min at room temperature, and then washed with PBS twice for 5 minutes each. This step was followed by 15 minutes fixation by using methanol and two washings with 0.05% TritonX in PBS followed by two washings of PBS. The brain sections were then blocked with 2.5% horse serum for 1 hour in moisture chamber. By using the same moisture chamber, I incubated them with rabbit anti BEP antibody (Peninsula Laboratories, Cat# T-4045) (1:1000) overnight at 4 oC. After overnight incubation, brain sections were washed once with 0.05% TritonX in PBS followed by two PBS washings at room temperature. Then, brain sections were incubated with FITC goat anti-rabbit (ThermoFisher, Alexa Fluor® 488) (1:1000) for one hour at room temperature in moisture chamber and in dark environment to protect the fluorescence from direct light. Sections were washed with two 0.05% TritonX in PBS washings followed by two PBS washings and mounted with DAPI and sealed by commercial nail polish. Pictures were taken by fluorescent microscope with 20x objective lens (Nikon Fluorescent Microscope). Positive cell numbers in each brain section were

manually counted as a ratio to the total cell number from all sections of one brain was calculated. The experimenter counting the immunopositive cells were blinded to the experimental conditions.

Fasting, during OGTT, and after HFD-STZ blood glucose and insulin measurements

I followed the same protocol mentioned above to conduct OGTT and GSIS and measuring blood glucose by AlphaTrak glucometer and strips and blood insulin using Ultrasensitive Rat Insulin ELISA kit (ChrystalChem).

4.3 RESULTS

Beta-endorphin is a potent immune-regulator and it can regulate the body immune response by its effect on HPA axis and immune-regulatory hormones that are controlled by regulatory nerve impulses. BEP is considered as a link between body stress and the response that should occur to cope it (Baumgarten et al., 2002). Since it is already found that PCAE could reduce that availability of BEP neurons in hypothalamic arcuate (Jabbar et al., 2016), I suggest that the abnormal glucose homeostasis I have seen in PCAE group that accompanied with high expression of inflammatory markers in pancreatic tissue could be related to PCAE effect on hypothalamic BEP. My results show that PCAE significantly decreased the total number of BEP neurons in hypothalamic arcuate in comparison with AD and PF groups (Figure 14).

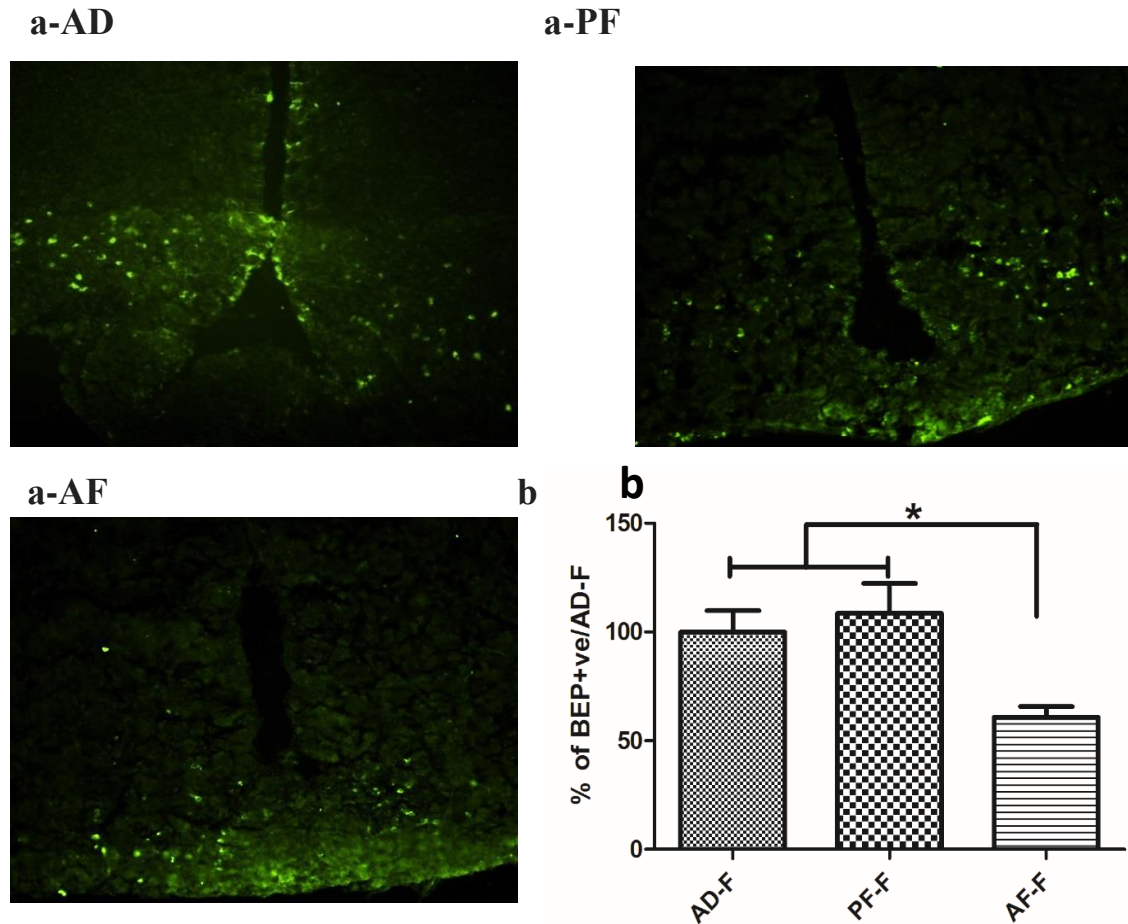


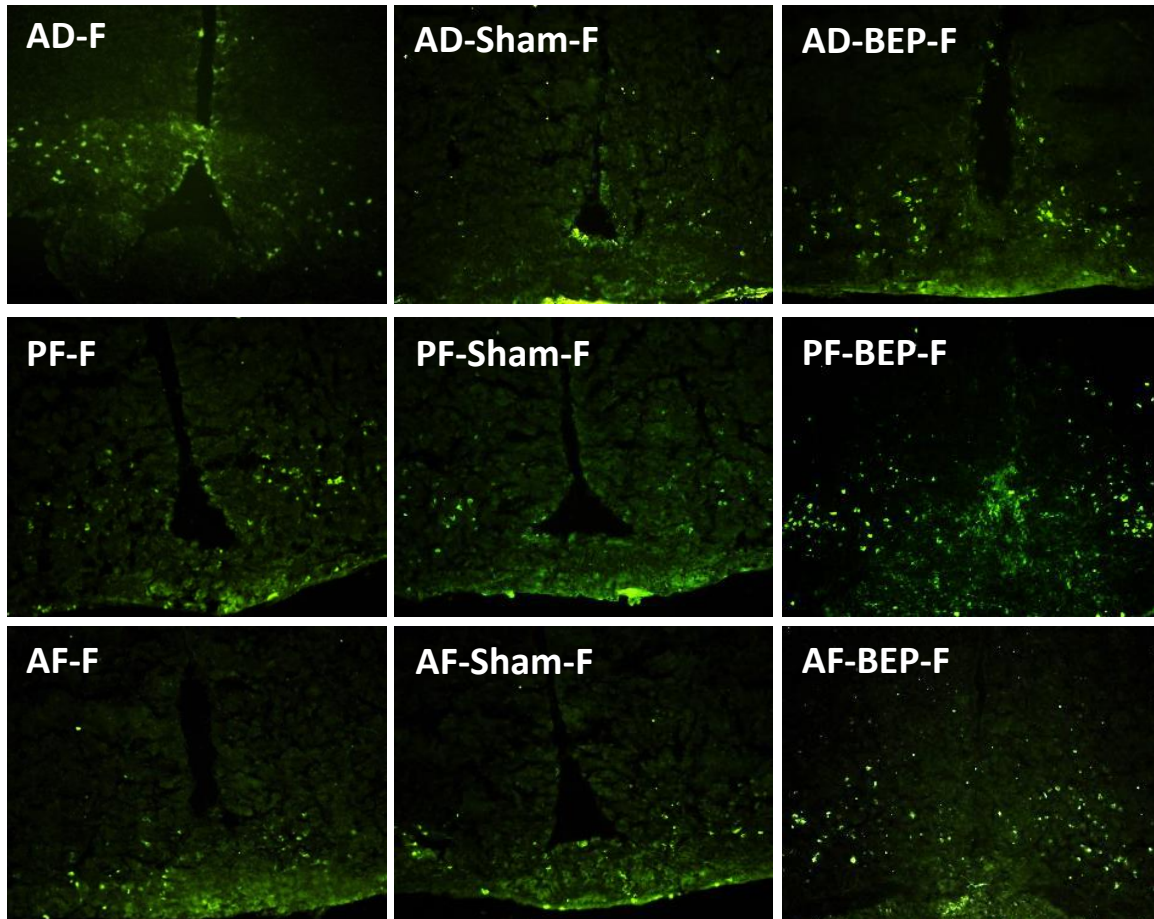
FIGURE 14: PCAE SIGNIFICANTLY ALTERS THE NORMAL EXPRESSION OF HYPOTHALAMIC BEP IN THE OFFSPRING

Preconception alcohol drinking reduces the number BEP positive cells. **a.** Immunostaining pictures of brain sections stained for BEP (green color -488 Alexa Fluor) in AD, PF, and AF groups using fluorescent microscope (20X magnification). **b.** Histogram showing the statistical analysis of means \pm SEM as a percentage of BEP positive cells in sham and BEP/BEP positive cells in control no-surgery groups of AD, PF, and AF. One-way ANOVA and Newman-Keuls Multiple Comparison Test. N=5-6, *P<0.05

At age of PND60, I did BEP replacement by ICV injection of nanosphere that have the ability to release cAMP to be considered as BEP groups, or injected with nanospheres without releasing cAMP to be represented as Sham groups. To confirm the accuracy of ICV injection, brain sections staining for BEP showed significant increase in

number of BEP neurons in BEP groups of AD, PF, and AF groups in comparison with no-surgery and Sham groups (Figure 15-a & b).

a-



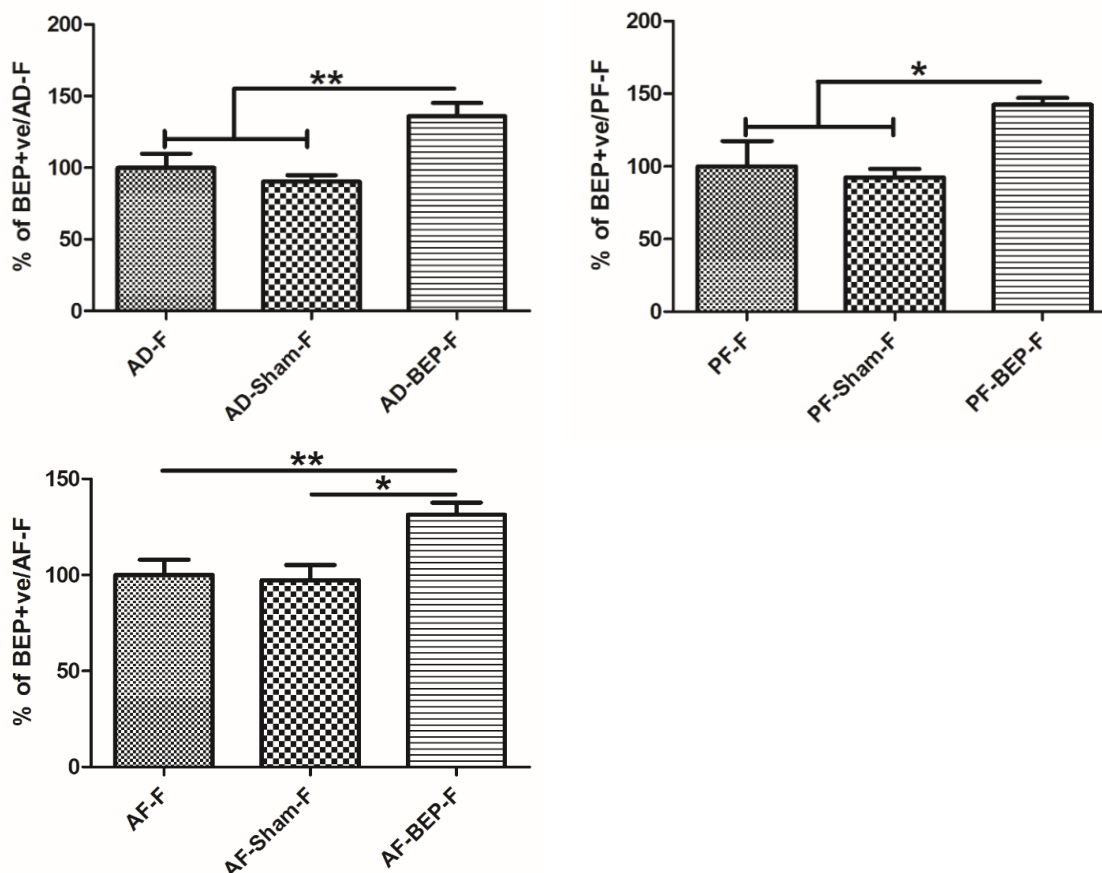


FIGURE 15: ICV INJECTION OF cAMP-DELIVERING NANOSPHERES INCREASES DIFFERENTIATION OF HYPOTHALAMIC BEP

Brain surgery was effectively increased the number of BEP +ve neurons in all of AD, PF, and AF groups. **a.** Histological pictures showing BEP expression in arcuate nucleus in no-surgery control, Sham surgery, and BEP-treated groups of AD, PF, and AF, respectively (green color -488 Alexa Fluor) by using fluorescent microscope (20X magnification). **b.** Histograms show the significant increment in BEP positive cells in sham and BEP groups of AD, PF, and AF as compared with BEP count in AD, PF, AF no-surgery groups by using fluorescent microscope (20X magnification) in AD, PF, and AF groups, respectively. N=6, One-way ANOVA and Newman-Keuls Multiple Comparison Test. *P<0.05 and **P<0.01.

At age of PND90 (one month after BEP replacement), I conducted fasting blood glucose, insulin, glucagon, and leptin in prediabetic model in control, Sham, and BEP

subgroups of AD, PF, and AF. In this experiment, I use three different sub-groups of AD, PF, and AF; the first group is without any surgery to be considered as a control group, 2nd group was injected with only nanospheres without cAMP to be considered as sham surgery (-Sham) group, and the third group was injected with nanospheres that have the ability to release cAMP (-BEP). Results of basal blood glucose and insulin in all of AD, PF, and AF subgroups showed that treatment with BEP incredibly decreases blood glucose and increases basal level of insulin in AF-BEP group (Figure 16: a-3 & b-3) without any significant change of both of glucose and insulin levels of AD and PF groups. (Figure 16-a-1, 2 & b-1, 2).

Since glucagon and leptin have an important role in regulating glucose homeostasis, I measured both of them using ELISA kits to detect and estimate the effect of BEP treatment on basal blood levels. My data revealed that treatment with BEP did not change the basal level of glucagon and leptin in all of AD and AF subgroups (Figure 17: a & b). Surprisingly, PF-BEP showed a significant increase in leptin level in comparison with PF and PF-Sham (Figure 17-b2) and that might explain why I did not find a significant increase in insulin level in PF-BEP at basal level. It is already found that the high level of leptin can suppress insulin production by pancreatic beta cells and it is showed that leptin can influence insulin secretion negatively by affecting KATP and Kv2.1 channel trafficking regulation and inhibit of β -cell excitability (Wu et al., 2015).

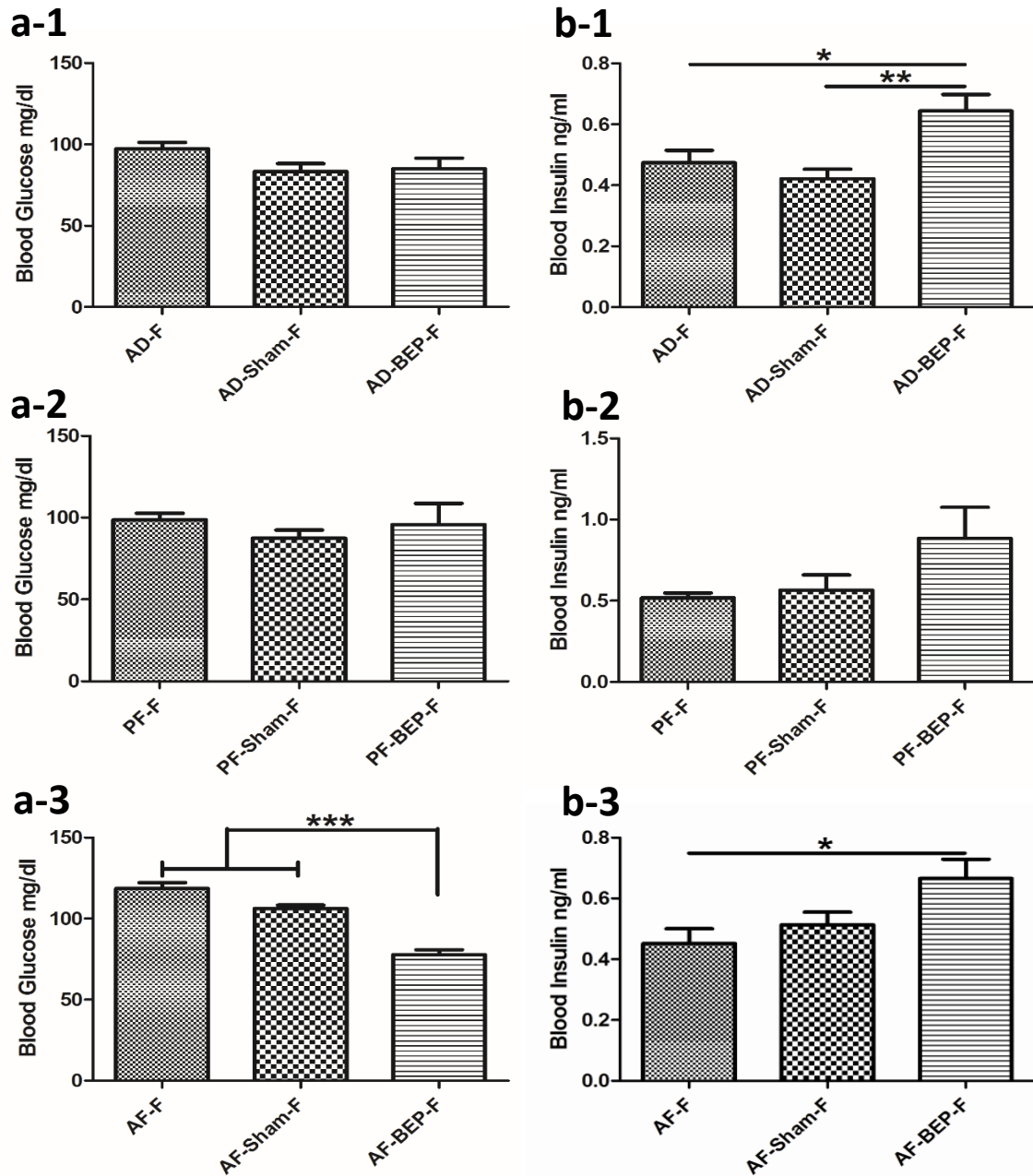


FIGURE 16: BEP REPLACEMENT HAS THE ABILITY TO CHANGE THE ABNORMAL BASAL BLOOD GLUCOSE AND INSULIN

BEP could significantly change the abnormal glucose and insulin that was seen in PCAE animals. **a(1-3)**. Basal blood glucose as mean \pm SEM using AlphaTrak glucometer and glucose strips. in AD,PF, and AF subgroups, respectively. **b (1-3)**. Histograms showing the basal blood insulin by using Ultrasensitive Insulin ELISA kit in AD, PF, and AF subgroups. One-way ANOVA and Newman-Keuls Multiple Comparison Test.. N=6-7. *P<0.05, **P<0.01, and ***P<0.001.

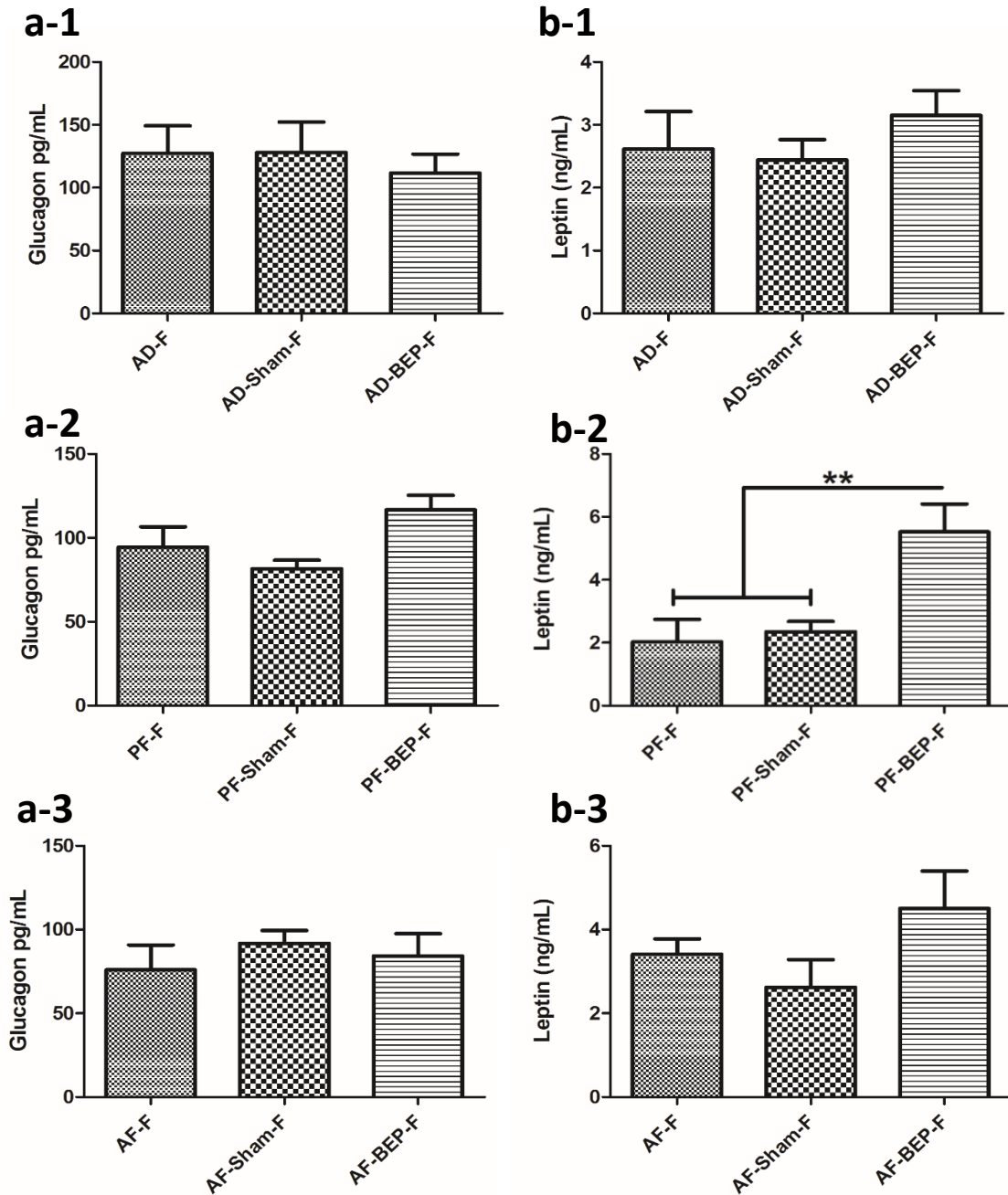


FIGURE 17: BEP REPLACEMENT DID NOT CHANGE THE BASAL LEVELS OF BLOOD GLUCAGON AND LEPTIN

BEP has no effect on basal blood leptin and glucagon. **a(1-3)**. Basal blood glucagon as mean \pm SEM using ELISA kit in AD, PF, and AF subgroups, respectively. **b(1-3)**. Histograms showing the basal blood leptin by using leptin ELISA kit in all of subgroups of AD, PF, and AF groups. One-way ANOVA and Newman-Keuls Multiple Comparison Test. N=6-7. **P<0.01.

OGTT data revealed that blood glucose was significantly lower in AF-BEP during the two hours of glucose challenge when it compared with AF and AF-Sham by using the area under the curve (AUC) ($P<0.001$) and ($P<0.01$), respectively (Figure 18-a-3 & b-3). Since the blood glucose homeostasis in AD and PF groups was normal, I did not find any significant changes among AD and PF subgroups suggesting that BEP treatment is very helpful in ameliorating abnormal status toward normal state without any side effects such as hypoglycemia in normal animals.

Glucose-stimulated insulin secretion (GSIS) is a very important test to reflect the body response to the available nutrients and it is the insulin produced as a response to the glucose solution that was given orally to the animals. The higher the insulin produced during OGTT, the healthier pancreatic function the animals have. GSIS shows that the body response to produce insulin during OGTT was significantly higher in –BEP subgroups of AD, PF, and AF in comparison with control and Sham subgroups as it is shown as a curves in (Figure 19-a) and as histograms of AUC in (Figure 19-b).

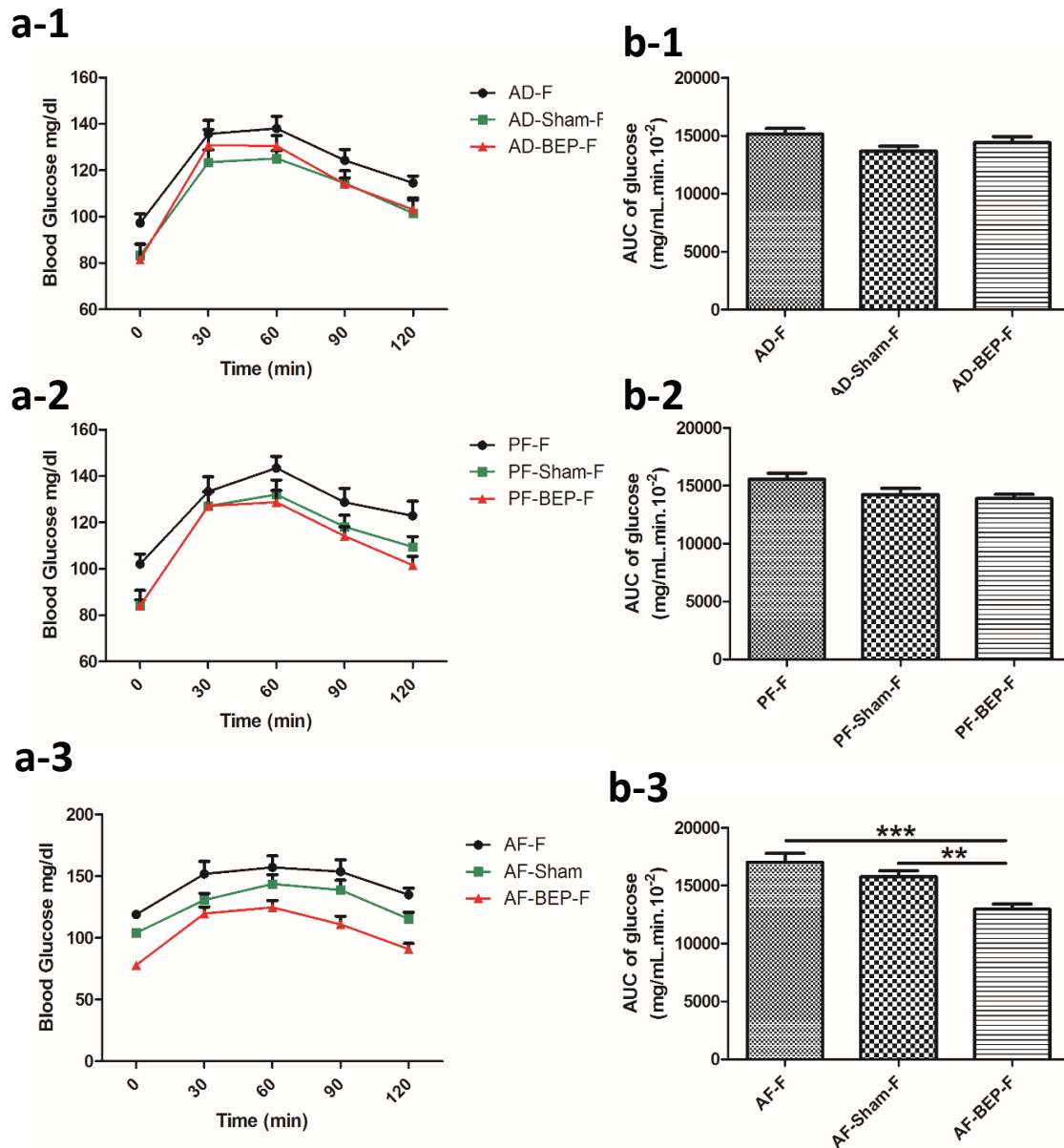


FIGURE 18: BEP TREATMENT HAS THE ABILITY TO AMELIORATE PCAE EFFECT ON GLUCOSE TOLERANCE

OGTT is significantly improved in AF animals that treated with BEP. **a(1-3)**. Curves represent blood glucose levels during OGTT in AD: Interaction: $F(8,72)=0.3063$, $P>0.05$, Time: $F(4,72)=77.85$, $P<0.0001$, and Treatment: $F(2,72)=1.379$, $P>0.05$, PF: Interaction: $F(8,68)=0.6606$, $P>0.05$, Time: $F(4,68)=60.37$, $P<0.0001$, and Treatment: $F(2,68)=3.758$, $P<0.05$, and AF: Interaction: $F(8,76)=0.5771$, $P>0.05$, Time: $F(4,76)=34.45$, $P<0.0001$, and Treatment: $F(2,76)=15.55$, $P<0.001$ subgroups. Control, -Sham, and -BEP subgroups are represented in three different colors; black, green, and red, respectively using two-way ANOVA and Bonferroni posttests **b(1-3)**. Histograms

showing the AUC of the OGTT in AD, PF, and AF (** $P < 0.01$ and *** $P < 0.001$). One-way ANOVA and Newman-Keuls Multiple Comparison Test. $N = 6-7$.

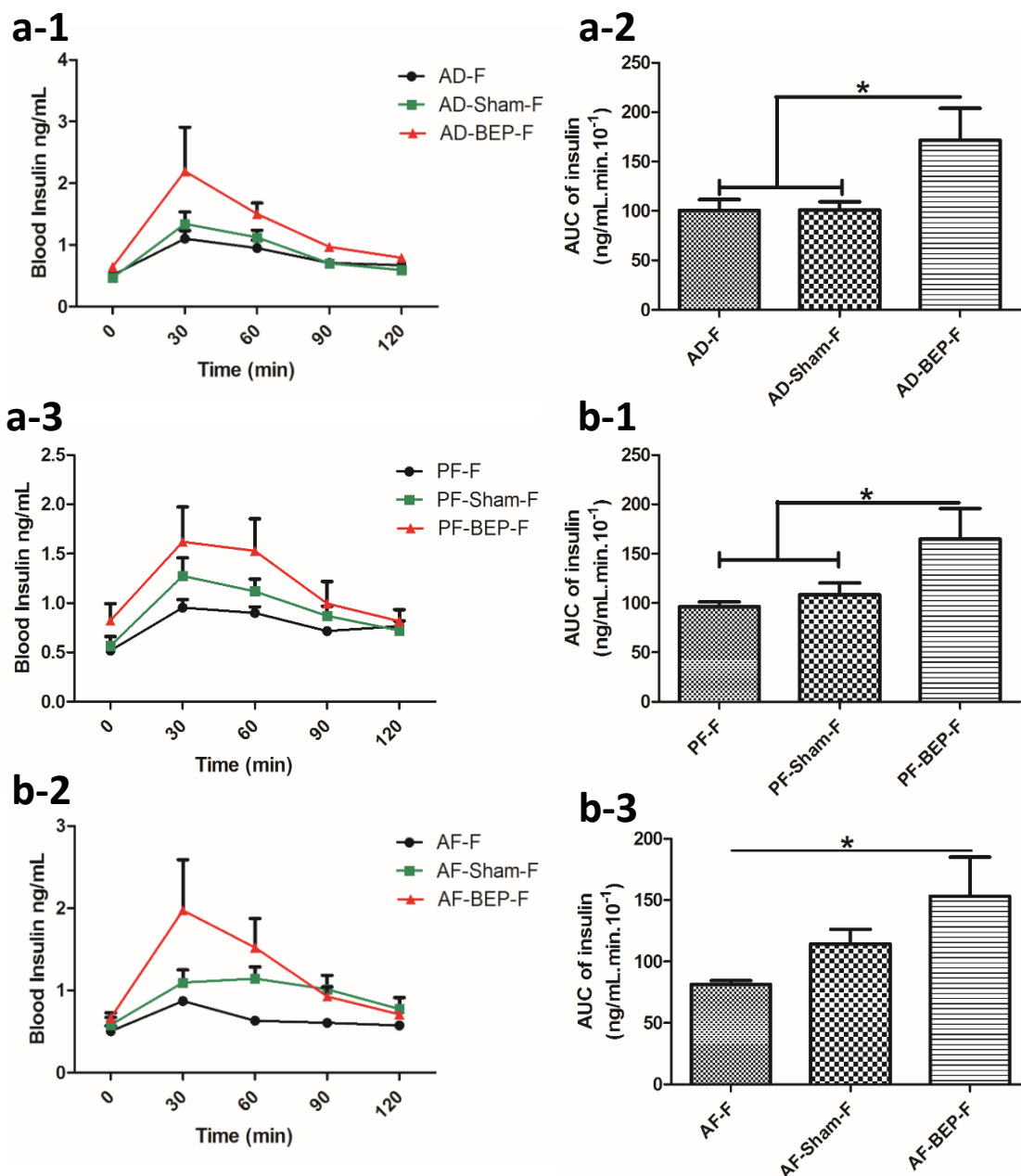


FIGURE 19: BODY RESPONSE TO OGTT IS IMPROVED AFTER TREATMENT WITH BEP

BEP could significantly enhance the GSIS. **a.** Curves show the blood insulin during OGTT in control no surgery (black), -Sham (green), and in -BEP (red) of AD:

Interaction: $F(8,72)= 1.403$, $P>0.05$, Time: $F(4,72)= 16.10$, $P< 0.0001$, and Treatment: $F(2,72)= 2.948$, $P>0.05$, PF: Interaction: $F(8,72)= 2.321$, $P<0.05$, Time: $F(4,72)= 29.87$, $P< 0.0001$, and Treatment: $F(2,72)= 1.751$, $P>0.05$, and AF: Interaction: $F(8,60)= 2.404$, $P<0.05$, Time: $F(4,60)= 9.925$, $P< 0.0001$, and Treatment: $F(2,60)= 3.304$, $P>0.05$. groups by using insulin ELISA kit. Two-way ANOVA and Bonferroni posttests. **b.** Histograms showing the AUC of GSIS in AD, PF, and AF. One-way ANOVA and Newman-Keuls Multiple Comparison Test. ($P<0.05$), $N=6-7$.

To examine the effect of BEP replacement in diabetic model, I induced diabetes in AD, PF, and AF groups by giving them 40% HFD for two weeks followed by STZ 40 mg/kg. The data showed that blood glucose in BEP-HFD-STZ group was significantly lower in comparison with HFD-STZ and Sham-HFD-STZ groups (Figure 20-a). Blood basal insulin in diabetic animals was detected by using insulin ELISA kit in all of AD, PF, and AF subgroups. My findings show that treatment with BEP significantly improved and increased blood insulin in diabetic animals of all of AD, PF, and AF subgroups (Figure 20-b). These data suggesting that BEP could significantly lower the high susceptibility to diabetes that I have seen in PCAE group when they exposed to HFD-STZ-induced diabetes.

In the same diabetic model, I also measured the blood glucagon and leptin levels in all of AD, PF, and AF subgroups diabetic animals to identify the role of BEP treatment on the basal levels of both of those hormones. BEP treatment did not change the levels of both of glucagon and leptin hormones in comparison with other control diabetic animals (Figure 21-a & b).

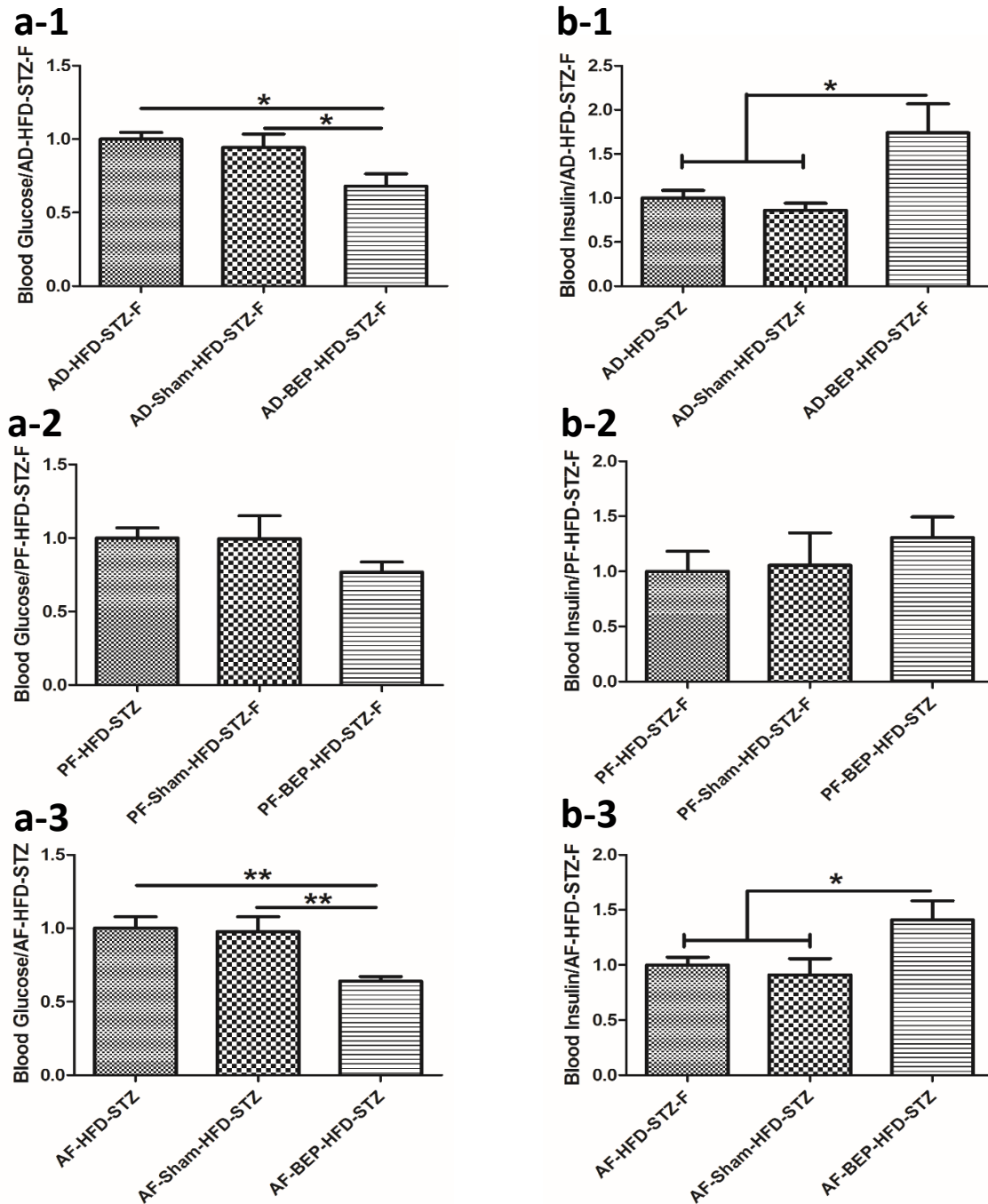


FIGURE 20: DIFFERENTIATED BEP REDUCES DIABETES SUSCEPTIBILITY IN PCAE ANIMALS

High susceptibility to diabetes in AF group could be ameliorated by BEP replacement. **a(1-3)**. Histograms show the blood glucose in AD, PF, and AF diabetic animals significantly. **B (1-3)**. BEP treatment decreases the susceptibility to diabetes that was seen in PCAE animals by lowering blood glucose significant. One-way ANOVA and Newman-Keuls Multiple Comparison Test. * $P < 0.05$ and ** $P < 0.01$, $N = 6-7$.

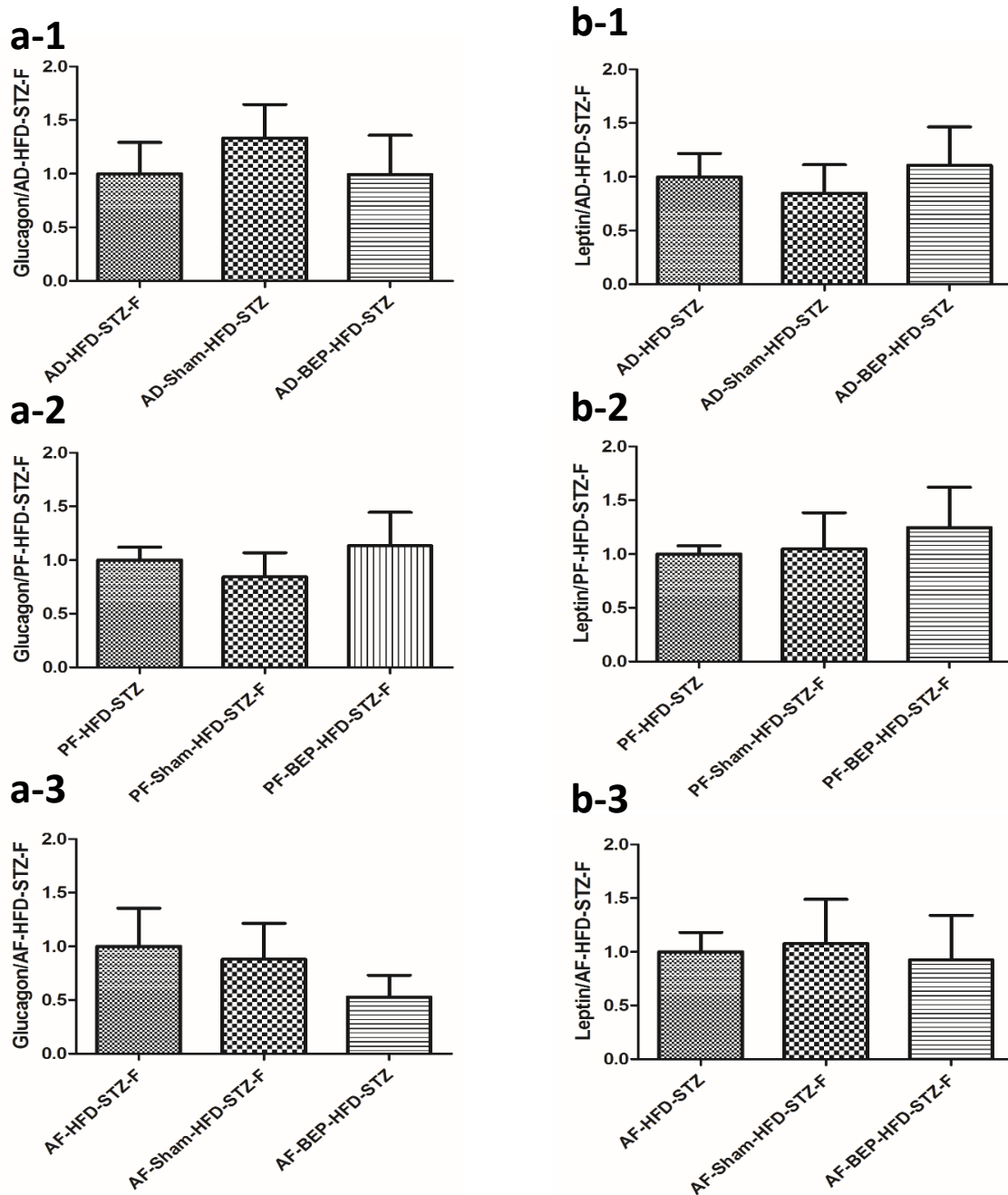


FIGURE 21: TREATMENT WITH BEP DID NOT CHANGE BLOOD GLUCAGON AND LEPTIN LEVELS IN DIABETIC ANIMALS

Glucagon and leptin levels did not show any change among diabetic groups. **A (1-3).** Histograms showing the basal glucagon level in diabetic animals. **b (1-3).** Histograms showing the blood leptin in diabetic AD, PF, and AF groups. One-way ANOVA and Newman-Keuls Multiple Comparison Test. Values represented as means \pm SEM as compared with no-surgery AD, PF, and AF subgroups. N=6-7. $P > 0.05$.

In conclusion, I suggest that the negative impact of alcohol drinking before pregnancy is mediated by the effect of PCAE on BEP expression in brain hypothalamic area and that could play a role in initiation of inflammatory process and results in abnormal pancreatic function and resulting abnormal glucose homeostasis in PCAE animals. To confirm that, measured the expression of BEP in PCAE animals' brains and I compared it with its normal expression in other AD and PF control groups and I have found the PCAE significantly lowered the BEP +ve cells number. To ameliorate that, I did BEP replacement and I found that PCAE animals treated with BEP showed better glucose homeostasis in prediabetic, lower blood glucose and higher insulin levels in diabetic models. Taken together, BEP treatment has the ability to reverse PCAE negative impact on prediabetic abnormal glucose homeostasis in addition to its ability to reduce the susceptibility to diabetes in AF animals and control animals as well.

4.4 DISCUSSION

The mechanism by which alcohol can exert its harming effects is still debatable. Our lab also found that one of the acceptable mechanisms is that exposure to alcohol, fetal alcohol, can significantly lead to initiate POMC/BEP apoptosis in hypothalamic arcuate nucleus (Sarkar et al, 2008).

POMC neurons, which are the precursor of endogenous opioid BEP in the hypothalamus, have a variety of actions to control many of body functions such as; energy metabolism, stress and immune functions. Prenatal and postnatal exposure to ethanol lead to disturb the normal response to stress in adulthood due to the effect of alcohol consumption and effect on HPA axis which might be caused by decreasing the expression of BEP producing neurons (Franklin, 2013).

In this study, I have found that PCAE could significantly reduce BEP in the offspring in comparison with the offspring of other AD and PF control groups. This finding is very similar to Jabbar et al., 2016, when they found that PCAE offspring had increased levels of hypothalamic CRF and reduced levels hypothalamic POMC/ β -endorphin that resulted in increased expression of CRF and Crfr1 mRNA levels which in turn, reflects and identifies an abnormal function in HPA axis that causes atypical stress response. They also concluded that PCAE significantly altered the neuroendocrine system that regulates HPA axis in the offspring.

When BEP neurons and POMC expression in the hypothalamus decreased, that will lead to disruption of the feedback regulation of the HPA axis and sympathetic nervous system (SNS). The hyper-activation of the HPA axis and SNS, accompanied

with hypo-activation of parasympathetic nervous system (PSNS). As a result of that, glucocorticoids and catecholamines production will be increased and will directly inhibit the activity of immune cells. Taken together, these changes caused by low expression of BEP will lead to stimulate immune cells to produce high amount of pro-inflammatory cytokines with low amount of anti-inflammatory cytokines (Zhang, 2013). That might give an explanation of why I found high inflammatory cytokines in AF animals in prediabetic and diabetic models in comparison with the normal stress/inflammatory response that I have seen in AD and PF control animals.

Boyadjieva et al., (2009) have found that BEP transplantation elevates BEP expression in the CNS without changing plasma BEP and that can play a role in inhibition of HPA hyper response in AF animals of fetal alcohol model. The CNS dysregulation of the HPA activation as a result of the reduction of BEP expressing neurons may have the ability to affect CRH release in the PVN. In other words, POMC/BEP neurons may also play a crucial controlling role in inflammatory signaling (Figure 20). In this study, I examined if that system is related to PCAE-induced high expression of inflammatory cytokines in pancreatic tissue and the high susceptibility to diabetes that I have seen in PCAE offspring.

To reverse that, I decided to conduct BEP replacement by ICV injection of dbcAMP delivering nanospheres to compensate the low expression of BEP that I have found in PCAE group. Our lab has shown that dbcAMP can be considered as a neurotropic factor for immature β -EP neurons (De et al., 1994).

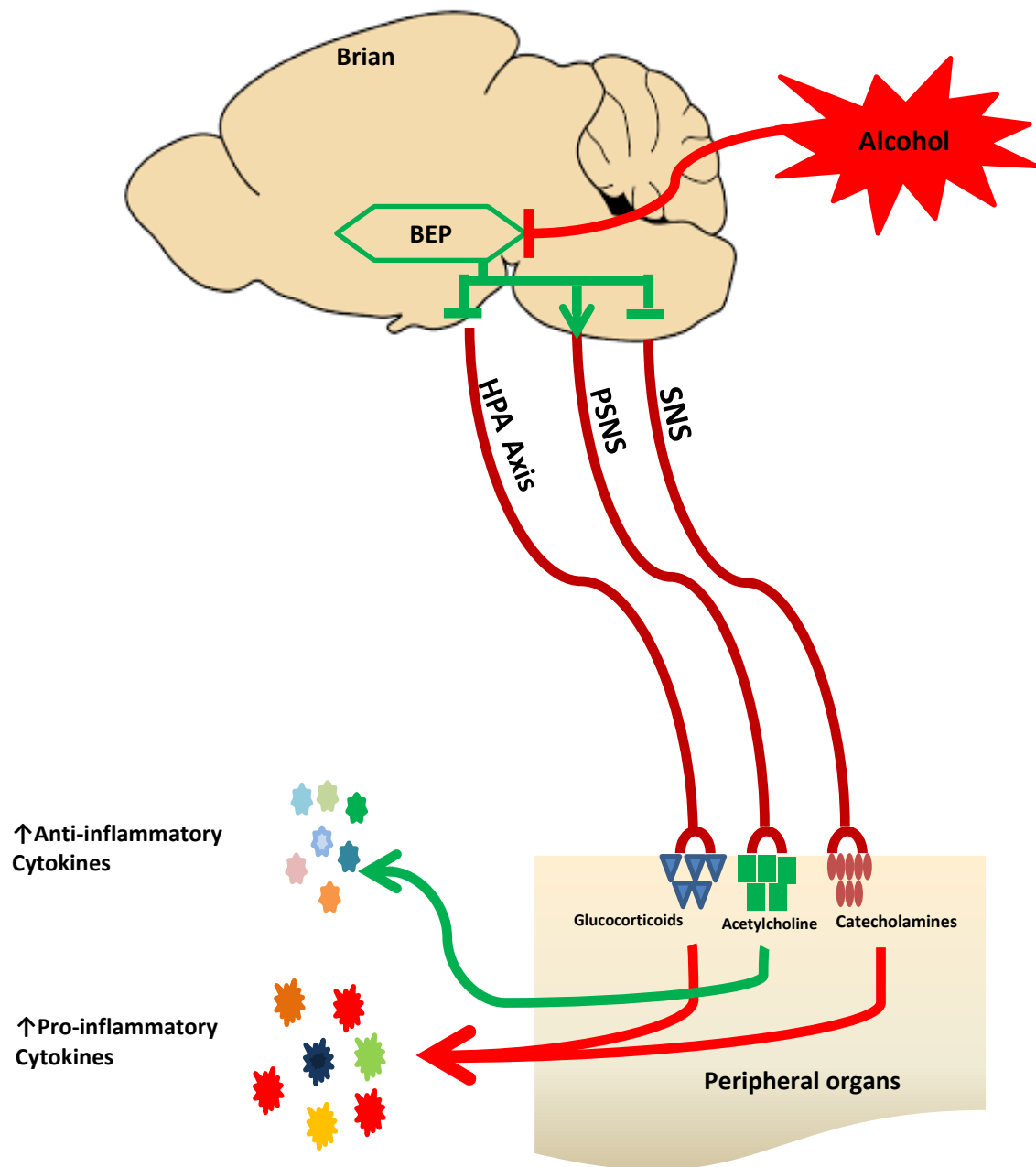


FIGURE 22: PROPOSED MODEL OF EFFECT OF ALCOHOL CONSUMPTION ON THE IMMUNE SYSTEM

Adapted from Sarkar et al., 2011.

Exposure to alcohol causes reduction of POMC/BEP neuronal numbers and expression in the hypothalamus, which in turn, decreases feedback regulation of the HPA axis and SNS. The hyperactivation of the HPA axis and SNS accompanied with hypo-activation of PSNS induces releasing of glucocorticoids and catecholamines, which directly inhibit the

activity of immune cells. As a result, these immune cells will produce a high number of pro-inflammatory cytokines, and low number of anti-inflammatory cytokines.

It is already found that dbcAMP has the ability to initiate and enhance the differentiation of NSCs to BEP neurons in culture and transplantation. The effect of dbcAMP in induction of BEP differentiation thought to be due to PKA pathway which up-regulates cAMP-responsive element binding protein (CREB), which is known to play an important role in regulating neuronal activity. In conclusion, the treated hypothalamic NSCs with dbcAMP showed high BEP positive immune-staining (Zhang, 2013).

These data are very parallel to my data after I did the ICV injection of dbcAMP-delivering nanosphers and I have found that the number of differentiated BEP is incredibly increased after dbcAMP injection. Since it already found and approved that hypothalamic BEP production in the hypothalamus can promote the activity of the ANS, immune function, and regulate the cytokine production, I suggest here that the normal OGTT and GSIS I have seen in prediabetic model of AF animals treated with dbcAMP could be promoted by the effect of BEP on HPA axis and its role in regulating inflammatory processes. That also can explain the low susceptibility to diabetes in AF-BEP animals in comparison with the highest susceptibility I have seen if AF when it compared to AD and PF diabetic models.

In final conclusion, I assume that the abnormal glucose homeostasis I have seen in AF in pre-diabetic model and the high AF susceptibility to diabetes is caused by the effect of PCAE on hypothalamic BEP. That was confirmed when I did BEP replacement and I have found that the blood glucose homeostasis and susceptibility went back to normal in AF group when it compared to AD and PF groups.

SUMMARY AND CONCLUSIONS

The main findings that I have found in this study can be concluded by that the PCAE could negatively and significantly alter the normal glucose homeostasis at different levels such as; histological and hematological basal and after challenge status indicating that drinking alcohol even before pregnancy can have intragenic (from F0 to F1) effect could affect the normal glucose homeostasis. The second important finding I have found is that PCAE induced inflammatory process in the offspring pancreatic tissue, which in turn, affect the normal pancreatic function and alters the normal glucose homeostasis. In addition, PCAE modifies liver glucose homeostasis by increasing glucose production by glycogenolysis and gluconeogenesis. The third finding I have observed was related to the mechanism by which PCAE can alter the normal glucose homeostasis and induce inflammation. I found that PCAE could significantly decrease BEP in the offspring hypothalamus. By this decrement, the innate immune system is disturbed and the inflammation is induced and the normal body function will be affected as a result. To connect all of those findings, I found that PCAE decreases the normal expression of BEP which is known to suppress CRH and cortisol that are known to be related to stress and low quality immune response. That can explain the induced pancreatitis and abnormal blood glucose homeostasis that I have seen in PCAE offspring and also show the similarity between PCAE and fetal alcoholism or the effect of alcohol on the metabolic status in alcoholics, suggesting that drinking alcohol even before pregnancy can affect the normal body health and leads to serious health problems. To ameliorate that deficiency in BEP, I decided to do BEP replacement to confirm that the decrement in BEP is responsible for PCAE negative impact regarding glucose homeostasis. AF animals that

treated with BEP incredibly showed improved glucose homeostasis and better basal and during glucose tolerance insulin production in comparison with non-treated and sham-surgery PCAE groups. The particular mechanism of POMC neuronal action on pancreatic beta-cell health is not elucidated here but previous studies from our group showed a strong relationship between POMC neuronal activation and the inhibition of sympathetic activity as well as activation of para-sympathetic neurons (Sarkar et al., 2011). The post-ganglionic fibers of the sympathetic nervous system terminate at every organ in the body including primary and secondary lymphoid tissues (lymph nodes, spleen, and bone marrow) as well as endocrine glands like the pancreas. POMC neurons produce β -endorphin which binds to μ -opioid receptors on post-synaptic parvocellular neurosecretory cells of the PVN that terminate in the spinal cord or in the rostral ventrolateral medulla where they can directly and indirectly modulate sympathetic nerve activity, respectively (Cechetto, & Saper, 1988; Swanson et al., 1980). Sympathetic innervations to the pancreas inhibit pancreatic beta-cell production and release of insulin, while parasympathetic innervation stimulates insulin release from pancreatic islets (Lubaczeuski et al., 2015 and Thorens, 2014). Therefore, POMC neuronal regulation of pancreatic function might involve suppression of sympathetic and activation of parasympathetic neuronal systems.

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