TURKISH REPUBLIC
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF SCINECE FACULITY
DEPARTMENT OF BIOLOGY

ROLE OF microRNAs IN ABORTION CAUSED BY DIABETES MELLITUS IN MICE MODEL

Prepared By
Khaleel Ibrahim Al-MASHHADANI

Supervisor
Prof. Dr. Mikail Akbulut
Co-Advisor
Dr. Md. Muhmodul Hasan SOHEL

M. Sc. Thesis

August 2018
KAYSERI

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SCIENTIFIC ETHICS SUITABILITY

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

Owner of thesis

Khaleel Ibrahim Al-MASHHADANI

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The M.Sc. thesis entitled **"Role of miRNAs in abortion caused by diabetes mellitus in mice model"** has been prepared in accordance with Erciyes University School of Natural and Applied Sciences Thesis Preparation and Writing Guide.

Student
Khaleel Ibrahim AL-MASHHADANI

Supervisor
Prof. Dr. Mikail AKBULUT

Head of the Department of Biology

Prof. Dr. Nusret AYYILDIZ

ACCEPTANCE AND APPROVAL PAGE

This study entitled "**Role of miRNAs in abortion caused by diabetes mellitus in mice model**" prepared by Khaleel Ibrahim AL-MASHHADANI under the supervision of Prof. Dr. Mikail AKBULUT was accepted by the jury as M.Sc. Thesis in Biology Department.

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JURY:

Supervisor : Prof. Dr. Mikail AKBULUT

Juror : Prof. Dr. Abdurrahman AYVAZ

Juror : Assist. Prof. Dr. Mona EL KHATIB

APPROVAL

That the acceptance of this thesis has been approved by the decision of the Institute's Board of Directors with the 18/09/2018 date and 2018/44-19 numbered decision.

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Prof. Dr. Mehmet AKKURT

Director of the Institute

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Khaleel Ibrahim AL-MASHHADANI

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ROLE OF miRNA IN ABORTION CAUSED BY DIABETES MELLITUS IN MICE MODEL

Khaleel Ibrahim AL-MASHHADANI

Erciyes University, Graduate School of Natural and Applied Sciences
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Thesis Supervisor: Prof. Dr. Mikail AKBULUT
Co-supervisor: Assist. Prof. Dr. Md. Muhmodul Hasan SOHEL

ABSTRACT

Diabetes mellitus is a chronic disease characterized by a relative or absolute lack of insulin, resulting in hyperglycemia. Chronic hyperglycemia can lead to a variety of complications, which may be life-threatening, if untreated. There is various complications caused by diabetes such as, heart disease, vascular diseases, renal failure, blindness, infertility and recurrent abortion (RA). Recurrent abortion, caused by diabetes, is a common health problem and may affect women in any reproductive age. Therefore, identifying risk factors of recurrent miscarriage is of great importance and might provide new therapeutic opportunities. More than 400 million people are diagnosed with diabetes worldwide and a significant proportion of these diabetic patients are suffering from abortion. Indeed, there are many studies about the relationship between diabetes and abortion, but the role of gene expression during the pregnancy period in diabetics is not precisely mentioned. However, to the best of our knowledge, there is no substantial study describes the effects of diabetes on the expression of microRNAs in an abortive mice model. Therefore, our study aims to investigate the differential expression of miRNAs in the uterus and ovary caused by diabetes in a diabetic mice model. For this, 18 mice are divided into two groups (Diabetic model & Control) where each group contain 6 female and three male mice. The diabetic mice model is induced by Streptozotocin (STZ). Ovary and uterus samples were collected form abortive and control mice and total RNA was extracted. miRNA expression was analyzed by using qPCR array technique to observe the differential expression of miRNA levels in these organs of diabetic mice model. Ultimately, the majority of miRNA expressions was downregulated in the uterus, while most of the miRNAs were upregulated in the ovary.

Keywords: Diabetes mellitus, abortion, Uterus, Ovary, miRNA, Gene expression, Female reproduction

DİYABET TARAFINDAN SEBEP OLUNAN DÜŞÜKLERDE MİKRO RNALAR'IN (miRNA) ROLÜ

Khaleel Ibrahim AL-MASHHADANI

Erciyes Üniversitesi, Fen Bilimleri Enstitüsü
Yüksek Lisans Tezi, Ağustos 2018
Tez Danışmanı: Prof. Dr. Mikail AKBULUT
İkinci Danışman: Assitant Prof. Dr. Md. Muhmodul Hasan SOHEL

ÖZET

Diabetes mellitus, hiperglisemiyle sonuçlanan, göreceli veya mutlak insülin eksikliği ile karakterize kronik bir hastalıktır. Kronik hiperglisemi, vücutta pek çok organda hastalık gibi çeşitli komplikasyonlara yol açabilir, ve tedavi edilmezse yaşamı tehdit edici olabilir. Çeşitli komplikasyonlar arasında alp hastalığı, damar hastalıkları, böbrek yetmezliği, körlük, kısırlık ve tekrarlayan düşük sayılabilir. Diyabet nedeniyle tekrarlayan düşük (RA), yaygın bir sağlık sorunu oup herhangi bir üreme çağındaki kadınları etkileyebilir. Tekrarlayan spontan düşük (RSA), fiziksel, duygusal ve ekonomik olarak çiftleri ve toplumları incitebilir. Bu nedenle, tekrarlayan düşüklerin risk faktörlerini belirlemek büyük önem taşımaktadır ve yeni tedavi fırsatları sunabilir. Dünya genelinde 400 milyondan fazla insana diyabet teşhisi konmuştur ve bu diyabetik hastaların önemli bir kısmı kürtajdan mustariptir. Gerçekten de, diyabet ve düşük arasındaki ilişki hakkında birçok çalışma vardır. Bununla birlikte, diyabetli gebelik sırasında gen ekspresyonunun rolü tam olarak araştırılmamıştır. Ayrıca, Mikro RNAlar en önemli gen ekspresyon düzenleyicileridir. Bildiğimiz kadarıyla, diyabetin abortif bir fare modelinde mikroRNA'ların ifadesi üzerindeki etkilerini tanımlayan çok sayıda çalışma yoktur. Bu nedenle, çalışmamız farelerde diyabetik modelde diyabet nedeniyle uterus ve overde miRNA'ların diferansiyel ekspresyonunu araştırmayı amaçlamaktadır. Bu amaçla 18 fare 6 dişi ve 3 erkek içerecek şekilde ikiye ayrılmıştır (Diyabetik model ve Kontrol). Diyabetik fare modeli Streptozotosin (STZ) tarafından indüklenmiştir. Abortif ve kontrol farelerinden over ve uterus örnekleri toplanmış ve total RNA ekstre edilmiştir. Diyabetik farelerin bu organlarındaki miRNA seviyelerinin diferansiyel ekspresyonunu gözlemlemek için qPCR dizin tekniği kullanılarak analiz edilmiştir. Nihayetinde, miRNA ekspresyonlarının çoğunluğu uterusta aşağı regüle edilirken, overde miRNA'ların çoğu yukarı regüle edilmiştir.

Anahtar kelimeler: Diabetes mellitus, düşük, uterus, yumurtalık, miRNA, Gen ifadesi, Dişi üreme

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LIST OF ABBREVIATIONS

RA	: Recurrent abortion
RSA	: Recurrent spontaneous abortion.
STZ	: Streptozotocin.
GDM	: Gestational diabetes mellitus.
IADPSG	: International Association of the Diabetes and Pregnancy.
COH	: Controlled ovarian hyperstimulation.
LADA	: Latent autoimmune diabetes in adults.
PCOS	: Polycystic ovary syndrome.
BMI	: Body mass index.
RPL	: Recurrent pregnancy loss.
UTR	: Untranslated region.
TNF	: Tumor necrosis factor.
LH	: Luteinizing hormone.
IVF	: In vitro fertilization.
CEMACH	: Confidential Enquiry into maternal and child health.
DM	: Diabetes mellitus.
IDDM	: Insulin-dependent diabetes mellitus.
NIDDM	: Non-insulin-dependent diabetes mellitus.
ART	: Assist reproductive technology.

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INTRODUCTION

Because of limited insulin production, diabetes causes hyperglycemia. It is categorized into two types: type 1 and type 2 [1]. Type 1 is a type of autoimmune disease that destroys beta cells of Langerhans islet in the pancreas that are responsible for insulin production. Therefore, type 1 diabetes is diagnosed in young adults or children due to their extremely limited production of insulin. As such, those who have this disease must use exogenous insulin to regulate their body's deficiency and the glucose levels within the blood in order to minimize the hyperglycemia. This is a genetic disorder that is also dependent on environmental conditions for the disease development. Identical twins have a 27 percent likelihood to develop type 1 diabetes in both individuals [2]. Globally 422 million adults were living with diabetes in 2014, compared to 108 million in 1980. The global prevalence (age-standardized) of diabetes has nearly quadrupled since 1980, rising from 4.7% to 8.5% in the adult population. This reflects an increase in associated risk factors such as being overweight or obesity. Over the past decade, diabetes prevalence has risen faster in low- and middle-income countries than in high-income countries according to the last statistics of WHO. Moreover, the estimated occurrence of type 1 diabetes in the UK is as low as 15 or as high as 20 per 100,000 people (0.015 or 0.02 percent) [3]. Its presence in Europe is increasing exponentially with the figures expected to be doubling in children under the age of five by 2020 [3]. Another type of diabetes, type 2, features beta cells that cannot effectively indemnify insulin and is often linked with morbid obesity [4]. Patients with type 2 diabetes are identified by their resistance to their naturally occurring insulin. This type is often manageable through an effective diet and staying physically active. Beyond the two normal classifications of diabetes, there is a third type that occurs during pregnancy known as gestational diabetes (GDM). It is classified as an intolerance to glucose that occurs alongside or is first recognized during pregnancy and most instances are resolved after labor. However, GDM is limited to such cases and not to those women who had an already undiagnosed

case of type 2 diabetes. Because of growing obesity rates and the habitual consumption of unhealthy food, type 2 diabetes is quite common in women who are at the age of childbearing. The number of women with type 2 diabetes in this age bracket has increased with high numbers of cases unrecognized until pregnancy. Upon intensive research by the International Association of the Diabetes and Pregnancy (IADPSG), it has been suggested that women already at risk for the disease should be checked for GDM during their first prenatal visitation. The American Diabetes Association (ADA) indicates that around seven percent of all pregnancies coincide with a diabetes diagnosis, or about 200,000 instances in the United States every year [5].

Although diabetes is manageable through diet, exercise, and exogenous insulin, it is also life-threatening if untreated. Several studies have demonstrated that this disease features an assortment of other related health issues including: blindness, heart disease, miscarriage and more [6]. Abortions onset by diabetes is a severe issue that is plaguing females. Diabetes related pregnancy disorder is an important medical situation because reproduction is a critical component of life. Despite the association of diabetes with female reproduction, there are only few studies that investigated the effects of diabetes in reproductive events and organs with a molecular point of view. Although insulin therapy has a positive impact on limiting the effects of such abnormalities, the risk for abnormal pregnancies and birth remains high in women with type 1 diabetes. Birth defects are common in such cases, including congenital malformation [7].

On the other hand, pregnancy loss is not completely based on diabetes, but rather on abnormalities in the paternal or maternal chromosomes. On the overall perspective, a total of 15 to 20 percent of all pregnancies result in loss of life within the first-trimester. Parents who carry abnormalities will therefore also have higher miscarriage rates. This is because genetic information often does not segregate into reproductive cells as it should. Such miscarriages can be caused by inversions, deletions, translocations, and/or duplications [6]. An estimated three to five percent of all pregnancy loss is based on genetics, while seven percent on defects in chromosomes, 15 percent on hormone problems, and 10 to 15 percent on defects in the development anatomy. The chromosomal abnormalities that result in miscarriage occur by chance [8]. In addition, studies revealed that genetic materials such as microRNA also play an important role in pregnancy loss [10].

MicroRNAs, also known as miRNAs, are tiny molecules of 18-22 nucleotides and are crucial regulators for the expression of genes transcriptionally. miRNAs typically regulate the expression of genes either by translational inhibition by binding at the 3'-end of the target genes or by mRNA destabilization. Numerous researches focused on assessing miRNA in a qualitative and quantitative manner have displayed a massive difference in miRNA expression associated with a number of diseases. Successful profiling of miRNA expression is crucial to diagnosing and treating diseases [9].

miRNA is associated with a great deal of reproductive functions, including conception, implanting and the development of embryos. Because of their important role in reproduction, studying miRNA can give researchers a more complete understanding of the roots of implantation and embryo progression issues. Controlled ovarian hyper stimulation (COH) triggers differential miRNA expression during IVF cycles. Because this suppresses target gene expression, the implantation process can be damaged. Scientists are now working on characterizing specific genes that are linked with implantation and how they correlate with miRNA [10]. However, it is not completely understood how miRNAs can be effective in the function of ovary and uterus under diabetic condition.

Therefore, this study aims to investigate the underlying molecular mechanisms, focusing on miRNA expression, and their biological relevance during ovarian and uterine dysfunction that could be associated with diabetes.

CHAPTER 1

LITERATURE REVIEW

1.1. History of Diabetes mellitus

Diabetes mellitus has been an established ailment since ancient times. While treatments first arose in the Middle Ages, it wasn't until the 20th Century that the disease had a firm classification and treatment consensus. Previous to then, type 2 diabetes was normally unrecognized and therefore undiagnosed. In 1889, researchers Mering and Minkowski discovered the pancreas' connection to diabetes upon experiments on dogs. They removed the dogs' pancreas and noticed that the animals developed symptoms associated with diabetes before dying. Then, in 1910, Sir Sharpey-Schafer reported that diabetes was onset by the lack of a chemical produced by the pancreas. This chemical was later named insulin [11]. The experiment of Mering and Minkowsi was repeated in 1921 by the two researchers Best and Banting. They, however, found it was possible to reverse the diabetic symptoms in the dogs by providing extracted materials from healthy dogs' pancreatic islets of Langerhans. This process moved research forward in terms of explaining how endocrine effects metabolism and insulin presence [5]. Through these experiments conducted at the University of Toronto where the scientists isolated insulin from bovine pancreases, an effective diabetes treatment was produced, with the first patient receiving treatment in 1922. Furthermore, type 1 and 2 diabetes were differentiated by Sir Harry Percival [11]. The above research led to the following important discoveries for diabetes: identifying sulfonylureas in 1942, the discovery of radioimmunoassay for insulin by Yallow and Berson, introducing metabolic syndrome in 1988 by Reaven, and identifying thiazolidinediones for antidiabetics in the 1990s [12].

1.2. The pancreas and insulin formation

A long organ, the pancreas is located near the tip of the small intestine, behind the stomach, and between the duodenum and spleen as shown in Figure 1.1. As both an endocrine and exocrine gland, the pancreas produces both hormones (like glucagon and insulin) and enzymes (like chymotrypsin and trypsin). The islets of Langerhans are contained within, and their alpha cells secrete glucagon and beta cells secrete insulin. They also have delta cells that secrete gastrin and F cells that produce the pancreatic polypeptide. These islets are strong in blood supply, providing blood to the pancreas' exocrine and endocrine sections. Although making up only one to two percent of the pancreas' mass, they receive nearly 10 to 15 percent of its blood flow [13].

The islet cells are not organized in a random order. The beta cells are on the center, and they are surrounded by the alpha, delta, and F cells. As previously mentioned, the beta cells are responsible for insulin production. Insulin is then stored as a granule within the pancreas. The pulsatile secretion of insulin is controlled by chemical, hormonal, and neuronal factors [13].

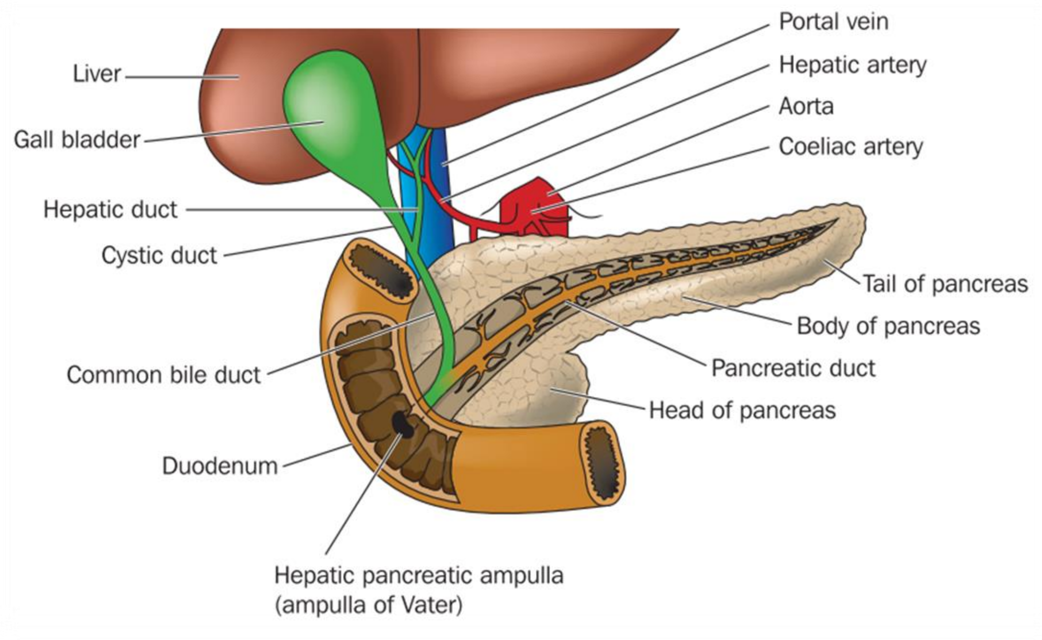


Figure 1.1. The Pancreas [13].

Additionally, they are innervated by sympathetic and parasympathetic nerves, which play a crucial role in the secretion of insulin. The islets of Langerhans have four types of hormone secreting cells. These are the:

● Alpha cells - secrete glucagon

● Beta cells - secrete insulin

● Delta cells - secrete gastrin

● F cells - produce pancreatic polypeptide.

These cells within an islet are not randomly distributed; beta cells occupy the central portion of the islet and are surrounded by alpha, delta and F cells Figure 1.2. The synthesis of insulin takes place in the beta cells and is stored in granule form in the pancreas. Pulsatile secretion of insulin is regulated by chemical, hormonal and neuronal control [13].

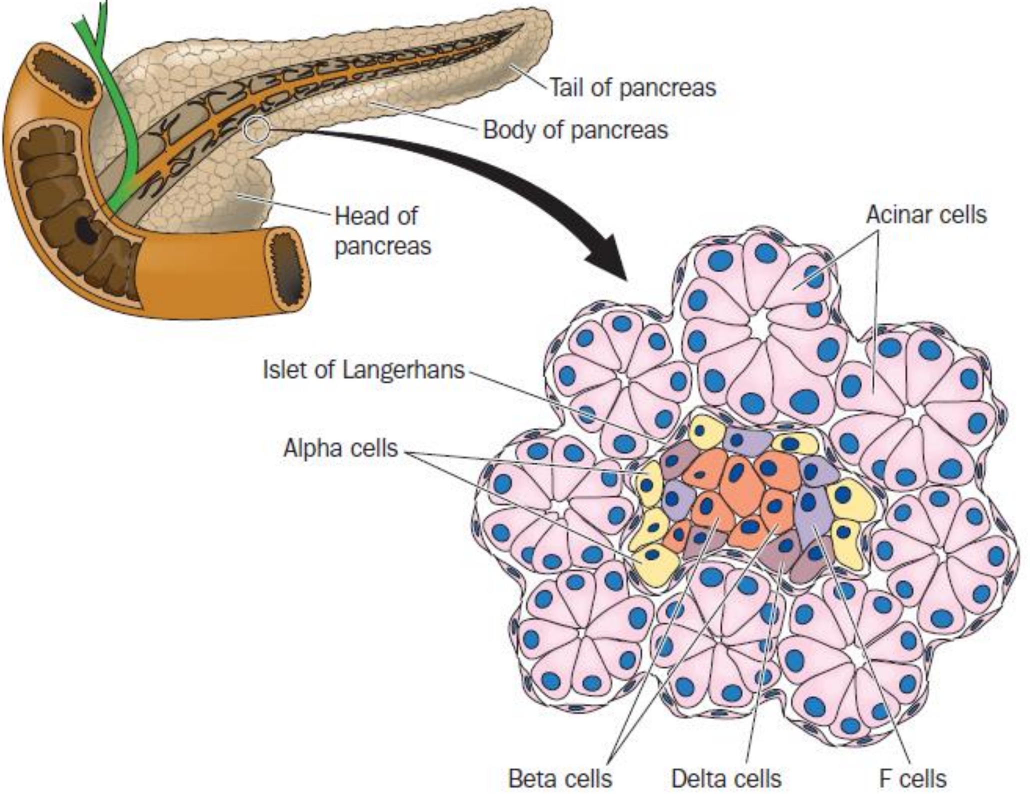
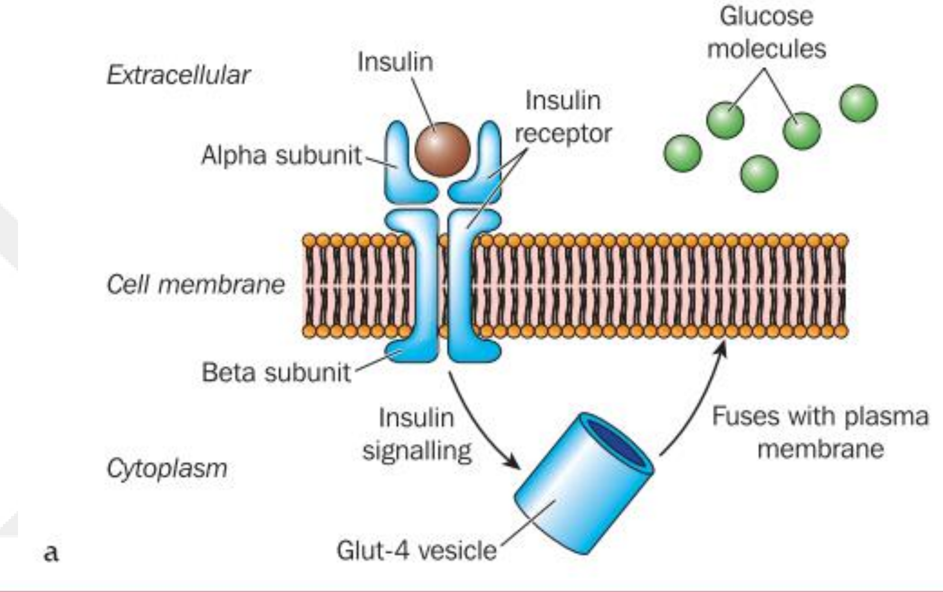


Figure 1.2. Islet of Langerhans [13].

1.3. Effects of insulin

Insulin is responsible for increasing the flow of glucose to the liver, the muscles of the skeleton, and the adipose tissue cells. Cells can only absorb glucose through facilitated diffusion using transporter proteins known as Glucose transporter type 4 (GLUT 4) as shown in Figure 1.3. When there is no insulin present, the GLUT 4 proteins remain inactive and are held within cytoplasmic vesicles [13].



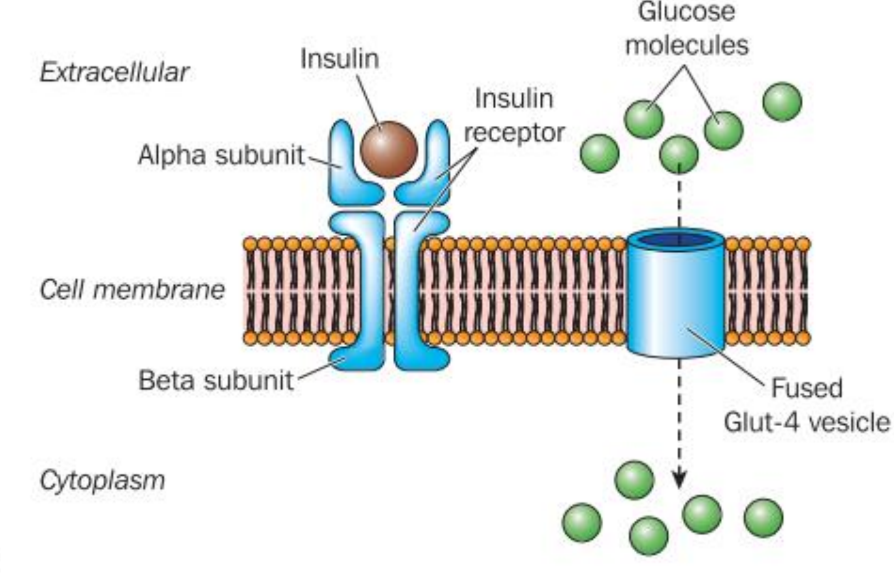


Figure 1.3. GLUT 4 protein transporter (a) and fused GLUT 4 to cell membrane (b). Extracellular [13].

1.4. Definition and description of Diabetes mellitus:

Diabetes includes an assortment of metabolic diseases featuring hyperglycemia that is caused by problems in secreting insulin, problems with insulin action, or both. Continued hyperglycemia from diabetes is linked to the damage or failure of many bodily organs, including: eyes, nerves, blood vessels, heart, and kidneys. A number of pathogenic processes can facilitate diabetes development. An autoimmune destruction of the β-cells within the pancreas can occur, but resistance to insulin action can be caused by several β-cell abnormalities. The foundation of irregularities in diabetic metabolism of protein, fat, and carbohydrate is insufficient action of insulin on targeted tissues. This insufficient insulin action arises from not enough insulin being secreted, lessened tissue reactions to insulin along the hormone action pathways, or both. Most diabetics experience both lessened insulin secretion and defected insulin action. As such, it is difficult to determine which of these issues is causing hyperglycemia, or if they are working in conjunction. Common hyperglycemia symptoms include: blurry vision, weight loss, polydipsia, and polyuria. Another common symptom of hyperglycemia is the heightened effect of some infections. Fatal diabetic issues include hyperglycemia with ketoacidosis and nonketotic hyperosmolar syndrome. There are several complications associated with prolonged diabetes that can significantly impair the diabetic's quality of life, including: vision loss, renal failure caused by nephropathy, peripheral neuropathy (causing Charcot joints, ulcers on the feet, and amputations), and autonomic neuropathy (leading to issues in the cardiovascular, gastrointestinal, and genitourinary systems or sexual dysfunction). Diabetics also carry a heightened risk of cerebrovascular, cardiovascular, and peripheral arterial diseases. Another risk for diabetics is hypertension and dysfunction of lipoprotein metabolism [5].

1.5. Classification of Diabetes mellitus and other categories of glucose regulation:

Diagnosing a particular type of diabetes is related to the symptoms they express at the time of discovery. Many diabetics don't find into a single category. It is entirely possible than a woman diagnosed with gestational diabetes continues with hyperglycemia after delivery and is later found to have type 2 diabetes. There is also an assortment of diabetes types related to the reason for diabetes occurring. Several of these types are mentioned below [5].

1.5.1. Type 1

Type 1 diabetes previously included the traditional type 1 as well as juvenile-onset and insulin-dependent diabetes. The cause of type 1 is the beta cells within the pancreas being destroyed WHO in 1998 [11]. The pace at which these cells are destroyed varies a great deal among some diabetics, as it is slow in some and quite rapid for others. It is more common for children to develop type 1 diabetes with rapidly deteriorating beta cells, but it is also possible for adults [14]. Because the slow deterioration is more common in adults, it is also known as Latent autoimmune diabetes in adults (LADA). For some type 1 diabetics, ketoacidosis is present at the earliest onset of the disease, which is more common for children and young adults [13].

For some patients, a modest hyperglycemia can develop into a severe state or even into ketoacidosis at the arrival of an infection or additional bodily stress. However, and particularly adults, retain residual function in their beta cells which is enough to avert ketoacidosis for a long period of time [11]. Because of the risk of ketoacidosis, diabetics with this type of type 1 are dependent on insulin to survive. When ketoacidosis occurs, there is minimal to no insulin secretion which is shown through unnoticeable plasma C-peptide levels. The vast majority of type 1 diabetics, upwards of 85 to 90 percent, experience immune destruction which includes islet cell antibodies and autoantibodies to insulin, as well as autoantibodies to GAD (glutamic acid decarboxylase) upon first detection of hyperglycemia [12]. Most such cases appear in children and young adults, but it is possible up until the latest stages of life. Autoimmune destruction of beta cells is primarily impacted by genetic factors. However, environmental factors play a key role as well, but they are still undefined through qualitative research. Type 1 diabetics are often not obese when their diabetes is detected, but the connection to obesity is also possible. These diabetics can also possess other autoimmune diseases, including Addison’s disease, Grave’s disease, and Hashimoto’s thyroiditis [5].

1.5.2. Type 2 Diabetes

Type 2 diabetes accounts for 90 to 95 percent of diabetic cases. As well as type 2 diabetes, it was also formerly known as NIDDM and adult onset diabetes. This category is often denoted by diabetics who have a resistance to insulin and minimal insulin deficiencies, rather than the total deficiency present in type 1. In their early stages with

the illness, type 2 diabetics do not rely on insulin for survival, and often live with the illness without any such treatment. Scientists have suggested a number of varying causes for type 2 diabetes. That said, the specific etiologies remain unknown even though beta cells are not destroyed, and other diabetic causes are not related. Many type 2 diabetics are obese. This is also true because obesity is another factor of resistance to the body's natural insulin. Those type 2 diabetics who are not obese can also have an abnormal distributed of body fat in the abdominal region. Ketoacidosis is rare for type 2, and if it does occur, it is often because of an infection or other bodily stresses. Type 2 often goes years without a proper diagnosis because there is not an evident appearance of hyperglycemia nor an outbreak of typical diabetes symptoms. Still, these diabetics carry an increased risk of complications in the macrovascular or microvascular systems. Diabetics with type 2 can display normal or high insulin levels, but this would be true even if the beta cells acted in their normal function because of the type 2 diabetic's other issues. Because of this, the secretion of insulin is malfunctioning and not enough to counteract type 2 diabetics' resistance to insulin. It is possible to improve this insulin resistance through weight loss or medicine, but it is rarely completely restored to normal. Those at risk for developing type 2 diabetes include: obese individuals, the elderly, those with minimal exercise, or those suffering for hypertension or dyslipidemia. The occurrence of type 2 diabetes is varied among different races and ethnicities. Type 2 diabetes has a stronger connection to genetic predisposition than type 1. Still, the genetic factors associated with this category are not yet fully understood nor defined [5].

1.5.3. Gestational Hyperglycemia and Diabetes

Gestational diabetes is an intolerance to carbohydrates that causes hyperglycemia of various levels of severity, either onset or first recognized during pregnancy. This is not related to women who have a previous diagnosis of another type of diabetes. Such women have different treatment before their pregnancy, during gestation, and after delivery. The concentration of fasting and postprandial glucose is lower for women in their first trimester than in women who aren't pregnant. Increased levels of either type of glucose during pregnancy can indicate a previous, undiagnosed, form of diabetes. When plasma glucose levels are higher than normal, it is important to manage the pregnant woman and an Oral Glucose Tolerance Test (OGTT) may be administered.

However, simply because glucose levels are normal during the early stages of pregnancy does not mean gestational diabetes will not develop at later stages. There are several types of women who have an elevated risk for this type: older women, women who have had issues with glucose intolerance, women with higher glucose levels (fasting, casual, or blood), and some ethnicities. Women belonging to these risk groups should be scanned for diabetes during their first trimester to see if there is an underlying undiagnosed case of diabetes. Formal testing usually occurs between 24 and 28 weeks [15].

1.6. Diabetes and pregnancy

Insulin sensitivity changes during pregnancy which can cause plasma glucose levels to also change. There exists a risk of delivery for women with any type of diabetes [16]. Before insulin was discovered, women with type 1 diabetes had an extremely minimal chance of delivering a healthy baby. Pregnancy loss remained high even after insulin treatment was invented. However, most of these were stillbirths and the previous risk of birth trauma, malformation, respiratory distress syndrome, and hypoglycemia were averted [17], [18]. Even with the stillbirths present in diabetic women, the figures have remained similar to those cases for women without diabetes [19]. Termination of pregnancy and neonatal death from serious congenital malfunctions constitute a large percentage of pregnancy loss among women with type 1 diabetes, and the latter are quite difficult to reduce [20]. Because obesity reached an epidemic status over the last 20 years, the development of type 2 diabetes in women of the age of childbearing has grown significantly. In some countries, the number of pregnant women with type 2 diabetes is far more than women with type 1 [21]. Some research has indicated higher stillbirth and congenital malformation rates in women with type 2 diabetes, which means that pregnancy for these diabetic women could be far worse than those who have type 1 [22].

Several factors highly the difference in neonatal loss between the two types of diabetes. Women who have type 2 tend to be older, obese, poorer, and minorities. These are all contributing factors to pregnancy difficulties on their own. On the other hand, women with type 1 have a higher rate of vascular complications caused by diabetes [7]. Research has indicated that obesity has stronger connections to pregnancy loss than diabetes alone and the loss rates between type 1 and 2 were similar (2.6 percent for type 1 and 3.7

percent for type 2). However, the cause of this pregnancy loss is often different. For type 1 diabetes, 75 percent of loss was linked to congenital malfunction or prematurity whereas 75 percent of loss in women with type 2 was caused by stillbirth or chorioamnionitis [7]. Because glycemic control was similar between pregnant women with type 1 and 2, there is no clear ruling on how important early referral has [7]. Also, maternal diabetes presents an unsavory environment for embryonic and fetoplacental development. Even through advancements in treatment, women who have diabetes predating their pregnancy continue to have higher risks for congenital anomalies, placental abnormalities, and complications in maternofetal and intrauterine [23].

Diabetes in pregnant women continues to have risk for both maternal and neonatal morbidity. As such, this type of diabetes has serious implications for medical researchers. Gestational diabetes is that onset during birth, while clinical diabetes refers to women who already had either type 1 or 2 before pregnancy. There are thresholds established by the American Diabetes Association for fasting and post-glucose loading levels. A ruling issued by the International Association of Diabetes in Pregnancy Study Groups stated the importance of pregnant women without preexisting diabetes to undergo a 75g oral glucose tolerance test during the 24 to 28-week period of gestation [24].

1.6.1. Diabetes and fertility issue in women

Both type 1 and 2 diabetes are important factors for infertility and menstrual abnormality. The menstruation cycle for diabetic women can become reduced and premature menopause is also common. The menstrual abnormalities oligomenorrhea and secondary amenorrhea have been linked to diabetes during the childbearing years. Research has shown that controlling glycemia and reducing complications associated with diabetes can improve the issues and fertility rates similar to those for non-diabetic women. When women who undergo the normal treatment continue to have complications, it is suggested that they undergo more complex evaluations. These include examining the hypothalamic-pituitary-ovarian axis and the status of present hormones, autoimmune thyroid disease, anti-ovarian autoantibodies, and hyperandrogenism. This is an important issue because more than 180 million people across the globe suffer from diabetes, with the figures predicted to double in the next decade [25]. The rate of growth of type 1 diabetes, a category which effects mostly

youth, is 3 percent each year. Every year, nearly 70,000 children 14 or younger develop diabetes. Even still, type 2 is also growing at an alarming rate in both developed and developing countries [26]. Women are affected by diabetes in an assortment of ways. These include:

1.6.2. Menarche & menstrual cycle disturbances in Type 1 diabetes

Before the first introduction of insulin in clinical care of Type 1 diabetes in 1922, menarche seldom occurred in girls with diabetes during childhood. When it did occur, menses usually stopped. Successful pregnancy was achieved in only two percent of Type 1 diabetic women [26]. Beginning insulin allowed for successful menstruation in the majority of diabetics, but the abnormalities in their menstrual system were not solved. Early research by the researcher Bergqvist in 1954 revealed delayed menarche in diabetic women who were diagnosed before menarche with menstrual issues continuing for 30 percent of the women in their 30s and 40s [26]. These disorders were proven to be three times more common in diabetics than in normal women [27]. Further research proved that diagnoses of diabetics in pre-menarche women, especially for those younger than 10, displayed delayed menstruation by nearly one year compared to the general population [28]. As such, study has shown that issues in menstruation is twice as common for diabetics than for other women (26 percent compared to 10.8 percent). What's more, women with type 1 diabetes have a higher probability of the following issues: longer cycles (more than 31 days), longer menstruation (more than six days), and larger loss of blood [26]. These issues were shown to subside as women reached the end of their reproductive age.

1.6.3. Correlation between Type 2 diabetes & fertility

The majority of women with type 2 diabetes are those past their menopause. However, because of the rising obesity rates and changes in eating patterns, the diagnosis of this type is growing in women in their reproductive years. A link exists between type 2 diabetes and fertility, menstrual cycle length changes, and menopausal age [29]. It is possible to explain this link due to the connection between type 2 diabetes and PCOS, which is the most distributed hormonal issue for reproductive-age women and a major reason for infertility. Obesity and insulin resistance are strong factors for PCOS development. The two diseases also share the risk factors, including obesity,

hypertension, hyperinsulinemia, and dyslipidemia. Hyperinsulinemia arrives from a resistance to insulin. It changes the IGFBP, SHBG, and IFG1 levels, which then secretes more androgen at the ovaries and adrenal gland, leading to anovulation. Women with PCOS are at-risk for defective tolerance to glucose and a diagnosis of type 2 diabetes, no matter their age or weight [15]. Obesity is normal for women with both PCOS and type 2 diabetes. General research has proven that obese women wait longer before conception, regardless of their age and cycle pattern [30]. A study showed that oocytes for high BMI women produces low quality blastocyst [31]. Current research analyzed the ovarian follicular environment of women across all BMIs through a measurement of their follicular fluid hormones and metabolites, gene expression, and granulosa [32]. It was found that obese women have higher intrafollicular insulin and triglycerides and a larger expression of lipoprotein receptors. BMI had no effect on gene expression [33].

1.6.4. Diabetes and Pregnancy losses.

Pregnancy loss related to diabetes is broken down into: medical abortions at 20-28 weeks, fetal death occurring at 28 weeks, or early neonatal death between a day and a month after delivery. The effects of miscarriages were not possible due to insufficient data. One of five reasons were considered for such deaths: chorioamnionitis, serious congenial irregularities, asphyxia during delivery, and prematurity. Although the frequency of pregnancy loss with type 1 and 2 diabetics was nearly the same, the overall cause was drastically different [34]. Congenital irregularities and neonatal issues resulted in the most deaths for type 1 diabetics, related to glycemic regulation during the early stages of pregnancy [35]. Effective counseling before conception reduced the congenital abnormalities a great deal [36]. One-fourth of deaths related to this issue were caused by fetal aneuploidy, which is not related to glycemic regulation. As such, if these figures were removed from the calculation, the rate of congenital anomalies in type 1 and 2 diabetic pregnancies is nearly identical (0.6 and 0.7 percent) [37]. The common pregnancy loss issues for type 2 diabetics were asphyxia, stillbirth, and chorioamnionitis, with the latter two far more common than in type 1. There were two clusters in unexplained stillbirths, and it is possible that some in the early cluster (20–29 weeks' gestation) were the result of unrecognized chorioamnionitis. Stillbirth is associated with greater maternal age [38]. But the difference in mean age between the

women with type 1 and women with type 2 diabetes was only 3 years. Maternal obesity is strongly associated with pregnancy loss [39]. For example, the risk of stillbirth and neonatal death was doubled in women with a mean BMI_30 kg/m2. The pre-pregnancy BMI exceeded this value in _70% of our subjects with type 2 diabetes. Maternal obesity, poverty, and hyperglycemia are all risk factors for chorioamnionitis [40]. However, the glycemic control of women with type 2 diabetes was like that of women with type 1 diabetes. Scientist argued that women with gestational diabetes who are shown to have diabetes on early postpartum testing should be considered as having newly recognized diabetes that likely antedated the pregnancy. Such pregnancies have the same risk of pregnancy loss and major congenital anomalies as established diabetes [41], [42]. eventually, poorly controlled diabetes in early pregnancy is associated with an increased risk of spontaneous abortion [43].

1.7. Genetic and molecular factors governing pregnancy loss:

Continual loss of pregnancy is more often the result of irregularities in parental chromosomes that lead to intrauterine damage to the fetus. Nearly 20 percent of all pregnancies end in spontaneous miscarriage during the first trimester. Irregularities in parental structure cause more miscarriages due to genetic information not being accurately segregated into the reproductive cells. The overall statistics related to continuing pregnancy loss are: 15 percent hormonal faults, 10 to 15 percent anatomy faults, seven percent chromosome faults, and three to five percent genetics. It is common for women to lose their fetus before reaching the 24-week gestation period, a time when the infant could survive out of the womb [44]. Although a few cases of recurrent pregnancy loss (RPL) are sporadic, most couples experience genetic basis as a challenging clinical dilemma. A variety of possible etiologies have been described for both sporadic and RPL [45]. Using the very sensitive pregnancy tests that are available now, it is known that one out of two pregnancies end in very early miscarriage [46]. In the past the majority of these would have been passed off as late or heavy menses. Even after a clinically diagnosed pregnancy, one out of 5- 6 pregnancies end in a miscarriage between 4th and 20th week of gestation [47]. genetic factors appear to be highly associated with reproductive loss [48]. Between 50 and 80 percent of loss of pregnancy results from chromosome irregularities. The rate differs based on the mother’s age and the period of gestation [49]. Erratic bodily occurrences lead to unusual embryos as far

as cytogenetics, and they can be either aneuploid or polyploid (from problems in fertilization). Incidence of numerical and structural chromosome abnormalities in spontaneous abortions is nearly half (49%), 10 percent polyploid, 8.6 percent 45 X, and 26.8 percent trisomic [50]. Autosomal trisomy's are involved in 50 percent of the cytogenetically irregular abortions in the first trimester. Triploidy and tetraploidy are connected to irregular fertilization and are not compatible with life. Triploidy is found in 16 percent of abortions, with fertilization of a normal haploid ovum by dispermy as the primary pathogenic mechanism [51]. Tetraploidy occurs in approximately eight percent of chromosomally abnormal abortions, caused by failure of an early cleavage division in an otherwise normal diploid zygote. Structural chromosomal abnormalities occur in approximately three percent of cytogenetically abnormal abortus. For men, this usually produces a low sperm count, unusual sperm count, or infertility [52]. When chromosomes are not distributed in balance, a partial trisomy occurs for one chromosome and a partial monosomy for the other.

The unbalanced distribution of the chromosomes involved in the translocation, leads to partial trisomy for one chromosome and partial monosomy for the other chromosome. Balanced translocations constitute the largest percentage of these karyotypic irregularities. They can cause pregnancy loss because segregation during meiosis results in gametes with duplication or deficiency of chromosome segments [46]. Furthermore, we can't forget the crucial role of the genes in the pregnant initiation and maintain this embryo and the most important particle that’s control the gene expression that is miRNA.

1.8. miRNA

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs), approximately 20 nucleotides in length, that regulate gene expression post transcriptionally by binding to 3_untranslated regions (UTR), coding sequences or 5_UTR of target messenger RNAs (mRNAs) and leading to inhibition of translation or mRNA degradation. Scientists estimate miRNA being responsible for nearly half of genomes that code protein. They are responsible for gene expression that relates to a number of important processes, such as differentiation, apoptosis, proliferation, and metastasis. MiRNA was unknown 20

years ago, and their importance was discovered in 1993. Until then, the scientific community focused on genes that codify for protein. Because it was assumed that DNA was transcribed into RNA and then into protein, all sequences not related to the coding of protein were ignored [53]. Before the discovery of miRNA, the non-coding regions in the genome of a species were thought of as 'junk' DNA. However, our understanding of these regions has turned upside down because of the discovery of lin-4, the first microRNA in Caenorhabditis elegans by Lee, Feinbaum and Ambros. The discovery of miRNAs has supplied much insight into the regulation of gene expression, which influence many biological activities. miRNAs are small non-coding RNAs work for negatively regulate translation of complementary RNAs by either repressing the translational machinery of the ribosome or by deadenylating and hence destabilizing the mRNA. More than 2000 mature human miRNAs sequences have been detected, and they are suggested to controlling gene expression of 50% of all genes that coding for protein [54]. Each miRNA has the ability to repress the expression of several target genes, So, miRNAs have a crucial role in many biological processes including cellular differentiation, proliferation and apoptosis [55].

1.8.1 Discovery of miRNA

Lin-4 was the first miRNA to be discovered, in 1993, by the joint efforts of Ambros's and Ruvkun's laboratories [56]. In the nematode Caenorhabditis elegans, heterochronic genes control the temporal development pattern of all larval stages. One of these genes is lin-4, discovered by the isolation of a null mutation that causes a failure in temporal development [57]. As such, lin-4, let-7 is a heterochronic gene of C. elegans. It was the second of found and recognized miRNA and was listed in the year 2000, seven years after the first was discovered. Reinhart and his team suggested that let-7 was a 21-nt RNA that regulates the L4 to adult change of developing larva [58].

1.8.2. miRNA biogenesis and miRNAs function

The miniscule RNA molecules are made up of 17-25 nucleotide RNA that don't code and limit messenger RNA expression. They are crucial to the regulation of reproductive and developing procedures after messages are transcribed. Their initial name of stRNA (small temporal) relates to the structured expression and their duty in circumstances related to development [59], [60], [61]. Three papers published in 2001 expounded

these mini RNAs and it was agreed that these small RNAs would be renamed “microRNAs [62]. miRNAs usually related to base pairing with the 3-untranslated region (3- UTR) of complementary messenger RNA targets, thus blocking the translation of select mRNA with or without subsequent mRNA degradation and effectively inhibiting gene expression [63]. The majority of miRNA are made from primary RNA being cut by RNAse III endonuclease, Drosha, and DGCR8 in the formation of 70 to 100 hairpin of nucleotides. Exportin 5 moves pre-miRNA away from the nucleus and to cytoplasm [64]. Dicer cleaves them and takes away the hairpin for the production of duplex miRNA. One of these strands are connected to miRISC that is primarily Argonaute for the ultimate miRNA. This process is called the canonical pathway. Noncanonical pathways also exist wherein miRNAs are created either without processing by Drosha/DGCR8 but still requiring Dicer or by passing directly from Drosha/DGCR8 to AGO2 thus bypassing Dicer [65]. Because it was originally thought that all miRNAs required Dicer for expression [66]. The information gained from RNAi was crucial to understand how miRNAs process and act [67]. In 2000 a study found that double-stranded RNA fragments of 21–23 (nt) were aiming at the mRNA cleavage. As such, the functional RNAi unit had the same dimensions of miRNAs. In the same year, two important studies suggested a participation of the RNAi pathway in the maturity of miRNAs [68]. Simultaneously, furthermore the other study found that let-7 pre-miRNA is cleaved by Dicer. In fact, when cells were transfected with siRNA duplex that correspond to human Dicer enzyme, pre-let- 7 accumulated in the cells [69].

Looking at this research, a connection was proven between RNAi and the miRNA pathway. In the miRNA biogenesis pathway, Drosha and Dicer are spatially separated, localized in the nucleus and cytoplasm [70]. In 2004 another study indicated that exportin 5 was the major mediator of effective nuclear transport of miRNA precursors. That said, several miRNAs are still only found in the nucleus. It was discovered in 2007 that several miRNAs have extra sequence components to regulate subcellular localization. As found, miR-29b has a terminal motif comprised of hexanucleotides to distribute the output to the nucleus [71]. The primary job of miRNA is the restriction of protein synthesis for genes that code protein by the degrading of mRNA or inhibiting the translation process. Beyond repressing mRNAs, miRNA also begin the mRNA translation [72]. Another study also showed that miRNAs can begin the translation

process. were the first to clearly demonstrate that, in some instances, miRNAs can work as translational activators. TNF_ AU-rich elements recruited miR-369-3 to facilitate translation upregulation, entirely through serum starvation. In addition, upon cell cycle arrest, let-7 and the synthetic miRNAcxcr4 induced translation, whereas they repressed translation in proliferating cells. As such, miRNAs can coordinate themselves to change to either repressing translation or activation [72]. In 2008 study displayed miR-373 aiming for the promoter of e cadherin and CSDC2 to initiate expression [73]. In 2007 study suggested that miRNA associates the location of targeted mRNA [74]. In 2008, a study reported that binding sites in coding sequences are abundant and experimentally showed that mouse Nanog, Oct4, and Sox2 genes have miRNA-binding sites in their coding sequences. MiRNAs targeting these genes modulate embryonic stem cell differentiation [75]. In 2010, researcher reported a remarkable finding for our understanding of how miRNAs function., in addition to miRNAs gene silencing activity through base pairing with mRNA targets, miRNAs also have decoy activity that interferes with the function of regulatory proteins [76]. In conclusion, these authors introduced the new concept that miRNAs can work as molecular decoys for RNA-binding proteins [60].

1.8.3. miRNAs in female reproductive function

The female reproductive tract is composed of the ovary, oviduct/fallopian tube, cervix and uterus. For sustained and successful reproduction, proper development and function of each component is imperative. For normal development and function, a myriad of gene transcription, translation and post-translational regulatory mechanisms must be invoked. Moreover, recent observations suggest that in addition to transcription, translation, and post-translational modifications, post-transcriptional gene regulation may play a more pronounced role in cell, tissue, and organ function. In the past several years, microRNAs (miRNAs) have been demonstrated to play a novel, yet not thoroughly defined, role in post-transcriptional regulation of gene expression and organ development [77]. Gene regulation after transcription is crucial for germ cell function in both men and women. For women, reproduction is successful through coordination of the hypothalamic pituitary gonadal axis and how it regulates the tissues of the cervix, uterus, and ovaries. The reproductive tract reacts to the pituitary cycle and hormones within the ovary for gamete transportation, producing a suitable site for implantation

and pregnancy, and protection against pathogens. The last four decades have been crucial towards understanding transcriptional gene regulation and cellular signaling pathways within reproductive tissues. That said, there is little understanding about how gene regulation works after transcription although it is crucial to protein synthesis and the general regulation of cell differentiation and proliferation. Post-transcriptional gene regulation includes the splicing, editing, transport, storage, turnover, and translation of mRNA, and can result in a difference in expression levels between the transcriptome (mRNA) and the proteome (proteins) of the cell [78]. All mRNA transcripts pass through some type of post-transcriptional processing, and although some post-transcriptional gene regulation may be considered almost constitutive in manner, other forms are dynamically regulated [79]. As such, the importance of regulating genes after transcription that coordinate gene expression variations becomes clear in the changing reproductive tract. In the female reproductive system several examples of post-transcriptional gene regulation have been shown to be involved in gonadal and reproductive tract function. In granulosa cells within the ovary, degrading the LH receptor transcript increases after the LH increase, and this is mediated by mevalonate kinase, an enzyme in the cholesterol biosynthetic pathway, that also acts as an RNA binding protein [80]. Connexin43 is also translationally repressed in granulosa cells after this increase, although the mechanisms that mediates this repression has not been identified. Additionally, estrogen treatment stabilized estrogen receptor α through the RNA-binding protein AUF1p45 within the uterus [81]. Although it is hard to prove, the high volume of expressed RNA-binding protein and miRNA indicate that gene regulation is crucial to tissues related to reproduction [82].

1.8.4. miRNAs in reproductive disease

Since miRNA play such a vital role in cell differentiation events, it is easy to envision how the dysregulation of miRNA expression could lead to a disease state. MicroRNA have been demonstrated to be important in multiple types of cancer including ovarian, endometrial, and cervical; as well as non-malignant pathologies, such as uterine fibroids and endometriosis.

In fact, miRNA have arguably been more thoroughly studied in the disease state of reproductive tissues than the normal state, and here we will briefly touch on some recent

findings in respect to miRNA and their dysregulation in diseased reproductive tissues of women Ovarian cancer is the sixth most common cancer in women, and miRNA expression has been studied in several types of ovarian cancer and a variety of cancer cell lines [83], [84]. To date, the predominant work focuses on elucidation of miRNA signatures that can be used for diagnosis. These studies compare cancerous tissue to either normal tissue or to immortalized cancer cell lines. Interestingly, a set of common miRNAs associated with ovarian cancer has not been generated, which may be due to the nature of the controls or reference samples used to compare the cancer tissues. In the uterus, endometrial carcinogenesis affects almost 40,000 women per year [85]. Recent study examined the expression of miRNA in endometrial adenocarcinomas versus normal endometrial tissue and identified thirteen miRNAs as differentially expressed and they also identified mRNA differences between the two tissues and found that 9% of the differentially expressed mRNA were predicted targets of the changed miRNA [85]. Uterine leiomyomas (i.e., fibroids) are a benign uterine pathology that affect 30 to 50% of women [86].

1.8.5. miRNA and pregnancy complication

MicroRNAs are lately discovered class of non-coding RNAs that are expressed in many cell types, where they responsible for regulation the complementary RNA production. miRNAs are predicted to regulate the expression of ~50 percent of all protein coding genes in mammals. Consequently, they engage effectively in all cellular processes investigated until now. Adjusted miRNAs expressions are linked to both physiological (such as pregnancy) and pathological processes (such as cancer). The maternal-fetal interface plays an important role in the maintenance of pregnant successfully. Investigation in this field has determined the existence and dysregulation of miRNAs that associated with pregnancy development [34]. From the time they were discovered, miRNAs have arisen as major bioregulatory molecules of a lot of essential processes that directly effect on pregnancy [87]. Pregnancy is a complicated process; Successful mammalian pregnancy rely on the establishment and maintenance of a sufficiently interface between mater and fetus. At first, the non-pregnant uterus must undergo alteration into a cellular and molecular circumstance that satisfactory to assist the implanting conceptus and fetal maintaining [88]. Former studies have reported the genes that coding for protein and its role in pregnancy progression [34].

1.8.6. Role of miRNAs in Embryo-Endometrial Cross Talk at Implantation

Successfully implanting during pregnancy is dependent on timing and the relationship between blastocyst and endometrium [89]. It is widely known that autocrine, paracrine, and endocrine factors work together to coordinate during implantation. A study found that expression of miR-503 increased in the late proliferative-phase samples when compared to the midsecretory phase samples while the expression level of miR-210, miR-29B, miR-29C, miR-30B, miR-30D, miR193A- 3P, miR-200C, and miR-31 was decreased in the late proliferative phase versus midsecretory phase [90]. It was surmised that hsa-miR-30b, hsa-miR-30d, hsa-miR-494, and hsa-miR-923 is crucial to gene reprogramming [91]. Moreover, a prospective analysis conducted on patients who received IVF treatment aimed to determine the effect of higher progesterone level on endometrial receptivity and found four downregulated miRNAs (hsa-miR-451, hsa-miR-424, hsa-miR-125b, and hsamiR- 30b) between normal and elevated progesterone groups that might explain the reduced pregnancy rate in patients with elevated progesterone [92]. There are several studies focusing on the importance of embryo-endometrial cross talk at implantation which seems to be mediated by exosomes released by the endometrium [93].

1.8.7. miRNAs in Recurrent Abortion

Recurring abortion is two or more successive pregnancy losses before the 20th gestational week or spontaneous abortion of a fetus weighing less than 500 grams. Around two percent of pregnancies across the globe are affected by it. It is incredibly challenging to find treatments for this issue and better diagnosis of the underlying causes is extremely important for current research [94]. Two variant alleles, rs41275794 and rs12976445, in pri-miR-125a have been recognized in recurrent abortion in a Chinese-Han population. They alter the production of miR-125a. Its decrease can increase LIFR and ERBB2, two of its target genes. This can alter implantation and decidualization [95]. Moreover, the rs6505162 C>A in the miR-423 coding region was also identified to be related to recurrent abortion. Besides, in the Chinese population, a study in the Korean population has also been conducted. They found that miR-196a2CC, miR-499AG+GG, and the miR-196a2CC/miR-499AG+GG combination were related to recurrent abortion in Korean pregnancies [96],[97]. In addition, miR-

34a, miR-155, miR-141, miR-125a, and miR-125b were found to be increased in the recurrent abortion women, while miR-24 was decreased in decidual natural killer cells [98]. However, the functional role of these aberrant miRNAs in recurrent abortion is unclear. A recent work has reported the potential of using plasma miRNAs as biomarkers for recurrent abortion. A total of 27 recurrent abortion patients and 28 normal early pregnancies patients were enrolled at 6–10 weeks of gestation. Based on miRNA microarrays and real-time quantitative reverse transcription polymerase chain reaction analysis, a total of 9 miRNAs were found to be increased while a total of 16 miRNAs were decreased. Further studies confirmed that miR-320b, miR-146b-5p, miR-221-3p, and miR-559 were upregulated, while miR-101-3p was downregulated [99].

1.9. Role of miRNA in reproductive system formation and function

1.9.1. Ovary dysfunction and role of miRNA during diabetes

The role of miRNA in the ovary is known through removing Dicer causing abnormalities of folliculogenesis, oocyte maturation, ovulation and infertility [100]. Piwi interacting RNAs (piRNAs), miRNA, and small interfering RNA (siRNA) are the major small RNAs present in the ovary [101]. Endogenous siRNA utilizes the well-known RNA interference pathway to regulate gene expression. In this regulatory system, endogenous double stranded RNAs\ (dsRNAs) are thought to derive from pseudogenes that encode a complementary mRNA allowing for formation of dsRNA templates, Dicer cleavage of the dsRNA then generates endogenous siRNAs. With respect to endogenous siRNAs, they have been identified to play a role in oocytes [102]. Knockout of DGCR8, which should specifically block the miRNA pathway, and leave the endogenous siRNA pathway intact helps to distinguish the effects of these closely related classes of RNA species (i.e., miRNA and siRNA). Targeted deletion of oocyte DGCR8, didn’t negatively impact oocyte maturity [103]. This shows a limited role of miRNA synthesized through this pathway. However, miRNA could also be produced by direct transcription of short hairpin RNAs and mirtrons, which short circuit the standard miRNA biogenesis process by eliminating the need for Drosha/DGCR8 mediated cleavage [100]. Ovaries during diabetes are also affected by this complicating. So, the diabetes mellitus patients need insulin which causes androgen synthesis.

Eighty-five women with type 1 diabetes mellitus were evaluated for symptoms and signs of hyperandrogenism. In 68 of the patients, several serum androgen and hormone concentrations were measured. The (PCOS) was defined by the presence of menstrual dysfunction, together with clinical and/or biochemical evidence of hyperandrogenism, and exclusion of other etiologies. The controls were 18 healthy women with regular menstruation cycles. Hyperandrogenic conditions were present in 33 of the women (39 percent), with 16 having PCOS and 17 with hirsutism [101]. Moreover, there is a very strong relationship between cancer and ovary during diabetic and Both cancer and diabetes share common risk factors such as age, race and obesity, making simple conclusions very difficult. The etiology of the association between diabetes and ovarian cancer is not clear, but there is ample evidence for biologic plausibility. Several studies have evaluated the effect of increased insulin-like growth factor (IGF)15. IGF-I and -II are overexpressed in many cancers, which may lead to increased proliferation as well as stimulation of pathways involved in invasion and metastasis [104]. Indeed, elevated IGF-I and –II levels have been associated with decreased survival in epithelial ovarian cancer [105]. Insulin resistance and diabetes are associated with decreased serum sex hormone binding globulin [106]. which can lead to elevated levels of free estrogen. While the evidence for elevated estrogen as a carcinogen is well-established in endometrial cancer, recent murine models suggest that it may also play a role in ovarian cancer. Another compelling link between diabetes and cancer development and progression is through inflammatory pathways. Adipose metabolic dysregulation is a hallmark of diabetes [107]. This can lead to increased levels of inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α24, in turn activating pathways involved in cell proliferation, invasion and evasion of antitumor immunity [107]. Recent studies have identified miRNAs as potential tissue specific biomarkers of disease and may provide a useful measure in the diagnosis of numerous pathologies [108]. In this study they showed that in comparison to normal ovaries, numerous miRNAs showed differential expression in epithelial ovarian cancer tissue. It was found in ovarian cancer tissue that the most significantly over-expressed miRNAs include miR-200a, miR-141, miR-200c, and miR-200b. The most down-regulated miRNAs are miR-199a, miR-140, miR-145, and miR-125b1 [109]. However, the potential application of using differential expression of miRNA as a biomarker for ovary cancer disease was investigated in this study [110].

1.9.2. Uterus dysfunction and role of miRNA during diabetes

Like the ovary and oviduct, the uterus undergoes drastic changes throughout the menstrual/ estrous cycle. The uterus transitions from a highly proliferative state that is nonreceptive to blastocyst implantation when estrogen is high, to a highly secretory state that is receptive to blastocyst implantation when progesterone is high. A global analysis of miRNA expressed both in the uterus and ovary suggests that the miRNA profiles of the two are very similar, indicating that similar mechanisms may be in place to regulate these cyclic changes [111]. A crucial point is discovering the roles of miRNAs in reproductive system. miRNAs have an essential role in endometrial receptivity, implantation, and even fetal losing [34]. In the uterus, majority of the physiological modification happening over the estrous/menstrual cycle and during pregnancy are guided by the ovarian hormones. In other organs and tissues, research has established that there are many chances to regulate miRNAs; their transcription, processing, RNA editing, function and intracellular localization can all be modified and thus affect miRNA binding of mRNAs within the uterus. Both estrogen and progesterone are able to adjust miRNA expression directly and indirectly by enhancing or repressing these processes [112]. Estrogen, one of the important reproductive hormones and have a good ability to regulate miRNA in the uterus and endometrial cells [113]. Both estrogen and progesterone are qualified to regulate miRNA expression in the uterus, mainly via interaction with the nuclear steroid receptors which then rapidly increase the bioavailability or expression of miRNA processing machinery and transporters. Ultimately, it visible that the role of the steroid hormones in the uterus expedites the conversion of pri- to pre-miRNAs, transportation of pre-miRs to the cytoplasm, and the processing of pre- to mature miRNAs. The controlling of miRNAs by E2 and progesterone hormones give an evidence that miRNAs are crucial mRNA regulators during pregnancy. Interestingly, the regulation of miRNA processing and expression is reported to be at least partly under ovarian steroid hormone control and generally, miRNA expression is repressed [114].

Furthermore, during diabetes there is also more than one drawback to safety pregnant, Epically the uterus and worldwide, diabetes in pregnancy is associated with significant fetal and maternal morbidity and mortality. The prevalence of births complicated by pre-existing diabetes has increased by 50% in under a decade [115]. There is agreement that

Caesarean sections are more common with diabetics, with a percentage reported of roughly 67 percent. The risk of emergency caesarian in pregnancy complicated by a diabetic pregnancy is 3-4 percent [116]. It was pointed out by CEMACH that more than half of C-sections were emergency operations. These operations in diabetics carries a two-and-a-half increased risk of infection, hemorrhage, and thrombosis [116]. Despite the significant attendant morbidity in diabetic C-sections, researchers still don’t fully understand the higher rates. It has been suggested that obesity plays a large role in the increased rates [117].

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Several chemicals and kits are used in accordance with the stage of experiment. Kits, tools, chemicals, and animals are purchased from different resources and different manufactures (table1). Sterilized tips and tubes were used for preparation of samples and qPCR work. The pH of solutions was adjusted with hydrochloric acid (HCl) or sodium hydroxide (NaOH).

Table 2.1. List of chemicals and kits

Chemicals and reagents	Manufacturer	Aim of use
Cotton	Medical office, Turkey	Swapping the injection surface with ethanol
Alcohol 70%	emsure / germany	Sterilization
50 mM sodium citrate buffer	Tekim\Turkey	Preparation of STZ
10% (w/v) sucrose (Sigma)	USA	Use with water after injection
sharp scissors, Forceps	Turkey	Sample collection
Petri dish	Medical office, Turkey	Sample isolation and chopping
1.5-ml microcentrifuge tubes	Isolab/ Spain	Preparation of STZ
1-ml syringes 25-G needles	Beybi/Turkey	I.V and I.P mice injection
Liqud nitrogen	Erciyes university	Sample collection
Pipette tip 200 µl- Box- sterile	Isolab/ Spain	routine laboratory work (genetic, molecular and cell culture)
Pipette tip 100 µl- Box- sterile	Isolab/ Spain	Routine laboratory work.
Pipette tip 10 µl- Box- sterile	Isolab/ Spain	Routine laboratorywork (genetic, molecular and cell culture)
0,5 ml PCR tube – sterile	Isolab/ Spain	Semi quantitative PCR (regular PCR) and laboratory use
Aluminum foil	Local market	Preparation of STZ
One Touch Basic blood glucose monitoring system	Contour\USA	Measure glucose level
Streptozotocin (STZ) 50 mg	Sigma S0130 \ USA	Diabetic mice induction
miScript SYBR® Green PCR Kit (1000)	Qiagen \Germany	Real-time quantitative PCR for target miRNA
miScript II RT Kit (50)	Qiagen \Germany	cDNA production for real-time quantitative PCR
miRNeasy mini kit (50)	Qiagen \Germany	Isolation of total RNA from cultured cells

Table 2.2. Buffers

Phosphate buffer saline 1X	8.00g of NaCl, 0.2g of KCl, 1.44g Na2HPO4, 0.24g KH2PO4 was added and completed to 1000 ml by distilled water
Sodium citrate (50 mM)	2.58 g of sodium citrate dissolved in 10ml distal water

2.2. Equipment's

Following equipment were used in the different phase of the experiment.

Table 2.3. Equipment

Name of equipment	Manufacturer
Centrifuges	hettich centrifuge, USA
Microcentrifuge	Beckman Coulter/ Germany
Thermocycler	Sensoquest labcycler /Germany
Quantitative real time PCR	Roche / Switzerland
Nanodrop 8000 spectrophotometer	Thermo Fisher Scientific, DE, USA
Ultra-low freezer (-80°C)	Labotect GmbH, Göttingen, Germany
Semi-micro Balance	Sanyo / Japan
lab shaker (vortex)	Thermofisher / USA
pH meter	Mettler Toledo/USA
Homogenizer	SCILOGEX\Germany

2.3. Methods

All animals were kept and sacrificed for this experiment after the permission was granted from Erciyes University Ethic Committee ((HADYEK, decision N0:17/055 in 14.6.2017). In this research there are three stages as follow:

2.3.1. First stage: Diabetic pregnant mice model development

2.3.1.1. Preparation of the animals

At the beginning of the experiment mice groups containing 1 male and two females (BALB\c) strain were housed together, after mating male mice were separated from female. A photoperiod of 12-hr light-dark cycle (light on at 8:00 and off at 20:00) was

applied and mice were allowed to access to food and water freely. The protocol detailed below is designed to minimize variability. For this experiment, group sizes of 12 mice are recommended. Given the morbidity associated with the STZ treatment and all mice are weighted before divided them into control and experimental groups. The number of mice should be equal for each group. On the first day of experiment, all food is removed from cages for all groups and provided water as normal 4 hr. prior to STZ treatment, [118].

2.3.1.2. Induction of type 1 diabetes mellitus in mice using a single high dose of streptozotocin

The STZ powder was weighted by microbalance in accordance with the calculated dose and kept in 3.0 ml tubes that covered with aluminum foil and kept on ice considering the STZ is very sensitive to the light. The STZ solution should be prepared fresh immediately before injection and injected within 5 minutes of being dissolved (Unstable). Immediately prior to injection, the STZ dissolved in sodium citrate buffer (pH 4.5) to a final concentration of 20 mg/ml and the dose of injection is calculated according to the mice body weight. STZ was injected I.P and the mice were returned to their cages and fed with regular diet, water and 10% sucrose and they were closely monitored every 2 hours for 12 hours for marked hypoactivity. Observed unresponsiveness of some mice mean they might die soon (within 24 hours) after receiving a high dose of STZ due to the rapid and massive β-cell necrosis resulting in the release of large quantities of insulin, causing fatal hypoglycemia. If the number of early deaths is higher than 20%, the remaining mice were injected I.P within 6 hr with 1 ml of 5% glucose solution instead of providing 10% sucrose water orally to prevent fatal hypoglycemia. On experimental day 3, the 10% sucrose water was replaced with regular water. On the day 3rd day of experiment, all the mice fasted for 6 hr. (e.g., from 7 a.m. to 1 p.m.), the blood glucose then measured to ensure hyperglycemia. Indeed, fasting glucose levels for mild hyperglycemia should be >150 mg/dl (8.3 mmol/liter) and higher hyperglycemia will be in the range of >300 to 600 mg/dl.

2.3.1.3. Blood sampling & Glucose measuring

Sampling from the tail vein is a simple procedure that can be carried out in any laboratory by using the sharp lancet to make a small puncture to get a 1-2 Drops of blood, and then blood is obtained by direct flow or by gently massaging the tail and collecting the blood in a capillary tube or another container Figure 2.1. Glucose-measuring was done by one touch blood glucose monitors. In this experiment, the whole blood monitor was preferred because it's easy to handle and another advantage of this device is that it requires small blood volumes typically 5 µl or less.

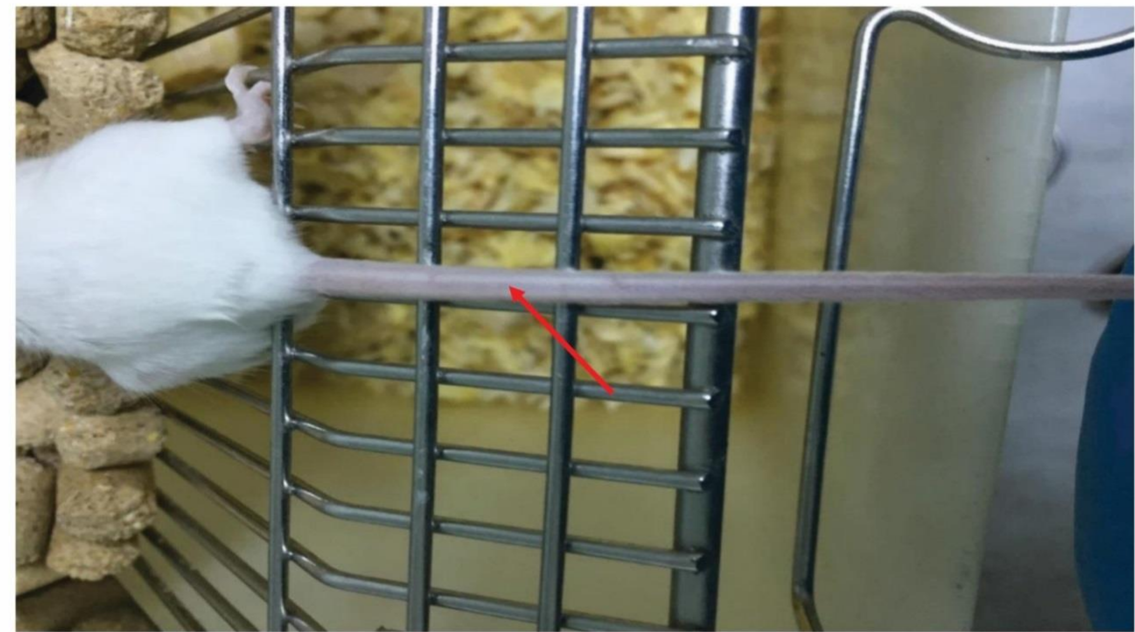


Figure 2.1. Mouse tail vein used for blood sampling.

2.2.1.4. Mice mating

Two female mice were housed with one male mouse (Treated group & Control group) during the dark cycle in the cage (Figure 2.2. The female mice were observed for the appearance of vaginal plug. A plug is hardened semen blocking the vagina and remain about 12 hours after mating. Mating is assumed to occur at the midpoint of the dark cycle (midnight under a 12 hour on/off cycle starting at 6), and thus noon of the next day is 0.5 days of gestation. On the other hand, if the mice don’t have vaginal plug they were isolated, and mating was repeated for one day or two days more.

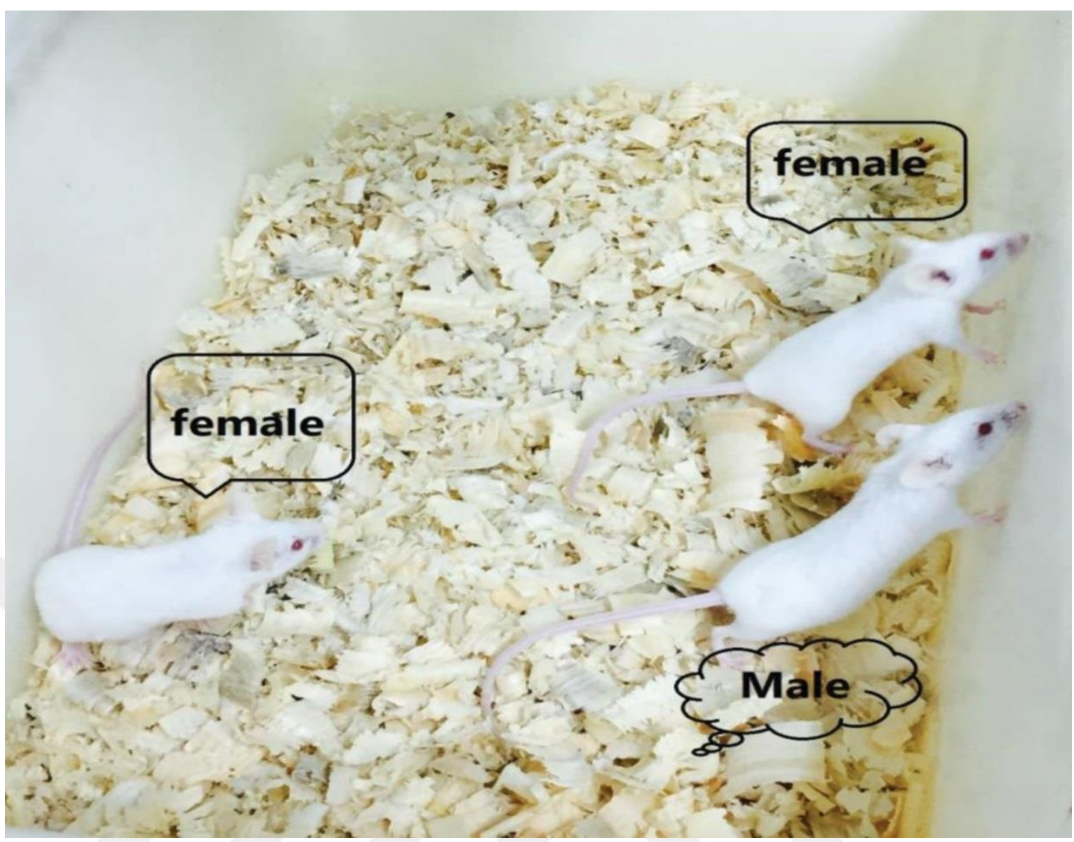


Figure 2.2. Every cage there are two female mice and one male mouse for mating during the dark cycle.

2.3.2. Second stage: Ovary and Uterus samples collection:

First, vaginal plug and later the increase of body weight are two indicators to make sure that the mice are pregnant. Mice were reared untill the 15th day of pregnancy and according to the experimental plan, the mice were sacrificed (treated and control group) on the exact day. After that the mice were dissected by the scissor and forceps and the uterus and ovary samples were isolated and they were washed with PBS solution in a petri dish (Figure (2.3) and Figure 2.4). The samples were then placed in a sterilized container and kept in liquid nitrogen box during transport to the lab and kept in -80°C until further use.

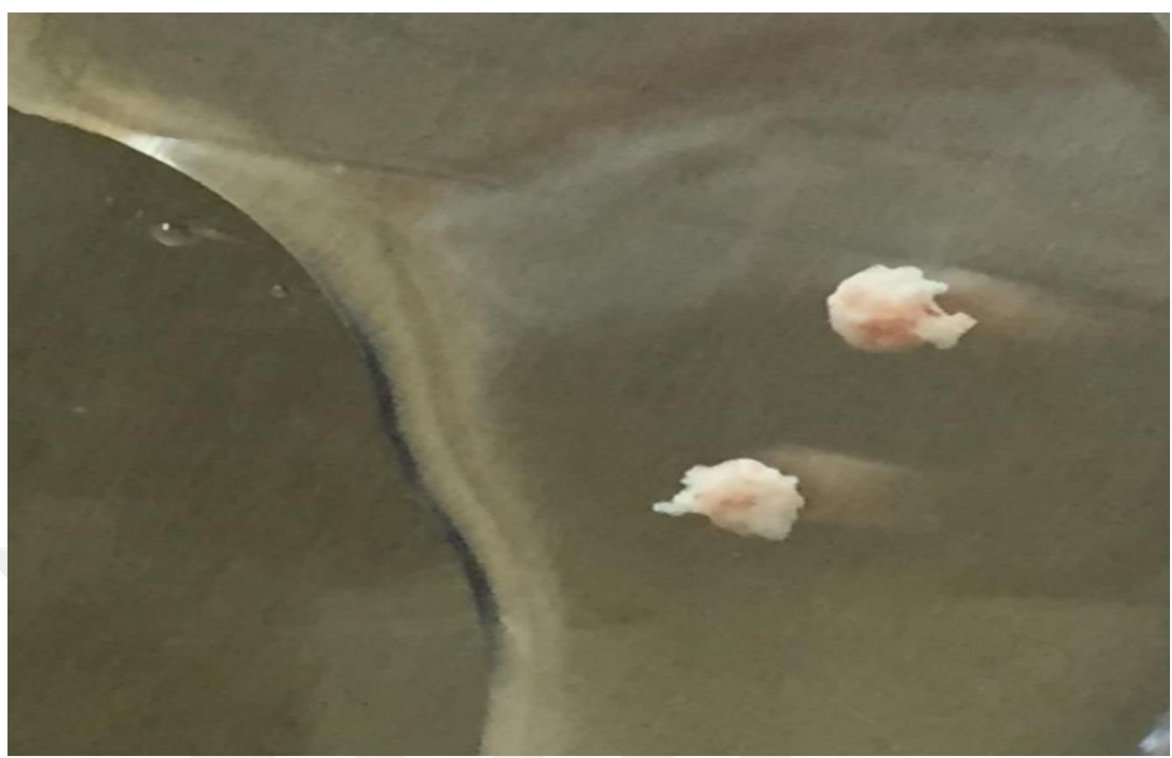


Figure 2.3. Ovaries samples that collected from experimental mice.

Figure 2.4. Uterus samples that gained from mice during the experiment.

2.3.3. Third stage: Total RNA Isolation and Quantitative real-time PCR work

2.3.3.1. Total RNA purification

Total RNA was isolated from uterus and ovary cells using the miRNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. Briefly, 30 mg of tissue was weighed and a drop of Qiazol lysis buffer was added to the tissue and chopped finely with scalpel in a petri dish and immediately place in the 2 ml Eppendorf tube containing 800ul of lysis buffer and homogenized by homogenizer for 5 minutes. After that homogenized samples were vortexed for complete digestion and incubated at room temperature (15-25°C) for 6-8 minutes. Following digestion and incubation, 160 µl of chloroform was added and shaked vigorously for 15 s and incubated for 5 min. The samples were then centrifuged at 12,000 × g for 15 min at 4°C. Clear aqueous phase containing total RNA was transferred to a new 2 ml micro-centrifuge tube. After retrieving the clear aqueous phase, 1.5 volume of 100% ethanol was added and mixed gently by pipetting. After this, 600 µl of sample was transferred to a miRNeasy spin column and centrifuged at 8,000 × g for 15 sec. The flow-through was discarded. Since the sample volume was exceeding 600 µl, remaining samples were centrifuged in the same column. Following this, 350 µl of RWT buffer was added to the spin column and centrifuged at 8,000 × g for 15 sec at room temperature. After that 80 µl (10 µl of DNase I + 70 µl of buffer RDD) of diluted DNase I solution was added to the RNeasy mini spin column membrane and incubated for 15 sec at room temperature on the benchtop. Following incubation, 350 µl of RWT buffer was added to the spin column and centrifuged at 8,000 × g for 15 sec at room temperature.

After removing the flow-through, 500 µl of buffer RPE was added to each spin column and centrifuged at 8,000 × g for 15 sec at room temperature. This step was repeated second time. The spin column was then transferred to a new 2 ml collection tube (supplied by company) and centrifuged at full speed for 1 min to eliminate any possible carryover of buffer RPE and residual flow-through that remained on the spin column. Following this, the spin column was transferred to a new 1.5 µl collection tube (supplied by company) and open the lid for 1 min that allowed the evaporation of residual ethanol portion from the spin column. Total RNA was eluted by adding 50-55 µl of RNase free water to the membrane of the spin column and incubated for 1 min before centrifugation

at 8000 × g for 1 min at room temperature. Finally using a NanoDrop ND-1000 spectrophotometer the total RNA concentration and purity were determined.

2.3.3.2. cDNA synthesis and analysis

Total RNA concentration was checked by spectrophotometer. For mRNA analysis 320 ng total RNA have been reverse transcribed to cDNA with miScript II RT kit (QIAGEN) in accordance with the manufacturer's protocol. In brief, the RNA samples were thawed on the ice and the 10x miscript nucleic mix & 5x miscript Hispec buffer was thawed at room temperature. Then, each solution was mixed by flicking the tubes and all these solutions were kept on ice. Thereafter, the reverse transcription master mix was prepared (4ul of the 5x miscript Hispec buffer, 10x miscript nucleic mix, Rnase free water up to 20, 2ul miscript reverse transcriptase mix, Template RNA 250-500 ng completed to 20ul. The next step, template RNA was added to each tube containing reverse-transcription master mix and mixed gently, centrifuged briefly and placed on ice and the sample incubated for 60 minutes at 37°C in a thermocycler and 5 minutes incubation at 95°C to inactive miscript RT mix and placed on ice. Finally, the samples were stored either at -20°C for short or -80°C for longer period.

2.3.3.3 Quantitative real-time PCR and expression analysis

Roche Lightcycler 480 (for software version1.2.9.11) PCR array instrument has been utilized, according to miscript SYBR Green PCR kit used with miscript miRNA PCR array (QIAGEN). The reaction mix for miscript 96-well. miRNA PCR array as follows; 1100 *u*l of 2x SYBR green, 220 *u*l of 10x miscript universal primer, 855*u*l of RNase-free water, 25 *u*l of Template cDNA) completed to a total volume of 2200 *u*l . Then, 20 *u*l from this mix was added to each well in 96-plate. The plates were carefully sealed by optical adhesive film tightly and centrifuged for 1 minutes at 1000 xg at room temperature. Subsequently, the real-time quantitative PCR was programmed. Firstly reaction is pre-incubated for 10 min at 95°C per 1 cycle for heat activation. The second step is amplification step and it was carried out for 45 cycles at 95°C and 60°C by holding 15 and 1 minutes, respectively. Following amplification the reaction mix was kept for 72°C for 30 seconds. Melt curve temperature was programmed 60°C with holding 15 seconds followed 95°C in continuous mode and 20 acquisitions (per °C). Relative expressions of each mRNA were analyzed using a comparative CT (2ΔΔCT)

method using web-based analysis tool provided by Qiagen (https://www.qiagen.com/it/shop/genes-and-pathways/data-analysis-center-overview-page/).

2.4. miRNA expression analysis

To examine differential expression of miRNAs in mice ovary and uterus, miRNA qPCR array analysis was performed to detect the global expression of miRNAs in these organs of Control (Non-diabetic and pregnant) and treatment group (Diabetic and aborted). The results of miRNAs was investigated in real-time PCR panel (with threshold cycle value of ≤40 in real-time PCR analysis) in ovary and uterus tissues. The first analysis was done by using (Data Analysis Center - QIAGEN). (www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/.

2.5. Identification and expression analysis of miRNA in ovary and uterus:

In this experiment 96 PCR array plate is used to study the expression of miRNAs in samples.

2.5.1. miRNA target prediction

To predict miRNA targets, we used target scan http://www.targetscan.org/vert_72/, which are containing experimentally validated targets of human, mouse miRNAs, for predicted miRNA targets and genes that were simultaneous targets of those miRNAs. Furthermore, only top genes were considered for each miRNA for further bioinformatics analysis by using DAVID 6.7 Bioinformatic Resource for validation of miRNA interaction (http://david.abcc.ncifcrf.gov/).

The predicted miRNA target genes were also analyzed by using the DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov/) server for Annotation, Visualization, and Integrated Discovery to identify the pathway distribution. These pathways were presented according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/ kegg/). Finally, in this study, we choose the more enriched pathways that are involved in uterus and ovary function or dysfunction.

2.6. Statistical analysis

All experiments were performed with a minimum of three biological replicates. Statistical differences between treatment group means (quantitative variables) were analyzed by one-way ANOVA followed by multiple pairwise comparisons (Tukey test, $\alpha=0.05$). For statistical analyses and graphs, SPSS version 20.0 and GraphPad Prism were used. Data are expressed as mean ± SD of three biological replicates. Differences were considered significant at $P<0.05$.

CHAPTER 3

RESULTS

3.1. Mice model for diabetes and pregnant

After administration of STZ, experimental animals were observed for the development of diabetes symptoms (increase of blood glucose level, body weight losing, Polyuria and aggressive behavior). The body weight loss was observed after 6 hours from injection and continued until the day 4. On day 4th, diabetic mice were mated and in next morning, female mice were examined for the vaginal plug as it the most important evidence of pregnancy (Figure 3.1). A significant change in body weight was observed in the pregnant mice on day 5th. The body weight gain or loss and changes of glucose level of mice in the control and treatment group were presented in Table 3.1 – 3.4.

Table 3.1. The body weight of Control mice group (gm).

Mice NO.	**Day 1**	Day **3**	Day **5**	**Day 7**	**Day 9**	**Day 12**	**Day 15**
Mice 1	19.4	22.0	24.2	26.0	29.2	31.0	32.5
Mice 2	19.5	22.1	24.1	26.4	29.7	31.5	33.0
Mice 3	20.1	23.0	24.2	27.0	30.0	32.6	34.2
Mice 4	18.7	20.8	23.8	25.2	28.3	31.5	33.2
Mice 5	22.0	23.5	26.1	28.3	31.6	33.5	35.0

Table 3.2. The level of glucose in control mice throughout the experiment (mg\dl).

Mice No.	**Day 1**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 12**	**Day 15**
Mouse 1	111	124	110	112	132	105	107
Mouse 2	123	127	130	120	117	129	124
Mouse 3	131	130	115	124	120	125	124
Mouse 4	117	119	108	128	109	127	119
Mouse 5	140	125	130	133	126	118	122

Table 3.3. The body weight of treated mice (gm).

Mice No.	Before injection	After 6 hr	After 12hr	After 24hr	Day 2	Day 5	Day 6	Day 8	Day 10	Day 12	Day 15
Mice 1	30.0	28.0	28.4	27.2	26.7	28.0	30.0	31.5	30.0	29.0	28.2
Mice 2	25.0	23.0	23.2	21.0	20.2	20.3	21.0	22.6	21.0	21.2	20.7
Mice 3	38.0	34.2	35.0	32.0	30.0	29.0	33.0	35.8	34.6	34.0	32.0
Mice 4	28.0	26.7	26.4	25.3	23.0	25.4	27.0	27.7	26.9	27.0	27.0
Mice 5	33.0	31.0	31.5	30.5	30.0	33.5	34.7	35.3	33.0	32.0	31.8
Mice 6	33.5	33.4	31.4	30.0	28.8	32.6	33.0	34.6	33.2	31.0	29.8

Table 3.4. The level of glucose in treated mice (mg/dl).

Mice No.	Before injection	After 6 hr	After 12hr	After 24hr	Day 2	Day 5	Day 6	Day 8	Day 10	Day 12	Day 15
Mice 1	114	164	256	300	350	392	514	473	550	539	590
Mice 2	123	180	450	404	468	500	480	570	572	486	460
Mice 3	131	400	485	448	461	531	500	561	582	483	570
Mice 4	117	149	225	437	369	335	370	440	480	509	440
Mice 5	150	152	265	300	308	340	322	400	385	373	420
Mice 6	140	180	288	310	275	341	477	367	392	440	435

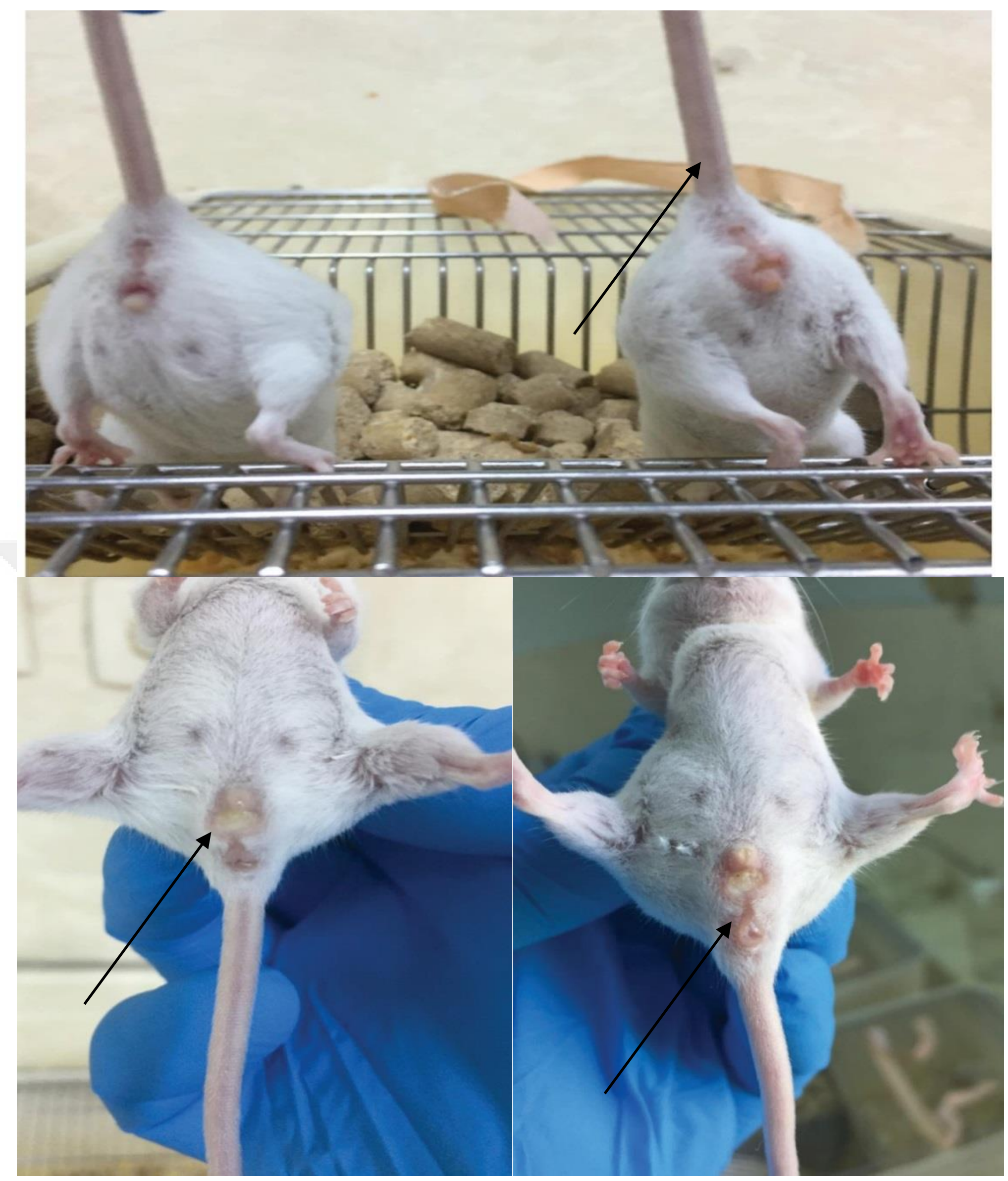


Figure 3.1. The vaginal plug after mating confirms pregnancy.

On 15th days of pregnancy, we observed that all of treated mice were aborted, the mice were sacrificed, and the sample had been collected for isolating RNA and PCR array.

3.2. DATA analysis

3.2.1. miRNA expression analysis in ovary:

To examine differential expression of miRNAs in mice ovary, miRNA qPCR array analysis was performed to detect the global expression of miRNAs in ovary by two groups; control group (Non-diabetic and pregnant) and the treated group (Diabetic and aborted). The

expression of miRNAs was investigated in real time PCR panel (with threshold cycle value of ≤40 in real time PCR analysis) in ovaries tissues. The first analysis was done by using (Data Analysis Center - QIAGEN), The results of analysis is shown in Table 3.5. and Figure 3.2. The results indicated that expression of (5) miRNAs (miR-29b-3p, miR-34a-5p, miR-193a-3p, miR-200a-3p, miR-1907). were significantly upregulated ($P < 0.05$) and (2) miRNAs was significantly downregulated (miR-21a-5p, miR-194-5p) ($P < 0.05$). These results suggest that miRNAs participate in regulating dynamic changes of miRNAs in ovary during diabetes.

Table 3.5. up and downregulation of miRNA in ovaries samples.

miRNA name	Fold change	P value
miR-1907	2.71	0.033969 ↑
miR-193a-3p	2.35	0.039821 ↑
miR-194-5p	3.48	0.045235 ↓
miR-200a-3p	4.46	0.047829 ↑
miR-21a-5p	3.51	0.041763 ↓
miR-29b-3p	2.54	0.033401 ↑
miR-34a-5p	9.78	0.026430 ↑

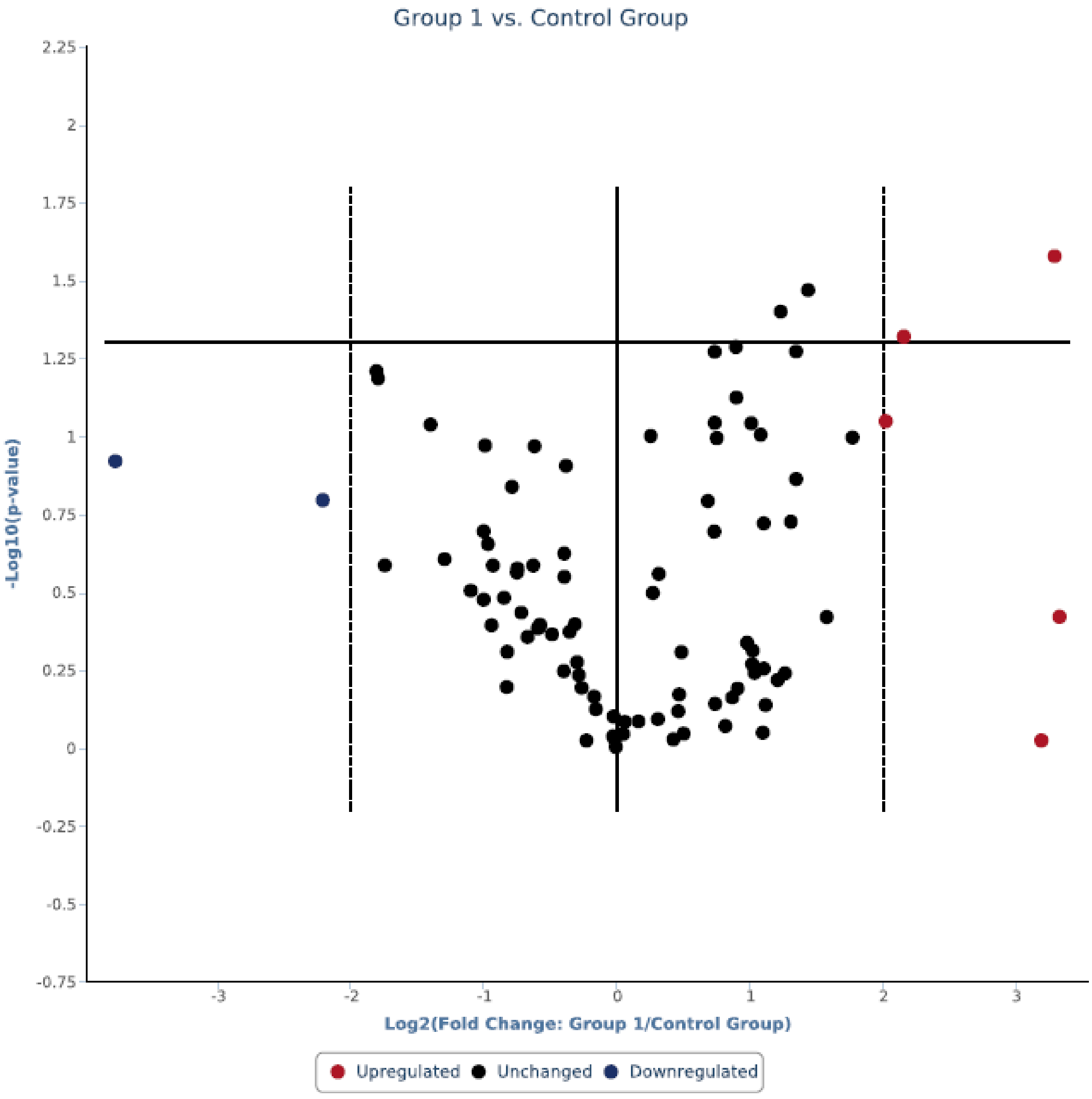


Figure 3.2. Ratio between the miRNA expression between control and treated group.

3.2.2. Analysis of miRNA and their expressions in ovary

In this experiment 96 well PCR array plate is used to study the regulation of miRNAs in ovary samples and five miRNAs are upregulated and two miRNAs are downregulated ($P < 0.05$). So, to identify putative targets of these 7 miRNAs in mice ovaries, Target scan, a database containing experimentally validated targets of human and mouse miRNAs were used. The results showed that a total of 7932 and 3573 genes were predicted to be targeted by up- and downregulated miRNAs. As shown in Figure 3.3.

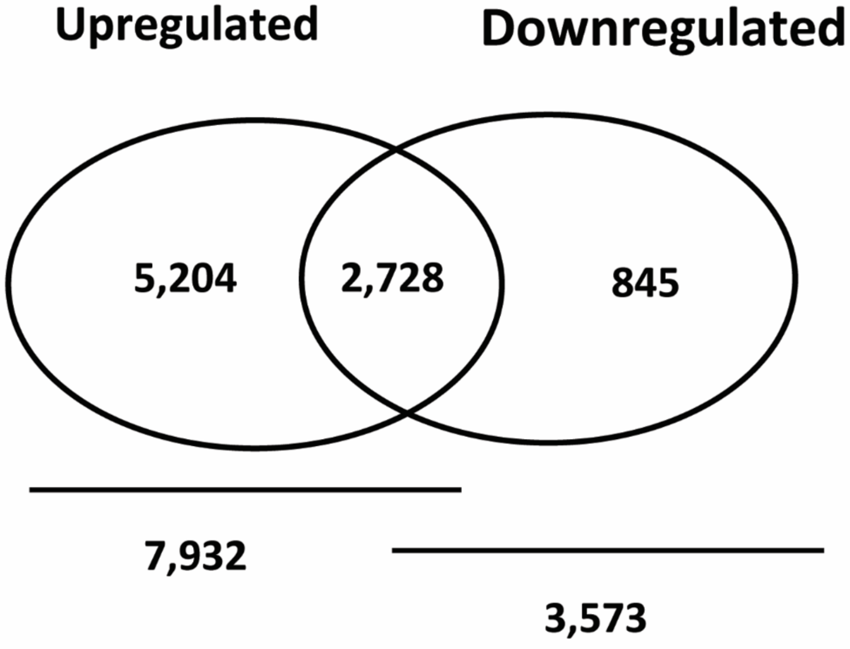


Figure 3.3. Predicted genes to be targeted by up and downregulated miRNAs in ovary.

Only top 100 genes were considered for each miRNA for further bioinformatics analysis by using DAVID 6.7 Bioinformatic Resource to identify potential biological relevance of the differently regulated miRNAs. After screening of the long list of genes, **2828** and **3000** genes were identified as potential targets of up- and down-regulated miRNAs, respectively.

Finally, the screened list of genes from ovary samples were subjected to a pathway analysis using NCBI DAVID Bioinformatic Resource 6.7 to identify the significantly enriched canonical pathway ($P < 0.05$). The genes targeted by upregulated miRNAs are involved in many signaling pathways which is listed in Table 3.6. Ras signaling pathway and pancreatic cancer pathway are the most enriched pathways potentially regulated by the differentially expressed miRNAs. Ras signaling pathway has an important role in regulation of cell proliferation, cell survival, migration and cell differentiation. The pancreatic cancer pathway is the most common malignancy of the pancreas. In addition, the second group of important enriched pathways were PI3K-Akt signaling pathway, protein digestions absorption and FoxO signaling pathway. Variations in the level of miRNAs in ovary might affect critical pathways involved in follicular development or in oocyte maturation and that lead to late of pregnant or infertility complications.

Table 3.6. List of enriched pathways (P <0.05). Upregulated genes predicted to be targeted by differentially expressed miRNAs (P <0.05) in ovary.

KEGG Pathway	Involved miRNAs	Count	P value	Gene symbol
PI3K-Akt signaling pathway	mmu-miR-29b-3p, mmu-miR-34a-5p, mmu-miR-193a-3p, mmu-miR-200a-3p, mmu-miR-1907.	54	5.39E-09	PDGFA, EFNA1, PPP2R5C, FASLG, FOXO3, PTEN, CCNE2, PDGFC, MYB, FGF1
Focal adhesion	mmu-miR-29b-3p, mmu-miR-34a-5p, mmu-miR-193a-3p, mmu-miR-200a-3p, mmu-miR-1907.	36	2.12E-07	PDGFA, GRB2, COL2A1, PTEN, ITGB1, LAMB4, BCL2, TNR, PAK4, COL27A1
Protein digestion and absorption	mmu-miR-29b-3p.	18	5.78E-05	COL4A4, COL4A3, COL4A1, COL15A1, COL2A1, COL5A3, COL5A2, COL4A6, COL5A1, COL4A5
Non-small cell lung cancer	mmu-miR-29b-3p, mmu-miR-34a-5p, mmu-miR-193a-3p, mmu-miR-200a-3p, mmu-miR-1907.	14	5.97E-05	E2F3, GRB2, TP53, RAF1, CDK6, FOXO3, KRAS, RASSF1, MAPK3, SOS2
Pancreatic cancer	mmu-miR-29b-3p, mmu-miR-34a-5p, mmu-miR-193a-3p, mmu-miR-200a-3p, mmu-miR-1907.	15	7.56E-05	E2F3, RALBP1, TP53, TGFB3, RAF1, CDK6, RALGDS, TGFB2, KRAS, VEGFA
Ras signaling pathway	mmu-miR-29b-3p, mmu-miR-34a-5p, mmu-miR-193a-3p, mmu-miR-200a-3p, mmu-miR-1907.	32	9.03E-05	PDGFA, EFNA1, GRB2, FASLG, GNG12, KRAS, GRIN2B, PAK4, SOS2, PDGFC
ECM-receptor interaction	mmu-miR-29b-3p.	17	1.74E-04	COL4A4, COL4A3, COL4A1, COL2A1, COL5A3, COL5A2, ITGB1, COL4A6, COL5A1, COL4A5
Prostate cancer	mmu-miR-29b-3p, mmu-miR-34a-5p, mmu-miR-193a-3p.	17	2.00E-04	E2F3, GRB2, PDGFA, TP53, RAF1, LEF1, PTEN, CCNE2, KRAS, BCL2
Renal cell carcinoma	mmu-miR-29b-3p, mmu-miR-34a-5p, mmu-miR-193a-3p, mmu-miR-200a-3p, mmu-miR-1907.	14	2.98E-04	GRB2, MET, TGFB3, RAF1, HGF, TGFB2, KRAS, PAK4, VEGFA, MAPK3
Pentose and glucuronate interconversions	mmu-miR-29b-3p, mmu-miR-200a-3p.	10	4.57E-04	UGT1A7, UGT1A10, UGT1A9, CRYL1, UGT1A3, KL, UGT1A5, UGT1A4, UGT1A1, UGP2
Endometrial cancer	mmu-miR-29b-3p, mmu-miR-193a-3p, mmu-miR-1907.	12	5.22E-04	KRAS, GRB2, MAPK3, SOS2, TP53, RAF1, LEF1, FOXO3, AXIN2, PTEN
FoxO signaling pathway	mmu-miR-29b-3p, mmu-miR-193a-3p, mmu-miR-200a-3p, mmu-miR-1907.	21	5.37E-04	SGK1, IRS2, GRB2, TGFB3, RAF1, FASLG, FOXO3, IL7R, CCNG2, PTEN
ErbB signaling pathway	mmu-miR-29b-3p, mmu-miR-34a-5p, mmu-miR-193a-3p, mmu-miR-200a-3p, mmu-miR-1907.	15	0.001727949	ERBB4, GRB2, CAMK2G, MAP2K4, RAF1, KRAS, PAK4, MAPK3, SOS2, TGFA
Gap junction	mmu-miR-29b-3p, mmu-miR-34a-5p, mmu-miR-193a-3p, mmu-miR-200a-3p, mmu-miR-1907.	15	0.001932578	GJD2, TUBB2A, GRB2, PDGFA, RAF1, PRKG2, KRAS, SOS2, MAPK3, TUBB6
Wnt signaling pathway	mmu-miR-29b-3p, mmu-miR-34a-5p, mmu-miR-193a-3p, mmu-miR-200a-3p, mmu-miR-1907.	19	0.004613824	TBL1XR1, BTRC, CAMK2G, TP53, PPP3R2, LEF1, CXXC4, SFRP5, SOST, CCND3

Furthermore, pathways affected by the genes targeted by downregulated miRNAs are presented in Table 3.7. MAPK signaling pathway and Chemokine signaling pathway are the most enriched pathways potentially regulated by the differentially expressed miRNAs.

Table 3.7. List of enriched pathways (P <0.05) affected by genes targeted by downregulated miRNAs in the ovary.

KEGG Pathway	Involved miRNAs	Count	P value	Gene symbol
MAPK signaling pathway	mmu-miR-21a-5p, mmu-miR-194-5p	25	6.04E-04	FGFR3, NTF3, MAP2K3, DUSP10, FASLG, MKNK1, PPP3R2, NR4A1, GNG12, MAPK10
Chemokine signaling pathway	mmu-miR-21a-5p,	20	8.36E-04	PDGFA, GRB2, COL2A1, PTEN, ITGB1, LAMB4, BCL2, TNR, PAK4, COL27A1
Toll-like receptor signaling pathway	mmu-miR-21a-5p,	14	0.00105	CCL3, IL6, MAP2K3, CXCL9, MAPK10, CXCL11, STAT1, CXCL10, CCL3L1, CCL3L3
Protein processing in endoplasmic reticulum	mmu-miR-21a-5p, mmu-miR-194-5p	17	0.004544	UBQLNL, MAN1A2, CRYAB, RNF185, EDEM3, MAPK10, SEC63, SSR1, BCL2, HSPA6
TNF signaling pathway	mmu-miR-21a-5p, mmu-miR-194-5p	12	0.009017	IL6, DNM1L, CCL20, CXCL3, MAP2K3, EDN1, CXCL2, IKBKG, MAPK10, PIK3R1
Cytokine-cytokine receptor interaction	mmu-miR-21a-5p,	20	0.009115	CCL3, IL6, IL6ST, IL9, CXCL9, FASLG, PF4, CNTFR, EDAR, IL6R
Chagas disease (American trypanosomiasis)	mmu-miR-21a-5p, mmu-miR-194-5p	11	0.020494	IL6, CCL3, CCL3L1, CCL3L3, IKBKG, IL12A, FASLG, MAPK10, PPP2R2C, PPP2R2D
Axon guidance	mmu-miR-21a-5p, mmu-miR-194-5p	12	0.030997	SEMA5A, EPHA4, NRP1, PAK2, CXCR4, CFL2, EFNB2, NTNG1, PPP3R2, PPP3CA
Hepatitis B	mmu-miR-21a-5p, mmu-miR-194-5p	13	0.033905	EGR3, IL6, CYCS, FASLG, MAPK10, STAT1, CCNE2, MAP3K1, BCL2, IKBKG
Jak-STAT signaling pathway	mmu-miR-21a-5p,	12	0.068826	IL6, SOCS2, IL6ST, IL9, SOS2, IL12A, CNTFR, IL6R, IL5RA, STAT1
HTLV-I infection	mmu-miR-21a-5p,	18	0.079235	WNT5A, DVL2, IL6, NRP1, TLN2, PPP3R2, MYBL1, FZD6, MSX1, ATF3
Measles	mmu-miR-194-5p	11	0.084132	CCNE2, IL6, TACR1, HSPA6, IL12A, FASLG, EIF2AK2, STAT1, PIK3R1, EIF2AK4
B cell receptor signaling pathway	mmu-miR-21a-5p, mmu-miR-194-5p	7	0.095613	CR2, SOS2, IKBKG, PPP3R2, PPP3CA, PIK3R1, BTK
Sphingolipid signaling pathway	mmu-miR-21a-5p, mmu-miR-194-5p	10	0.099201	CERS2, SGPP1, BCL2, PPP2R5E, MAPK10, NSMAF, PPP2R2C, PPP2R2D, PIK3R1, DEGS1
Neurotrophin signaling pathway	mmu-miR-21a-5p, mmu-miR-194-5p	10	0.099201	NTF3, MAP3K3, BCL2, MAP3K1, PSEN2, SOS2, FASLG, MAPK10, CRK, PIK3R1

3.3. miRNA expression analysis in uterus

Differential expression of miRNAs was also examined in the uterus of female mice. miRNA qPCR array analysis was performed to detect the global expression of miRNAs in uterus of control (Non-diabetic and pregnant) and treated group (Diabetic and aborted). The expression of miRNAs investigated in real time PCR panel (threshold cycle value

Of ≤40) in uterus tissues. The first analysis was done by using web-based data analysis software provided by QIAGEN. As shown in Table 3.8. and Figure 3.4. the expression of (6) miRNAs was significantly upregulated ($P < 0.05$). Those miRNAs are miR-26a-5p, miR-27a-3p, miR-30c-5p, miR-150-5p, miR-335-3p, and miR-365-3p. On the other hand, the (16) miRNAs (miR-126a-5p, miR-127-3p, miR-129-2-3p, miR-129-5p, miR-135b-5p, miR-199a-5p, miR-201-5p, miR-296-5p, miR-325-3p, miR-375-3p, miR-377-3p, miR-380-5p, miR-433-3p, miR-450a-5p, miR-669b-3p, miR-669f-3p) were significantly ($P < 0.05$) downregulated. Differential expression of miRNAs in the diabetic uterus clearly indicates that diabetic condition could modulate the expression of miRNAs which are important in saving the pregnancy in mice.

Table 3.8. Data analysis by QIAGEN (up and downregulation of miRNA).

miRNA name	Fold change	P value
miR-201-5p	0.1	0.001335 ↓
miR-325-3p	0.1	0.001335 ↓
miR-450a-5p	0.08	0.003150 ↓
miR-129-5p	0.29	0.003477 ↓
miR-129-2-3p	0.14	0.003937 ↓
miR-127-3p	0.1	0.005019 ↓
miR-375-3p	0.06	0.006506 ↓
miR-296-5p	0.28	0.006885 ↓
miR-199a-5p	0.23	0.007532 ↓
miR-377-3p	0.24	0.008748 ↓
miR-669b-3p	0.25	0.011315 ↓
miR-669f-3p	0.28	0.011907 ↓
miR-433-3p	0.09	0.016533 ↓
miR-135b-5p	0.11	0.018927 ↓
miR-126a-5p	0.35	0.023631 ↓
miR-380-5p	0.09	0.026457 ↓
miR-365-3p	6.01	0.027985 ↑
miR-30c-5p	2.35	0.030365 ↑
miR-27a-3p	3.34	0.032806 ↑
miR-150-5p	5.37	0.033137 ↑
miR-335-5p	2.28	0.037953 ↑
miR-26a-5p	7.77	0.041325 ↑

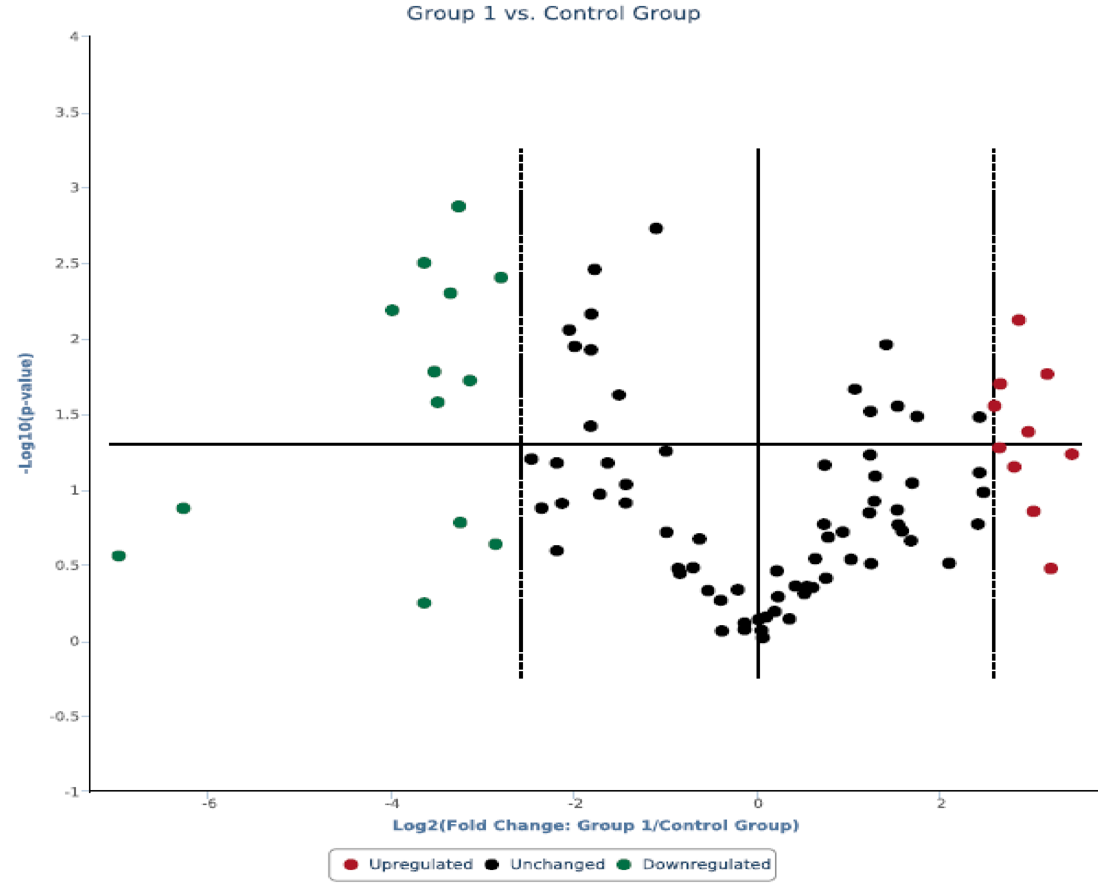


Figure 3.4. Volcano diagram showing the miRNA expression in treated group compared to control group.

3.3.1. Identification and expression analysis miRNA in uterus

In this experiment 96 well PCR array plate is used to study the regulation of miRNAs in uterine samples. Six miRNAs are upregulated, and sixteen miRNAs are downregulated ($p < 0.05$). Target scan database are used, to identify putative targets of these 22 miRNAs in mice uterus, because this database containing experimentally validated targets of human and mouse miRNAs. Target prediction revealed that a total of 2547 and **7860** genes were predicted to be targeted by up and downregulated miRNAs, respectively Figure 3.5.

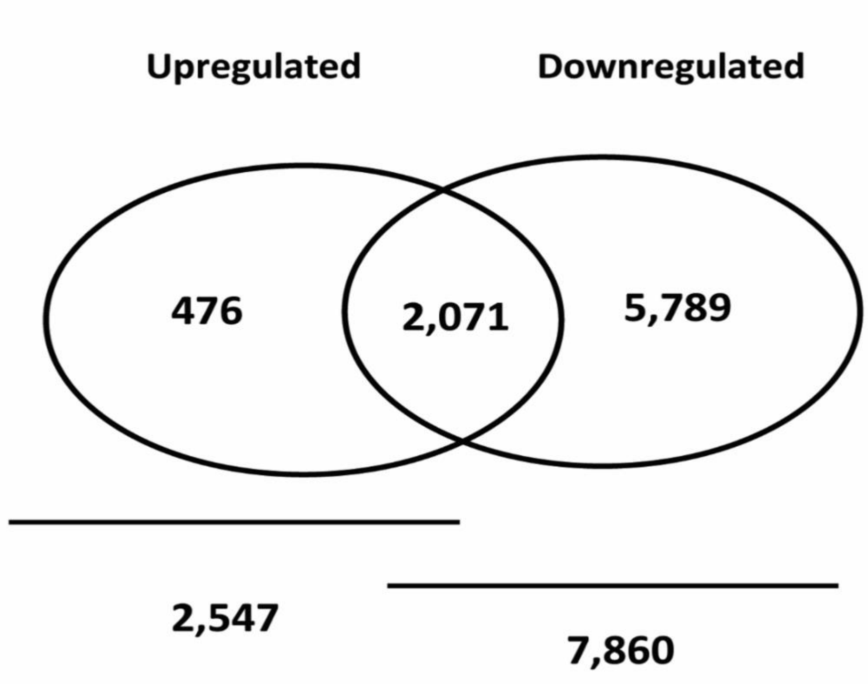


Figure 3.5. Van diagram showing the number of target genes predicted to be targeted by up- and downregulated miRNAs.

Only top 100 genes were considered for each miRNA for further bioinformatic analysis by using DAVID 6.7 Bioinformatic Resource for validation of miRNA interactions. After screening of the long list of genes, 2629 and 2894 genes were identified as potential targets of up- and down-regulated miRNAs, respectively.

Finally, the screened list of genes was subjected to a pathway analysis using DAVID Bioinformatic Resource 6.7 to identify the significantly enriched canonical pathway (P < 0.05) (Table 3.9. and Table 3.10).

Table 3.9. List of enriched pathways (P<0.05). Upregulated genes were predicted to be targeted by differentially expressed miRNAs (P<0.05) in uterus.

KEGG Pathway	Involved miRNAs	Count	P value	Gene symbol
Wnt signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-30c-5p, mmu-miR-150-5p.	44	6.69E-09	WNT5A, PPP3R1, DAAM1, TCF7L2, GPC4, CHD8, PLCB4, CACYBP, PPP3CB, CAMK2D
Axon guidance	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-30c-5p, mmu-miR-150-5p.	41	1.61E-08	NRP1, GNAI2, PLXNA2, EFNA3, PPP3R1, EPHB2, KRAS, PAK2, ROBO1, SEMA7A
Thyroid hormone signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-150-5p, mmu-miR-365-3p.	38	2.29E-08	ATP1B1, THRB, PFKFB2, FOXO1, ITGB3, MED12L, PDPK1, PLCB4, KRAS, GATA4,
cGMP-PKG signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-30c-5p, mmu-miR-365-3p.	47	1.12E-07	GNA13, ADCY3, MEF2C, ATP1B1, GNAI2, ADCY5, ADCY6, PPP3R1, PRKG1, KCNMB1
FoxO signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-30c-5p, mmu-miR-365-3p.	40	2.60E-07	GRB2, STK11, PRKAG2, FOXO1, FOXO3, FOXO4, CCNG2, PTEN, IL10, PDPK1
Signaling pathways regulating pluripotency of stem cells	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-30c-5p, mmu-miR-150-5p, mmu-miR-365-3p.	40	9.03E-07	BMI1, WNT5A, FGFR3, GRB2, BMPR2, PAX6, MEIS1, ACVR1C, PCGF5, PCGF3
PI3K-Akt signaling pathway	mmu-miR-30c-5p, mmu-miR-150-5p, mmu-miR-365-3p.	75	1.91E-06	FGF14, PGF, EFNA3, FOXO3, PTEN, CCNE2, PDPK1, PIK3AP1, FGF1, MYB
Neurotrophin signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-30c-5p, mmu-miR-365-3p.	35	2.98E-06	GRB2, FOXO3, BDNF, PDPK1, MAP3K5, KRAS, MAP3K3, BCL2, MAP3K1, SOS1
Transcriptional misregulation in cancer	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-335-3p, mmu-miR-365-3p.	43	7.47E-06	SLC45A3, BMI1, MEF2C, KDM6A, KMT2A, UTY, PPARG, PAX5, FOXO1, ZBTB16
mTOR signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-150-5p, mmu-miR-365-3p.	21	1.44E-05	PRKCA, TNF, STK11, PIK3CD, IGF1, RICTOR, RRAGD, IRS1, PTEN, RPS6KA6
ErbB signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-30c-5p, mmu-miR-365-3p.	27	1.54E-05	NRG3, ERBB4, GRB2, KRAS, PAK2, SOS1, CAMK2D, NRG1, PIK3R3, NRG2
MAPK signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-30c-5p,	57	1.71E-05	MEF2C, FGF14, MAX, BDNF, MAP3K5, PAK2, MAPT, PRKACA, FGF1, MAP2K7
Hippo signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-150-5p,	39	1.72E-05	WNT5A, YWHAZ, MOB1A, BMPR2, GLI2, TCF7L2, LATS2, FRMD6, CTGF, PPP2CB
AMPK signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-365-3p	33	3.27E-05	PFKFB3, STK11, PFKFB2, LEPR, PRKAG2, PPARG, FOXO1, FOXO3, CAMKK2, PDPK1
Protein processing in endoplasmic reticulum	mmu-miR-26a-5p, mmu-miR-30c-5p,	41	4.81E-05	RAD23B, SEC31B, DERL2, TUSC3, SEC24A, UBE2G1, MAN1B1, PDIA6, EDEM3, LMAN1
Oxytocin signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-30c-5p, mmu-miR-335-3p,	39	5.10E-05	ADCY3, MEF2C, GNAI2, PTGS2, ADCY5, ADCY6, PRKAG2, PPP3R1, CACNB2, CACNB4
Focal adhesion	mmu-miR-27a-3p, mmu-miR-30c-5p, mmu-miR-365-3p	47	6.30E-05	XIAP, GRB2, PGF, TNC, ITGB3, PTEN, PXN, PDPK1, PAK2, BCL2
Estrogen signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-335-3p, mmu-miR-365-3p.	28	6.44E-05	ADCY3, GNAI2, GRB2, FKBP5, ADCY5, ADCY6, ATF2, KRAS, PLCB4, SOS1
Insulin signaling pathway	mmu-miR-27a-3p, mmu-miR-365-3p.	35	7.51E-05	GRB2, PRKAG2, MKNK2, FOXO1, PRKAR2A, PDPK1, KRAS, SOS1, FASN, PRKACA
GnRH signaling pathway	mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-335-3p,	28	7.79E-05	ADCY3, GRB2, ADCY5, ADCY6, KRAS, PLCB4, MAP3K3, MAP3K2, SOS1, MAP3K1

The most enriched pathways in the upregulated expression of miRNA were signaling pathways regulating pluripotency of stem cells. (PSCs) are basic cells with an indefinite self-renewal capacity and the potential to generate all the cell types of the three germinal layers include embryonic stem cell that are derived from the inner cell mass of blastocyst stage embryo. In addition, there are another's important enriched pathways such as, Transcriptional misregulation in cancer and Insulin signaling pathway. Furthermore, very important hormone that’s responsible for embryo implantation and pregnancy maintenance including GnRH signaling pathway, Estrogen signaling pathway and Thyroid hormone signaling pathway are also enriched. Finally, Wnt signaling pathway, Focal adhesion, MAPK signaling pathway, Hippo signaling pathway are detected as an enriched and important pathways.

The most enriched pathways in the downregulated expression of miRNAs was employed in table 3.10. The most enriched pathways in this case was found to be Retrograde endocannabinoid signaling and TGF-beta signaling pathway. Furthermore, the Signaling pathways regulating pluripotency of stem cells, Wnt signaling pathway, cAMP signaling pathway and FoxO signaling pathway are enriched pathways which influence uterus function.

Table 3.10. List of enriched pathways (p<0.05), in which downregulated genes predicted to be targeted by differentially expressed miRNAs (p<0.05) in uterus.

KEGG Pathway	Involved miRNAs	Count	P value	Gene symbol
Pathways in cancer	mmu-miR-126a-5p, mmu-miR-135b-5p, mmu-miR-199a-5p, mmu-miR-201-5p, mmu-miR-377-3p, mmu-miR-380-5p, mmu-miR-669b-3p, mmu-miR-669f-3p.	90	5.57E-07	GNA13, E2F1, ADCY4, E2F3, ADCY7, ADCY5, FGF16, FGF10, FOXO1, CXCL12
Hippo signaling pathway	mmu-miR-126a-5p, mmu-miR-135b-5p, mmu-miR-199a-5p, mmu-miR-201-5p, mmu-miR-375-3p, mmu-miR-380-5p, mmu-miR-669b-3p, mmu-miR-669f-3p.	43	3.40E-06	WNT5A, MOB1A, BTRC, SAV1, SOX2, BMPR2, TCF7L2, LATS2, TGFB1, SCRIB
MAPK signaling pathway	mmu-miR-126a-5p, mmu-miR-129-2-3p, mmu-miR-199a-5p, mmu-miR-201-5p, mmu-miR-296-5p, mmu-miR-377-3p, mmu-miR-380-5p, mmu-miR-433-3p, mmu-miR-669b-3p, mmu-miR-669f-3p.	62	5.93E-06	MEF2C, FGF16, FGF10, TGFB1, TGFB2, MAP3K7, MAX, MAP3K5, BDNF, MAP3K4
Signaling pathways regulating pluripotency of stem cells	mmu-miR-126a-5p, mmu-miR-129-2-3p, mmu-miR-135b-5p, mmu-miR-199a-5p, mmu-miR-201-5p, mmu-miR-375-3p, mmu-miR-377-3p, mmu-miR-380-5p, mmu-miR-669b-3p, mmu-miR-669f-3p.	40	7.28E-06	SMARCAD1, WNT5A, IL6ST, GRB2, SOX2, BMPR2, PAX6, MEIS1, WNT2, PCGF5
Axon guidance	mmu-miR-126a-5p, mmu-miR-129-2-3p, mmu-miR-135b-5p, mmu-miR-199a-5p, mmu-miR-380-5p, mmu-miR-433-3p, mmu-miR-669b-3p, mmu-miR-669f-3p.	37	1.07E-05	ABLIM1, NRP1, GNAI2, PLXNA2, ABLIM3, PPP3R1, PPP3R2, LRRC4C, CXCL12, EPHB1
Wnt signaling pathway	mmu-miR-126a-5p, mmu-miR-129-2-3p, mmu-miR-135b-5p, mmu-miR-199a-5p, mmu-miR-201-5p, mmu-miR-375-3p, mmu-miR-377-3p, mmu-miR-669b-3p, mmu-miR-669f-3p.	37	7.53E-05	WNT5A, BTRC, PPP3R1, PPP3R2, CXXC4, TCF7L2, MAP3K7, CSNK2A2, WNT2, CSNK2A1
TGF-beta signaling pathway	mmu-miR-126a-5p, mmu-miR-135b-5p, mmu-miR-199a-5p, mmu-miR-377-3p, mmu-miR-380-5p, mmu-miR-669b-3p, mmu-miR-669f-3p.	26	9.89E-05	NOG, E2F5, FST, BMPR2, TGFB1, TGFB2, IFNG, CUL1, PITX2, BMP4
Ubiquitin mediated proteolysis	mmu-miR-325-3p, mmu-miR-377-3p.	35	3.32E-04	SYVN1, XIAP, UBE3A, UBE2G1, BTRC, UBA7, FBXW7, UBE2D2, MAP3K1, KLHL9
FoxO signaling pathway	mmu-miR-126a-5p, mmu-miR-199a-5p, mmu-miR-201-5p, mmu-miR-433-3p, mmu-miR-669b-3p, mmu-miR-669f-3p.	34	4.69E-04	ATG12, GRB2, BNIP3, FOXO1, IL7R, FOXO4, CCNG2, FOXO6, TGFB1, TGFB2
Adrenergic signaling in cardiomyocytes	mmu-miR-126a-5p, mmu-miR-129-2-3p, mmu-miR-135b-5p, mmu-miR-201-5p, mmu-miR-325-3p, mmu-miR-669b-3p, mmu-miR-669f-3p.	36	5.49E-04	ADCY4, ATP1B1, ATP1B3, ADCY7, GNAI2, ADCY5, PPP2R5C, CACNB2, CACNB4, ATP2B1
cAMP signaling pathway	mmu-miR-126a-5p, mmu-miR-129-2-3p, mmu-miR-135b-5p, mmu-miR-201-5p, mmu-miR-380-5p, mmu-miR-669b-3p, mmu-miR-669f-3p.	45	6.66E-04	ADCY4, ACOX1, ATP1B1, ATP1B3, ADCY7, GNAI2, ADCY5, NFKBIA, GABBR2, ATP2B1
Basal cell carcinoma	mmu-miR-199a-5p, mmu-miR-669b-3p, mmu-miR-669f-3p.	18	7.79E-04	WNT5A, BMP4, FZD8, WNT10B, FZD4, TCF7L2, SHH, SUFU, FZD6, WNT2
Retrograde endocannabinoid signaling	mmu-miR-126a-5p, mmu-miR-201-5p, mmu-miR-325-3p, mmu-miR-380-5p, mmu-miR-669b-3p, mmu-miR-669f-3p.	27	9.01E-04	ADCY4, GNAI2, ADCY7, GABRB2, ADCY5, RIMS1, CNR1, PRKACA, GNG4, PLCB1
Protein processing in endoplasmic reticulum	mmu-miR-296-5p, mmu-miR-325-3p.	39	0.001203	RAD23B, SEC31B, SYVN1, GANAB, UBE2G1, EDEM3, EDEM2, SEC62, SEC63, SSR1
Oxytocin signaling pathway	mmu-miR-129-2-3p, mmu-miR-135b-5p, mmu-miR-201-5p, mmu-miR-325-3p, mmu-miR-377-3p, mmu-miR-380-5p, mmu-miR-669b-3p, mmu-miR-669f-3p.	37	0.001249	MEF2C, ADCY4, ADCY7, GNAI2, ADCY5, PPP1R12C, PPP3R1, CACNB2, PPP3R2, CACNB4
p53 signaling pathway	mmu-miR-201-5p.	20	0.001263	STEAP3, CYCS, IGF1, CDK6, SFN, CHEK2, PMAIP1, CCNG2, SESN1, SESN3
Pancreatic cancer	mmu-miR-135b-5p, mmu-miR-201-5p, mmu-miR-380-5p, mmu-miR-433-3p, miR-669b-3p mmu-miR-669f-3p.	19	0.002252	E2F1, E2F3, RALBP1, ARHGEF6, TGFBR1, TGFBR2, SMAD3, CDK6, BCL2L1, MAPK10
Morphine addiction	mmu-miR-126a-5p, mmu-miR-201-5p, mmu-miR-380-5p, miR-669b-3p, mmu-miR-669f-3p.	24	0.002263	PRKCA, ADCY4, GABRG3, GABRA1, ADCY7, GNAI2, GABRB2, GABRA6, ADCY5, GABRA5
cGMP-PKG signaling pathway	mmu-miR-126a-5p, mmu-miR-129-2-3p, mmu-miR-135b-5p, mmu-miR-201-5p, mmu-miR-325-3p, mmu-miR-380-5p, mmu-miR-450a-5p, miR-669b-3p, mmu-miR-669f-3p.	37	0.003087	GNA13, MEF2C, ADCY4, ATP1B1, MEF2A, ATP1B3, ADCY7, GNAI2, ADCY5, PPP3R1
Thyroid hormone signaling pathway	mmu-miR-126a-5p, mmu-miR-129-2-3p, mmu-miR-135b-5p, mmu-miR-199a-5p, mmu-miR-201-5p, mmu-miR-325-3p, mmu-miR-375-3p, mmu-miR-380-5p, miR-669b-3p, mmu-miR-669f-3p.	27	0.00555	ATP1B1, THRA, ATP1B3, THRB, PFKFB2, FOXO1, ACTG1, PDPK1, KRAS, TBC1D4

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion

In this study, the role of miRNAs in Diabetes mellitus induced abortion was investigated by using diabetic mice model. For this purpose, using miRNA qPCR array platform we studied the expression of miRNAs mostly related to diabetes in ovarian and uterine tissues. We found several miRNAs are differently regulated in both ovarian and uterine tissue in diabetic mice compared to that of control mice.

Diabetes mellitus (DM), is a group of metabolic diseases in which a person has high blood sugar because the body does not produce enough insulin. Diabetes is divided into three main types: Type 1 DM , Type 2 DM and gestational diabetes [14]. The mortality statistics related to diabetes for fetus is roughly 70 percent while it is nearly 40 percent for mothers [119]. Unfortunately, the percentage of abortion in diabetic patients is about 10% higher than that of in the general population (3%) and appears to be on the rise [120]. In addition, genetic factors also play a role in abortion or maintenance of pregnancy. For instance, miRNAs play a significant role in regulation of many events such as inflammatory and immune responses, cell-cycle progression, differentiation, apoptosis, and tissue remodeling. The precise regulation of the expression of these genes is fundamental for normal ovarian and endometrial functions. Therefore, differential expression of certain miRNAs is related to certain gynecological pathological processes or pregnancy complications [121].

Furthermore, there is a strong relationship between diabetes and pregnancy complications. So, in our experiment, we focused on the female reproductive system and its complication by choosing the uterus and ovary. Because uterus plays an essential role through implantation and the pregnancy progress through the dialogue between gene expression and hormonal change. In addition, diabetic intrauterine environment

affects the offspring of women in all types of diabetes mellitus. Furthermore, the ovary is very important in fertility studies because it is responsible for ovulation and oocyte maturation for the pregnancy.

To induce diabetes, we used a single high dose of STZ (200 mg/kg) which is directly toxic to pancreatic β-cells, causing the rapid onset of diabetes, with blood glucose levels of>500 mg/dl within 48 hours [122]. Although multiple low doses of STZ are associated with fewer toxic effects than a single high dose of STZ, many investigators still prefer the single high-dose STZ approach for generating diabetic animals. Animal models have enormously contributed to the study of diabetes mellitus, a metabolic disease with abnormal glucose homeostasis, due to some defect in the secretion or the action of insulin. This allow researchers to control *in vivo* the genetic and environmental factors that may influence the development of the disease and establishment of their complications. Most experiments are carried out on rodents, even though other species with human-like biological characteristics are also used. Animal models develop diabetes either spontaneously or by using chemical, surgical, genetic or other techniques, and depict many clinical features or related phenotypes of the disease [123].

In this study diabetic mice model was developed to study the effect of diabetes on abortion. For that purpose, we collected the uterus and ovary samples to investigate the differential expression of miRNA during the pregnancy. miRNA expression analysis revealed a differential expression of miRNAs in uterus and ovary. The target genes of those miRNA were also determined by Target scan (7.2) and significantly enriched pathways were determined by DAVID bioinformatics resource.

In the uterus, the expression of six miRNAs which are significantly upregulated ($P < 0.05$), these are miR-26a-5p, miR-27a-3p, miR-30c-5p, miR-150-5p, miR-335-3p, miR-365-3p. On the other hand, 16 miRNAs were significantly downregulated (miR-126a-5p, miR-127-3p, miR-129-2-3p, miR-129-5p, miR-135b-5p, miR-199a-5p, miR-201-5p, miR-296-5p, miR-325-3p, miR-375-3p, miR-377-3p, miR-380-5p, miR-433-3p, miR-450a-5p, miR-669b-3p, miR-669f-3p). Overall, these results suggest that miRNAs participate in differential expression of miRNAs in the uterus during diabetes. To understand the biological relevance of these differentially regulated miRNAs, we

predicted the target genes and their biological pathways. So, we found signaling pathways regulating pluripotency of stem cells which is the most enriched pathway potentially regulated by the differentially expressed miRNAs. PSCs are basic cells with an indefinite self-renewal capacity and they have potential to generate all the cell types of the three germinal layers including embryonic stem cells that are derived from the inner cell mass of blastocyst stage embryo. In addition, there are other important enriched pathways such as transcriptional misregulation in cancer and the insulin signaling pathway. They are involved in mutations such as amplification, deletion, translocation and inversion or subjected to point mutations that result in a gain or loss of function. Furthermore, many other important pathways are enriched which are responsible for embryo implantation and pregnancy maintenance, including GnRH signaling pathway, estrogen signaling pathway, and thyroid hormone signaling pathway**.** Some other pathways such as Wnt signaling pathway, focal adhesion, and AMPK signaling pathway which are responsible for basic development process including cell motility, cell proliferation, cell differentiation and regulation of gene expression and cell survival were also enriched.

In turn, the 16 miRNAs were significantly downregulated in the uterus. We examined the pathways that were affected by those miRNAs. TGF-beta signaling pathway and retrograde endocannabinoid signaling are the most enriched pathways potentially regulated by the differentially expressed miRNAs. The TGF-beta signaling pathway is highly conserved and affect the wide spectrum of cellular function such as cell proliferation, apoptosis, differentiation, and migration. The main function of this pathway is to serve as retrograde messengers at synapses in various regions of the brain. Besides, more enriched pathways such as signaling pathways regulating pluripotency of stem cells, Wnt signaling pathway, cAMP signaling pathway were also detected. PSCs are basic cells with an indefinite self-renewal capacity and they have potential to generate all the cell types of the three germinal layers including embryonic stem cells which are derived from the inner cell mass of blastocyst stage embryo. Wnt proteins secretes morphogens that are required for basic developmental processes, such as cell-fate specification, progenitor-cell proliferation and the control of asymmetric cell division in many different species and organs. cAMP signaling pathway is involved in one of the most and common second messengers. Finally, pathways in cancer, MAPK signaling pathway, FoxO signaling pathway, Hippo signaling

pathway were also detected as an enriched pathway and might influence uterus function since those pathways involved in various cellular function including cell proliferation, differentiation, and migration. FoxO signaling pathway is a family of transcription factors that regulate the gene expression in cellular physiological events including apoptosis, cell cycle control, glucose metabolism, oxidative stress resistance. Hippo signaling pathway is detected as an enriched and important pathway which is involved in organ size during development from flies to human.

In addition, we detected several miRNAs and their pathways with important role in abortion or interrupt the pregnancy. Many studies suggested that the level of apoptosis is critically important for the successful development of normal pregnancy [124]. For instance, FoxO signaling pathway involved in apoptosis is enriched in this study. A high level of apoptosis may result in miscarriage [125]. Previous research has reported that a series of genes coordinately regulate apoptosis and proliferation during pregnancy [126].

In a previous study the miR-199a were reported to be spatiotemporally expressed in the mouse uterus during implantation and post-transcriptionally regulate the expression of cyclooxygenase-2 [127]. In accordance with this study we also found this miRNA is down-regulated and this could affect embryo implantation or pregnancy maintanance. In a study, six differentially expressed miRNAs were identified to be potential utility in endometriosis diagnosis. Among them, miR-199a and miR-542-3p were found to be particularly useful, when combined reaching a sensitivity and a specificity in diagnosing endometriosis of up to 96.61% and 79.66%, respectively [128]. In addition, miR-199a is correlated with pelvic adhesion and lesion distribution as well as hormone-mediated signaling pathways, demonstrating that it may play an important role in the disease progression [129]. In the endometrium, miRNAs are involved in the dynamic changes associated with the menstrual cycle, implicated in implantation and in reproductive disorders. Adequate endometrial receptivity and embryo development are essential for the pursued goal of increased pregnancy rates and reduced early pregnancy loss [126]. The normal pregnancy is depending on the cooperative interactions between different cell types and various growth factors in the processes of implantation, embryonic development and placentation. Those are also required for normal functioning vascular

network development [130]. It is thus likely that miRNAs are necessary to regulate genes involved in a pregnancy [131].

Furthermore, we suggest the miRNA 126 may has an important role in embryo development because of its role in angiogenesis. This miRNA is a key regulator of vascular endothelial growth factor and fibroblast growth factor signaling in endothelial cells [132]. It is a common knowledge that autocrine, paracrine, and endocrine factors are working closely, coordinating their effects during embryo implantation. It has been suggested that, among this multitude of players, miRNAs might also contribute knowing that their expression throughout the menstrual cycle is sex hormone dependent [93].

In addition, the importance of miRNAs in embryo implantation has been demonstrated by comparing miRNA expression patterns between the implantation and inter-implantation sites. In a mouse model, increased expression of 30 miRNAs, including miR-28, miR-292-3p, miR-96, miR-30b, miR-429 and miR-135b, reduced expression of 42 miRNAs, including miR-292-5p, miR-1956, miR-290-3p and miR-122, was shown at the implantation compared with inter implantation site at Day 5 of gestation [133]. Altered expression of miRNAs in endometrial tissue could cause embryo implantation failure and thus miRNAs could be implicated in endometrial receptivity and embryo implantation. Altered expression of miRNAs associated with cell adhesion, Wnt signaling, p53 signaling and cell cycle pathways in the secretory phase of the endometrium in women with repeated implantation failure could be one example [134]. The pathways which are mentioned above was also shown to be enriched pathways in our study.

A recent study about the miRNA regulation of angiogenesis has assessed the therapeutic efficacy of miR-126, a pro-angiogenic miRNA, in reversing the inadequate vasculogenesis that occurs in pre-eclampsia. In a pre-eclamptic rat model, miR-126 replacement therapy increased placental area, weight, and microvessel density compared to saline-injected control animals [135]. The results of this study suggest that there might be significant role of this miRNA in maintenance of pregnancy. miRNA-129 was downregulated in our samples and that could be an important reason for abortion.

Finally, in our study, we found the Estrogen signaling pathway is one of the highly affected important pathway and we suppose it has a critical role in abortion or maintenance of pregnancy. Previous studies were demonstrated that Estrogen is one of the major reproductive hormones that regulates miRNA in endometrial cell and uterus. It can directly regulate miRNAs by binding estrogen receptor 1 and alters the consortium of cofactors recruited, either promoting or suppressing their transcription [112],[90]. Ultimately, it seems that the steroid hormones in the uterus accelerate the conversion of pri to pre-miRNAs, the transport of pre-miRNAs to the cytoplasm, and the processing of pre to mature miRNAs. The regulation of miRNAs by both gonadal hormones suggests that miRNAs are important regulators of miRNA translation in the reproductive system during pregnancy.

On the other hand, in ovary samples 5 miRNAs were significantly upregulated in this study ($P < 0.05$). Those miRNAs were miR-29b-3p, miR-34a-5p, miR-193a-3p, miR-200a-3p, and miR-1907. The 2 miRNAs were significantly downregulated (miR-21a-5p, and miR-194-5p) ($P < 0.05$). These results proposed that miRNAs participate in regulating dynamic changes of miRNAs in the ovary during diabetes and they could affect the oocyte maturation or ovulation. The list of genes from ovary samples was subjected to a pathway analysis using NCBI DAVID Bioinformatic Resource 6.7 to identify the significantly enriched canonical pathway ($P < 0.05$). Significantly upregulated 5 miRNAs and their pathways were analyzed. As a result, the Ras signaling pathway and pancreatic cancer pathway were the most enriched pathways potentially regulated by the differentially expressed miRNAs. Ras signaling pathway have an important role in regulating cell proliferation, cell survival, migration and cell differentiation and the pancreatic cancer pathway. In addition, the second group of important enriched pathways were PI3K-Akt signaling pathway, protein digestion and absorption, and FoxO signaling pathway. They are involved in regulating fundamental cellular functions such as transcription, translation, cell proliferation, cell growth and cell survival. FoxO is a big family of transcription factors that regulate gene expression in cellular physiological events including apoptosis, cell cycle control and oxidative stress. Differential expression of miRNAs in ovary might affect critical pathways involved in follicular development and oocyte maturation or late of pregnancy.

We also studied the downregulated expression of miRNA and among the lists of pathways. MAPK signaling pathway and Chemokine signaling pathway are the most enriched pathways potentially regulated by the differentially expressed miRNAs. The MAPK signaling pathway is a highly conserved module that is involved in various cellular functions, including cell division, cell proliferation, differentiation, apoptosis, and migration. The main function of Chemokine signaling pathway this pathway is the attract an immune system cell. Indeed, the trouble in these two pathways also might associate with the dysfunction of the ovary.

In this study, miR-21 was identified as one of the three highly LH-induced miRNAs in murine granulosa cells. The function of miR-21 within granulosa cells during transition to luteal cells has been examined. Similarly, the depletion of miR-21 activity in vivo in mice treated by a phosphorothioate-modified LNA-21 oligonucleotide has led to increased apoptosis in the granulosa cells along with decreased ovulation rates in the treated ovary compared to their contralateral controls [136]. So, in our study, we found that miRNA-21 was downregulated during diabetes and this result could be a good evidence for late pregnancy or abortion. Various studies demonstrated how these small noncoding RNAs can impact ovarian function and examine the role of miRNAs in the regulation of follicle growth have focused on granulosa cells [137]. So, another miRNA shown to be significantly and rapidly up-regulated in granulosa cells following in *vivo* hCG/LH stimulation was miR-21 [138],[112]. miRNA-21 is up-regulated in many cancers and tumors. It has been heavily studied for a possible role in carcinogenesis, and it is highly expressed in human granulosa cells [112]. Knockdown of miR-21 using specific inhibitors causes granulosa cell apoptosis, suggesting a role for miR-21 in the maintenance of granulosa cells within the preovulatory follicle [112]. Most importantly, in *vivo* blockage of miR-21 action by administration of a blocking oligonucleotide into the ovarian bursa prevents ovulation [139]. Specifically, in the ovary, miR-21 promotes cell survival during luteinization [136].

Besides, the increasing miR-200a levels in the human cervix could increase levels of the progesterone metabolizing enzyme 20a-hydroxysteroid dehydrogenase by reducing the signal transducer and activator of transcription (STAT) 5, leading to cervical non-responsiveness to implantation [133]. In our study, we observed how miRNA-200a was

downregulated and this downregulation could also lead to non-responsiveness to implantation.

The role of miRNAs was studied to investigate Decidual Natural Killer Cells with Unexplained Recurrent Spontaneous Abortion. They assumed the miRNA-34 family (miR-34a, miR-34b, and miR-34c) are direct transcriptional targets of the tumor suppressor protein p53, with the potential to regulate both apoptosis and cell proliferation. miR-141, a member of the miR-200 family, is reportedly associated with various human malignancies. For instance, miR-141 is upregulated in ovarian cancer [121], but downregulated in hepatocellular and prostate cancers in K. P. Porkka et al. in 2007 [140]. In addition, we found the miRNAs (34a, 200) are upregulated and this was a good indicator of the role of those miRNAs in the ovary function.

Finally, in our study, we observed that a huge number of pathways were affected because there is more than one pathway that influences the ovary function such as, the MAPK and WNT signaling pathway. In this study we suggest that the MAPK signaling and insulin signaling pathways have a serious impact on ovarian function. All these pathways are known to be involved in ovarian follicular growth and many developmental processes. The ubiquitin-mediated pathway is known to modulate oocyte meiotic maturation [141]. Early mitotic division in developing embryos plays important role in many cellular processes [142]. The MAPK signaling mediates LH-induced oocyte maturation and its activation in cumulus cells appears to require the permissive effect of the oocyte itself [143]. WNT molecules are glycoproteins involved in the fetal ovarian development and adult ovarian function including follicular growth, oocyte growth or maturation, steroidogenesis, ovulation and luteinization [144]. Our study suggest that diabetes may cause abortion by regulating the expression of miRNAs in the ovary and uterus.

4.2. Conclusion

Pregnancy with diabetes constitutes an unfavorable environment for embryo development and increasing the chance for congenital malformation. Furthermore, in mammalian cells, microRNAs are key post-transcriptional regulators and function by

modulating translation or degradation of their target mRNA. In this experiment we investigated the miRNA expression in uterus and ovary during diabetes mellitus by using mice model for this purpose.

The main focus of this study was to analyze the miRNA expressions in uterus and ovary during diabetes and we found 7 miRNAs that were expressed differentially in ovary during diabetes and according to data analysis of these miRNAs and their targets could have an important role in ovary function. In addition, in uterus we observed 22 miRNAs which are differentially expressed during diabetes. The targets of those miRNAs are also investigated and a significant role of this miRNAs in the function of uterus like an implantation receptivity and embryo implantation and development is suggested. Indeed, the ability to enhance reproductive efficiency or inhibit reproductive activity is dependent upon a solid understanding of reproductive physiology. Early diagnosis could allow medical management/intervention that could improve outcomes for both the mother and fetus. Further experimentation is required to investigate the functional implications of miRNAs in effective pregnancy. Moreover, these miRNAs have the ability to be important biomarkers for abortion or any complication during diabetes. However, less is known about post-transcriptional gene regulation in these tissues, even though they play essential roles in the synthesis of proteins and is known to be important in the general regulation of cell differentiation and proliferation.

4.3. Our suggestions for future studies

An important goal in eliminating birth defects is to develop therapeutic interventions that can protect embryos from hyperglycemic insult. This goal can only be achieved by understanding the cellular and molecular mechanisms underlying diabetic embryopathy.

On the other hand, according to dysregulation of gene expression in the tissues give ability to develop novel miRNA biomarkers to detect diseases of reproductive tissues and possibly to aid in the selection of oocytes and embryos for ART technique.

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CURRICULUM VITAE

Name and Surname: Khaleel Ibrahim AL-MASHHADANI

Nationality: Iraqi

Birth Date and Place: September 04, 1986, Baghdad

E-mail: khalilgazwan@gmail.com

EDUCATION

Degree	Institute	Date of Graduate
Bachelors	Baghdad university College of Sciences Biology department	2008
Highschool	Al-Kindy	2004

Language:
Arabic, English, Turkish

Hobbies:
Reading books, Human resource development.

Interesting in:
Genetics, PGD, Infertility.