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Influence of Metabolic Dysfunction in Type 2 Diabetic Females on Their Fertility: Polymorphism Analysis in Alpha (α)-Estrogen Receptors Gene

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Abstract

Type 2 diabetes mellitus (T2DM) could be important in the early diagnosis of menstrual abnormalities and female infertility (FI). Here, we assess the association between T2DM-associated metabolic disturbances and FI. This study included 300 females divided into five groups; each group was made up of 60 individuals. Fasting blood samples was taken for DNA extraction & genotyping analysis and the other part for separating serum to estimate total cholesterol, triacylglycerols, insulin, luteinizing hormone (LH), follicle stimulating hormone (FSH), dehydroepiandrosterone sulfate (DHEA-S), estradiol, progesterone, testosterone and leptin levels. Present data showed significant high levels of LH, estradiol, testosterone, leptin, and BMI in obese T2DM (fertile or infertile) compared to non-obese T2DM (fertile or infertile) and in obese T2DM infertile female than obese T2DM fertile female. On the other hand, results showed significant low levels of FSH, progesterone and DHEA-S levels in obese T2DM (fertile or infertile) compared to their counter parts in non-obese T2DM (fertile or infertile), and in obese T2DM infertile female than obese T2DM fertile one. In T2DM population, the XbaI and PvuII polymorphisms in estrogen receptors-alpha (ESRα) gene were shown to be associated with risk of FI. The FI is reported to be associated with obesity and T2DM complications.

Keywords: Type 2 diabetes; Hyperinsulinemia; Hyperleptinemia; Genetic Polymorphism; Estrogen; Female infertility

1. Introduction

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes. Individuals can have T2DM at any age, which differ depending on one's genetic makeup, health, family history, ethnicity, and environmental factors **(1)**. T2DM is associated with insulin resistance (IR), a condition where glucose cannot be transported into adipocytes and skeletal muscles due to defect in insulin receptors in tissue membranes. Within hepatocytes, glucose oxidation (by glucokinase) is also affected by IR T2DM is also associated with hyperinsulinemia. **(2).**

Hyperinsulinemia is known to cause hyperandrogenemia that then results in an imbalance of female sex hormone levels and may negatively impact sexual function **(3)**. Insulin is reported to act on steroidogenesis through insulin receptors on ovary that activates the release of androgens from the theca cells. In obese women, increase in the peripheral aromatization of androgens to estrogens influence the secretion of gonadotropin and thus disturbing the hypothalamic-pituitary-ovarian axis. Both hyperinsulinemia and hyperandrogenemia inhibit the regular ovulation in obese women **(4, 5)**.

IR is reported to be caused by estrogen which interferes with binding of insulin to its receptor **(3)**. Estrogen receptors (ESR) mediate estrogen activity on both peripheral and central nervous system, as many genes are part of the complex process of reproduction

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(6). There are at least two ESR isoforms (ESRα and ESRβ) that have been shown to be expressed in human germ cells at various stages **(7)**. ESRα gene consists of eight exons separated by seven intronic regions and is reported to have a size of 140 kband to be located on

chromosome number 6q25. This gene codes for 595 amino acid proteins. Common polymorphisms of ESRα including PvuII (rs2234693) and XbaI (rs9340799) has been reported in T2DM infertile females. These polymorphisms are found on intron 1 and are only separated by 46 basic pairs (bp). The polymorphism of PvuII (T397C) takes place on the first intron due to T/C transition, however the polymorphism of XbaI (G351A) is induced by a G/A transition located 50 bp downstream of the PvuII polymorphic site **(8)**.

The obesity is associated with ovarian abnormalities, through hormonal mechanisms that negatively impact neuroendocrine and ovarian functions, as well as the homeostatic ovulation **(9)**. Polycystic ovary syndrome (PCOS) is known to be associated with obesity and with clear signs of hyperandrogenemia, hyperinsulinemia, IR, ovulatory dysfunction, and polycystic ovaries that often lead to infertility **(10)**. PCOS is therefore a condition where female sex hormones are imbalanced and is characterized by anovulation, high androgen levels and cyst on one or both ovaries that can be diagnosed by ultrasound **(11)**.

Leptin is the first discovered adipokine that maintains the endocrine functions in adipose tissue and is strongly related to obesity. It is secreted mainly by adipocytes and in proportion to the fat mass in individuals with large visceral fat. It inhibits dietary consumption and, through its direct effects on the hypothalamic signals, it increases energy expenditure and is therefore believed to be an anti-obesity agent **(12)**.

In the present study, we investigated the effect of the T2DM altered metabolic conditions on women's fertility. We also provided insights into the impacts of obesity and two of ESRα genetic polymorphisms on female fertility and concluded with proposing a possible biochemical mechanism of obesity and diabetes on FI.

2. Patients and methods

This study was approved by Ahram Canadian University (ACU) Human Ethics Committee and conducted according to the specific national laws where applicable (ACU PBC003) (Giza, Egypt). All methods were performed in accordance with the relevant guidelines and regulations. All participants were asked for and provided their informed consents.

Experimental Design

This study involved three hundred female subjects. They were divided into five groups (n = 60). Group [1] Healthy fertile women as a control. Group [2] Non-obese fertile women with T2DM. Group [3] Obese fertile women with T2DM. Group [4] Non-obese infertile women with T2DM. Group [5] Obese infertile women with T2DM.

All patients were at the outpatient clinic, department of internal medicine, Zagazig university hospital. All participants have completed detailed questionnaires with information about their medical and surgical history, lifestyle habits, exposure to gonadotrophins and family history.

Inclusion Criteria

The inclusion and exclusion criteria were set to carefully evaluate patients to determine the exact causes of female infertility and to select only primary infertile females. Adult Female patients from 18-40 years old were included. With fasting blood glucose level ≥ 140 mg/dl, patients have been diagnosed as diabetic with T2DM whereas if their BMI is more than 30 kg/m^2, and their abdominal circumference was ≥ 88cm, patients have been considered as obese.

The FI was diagnosed by the absence of ovulation and inability to deliver a child after one-year time period of having un-protected sexual intercourse **(13)**. The presence of ovulation was confirmed by assessing serum progesterone in mid-luteal phase. Tubal patency was also investigated by hysterosalpingography. The infertile females live a sedentary life, and they were not athletes.

Classifications of patients, according to the causes of infertility are described in **Table 1**. The major causes of infertility in infertile females, were endometriosis =18 (15 %), followed by PCOS (18.33 %), then due to unexplained causes (23.33 %).

Exclusion Criteria

Generally, patients on chemotherapy, hormonal therapy, and radiotherapy were excluded. Individuals with history of karyotypes abnormality, cystic fibrosis, AIDS, liver diseases, renal diseases, general illness, or any other contagious/viral infections were also excluded. Women with history of corrective surgery of vagina and uterus, who take drugs which affect their appetite and performance, as well as smokers were all excluded.

TABLE 1. Classification of patients based on various types of female infertility.

Category	Type	No. of patients	Frequency (%)
Infertile females	Unexplained	28	23.33
	PCOS	22	18.33
	Endometriosis	18	15
	Dysmenorrhea	12	10
	Sec tubal factor	10	8.33
	Irregular cycles	8	6.67
	Tubal factor	8	6.67
	Primary endo III	6	5
	Ostreum blocked	4	3.33
	Lactoria	4	3.33
	Total	120	

Methods

Sample Collection

Blood samples were drawn under complete aseptic conditions. Each subject donated 6 ml of blood in the two different tubes. Two mls of blood was taken in a sterile EDTA coated vacutainer (BD Vacutainer®, Catalog number: 367841) for DNA extraction and & genotyping. The rest of the donated blood (mls) was collected into sterile tubes without any anticoagulant for separating the serum (BD Vacutainer®, Catalog no. 368774). Separated serum was immediately stored at -80 °C until hormone profiling whereas blood vials were properly labeled and stored at -80 °C. To reduce the hormonal variation during menstruation cycle, blood samples from all females were collected within a narrow window (20^{th}-24^{th} day of the cycle) when most women were in mid-luteal phase.

TABLE 2. Primers, PCR conditions and restriction enzymes for estrogen receptor polymorphisms.

Variants	PCR primer sequences	Annealing condition	Extension condition	Enzymes	Enzymes digests (bp)
***Pvu*II**	F:5'CTGCCACCCTATCTGTA TCTTTTCCTATTCTCC-3' R:5'TCTTTCTCTGCCACCCT GGCG TCGATTATCTGA-3'	64.5ºC for 1.10 mins	72ºC for 1.50 mins	*Pvu*II	T= 1374 C= 937+438
***Xba*I**	F:5'CTGCCACCCTATCTGTA TCTTTTCCTATTCTCC-3' R:5'TCTTTCTCTGCCACCCT GGCG TCGATTATCTGA-3'	64.5ºC for 1.10 mins	72ºC for 1.50 mins	*Xba*I	G = 1374 A= 983+393

In all PCR experiments, pre-denaturation step was of 5 mins at 95 °C, denaturation of 50 seconds at 95 °C, 33 cycles and final extension of 5 mins at 72 °C.

Biochemical Analysis

Extraction of genomic DNA

To isolate genomic DNA from whole blood, Wizard® Genomic DNA Purification Kit (Promega) was used and the manufacturer recommendations were followed for isolation of DNA.

ESR-α genotype determination

For the determination of ESRα genotype, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis was performed. For determination of PvuII (C.454 –397 T > C) and XbaI (C.454 –351 A>G) polymorphisms, PCR amplification of the polymorphic regions was performed using oligonucleotide primers as described by **Safarinejad and colleagues (8)**. For PCR amplification, total volume of the reaction mixture was 50 µl which consisted of 25 µl master mix (Fermentas #K0171), 1 µl (10 pmol) of each forward and reverse primer and 5 µl of template DNA. To differentiate C.454–397 T/C (PvuII) and C.454–351 A/G (XbaI) polymorphisms, digestion of

the amplified PCR fragment of 1372 bp was separately performed using PvuII (Fermentas#ER0631) and XbaI (Fermentas#ER0681) restriction enzymes. The reaction solution was mixed gently and spun down for a few seconds. Then the reaction vials were kept at 37 °C water bath for 1 hour and stored at 20 °C until visualization on ethidium bromide stained 2.0 % agarose gel by gel doc. The primers, PCR

conditions, and restriction enzymes used for ESRα polymorphism are shown in **Table 2**.

Determination of cholesterol and triglyceride levels

Diagnostic kit was used for determination of serum levels of total cholesterol (TC, Cat. no. 10007640) and TAGs (Cat. no. 10010303), purchased from the Cayman Chemical Company, USA.

Determination of leptin levels

Leptin level was estimated in serum by using a commercial enzyme linked immunosorbent assay kit (Leptin Sandwich ELISA RUO, SKU = EIA2395R; DRG Instruments, Marburg, Germany).

Determination of glucose and glycosylated hemoglobin

Fasting blood glucose levels were measured immediately by enzymatic colorimetric method, using kits of Centronic Co. (Wartenberg, Germany), according to the method of **Trinder (14)**. Glycosylated hemoglobin (HbA1c) was measured by the calorimetric method of **Standefer and Eaton (15)**.

Determination of endocrine hormones

All endocrine hormones were estimated in serum samples using a commercial enzyme linked immunosorbent assay kits (DRG Instruments, Marburg, Germany). Insulin levels were measured by insulin ELISA, SKU = EIA2935, serum estradiol levels were measured by estradiol sensitive ELISA, SKU = EIA4399, serum progesterone levels were measured by progesterone ELISA, SKU = EIA1561, serum free testosterone levels were measured by free testosterone ELISA, SKU = EIA6101, serum dehydroepiandrosterone sulfate levels were measured by DHEA-S ELISA, SKU = EIA1562, LH levels were measured by LH-Serum ELISA, SKU = EIA1289, and FSH levels were measured by FSH ELISA, SKU = EIA1288.

Determination of HOMA-IR

Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated from this equation: HOMA-IR = fasting glucose (mg/dl) × fasting insulin (μIU/ml)/405, according to **Mathews et al. (16)**.

Statistical Analysis

The clinical data are shown as Mean ± SEM and compared among groups by ANOVA. The genotype and allelic frequencies were evaluated by Hardy-Weinberg equilibrium (HWE) and compared by Chi-square test and Fisher's exact test, by calculating the Odds ratio (OR) and 95% confidence interval (CI) by using GraphPad Prism (version 7.00). The correlation between different parameters was performed in obese infertile diabetic females. P values < 0.05 were regarded as statistically significant.

3. Results

Effects of T2DM and Obesity on both the Glycemic Index and Insulin Levels

Our results showed significantly ($P < 0.05$) higher levels in FBS, HBA1c, HOMA-IR and fasting insulin levels in obese (fertile and infertile) compared to either non-obese (fertile and infertile), or control groups (Table 3). Also, there were significantly ($P < 0.05$) higher levels of FBS, HBA1c, HOMA-IR and fasting insulin levels in infertile obese female compared to fertile obese female or to the control group.

Effects of T2DM and Obesity on Leptin, Cholesterol and Triglycerides (TAGs) Levels

Tabulated data showed significantly ($P < 0.05$) higher levels of insulin and leptin in infertile obese females compared to infertile non-obese females. In addition, TAGs and TC levels were significantly ($P < 0.05$) higher in obese (fertile or infertile) compared to either non-obese (fertile or infertile), or control groups (Table 4). Similarly, significantly ($P < 0.05$) higher levels of TAGs and TC in obese infertile female were demonstrated compared to the obese fertile female or the control group.

Effect of T2DM and Obesity on LH, FSH, DHEA-S, Free Testosterone, Estradiol, and Progesterone Levels

Levels of LH, estradiol, and free testosterone in obese (fertile or infertile) female subjects were significantly ($P < 0.05$) higher than their counterparts in either non-obese (fertile or infertile) or control groups (Table 5). The same was true for LH, estradiol and free testosterone levels in obese infertile female compared to fertile obese female or control group.

On the other hand, we showed significantly ($P < 0.05$) lower levels of FSH, progesterone and DHEA-S levels reported in obese (fertile or infertile) females compared to either non-obese (fertile or infertile) or control female groups (Table 5). Lower levels of FSH, progesterone and DHEA-S were significantly ($P < 0.05$) recorded in obese infertile female compared to fertile obese female or control group.

Correlation between Studied Biochemical Parameters

There were three significant positive co-linear relationships reported in diabetic infertile obese

females. Those were manifest in insulin and estradiol levels (r = 0.34; p = 0.007), leptin and free testosterone levels (r = 0.36; P = 0.0045), and in HOMA-IR versus estrogen (r= 0.3, P= 0.0194) (Figure 1).

ESR-α Genotyping

Genotype of ESR-α XbaI

Genotype distribution and allele frequencies of the A/G polymorphism in ESR-α XbaI gene rs9340799 for diabetic infertile patients' groups.

The observed genotype distributions of the A/G polymorphism presented in Table 6A indicated that there is a significant difference in the frequencies of rs9340799 AG+GG genotype (90.8 %) in diabetic infertile patients compared to AA genotype (9.2 %) with odds ratio (OR= 5.34) at X2= 18.26, CI= 2.36- 12.07, P = < 0.0001. Also, there is a significant difference in AG genotype (75.8 %) with OR= 13.36 at X2= 36.83, CI= 5.26- 33.97, P= < 0.0001 compared to AA allele. Meanwhile, there was no significant (P= 0.17) variation of A allele compared to G allele (OR= 1.57, X2= 1.88, CI= 0.82- 3.00).

Genotype of ESR-α PvuII

TABLE 3. Comparison among HBA_{1C}, FBS, and insulin levels in all studied groups (diabetic groups were treated with different oral hypoglycemic drugs and the duration of diabetes is 3 years).

Groups/ Variables	FBS (mg/dl)	$HBA1_C$ (%)	Insulin (µU/ml)	HOMA-IR
Control (n = 60)	94.58±0.65	4.97±0.05	9.69±0.04	2.26±0.02
Diabetic Fertile Non-Obese (n = 60)	223.6±1.98 [a]	9.06±0.04 [a]	14.02±0.03 [a]	7.74±0.07 [a]
Diabetic Fertile Obese (n = 60)	307.5±1.68 [ab]	9.59±0.04 [ab]	18.4±0.22 [ab]	13.99±0.21 [ab]
Diabetic Infertile Non-Obese (n = 60)	345.2±1.64 [abc]	11.02±0.03 [abc]	18.34±0.22 [ab]	15.62±0.18[abc]
Diabetic Infertile Obese (n = 60)	403.2±2.06 [abcd]	12.02±0.04 [abcd]	21.46±0.17 [abcd]	21.37±0.21 [abcd]

Data was presented as Mean ± SED. HBA1c = glycated hemoglobin, FBS = fasting blood sugar, HOMA-IR = Homeostatic Model Assessment of Insulin Resistance, a = significant when compare all groups to control P < 0.05, b = significant when compare all groups to diabetic fertile non-obese P < 0.05, c = significant when compare diabetic . infertile (obese or non-obese) groups to diabetic fertile obese P < 0.05, d= significant when compare diabetic infertile obese to diabetic infertile non-obese P < 0.05.

Genotype distribution and allele frequencies of the T/C polymorphism in ESR-α PvuII gene rs2234693 for Diabetic Infertile patients' groups

The observed genotype distributions of the T/C polymorphism presented in Table 6B indicated that there was a significant (P = 0.0001) difference in the frequencies of rs2234693 TC+CC genotype (86.7 %) in diabetic infertile patients compared to TT genotype (13.3 %) with OR= 4.04, at X2= 14.73, CI= 1.93- 8.47.

Also, there was a significant (P = 0.0001) difference in TC genotype (76.7 %) with OR= 6.96 at X2= 25.08, CI= 3.11- 15.6 compared to TT allele. However, no significant (P= 0.67) variation of T allele compared to C allele (OR= 0.87, X2= 0.18, CI= 0.47- 1.63) was reported.

TABLE 4. Comparison among leptin, cholesterol, and tags levels in all studied groups.

Groups/ Variables	BMI (kg/m^2)	Leptin (ng/ml)	Cholesterol (mg/dl)	TAGs (mg/dl)
Control (n = 60)	18.57±0.25	21.67±0.19	160.1±1.29	72.72±1.76
Diabetic Fertile Non-Obese (n = 60)	18.4±0.25 [a]	22.73±0.22 [a]	223.1± 1.51 [a]	129.4±1.9 [a]
Diabetic Fertile Obese (n = 60)	33.65±0.36 [ab]	35.22±0.33 [ab]	263.8±1.5 [ab]	173.7±1.1 [ab]
Diabetic Infertile Non-Obese (n = 60)	22.73±0.22 [abc]	25.73±0.28 [abc]	249.2±0.96 [abc]	147.6±1.69 [abc]
Diabetic Infertile Obese (n = 60)	37.83±0.29 [abcd]	42.87±0.43 [abcd]	334.6±2.88 [abcd]	249.2±0.96 [abcd]

Data was presented as Mean ± SED. BMI = body mass index, TAGs = triacylglycerols. a = significant when compare all groups to control P < 0.05, b = significant when compare all groups to diabetic fertile non-obese P < 0.05, c = significant when compare diabetic infertile (obese or non-obese) groups to diabetic fertile obese P < 0.05, d= significant when compare diabetic infertile obese to diabetic infertile non-obese P < 0.05.

TABLE 5. Comparison among reproductive hormones in all studied groups.

Groups/ Variables	LH (IU/ml)	FSH (IU/ml)	Estradiol (IU/ml)	Progesterone (IU/ml)	DHEA-S (µg/dl)	Free Testosterone (nmol/L)
Control (n = 60)	3.75±0.02	7.77±0.02	77.17±0.2	11.96±0.03	192.2±0.74	1.56±0.004
Diabetic Fertile Non-Obese (n = 60)	3.8±0.01	7.31±0.01 [a]	80.1±0.1 [a]	11.66±0.01 [a]	187.8±1.16 [a]	1.7±0.004 [a]
Diabetic Fertile Obese (n = 60)	4.39±0.02 [ab]	6.14±0.01 [ab]	95.35±0.08 [ab]	10.51±0.01 [ab]	181.6±0.39 [ab]	1.83±0.004 [ab]
Diabetic Infertile Non-Obese (n = 60)	4.95±0.04 [abc]	5.83±0.01 [abc]	100.1±0.08 [abc]	9.25±0.02 [abc]	176.2±0.73 [abc]	1.88±0.002 [abc]
Diabetic Infertile Obese (n = 60)	5.65±0.02 [abcd]	4.93±0.04 [abcd]	106.2±0.08 [abcd]	7.3±0.01 [abcd]	166.2±0.88 [abcd]	2.16±0.01 [abcd]

Data was presented as Mean ± SED. LH = luteinizing hormone, FSH = follicle stimulating hormones, DHEA-S = dehydroepiandrosterone sulfate, a = significant when compare all groups to control $P < 0.05$, b = significant when compare all groups to diabetic fertile non-obese $P < 0.05$, c = significant when compare diabetic infertile (obese or non-obese) groups to diabetic fertile obese $P < 0.05$, d= significant when compare diabetic infertile obese to diabetic infertile non-obese $P < 0.05$.

4. Discussion

The present investigation was set to assess the effect of both obesity and T2DM on fertility in diabetic females via their effects on reproductive hormones and selected metabolic parameters. The goal of such study as to explore possible mechanisms attributed to the pathogenesis of infertility in obese diabetic females.

In the current study,results showed that the fasting insulin levels were twice as high as normal, especially, in the infertile obese group. Similar results have been reported showing that hyperglycemia can aggravate IR, including hepatic IR (**Aguayo-Mazzucato et al. (17)**). Some authors showed that due to hepatic IR the increased level of serum insulin cannot decrease the hepatic gluconeogenesis leading to peripheral hyperglycemia and further insulin secretion.The present fasting hyperinsulinemia may serve as an inhibitor of lipolysis, promotes lipogenesis which lead to obesity and selective IR,. Interestingly, **Corkey (18)** has argued that hyperinsulinemia is potentially a risk factor for obesity. Beside obesity the fasting hyperinsulinemia and IR are also known as underlying causes of PCOS and induced oxidative stress that typically result in the deterioration of fetal development and FI **(19, 20)**.

The variations of FBS, HBA_{1C}, HOMA-IR and fasting insulin reported here in the obese groups (fertile and infertile) versus their non-obese counterparts or the control groups consistent with **Diamanti-Kandarakis et al. (21).** They concluded that the diabetic infertile females have an increased level of HbA1c. The hyperglycemia leads to the accumulation of advanced glycation products that impair ovarian function **(17)**. This hypothesis was supported by the present positive correlations between hyperinsulinemia and hyperestrogenemia (**Figure 1 A**) and between the induced HOMA-IR and hyperestrogenemia (**Figure 1 C**).

Leptin is considered as hormone/proinflammatory protein and it is positively correlated with BMI, which in turn may lead to a metabolic disease **(22)** and therefore, obesity is related to high leptin levels in serum and follicular fluid (**23**).

The current results showed significantly higher leptin levels in obese than non-obese or control groups. This increase was observed in infertile obese female than in fertile obese one.

In line with our findings, **Olszanecka-Glinianowicz et al. (24),** observed that the serum leptin levels were significantly higher in obese PCOS patients compared to lean PCOS and obese non-PCOS patients. In previous studies, they proved that both hyperleptinemia and high BMI impair the follicle development, ovulation, and oocyte maturation in rodent models. This illustrates the failure of pregnancy in obese women with in-vitro fertilization **(25, 26)**. So, these results indicate that the diabetes and obesity are associated with FI likely due to hyperleptinemia. This hypothesis can be evidenced by a significant positive correlation between hyperleptinemia and hyperandrogenemia (increased testosterone) (**Figure 1 B**). The results of the current study showed significantly higher levels in TAGs and TC levels in obese than non-obese, or control groups, particularly in infertile obese female than fertile obese one. The results indicates that the DM and FI are associated with hypercholesterolemia and hypertriglyceridemia. In line with our findings, **Dal and Sigrist, (27)** concluded that increased plasma TAGs levels in the form of VLDL, are a popular property of IR and are associated with T2DM. Elevated TAGs levels may also further increase leptin resistance **(28)**.

TABLE 6. Frequency distribution of ESR*α* genotype, alleles and their Association with Hardy Weinberg Equilibrium (HWE) (n=300; each group = 60).

Genotyping		OR	95% CI	X^2	P value
Additive	AA	1.00 (ref)			
	AG	13.36	5.26-33.97	36.83	<0.0001*
	GG	0.96	0.36-2.53	0.007	0.93
Dominant	AA	5.34	2.36-12.07	18.26	<0.0001*
	AG+GG				
Allelic	A	1.57	0.82-3.00	1.88	0.17
	G				

Table 6A ESR-*α Xba*I.

CI = confidence interval; OR = odds ratio. X^2 = deviation from HWE. *A p value of < 0.05 is considered to be statistically significant

.Table 6B ESR-*α Pvu*II.

Genotyping		OR	95% CI	X^2	P value
Additive	TT	1.00 (ref)			
	TC	6.96	3.11-15.6	25.08	<0.0001*
	CC	0.96	0.36-2.53	0.007	0.93
Dominant	TT	4.04	1.93-8.47	14.73	<0.0001*
	TC+CC				
Allelic	T	0.87	0.47-1.63	0.18	0.67
	C				

CI = confidence interval; OR = odds ratio. X2 = deviation from HWE.
*A p value of < 0.05 is considered to be statistically significant.

Additional results of the current study showed significant higher levels of free testosterone, estradiol, and LH in obese than non-obese and in infertile obese than in the fertile obese group.

These results are consistent with the studies of **Bui et al. (29) and Tosi et al. (30)** who reported that levels of free testosterone (FT) are always greater than normal in obese premenopausal females with lower circulating levels of dehydroepiandrosterone-sulphate (DHEA-S) and this elevated FT levels may decrease the potential for fertility. The Free Androgen Index (FAI) was greater in overweight and obese females than in natural-weight ones and was positively correlated with the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) **(29, 30)**.

Therefore, the IR and hyperinsulinemia in obese women lead to hyperandrogenemia as reported by **Sirotkin (31)**. Hyperandrogenism accounts for the etiology of PCOS, and it can disturb the steroidogenesis process **(32).** Both hyperandrogenism and hyperinsulinemia inhibit the regular function of ovary in both obese and non-obese women. These hormonal changes resulted in severe disturbance of neuro-regulation derangement of the hypothalamic-pituitary-ovarian (HPO) axis in obese women; thus increases the risk of abortion, unfavorable pregnancy outcomes, reduced fetal well-being, impaired ovulatory function as well as the reproductive health **(5)**.

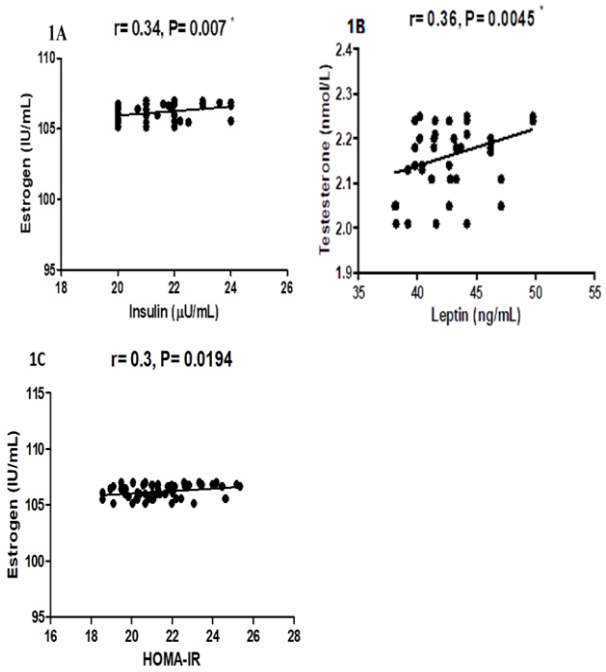

FIGURE 1. A Scatter plot of positive linear correlation between insulin and estrogen of diabetic infertile obese female subjects (1A). Scatter plot of positive linear correlation between leptin and free testosterone of diabetic infertile obese female subjects (1B). Scatter plot of positive linear correlation between HOMA-IR and estrogen of diabetic infertile obese female subjects (1C).

In the current study, the increased level of estradiol in the infertile obese females is consistent with the study of **Gibb et al. (33)** who stated that obesity influences the HPG axis by raising the levels of free estrogen

caused by accelerating peripheral transformation of androgens to estrogens in adipose tissues. It has been shown that the development of estrogens in granulosa cells is induced by insulin via stimulation of aromatase. Increased estrogen leads to a decrease in gonadotropin-releasing hormone (GnRH) by negative feedback. Consequently, this affects HPG axis inducing abnormal or anovulatory phases. **(34)**.

Souter et al. (35) is not consistent with the current results, who stated that BMI was negatively correlated with estradiol concentrations released per preovulatory follicle, leading to reduced amounts of estradiol with an increase in BMI.

Increased LH levels in obese infertile females in the current results may be because of both obesity and hyperleptinemia. LH induces steroidogenesis and inhibits more division and ultimate proliferation of granulosa cells in the pre-ovulatory follicle **(36)**. The impact of LH on granulosa cells in individuals with PCOS is exacerbated by the existence of hyperinsulinemia. Which triggers the development of the follicle to be ended **(37)**.

Other studies are not consistent with the current results regarding LH, estradiol, and testosterone. In eumenorrheic obese women, hyperleptinemia with high BMI suppress insulin stimulated ovarian steroidogenesis and prevent LH induced estradiol synthesis by the granulosa cells **(38,39)**. Another study has demonstrated that uncontrolled DM is resulting in a profound hypogonadotropic condition, which is represented by reduced basal concentrations of sex steroids, gonadotropins, and reduced LH pulsatility **(40)**. Also, the current results are not consistent with **Arrais and Dib (41)** who concluded the decreased level of LH in diabetic females with infertility.

The results of the current study showed significantly lower levels in progesterone, DHEA-S, and FSH in obese than in non-obese, and in infertile obese than in the fertile obese groups. The reduced level of progesterone is due to higher serum leptin levels in obese females associated with increased leptin levels in the follicular fluid (**23**).

The current results of DHEA are in agreement with many studies, which also reported an inverse relationship between DHEA levels and whole-body lipids (**42, 43**). In addition, these results were documented by **Kauffman et al (42) and Moran et al (43)**. Also, another experiment confirmed the diminished DHEA-S manufacturing in the existence of hyperinsulinemia in normal women **(44)**. On the other hand, there is another study has not succeeded to prove any correlation between DHEA-S levels and insulin in women with PCOS **(45)**.

Concerning FSH, our results are in agreement with **Arrais and Dib (41)** who concluded that infertile diabetic females have decreased levels of FSH. In addition, patients with PCOS often have elevated plasma LH levels and decreased FSH levels. These abnormalities may be due to the defect in the GnRH pulse generator that shows a low susceptibility to ovarian steroids' suppression **(46)**. FSH engages in the steroidogenesis cycle, oocyte maturation and follicle formation via FSHR localized on the surface of granulosa cells in ovarian tissues **(47)**.

There are statistically significant (not biologically significant) differences between diabetic fertile (either non-obese or obese) and control groups in all studied reproductive hormone levels except LH, this possibility because of diabetes and/or obesity effect on female sex hormone levels. In addition, there is no significant difference in LH levels between diabetic fertile non-obese group and control group, indicating the effect of obesity and hyperleptinemia on LH levels in obese groups.

The common genetic polymorphisms of ESRα including PvuII (rs2234693) and XbaI (rs9340799) are reported in T2DM infertile females in a limited number of studies. Therefore, in the current study, the relationship between these polymorphisms and FI in obese diabetic females was explored.

ESR is a group of protein receptors that are triggered by the hormone estrogen (17 β-estradiol). There are two categories of ESR: ESRα and ESRβ genes. They have a role in controlling the physiological response to estrogen **(48)**.

Our results showed that in T2DM population, the XbaI and PvuII polymorphisms in ESRα gene are strongly associated with risk of FI. The ESR1 PvuII polymorphism strongly reduced the pregnancy rate after in vitro fertilization (IVF), it was associated with the predisposition to endometriosis and controlled ovarian hyperstimulation **(49)**.

Expected Mechanistic Correlation between T2DM and FI

Based on the results of the current study and linking it with the results of previous researchers' studies in the same field, we try to explain the relationship between metabolic dysfunction in obese T2DM and FI. **Figure 2** illustrates this relationship, which depends on the fasting hyperinsulinemia that leads to two prominent outcomes. The first is the

increase of adipose steroidogenesis that results in hyperandrogenism (↑ FT) and hyperestrogenemia(↑ estradiol) due to the stimulation of aromatase. The second is the obesity, which leads to three pathways, 1) IR that causes hyperglycemia, 2) a decrease of DHEA-S and FSH, and an increase in LH, and 3) hyperleptinemia, which leads to a decrease of progesterone. This entire hormonal imbalance mostly led to PCOS, inhibition of ovulation and consequently FI.

The novelty and innovation in the current research are the clarification of the pathological mechanism of infertility in obese T2DM females, through the linking of the metabolic imbalance caused by diabetes and obesity and being indirect causes of FI.

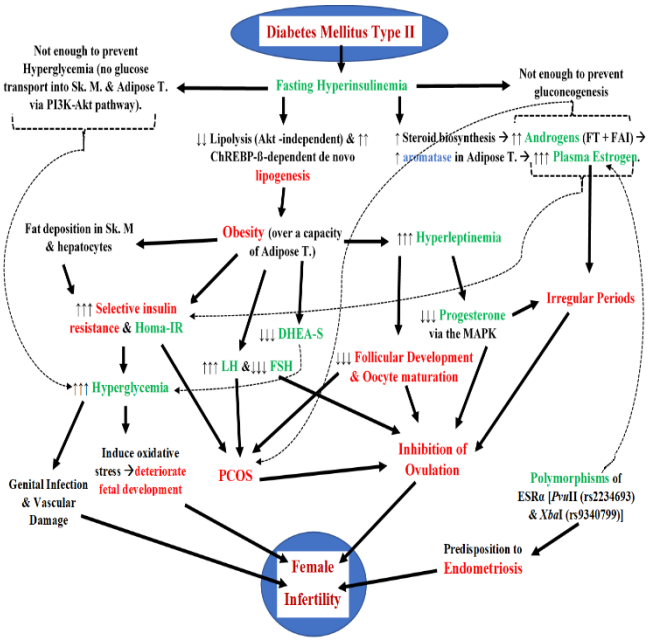

FIGURE 2. A diagrammatic illustration of the relationship of metabolic dysfunction in type 2 diabetic females and their infertility depending on the links between the results of the current study and researchers' previous studies.

5. Study Limitations

The current study was conducted on T2DM infertile females before starting the treatment of infertility to investigate the metabolic impacts of obesity and T2DM on some metabolic parameters and reproductive hormones without the effects of those drugs that can affect the reproductive hormones under study. These diabetic females were recently diagnosed as an infertile.

6. Conclusion

Infertility incidence in women has been closely linked to the risks and complications of T2DM due to fasting hyperleptinemia, hyperinsulinemia and hyperlipidemia, which subsequently leads to reproductive hormone abnormalities and prevention of ovulation. In our T2DM population, the XbaI and PvuII forms in the ESRα gene are highly correlated with the risk of female infertility.
More future studies may be required to investigate the possible impacts of oral hypoglycemic, anti-hypertensive, hypolipidemic and weight losing drugs for female fertility.

7. Abbreviations

BMI = body mass index, DHEA-S= dehydroepiandrosterone sulfate, ESRα = estrogen receptor alpha, FAI = free androgen index, FBS = fasting blood sugar, FI = female infertility, FSH = follicle stimulating hormone, FSHR = follicle stimulating hormone receptor, FT = free testosterone, GLUT1 & 4 = glucose transporter 1 & 4, GnRH = gonadotropin-releasing hormone, HbA1c = glycosylated hemoglobin A1c, HOMA-IR = homeostatic model assessment of insulin resistance , HPG = hypothalamic pituitary gonadal, IR = insulin resistance, IVF = in vitro fertilization, LH = luteinizing hormone, PCOS = Polycystic ovary syndrome, PCR = polymerase chain reaction, T2DM = type 2 diabetes mellitus, TAGs = triacylglycerols, and VLDL = very low-density lipoprotein.

8. Data Availability Statements

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

9. Ethics Statements

This study was approved by Ahram Canadian University (ACU) Human Ethics Committee and conducted according to the specific national laws where applicable (ACU PBC003) (Giza, Egypt). All methods were performed in accordance with the relevant guidelines and regulations. All participants were asked for and provided their informed consent.

10. Author Contributions

All authors have made considerable contribution to the work and approved the final version of the manuscript for the publication. All authors greatly contribute to experimenting, acquiring, designing, analyzing, and drafting the manuscript.

11. Conflict Of Interest

There is no conflict of interest.

12. Acknowledgments

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13. References

1. Kourtoglou GI. Insulin therapy and exercise. Diabetes Res Clin Pract (2011) 93 (1): 73-77.
2. Samuel VT, Shulman GI. Mechanisms for Insulin Resistance: Common Threads and Missing Links. Cell (2012) 148 (5): 852-871.
3. Keene KL, Mychaleckyj JC, Leak TS, Smith SG, Perlegas PS, Divers J, et al. Exploration of the utility of ancestry informative markers for genetic association studies of African Americans with type 2 diabetes and end stage renal disease. Hum Genet (2008) 124 (2):147-154.
4. Nandi A, Poretsky L. Diabetes and the female reproductive system. Endocrinol Metab Clin North Am (2013) 42 (4): 915-946.
5. Talmor AJ, Dunphy B. Female obesity and infertility. Best Practice & Research: Clinical Obstetrics & Gynaecology (2015); 29 (4): 498-506.
6. Jarvi K, Lo K, Grober E, Mak V, Fischer A, Grantmyre J, et al. The workup and management of azoospermic males. Can Urol Assoc J (2015) 9 (7-8): 229-235. doi: 10.5489/cuaj.3209
7. Cooke PS, Nanjappa MK, Ko C, Prins GS, Hess RA. Estrogens in Male Physiology. Physiol Rev. (2017) 97(3): 995- 1043.
8. Safarinejad MR, Shafiei N, Safarinejad S. Association of polymorphisms in the estrogen receptors alpha, and beta (ESR1, ESR2) with the occurrence of male infertility and semen parameters. J Steroid Biochem (2010) 122: 193-203.
9. ASRM - American Society for Reproductive Medicine. Practice Committee of American Society for Reproductive Medicine. Obesity and reproduction: an educational bulletin. Fertil Steril (2008) 90: S21-S29.
10. Petraglia FW, Farber SH, Gramer R, Verla T, Wang F, Thomas S, et al. The Incidence of Spinal Cord Injury in Implantation of Percutaneous and Paddle Electrodes for Spinal Cord Stimulation. Neuromodulation (2016) 19 (1): 85-90.
11. Catalano PM. Trying to understand gestational diabetes. Diabet Med (2014) 31 (3): 273-281.
12. Budak E, Fernández Sánchez M, Bellver J, Cerveró A, Simón C, Pellicer A. Interactions of the hormones leptin, ghrelin, adiponectin, resistin, and PYY3-36 with the reproductive system. Fertil Steril (2006) 85 (6): 1563-1581.
13. Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, et al. International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology. Fertil Steril (2009) 92 (5): 1520-1524.
14. Trinder P. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann Clin Biochem (1969) 6: 24–27.
15. Standefer JC, Eaton RP. Evaluation of a calorimetric determination of glycosylated hemoglobin. J Clin Chem (1983) 29: 135- 140.
16. Mathews DR, Hoker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia (1985) 28: 412–419.
17. Aguayo-Mazzucato C, van Haaren M, Mruk M, Lee TB Jr, Crawford C, Hollister-Lock J, et al. β Cell Aging Markers Have Heterogeneous Distribution and Are Induced by Insulin Resistance. Cell Metab (2017) 4; 25 (4): 898-910.e5.
18. Corkey BE. Banting lecture: hyperinsulinemia: cause or consequence? Diabetes (2012) 61 (1): 4-13.
19. Samara-Boustani D, Colmenares A, Elie C, Dabbas M, Beltrand J, Caron V, et al. High prevalence of hirsutism and menstrual disorders in obese adolescent girls and adolescent girls with type 1 diabetes mellitus despite different hormonal profiles. Eur J Endocrinol (2012) 166 (2): 307-316.
20. Chen X, Shi YD, Tie L. Rho-associated protein kinase: significant role in diabetic complications. Sheng Li Ke Xue Jin Zhan. (2015) 46 (6): 433-438.
21. Diamanti-Kandarakis E, Christakou C, Kandarakis H. Polycystic ovarian syndrome: the commonest cause of hyperandrogenemia in women as a risk factor for metabolic syndrome. Minerva Endocrinol (2007) 32 (1): 35-47.
22. Bays HE, González-Campoy JM, Henry RR, Bergman DA, Kitabchi AE, Schorr AB, et al.. Adiposopathy Working Group. Is adiposopathy (sick fat) an endocrine disease? Int J Clin Pract (2008) 62 (10): 1474-1483.
23. Li MG, Ding GL, Chen XJ., et al., "Association

of serum and follicular fluid leptin concentrations with granulosa cell phosphorylated signal transducer and activator of transcription 3 expression in fertile patients with polycystic ovarian syndrome," Journal of Clinical Endocrinology and Metabolism (2007) 92 (12): 4771–4776.
24. Olszanecka-Glinianowicz M, Madej P, Nylec M, Owczarek A, Szanecki W, Skałba P, et al. Circulating apelin level in relation to nutritional status in polycystic ovary syndrome and its association with metabolic and hormonal disturbances. Clinical Endocrinology. (2013) 79 (2): 238–242.
25. Lainez NM, Coss D. Obesity, Neuroinflammation, and Reproductive Function. Endocrinology (2019) 160 (11): 2719- 2736.
26. Magariños MP, Sánchez-Margalet V, Kotler M, Calvo JC, Varone CL. Leptin promotes cell proliferation and survival of trophoblastic cells. Biol Reprod (2007) 76 (2): 203-210.
27. Dal S, Sigrist S. The Protective Effect of Antioxidants Consumption on Diabetes and Vascular Complications. Diseases. (2016) 4 (3): 24.
28. Farooqui AA, Farooqui T, Panza F, Frisardi V. Metabolic syndrome as a risk factor for neurological disorders. Cell Mol Life Sci (2012) 69 (5): 741-762.
29. Bui HN, Sluss PM, Hayes FJ, Blincko S, Knol DL, Blankenstein MA, et al. Testosterone, free testosterone, and free androgen index in women: Reference intervals, biological variation, and diagnostic value in polycystic ovary syndrome. Clin Chim Acta (2015) 23; 450: 227-232.
30. Tosi F, Fiers T, Kaufman JM, Dall'Alda M, Moretta R, Giagulli VA, et al. Implications of Androgen Assay Accuracy in the Phenotyping of Women With Polycystic Ovary Syndrome. J Clin Endocrinol Metab (2016) 101 (2): 610-618.
31. Sirotkin AV. Growth factors controlling ovarian functions. J Cell Physiol (2011) 226 (9): 2222-2225.
32. Barbar E, Lehoux JG, Lavigne P. Toward the NMR structure of StAR. Molecular and Cellular Endocrinology (2009) 300: 89–93.
33. Gibb FW, Homer NZ, Faqehi AM, Upreti R, Livingstone DE, McInnes KJ, et al. Aromatase Inhibition Reduces Insulin Sensitivity in Healthy Men. J Clin Endocrinol Metab (2016) 101 (5): 2040-2046.
34. Bulun SE, Lin Z, Imir G, Amin S, Demura M, Yilmaz B, et al. Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment. Pharmacol Rev (2005) 57 (3): 359-383.
35. Souter I, Baltagi LM, Kuleta D, Meeker JD, Petrozza JC. Women, weight, and fertility: the effect of body mass index on the outcome of superovulation/intrauterine insemination cycles. Fertil Steril (2011) 95 (3): 1042-1047.
36. Raju GA, Chavan R, Deenadayal M, Gunasheela D, Gutgutia R, Haripriya G, et al. Luteinizing hormone and follicle stimulating hormone synergy: A review of role in controlled ovarian hyper-stimulation. J Hum Reprod Sci (2013) 6 (4): 227–234.
37. Rice S, Christoforidis N, Gadd C, Nikolaou D, Seyani L, Donaldson A, et al. Impaired insulin-dependent glucose metabolism in granulosa-lutein cells from anovulatory women with polycystic ovaries. Hum Reprod (2005) 20 (2): 373-381.
38. Lin O, Poon SL, Chen J, HoYuen B, Leung PC. Leptin interferes with 3'5'−cyclic adenosine monophosphate (Camp) signaling to inhibit steroidogenesis in human granulosa cells. Reprod Biol Endocrinol (2009) 7: 115.
39. Jain A, Polotsky AJ, Rochester D, Berga SL, Loucks T, Zeitlian G, et al. Pulsatile luteinizing hormone amplitude and progesterone metabolite excretion are reduced in obese women. J Clin Endocrinol Metab (2007) 92 (7): 2468-2473.
40. Esposito K, Chiodini P, Bellastella G, Maiorino MI, Giugliano D. Proportion of patients at HbA1c target <7% with eight classes of antidiabetic drugs in type 2 diabetes: systematic review of 218 randomized controlled trials with 78 945 patients. Diabetes Obes Metab (2012) 14 (3): 228-233.
41. Arrais RF, Dib SA. The hypothalamus–pituitary–ovary axis and type 1 diabetes mellitus: a mini review. Human Reproduction (2006) 21(2): 327–337.
42. Kauffman RP, Baker VM, DiMarino P, Castracane VD. Hyperinsulinemia and circulating dehydroepiandrosterone sulfate in white and Mexican American women with polycystic ovary syndrome. Fertil Steril (2006) 85 (4): 1010-1016.
43. Moran LJ, Norman RJ, Teede HJ. Metabolic risks in PCOS phenotype and adiposity impact. Trends Endocrinol n Metab (2015) 26 (3): 136−143.
44. Brennan K, Huang A, Azziz R. Dehydroepiandrosterone sulfate and insulin resistance in patients with polycystic ovary syndrome. Fertil Steril (2009) 91 (5): 1848-1852.
45. Ehrmann DA, Kasza K, Azziz R, Legro RS, Ghazzi MN. PCOS/Troglitazone Study Group. Effects of race and family history of type 2 diabetes on metabolic status of women with polycystic ovary syndrome. J Clin Endocrinol Metab (2005) 90 (1): 66-71.
46. Kosova G, Urbanek M. Genetics of the polycystic ovary syndrome. Mol Cell Endocrinol (2013) 373

(1-2): 29-38.
47. Kim JJ, Choi YM, Hong MA, Chae SJ, Hwang K, Yoon SH, et al. FSH receptor gene p. Thr307Ala and p. Asn680Ser polymorphisms are associated with the risk of polycystic ovary syndrome. Journal of Assisted Reproduction and Genetics (2017) 34: 1087–1093.
48. Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, et al. Estrogen receptors: how do they signal and what are their targets. Physiol Rev (2007) 87 (3): 905-931.
49. Ereqat S, Cauchi S, Eweidat K, Elqadi M, Nasereddin A. Estrogen receptor 1 gene polymorphisms (PvuII and XbaI) are associated with type 2 diabetes in Palestinian women. Peer J (2019) 7: e7164.