Metabolic Effects of Estrogen and / or Insulin in Ovariectomized Experimentally Diabetic Rats

Aziza S. El-Nasr, Faten M.A. Diab, Nihal M. Bahgat, * Mona A. Ahmed, Sahar S. Thabet and Soha M.Y. El-Dakkak

Department of Physiology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
*drmona_agha70@yahoo.com

Abstract: Postmenopausal adverse metabolic changes increase cardiovascular risk and impair quality of life. This study was planned to evaluate the benefits gained by estradiol treatment alone and insulin treatment alone versus combination of these two hormonal therapies on the metabolic derangements accompanying estrogen deficiency with diabetes. Rats were divided into five groups: control sham-operated group, ovariectomized streptozotocin diabetic group (OVX–STZ), estradiol-treated OVX–STZ diabetic group that received daily subcutaneous injection of estradiol (50µg/kg) for 4 weeks, insulin-treated OVX–STZ diabetic group that received daily subcutaneous injection of insulin (10 or 20 IU/kg) for 2 weeks and combined estradiol-treated, insulin-treated OVX–STZ diabetic group. Rats in all groups were subjected to determination of body weight, body mass index (BMI), blood glucose, plasma levels of total cholesterol, triglycerides, HDL-c, insulin, estradiol, leptin and malondialdehyde (MDA). In addition, *in vitro* glucose uptake by the diaphragm and glucose output by both kidneys were measured. Insulin treatment alone increased peripheral glucose uptake, reduced renal gluconeogenesis, normalized blood glucose and plasma total cholesterol, decreased triglycerides, LDL-c and atherogenic index and increased HDL-c. Plasma MDA was reduced however, still higher than controls. Estrogen therapy alone lowered blood glucose although not fully normalized, increased peripheral glucose uptake and decreased renal gluconeogenesis, reduced plasma triglycerides, total cholesterol, LDL-c and MDA and elevated HDL-c as compared to untreated groups, yet, not completely normalized. Combined estradiol and insulin therapy returned all measured parameters towards control values with complete normalization of peripheral glucose uptake and blood glucose levels as well as plasma triglycerides, HDL-c, atherogenic index and MDA, while BMI, gluconeogenesis, total cholesterol and LDL-c approached control values although still not fully normalized. It is concluded that either insulin or estrogen therapy provided only partial improvement of the metabolic error of estrogen deficiency with diabetes while the best cure was found with combined estradiol and insulin therapy which achieved successful optimization of weight gain, reduced adiposity, tight glycemic control, alleviated dyslipidemia and normal oxidative state. Thus, insulin therapy together with hormonal replacement therapy as a coadjuvant might be the most advisable line of treatment in postmenopausal diabetic women.

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1. Introduction:

Many postmenopausal women live with diabetes mellitus; however, little information is available about how the changes that occur around the time of menopause might uniquely affect management of diabetes mellitus [(1)].

As noticed in the literature, postmenopausal diabetic patients encountered the reality of increased atherogenic lipid profile [(2)], as well as redox imbalance [(3)], and thereby increased cardiovascular risk factors. However, the role of hormonal replacement therapy in reversing such threats remains a subject of debate.

There are conflicting data on the effect of hormonal replacement therapy in postmenopausal diabetic women. On one hand, Borissova *et al.* [(4)] recommended the use of hormonal replacement therapy in diabetic postmenopausal women because of its favorable effect. On the other hand, Feher and Isaacs [(5)] denied the potential benefits of hormonal replacement therapy. In addition, some evidence suggests that estrogen therapy may improve insulin sensitivity [(6, 7)].

It was therefore, worthwhile to investigate the effect of estrogen treatment alone and insulin treatment alone versus combination of these two hormonal therapies on modulating the metabolic error presented by altered glycemic and lipid metabolism in estrogen deficient experimentally diabetic rats.

2. Materials and Methods

Experimental animals:

The present study was performed on 92 female Wistar rats. The rats were purchased from Research

Institute of Ophthalmology (Giza) and were maintained in Physiology Department Animal House, under standard conditions of boarding and given regular diet consisting of bread, vegetables and milk with free access to water.

Experimental protocol:

Rats were divided into 5 groups:

Group 1: Sham-operated control rats (Sham) (n =16). Rats in this group were subjected to all surgical procedures of ovariectomy except for removal of ovaries. Two weeks later, they received a single i.p. injection of 0.05 M citrate buffer (1 ml/kg) and were studied 2 weeks later.

Group 2: Ovariectomized streptozotocin diabetic rats (OVX–STZ) (n =32). Rats in this group were subjected to bilateral ovariectomy [8]. Two weeks after the operation, they received a single i.p. injection of STZ (Sigma, USA) in a dose of 40 mg/kg [9] and were studied after 2 weeks.

Group 3: Estradiol-treated ovariectomized STZ diabetic rats (E_2+OVX–STZ) (n =16). Rats in this group were subjected to bilateral ovariectomy and on the next day, they received subcutaneous injection of estradiol (Sigma, USA) in a dose of 50 µg/kg, daily 6 days/week for 4 weeks [10]. Two weeks later, they received a single i.p. injection of STZ then studied 2 weeks later.

Group 4: Insulin-treated ovariectomized STZ diabetic rats (Insulin+OVX–STZ) (n =13). Rats in this group were subjected to bilateral ovariectomy. Two weeks later, they were rendered diabetic then received daily subcutaneous injection of insulin (Lilly, Egypt) in a dose of 10 or 20 IU/kg/day, 6 days/week for 2 weeks according to their blood glucose level [11].

Group 5: Estradiol-treated, Insulin-treated ovariectomized STZ diabetic rats (E_2 + Insulin + OVX – STZ) (n =15). Rats in this group were subjected to bilateral ovariectomy followed on the next day by subcutaneous injection of estradiol for 4 weeks. Two weeks after the operation, rats were rendered diabetic then treated with daily subcutaneous injection of insulin for 2 weeks.

Experimental Procedure

On the day of experiment, overnight fasted rats were tested for re-estimation of blood glucose level via rat tail sampling using blood glucose test strips. Then, rats were weighed and anaesthetized i.p with thiopental sodium 40 mg/kg (Sandoz, Austria). The length of the anaesthetized rat was measured from tip of the nose to the anus to calculate body mass index (BMI) according to the following equation:

BMI = Body weight (kg) / length (m^2).

A midline abdominal incision was made and blood samples from the abdominal aorta were collected into two plastic tubes. One tube containing sodium fluoride / potassium oxalate, for immediate determination of blood glucose concentration. The other tube containing EDTA, for preparation of plasma which was stored at – 20 °C for later determination of plasma leptin, estradiol, insulin, malondialdehyde (MDA) and lipid profile (total cholesterol, triglyceride and high density lipoprotein-cholesterol (HDL-c).

Immediately after blood collection, both kidneys were exposed and excised from the renal pedicle and placed separately in ice cold Krebs Ringer solution for 10 minutes after which cortical kidney slices were prepared for *in vitro* estimation of glucose output by both kidneys. Then, the diaphragm was exposed, quickly and carefully excised then immediately placed in ice cold Krebs' solution for *in vitro* estimation of glucose uptake by diaphragm.

Methods:

I. Biochemical studies

Blood glucose was determined by glucose oxidase enzymatic colorimetric technique, according to the method described by Trinder [12], using kits supplied by Stanbio, USA. Plasma lipids (triglycerides, total cholesterol and HDL-cholesterol) were measured by quantitative enzymatic colorimetric methods [13, 14] using kits supplied by Stanbio-laboratory, Texas, U.S.A. Plasma LDL-cholesterol and atherogenic index (AI) were calculated according to Friderwald *et al*. [13] and Grundy *et al*. [15] respectively as follows:

LDL-c = Total cholesterol – (triglyceride/5 + HDL-c)

$$\text{Atherogenic index} = \frac{\text{Total cholesterol}}{\text{HDL-c}}$$

Malondialdehyde (MDA) was assayed in plasma, according to the method of Esterbauer and Cheeseman [16], as thiobarbituric acid reactive substance.

Plasma estradiol was estimated by radioimmunoassay using RIA estradiol kit, supplied by Immunotech, France. Plasma insulin was measured quantitatively by immunoradiometric assay using Insulin (e) IRMA kit supplied by Immunotech, Czech Republic. The measurements of plasma estradiol and insulin levels were performed in Middle Eastern Regional Radioisotope Center for Arab Countries, Cairo. Plasma leptin was determined quantitatively by Elisa technique using leptin (sandwich) ELISA kit supplied by DRG, Germany. The measurement was performed in Oncology Diagnostic Unit, Biochemistry Department, Faculty of Medicine, Ain Shams University.

II. *In vitro* determination of glucose uptake by the diaphragm was performed according to the method described by Mohamed *et al*. [17].

III. *In vitro* determination of glucose output by both kidneys

Cortical kidney slices from both right and left kidneys were separately used to measure glucose output by the kidney according to the method described by Randall [18] with few modifications.

Statistical Analysis:

Results were statistically analyzed by one-way ANOVA for differences between means of different groups. Further analysis was made by least significant difference (LSD) to find inter-groupal significance. For differences within the same group, analysis was determined by Student's't' test for paired data. Correlations and lines of regression were calculated by linear regression analysis using the Least Square Method. All data were analyzed using SPSS statistical package (SPSS Inc.) version 8.0.1. A probability of $P<0.05$ was considered statistically significant.

3. Results:

Body weight and body mass index (BMI) changes

In table (1), treatment of OVX-STZ diabetic rats with estrogen alone, insulin alone as well as with combined estradiol and insulin resulted in significant increase in final body weights as compared to their initial values ($P<0.001$).

The final body weight was significantly increased in insulin-treated OVX-STZ rats as compared to OVX-STZ, estradiol-treated and sham-operated groups ($P<0.001$, $P<0.001$ & $P<0.01$ respectively). Estradiol-treated OVX-STZ diabetic group demonstrated non significant difference as compared to OVX-STZ group. Combined treatment with estradiol and insulin showed non significant difference as compared to OVX-STZ and estradiol-treated groups but significant decrease as compared to insulin-treated group ($P<0.001$).

BMI showed significant increase in insulin-treated OVX-STZ diabetic rats as compared to OVX-STZ ($P<0.001$) and estradiol–treated ($P<0.001$) groups. Combined treatment with estradiol and insulin showed significant decrease in BMI as compared to insulin-treated OVX-STZ rats ($P<0.001$) while non significant difference as compared to both OVX-STZ and E_2+OVX-STZ groups.

Glycemic parameters

As shown in table (2), OVX-STZ diabetic rats demonstrated significant increase in blood glucose ($P<0.001$) as well as in glucose output by both right and left kidneys ($P<0.001$) but significant decrease in glucose uptake by the diaphragm ($P<0.001$) as compared to sham control rats.

Treatment with either estradiol alone or insulin alone as well as combined therapy with estradiol and insulin resulted in significant decrease in blood glucose ($P<0.001$) and glucose output by right and left kidneys ($P<0.001$) and significant increase in glucose uptake by the diaphragm ($P<0.001$) as compared to OVX-STZ rats. Compared to estradiol treatment, insulin treatment caused significant decrease in blood glucose ($P<0.001$) and in right and left kidneys glucose output ($P<0.05$ & $P<0.001$ respectively) but a similar effect on diaphragmatic glucose uptake. Rats receiving combined treatment with estradiol and insulin demonstrated significantly lower blood glucose ($P<0.001$) and glucose output of both kidneys ($P<0.001$) together with significantly higher glucose uptake ($P<0.001$) than rats receiving estradiol treatment alone as well as significantly higher glucose uptake ($P<0.001$) than rats receiving insulin treatment alone.

Lipid profile

OVX–STZ group showed significant increase in plasma triglycerides ($P<0.001$), total cholesterol ($P<0.001$), LDL-c ($P<0.001$) and atherogenic index ($P<0.001$) but significant decrease in HDL-c ($P<0.001$) as compared to sham-operated group.

In estradiol-treated OVX–STZ diabetic rats, plasma triglycerides, total cholesterol and LDL-c showed significant decrease as compared to OVX-STZ group ($P<0.001$) but significant increase as compared to sham control group ($P<0.05$, $P<0.001$ and $P<0.001$, respectively), the atherogenic index was decreased as compared to OVX-STZ group ($P<0.001$) but was insignificant from sham control group whereas plasma HDL-c was increased as compared to OVX-STZ group ($P<0.001$) but decreased as compared to sham control rats ($P<0.05$).

In insulin-treated OVX-STZ diabetic rats, plasma triglycerides was lower than in OVX-STZ group ($P<0.001$) but higher than in estradiol-treated ($P<0.01$) and sham control ($P<0.001$) rats, total cholesterol was lower than in both OVX-STZ ($P<0.001$) and E_2-OVX-STZ ($P<0.01$) rats and plasma HDL-c was higher than in OVX-STZ group ($P<0.001$) but lower than in sham-control ($P<0.001$) and estradiol-treated ($P<0.001$) groups. Plasma LDL-c and atherogenic index were lower than in OVX-STZ group ($P<0.001$) but were higher than in sham-control group ($P<0.001$).

In combined estradiol and insulin treated ovariectomized diabetic group, plasma triglycerides showed significant decrease when compared to non-treated OVX-STZ ($P<0.001$) and insulin-treated groups ($P<0.001$), total cholesterol was decreased as compared to OVX-STZ rats ($P<0.001$) but was increased as compared to sham-operated control ($P<0.01$) and insulin-treated ($P<0.05$) groups, HDL-c was increased when compared to OVX-STZ ($P<0.001$) and insulin-treated ovariectomized diabetic ($P<0.001$)

groups, LDL-c was decreased as compared to OVX-STZ group (P<0.001) but was insignificant from either estradiol treatment or insulin treatment alone and the atherogenic index was significantly decreased as compared to OVX-STZ (P<0.001) and insulin-treated (P<0.05) groups but was insignificant from estradiol-treated and sham control groups (table 3).

Plasma levels of malondialdehyde, estradiol, insulin and leptin are shown in figure 1.

Correlation studies among the experimental groups:

Correlations of plasma estradiol levels and plasma insulin levels versus other parameters in untreated ovariectomized STZ-diabetic (OVX-STZ), estradiol-treated ovariectomized diabetic (E_2+OVX–STZ), insulin-treated ovariectomized diabetic (Insulin+OVX–STZ) and estradiol-treated, insulin-treated ovariectomized diabetic (E_2 + Insulin + OVX – STZ) groups are displayed in tables 4 & 5; figures 2 &3.

Table (1): Initial and final body weights (BW) and body mass index (BMI) in the groups studied.

		Initial BW (g)	Final BW (g)	BMI (Kg/m²)
Sham	(16)	181 ± 11.5	214 ± 13.3 *	5.6 ± 0.2
OVX -STZ	(32)	195 ± 5.0	195 ± 4.8	5.1± 0.09 [a]
E_2 + OVX – STZ	(16)	174 ± 5.7	188 ± 6.6 * [a]	5.2 ± 0.12
Insulin+ OVX – STZ	(13)	212 ± 4.6	258 ± 5.9 * [a b c]	6.3 ± 0.13 [a b c]
E_2+Insulin +OVX – STZ	(15)	164 ± 5.1	198 ± 5.2 * [d]	5.1 ± 0.06 [a d]

Number in parenthesis is the number of rats in each group. Results are expressed as means ±SEM. *: Significance by Student's't' test at P<0.05 from respective baseline value for paired data. a: significance from sham group by LSD at P<0.05. b: significance from OVX-STZ group by LSD at P<0.05. c: significance from E_2 + OVX-STZ group by LSD at P<0.05. d: significance from insulin + OVX-STZ group by LSD at P<0.05.

Table (2): Blood glucose, glucose uptake by the diaphragm, glucose output by the right kidney and glucose output by the left kidney in the studied groups.

	Blood glucose (mg/dl)	Glucose uptake by diaphragm (mg/g/90min)	Glucose output by right kidney (mg/g/hr)	Glucose output by left kidney (mg/g/hr)
Sham	78 ± 2.6 (16)	6.7 ± 0.2 (16)	3.4 ± 0.15 (15)	3 ± 0.2 (16)
OVX – STZ	480 ± 15.4 [a] (32)	1.3 ± 0.04 [a] (32)	9.1 ± 0.32 [a] (32)	9.1 ± 0.23 [a] (32)
E_2 + OVX – STZ	213 ± 4.2 [a b] (16)	3.9 ± 0.18 [a b] (16)	6.1 ± 0.22 [a b] (16)	6.5 ± 0.25 [a b] (16)
Insulin + OVX – STZ	97 ± 4.5 [b c] (13)	3.7 ± 0.23 [a b] (13)	4.9 ± 0.19 [a b c] (13)	4.8 ± 0.32 [a b c] (13)
E_2 + Insulin + OVX– STZ	94 ± 2.7 [b c] (15)	6.4 ± 0.23 [b c d] (15)	4.3 ± 0.11 [b c] (15)	4.4 ± 0.18 [a b c] (15)

Number in parenthesis is the number of rats in each group. Results are expressed as means ±SEM. a: significance from sham group by LSD at P<0.05. b: significance from OVX-STZ group by LSD at P<0.05. c: significance from E_2 + OVX-STZ group by LSD at P<0.05. d: significance from insulin + OVX-STZ group by LSD at P<0.05.

Table (3): Plasma triglycerides (TG), plasma total cholesterol (TC), plasma high density lipoprotein cholesterol (HDL-c), plasma low density lipoprotein cholesterol (LDL-c) and atherogenic index (AI) in the studied groups.

		TG (mg/dl)	TC (mg/dl)	HDL-c (mg/dl)	LDL-c (mg/dl)	AI
Sham	(16)	46 ± 1.40	85 ± 1.1	52 ± 0.93	24 ± 1.19	1.6 ± 0.05
OVX –STZ	(32)	107 ± 1.9 [a]	129 ± 1.1 [a]	26 ± 0.57 [a]	82 ± 1.33 [a]	5.1 ± 0.12 [a]
E_2 + OVX–STZ	(16)	56 ± 1.14 [a b]	98 ± 2.4 [a b]	48 ± 2.1 [a b]	39 ± 1.03 [a b]	2.1 ± 0.05 [b]
Insulin + OVX–STZ	(13)	69 ± 3.89 [a b c]	88 ± 1.4 [b c]	37 ± 1.19 [a b c]	37 ± 1.28 [a b]	2.4 ± 0.06 [a b]
E_2 + Insulin + OVX–STZ	(15)	52 ± 1.11 [b d]	94 ± 1.5 [a b d]	49 ± 0.98 [b d]	34 ± 1.37 [a b]	1.9 ± 0.03 [b d]

Number in parenthesis is the number of rats in each group. Results are expressed as means ±SEM. a: significance from sham group by LSD at P<0.05. b: significance from OVX-STZ group by LSD at P<0.05. c: significance from E_2 + OVX-STZ group by LSD at P<0.05. d: significance from insulin + OVX-STZ group by LSD at P<0.05.

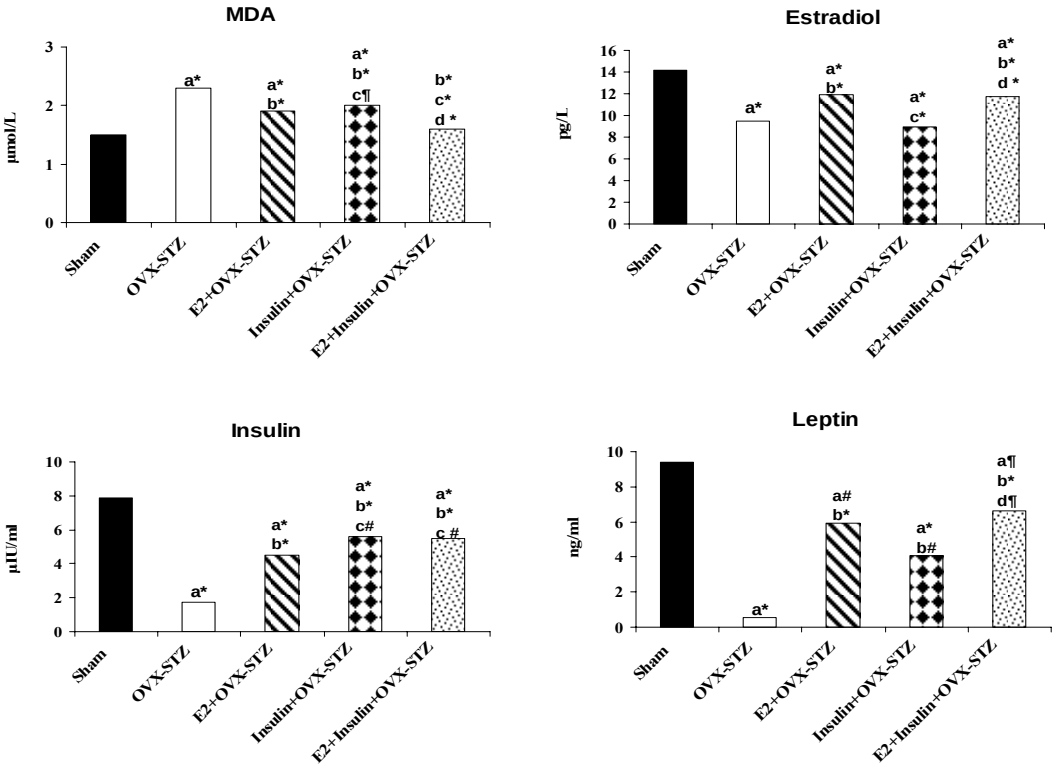

Figure (1): Mean values of plasma levels of malondialdehyde (MDA), estradiol, insulin and leptin in the different groups studied.

¶: P<0.05, #: P<0.01, *: P<0.001, a: significance from sham group by LSD. b: significance from OVX-STZ group by LSD. c: significance from E_2 + OVX-STZ group by LSD. d: significance from insulin + OVX-STZ group by LSD.

Table (4): Correlation coefficients (r) between plasma estradiol levels and other parameters in the groups of rats studied.

Parameters	OVX – STZ + Sham		E_2 + OVX – STZ + Sham		Insulin + OVX –STZ + Sham		E_2 + Insulin + OVX–STZ + Sham	
	r	P	r	P	r	P	r	P
Blood glucose	-0.88 (28)	<0.001	-0.86 (24)	<0.001	-0.57 (22)	<0.01	-0.63 (23)	<0.01
BMI	0.22 (28)	NS	0.13 (24)	NS	-0.63 (22)	<0.01	0.36 (23)	NS
Plasma TG	-0.91 (28)	<0.001	-0.62 (24)	<0.01	-0.54 (22)	<0.05	-0.46 (23)	<0.05
Plasma TC	-0.85 (28)	<0.001	-0.42 (24)	<0.05	-0.20 (22)	NS	-0.72 (23)	<0.001
Plasma HDL-c	0.88 (28)	<0.001	0.49 (24)	<0.05	0.82 (22)	<0.001	0.30 (23)	NS
Plasma LDL-c	-0.86 (28)	<0.001	-0.76 (24)	<0.001	-0.81 (22)	<0.001	-0.72 (23)	<0.001
Atherogenic index	-0.87 (28)	<0.001	-0.75 (24)	<0.001	-0.82 (22)	<0.001	-0.61 (23)	<0.01
Plasma Insulin	0.86 (19)	<0.001	0.80 (19)	<0.001	0.59 (17)	<0.05	0.70 (18)	<0.01
Plasma Leptin	0.76 (18)	<0.001	0.28 (15)	NS	0.50 (15)	NS	0.06 (17)	NS
Plasma MDA	-0.83 (28)	<0.001	-0.68 (24)	<0.001	-0.87 (22)	<0.001	-0.34 (23)	NS

In parenthesis is the number of observations. NS: not significant

Table (5): Correlation coefficients (r) between plasma insulin levels and other parameters in the groups of rats studied.

Parameters	OVX – STZ + Sham		E_2 + OVX – STZ + Sham		Insulin + OVX –STZ + Sham		E_2 + Insulin + OVX–STZ + Sham	
	r	P	r	P	r	P	r	P
Blood glucose	-0.91 (28)	<0.001	-0.90 (24)	<0.001	-0.49 (22)	<0.05	-0.69 (22)	<0.001
Glucose uptake by diaphragm	0.97 (28)	<0.001	0.89 (24)	<0.001	0.79 (22)	<0.001	0.47 (22)	<0.05
Glucose output by right kidney	-0.86 (28)	<0.001	-0.74 (24)	<0.001	-0.48 (22)	<0.05	-0.47 (22)	<0.05
Glucose output by left kidney	-0.94 (28)	<0.001	-0.83 (24)	<0.001	-0.61 (22)	<0.01	-0.65 (22)	<0.01
BMI	0.19 (28)	NS	-0.09 (24)	NS	- 0.62 (22)	<0.01	-0.03 (22)	NS
Plasma TG	-0.91 (28)	<0.001	-0.66 (24)	<0.001	-0.59 (22)	<0.01	-0.40 (22)	NS
Plasma TC	-0.93 (28)	<0.001	-0.56 (24)	<0.01	-0.23 (22)	NS	-0.48 (22)	<0.05
Plasma HDL-c	0.94 (28)	<0.001	0.31 (24)	NS	0.62 (22)	<0.01	0.29 (22)	NS
Plasma LDL-c	-0.94 (28)	<0.001	-0.71 (24)	<0.001	-0.54 (22)	<0.05	-0.49 (22)	<0.05
Atherogenic index	-0.93 (28)	<0.001	-0.68 (24)	<0.001	-0.61 (22)	<0.01	-0.51 (22)	<0.05
Plasma Leptin	0.88 (27)	<0.001	0.61 (20)	<0.01	0.75 (20)	<0.001	0.55 (20)	<0.05
Plasma MDA	-0.87 (28)	<0.001	-0.71 (24)	<0.001	-0.65 (22)	<0.01	-0.21 (22)	NS

In parenthesis is the number of observations. NS: not significant

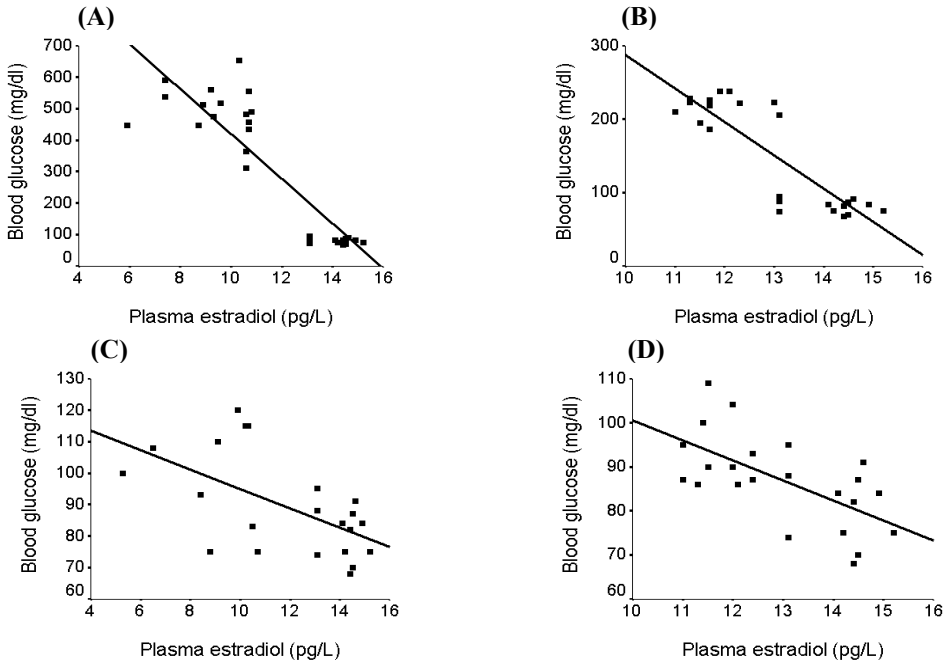

Figure (2): Graphs showing relationships between plasma estradiol and blood glucose in
(A) Untreated OVX-STZ diabetic group + Sham-operated controls (r = -0.88, P<0.001, n= 28).
(B) Estradiol-treated OVX-STZ diabetic group + Sham-operated controls (r =-0.86, P<0.001, n=24).
(C) Insulin-treated OVX-STZ diabetic group + Sham-operated controls (r = -0.57, P<0.01, n=22).
(D) Combined estradiol and insulin–treated OVX-STZ diabetic group + Sham-operated controls (r = -0.63, P<0.01, n=23).

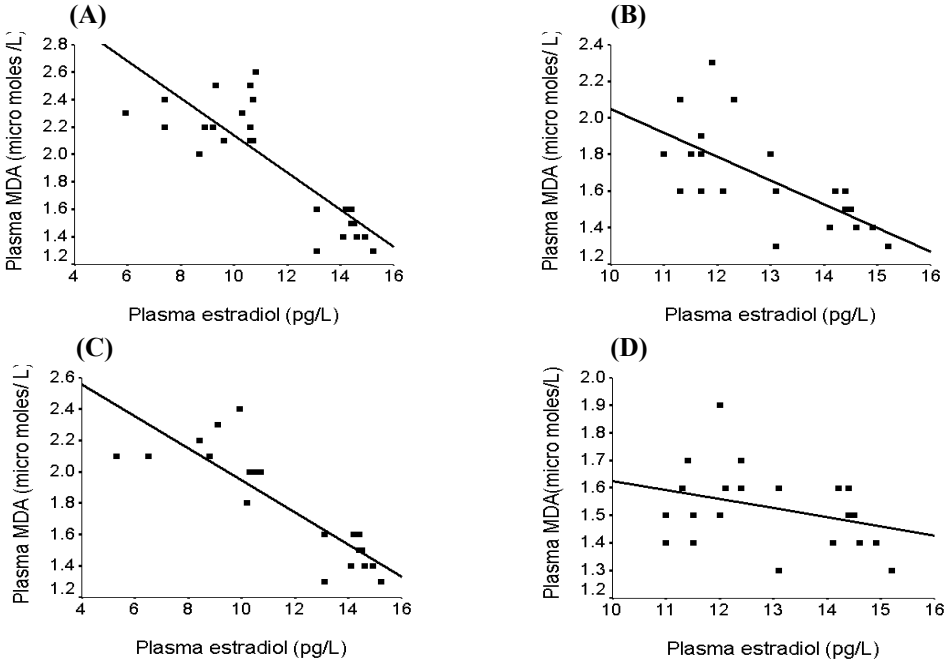

Figure (3): Graphs showing relationships between plasma estradiol and plasma MDA in
(A) Untreated OVX-STZ diabetic group + Sham-operated controls (r = -0.83, P<0.001, n=28).
(B) Estradiol-treated OVX-STZ diabetic group + Sham-operated controls (r = -0.68, P<0.001, n=24).
(C) Insulin-treated OVX-STZ diabetic group + Sham-operated controls (r = -0.87, P<0.001, n=22).
(D) Combined estradiol and insulin –treated OVX-STZ diabetic group + Sham-operated controls (r = -0.34, NS, n=23).

4. Discussion:

The present work studied the extent by which either estradiol treatment or insulin treatment can improve the metabolic derangements in estrogen deficiency with diabetes and finally evaluated the gain achieved by combination of these two hormonal therapies.

Estradiol treatment was found to exert an obvious effect in improving adiposity, dyslipidemia, oxidative stress and hyperglycemia, yet still diabetic; however, with the exception of adiposity, none of the metabolic parameters were back to control values. Insulin therapy successfully normalized blood glucose, reduced dyslipidemia and oxidative stress; however, estradiol therapy was more effective in reducing oxidative stress and adiposity compared to insulin therapy. Combination of both hormonal therapies in estradiol-treated, insulin-treated ovariectomized diabetic group offered the most beneficial effect as it successfully resulted in optimization of weight gain, reduced adiposity, good glycemic control, reduced dyslipidemia and normal oxidative state.

In insulin-treated group, blood glucose was normalized, diaphragmatic glucose uptake was increased as compared to untreated group but did not differ from estradiol-treated group and was still significantly lower than the control values. Renal glucose output was decreased as compared to untreated and estradiol treated groups but still significantly higher than control values. It seems that insulin therapy exerted better euglycemia compared to estrogen therapy and this effect is probably via decreased gluconeogenesis rather than complete normalization of peripheral glucose uptake which could be due to insufficient dose or duration.

More favorable lipid profile was seen following insulin control of hyperglycemia, where it had prominent effect in lowering total cholesterol towards normal as well as improving triglycerides, HDL-c, LDL-c and AI as compared to untreated ovariectomized diabetic group but not back to control values. When compared to estradiol-treated group significant increase in triglycerides and decrease in total cholesterol as well as HDL-c was recorded together with non significant difference in LDL-c and AI. Insulin is the major hormone to inhibit hydrolysis of triglycerides (TG) in adipose cells into glycerol and free fatty acids (FFAs). Together with glucose, insulin may also play a role in the reesterification of FFAs in adipose cells, promoting TG storage [(19)]. By these mechanisms, insulin lowers plasma FFA levels which are the major substrates for TG synthesis in the liver, and they stimulate apoB secretion from the liver [(20)]. Thus, the ability of insulin to suppress plasma FFA concentrations plays a major role in hepatic VLDL TG synthesis and secretion, apoB secretion [(20)], and plasma VLDL cholesterol and apoB concentrations [(21)].

Following insulin treatment body weight and BMI were significantly high, this could be attributed to insulin role in promoting lipogenesis as well as its inhibitory role on protein catabolism (positive nitrogen balance) at the same time, the persisted estradiol deficiency promoted central fat deposition, so it is most likely that the increase in weight is mostly due to fat deposition as confirmed by increased BMI. The associated increase in plasma leptin level seems to be insulin-induced as insulin was reported to stimulate leptin mRNA and protein expression, due to increased activation of the leptin gene promoter [(22)].

Suppression of oxidative stress was one of the goals achieved by insulin-mediated metabolic control. Hyperglycemia is the major causative factor of raised oxidative stress in diabetes, but in this group of rats, the insulin treated ovariectomized diabetic, estrogen deficiency also participates; so administration of insulin alone reversed hyperglycemia with no obvious effect on estradiol values which led to partial improvement of oxidative stress, yet not back to normal values. It is worthmentioning here that, estradiol treatment showed more effective role in reducing oxidative stress than do insulin treatment although none of them succeeded to get it back to normal control values.

Unexpectedly, insulin treatment did not enhance aromatase activity and thereby estradiol production, instead estradiol values were very close to untreated ovariectomized diabetic rats. This could be attributed to the duration of insulin therapy or less than needed dose further investigations are needed to clarify this point.

Estradiol replacement was another line of treatment in the present study, the overall judgment about how much estradiol treatment was able to improve the metabolic state is that, although most of the measured parameters were significantly improved when compared to untreated ovariectomized diabetic rats, yet they were still significantly different from control values, a result that make it possible to say that hormonal replacement therapy per se is not sufficient to maintain good glycemic and metabolic control in postmenopausal women who developed diabetes.

The significant decrease in weight gain following estradiol treatment seems to be a combined effect of estradiol and diabetes, where besides the diabetic effect on lipid and protein metabolism, estradiol goes through different pathways to achieve an obesity reducing property, where it is known that estrogen decreases central adiposity [(23)]. This represents a

major health problem because abdominal visceral fat shows greater lipolytic sensitivity than femoral and gluteal subcutaneous fat due to fewer inhibitory alpha adrenergic receptors in abdominal regions and greater alpha adrenergic receptors in gluteal and femoral regions [24].

Estradiol treatment was associated with significant rise in plasma leptin, this could be another mechanism decreasing food intake and increasing energy expenditure and thereby decreasing body weight. Our results are in agreement with Shimizu *et al*. [25] who experimentally proved that estradiol supplementation reversed the inhibitory effect of ovariectomy on ob gene expression and circulating leptin levels and that serum leptin concentration was higher in premenopausal women than in men and postmenopausal women; this allowed them to declare that estrogen increased *in vivo* leptin production in rats and human subjects. However, studies evaluating the effect of estrogen replacement therapy on leptin levels were contradictory, with some authors supporting a stimulatory effect of estrogen whereas others suggested that estrogens do not have a stimulatory action on leptin in humans [26, 27].

Estradiol relation to ghrelin hormone provides another mechanism explaining the decrease in weight gain in this group, where, estradiol was found to attenuate the orexigenic action of ghrelin [28] and the drop of estrogen levels following ovariectomy was associated with an increase in plasma ghrelin that was associated with increased food intake, body weight, and hypothalamic neuropeptide Y [28]. From the above mentioned estradiol hormonal interactions, it could be suggested that estrogen, indeed provides protection against weight gain.

Better glycemic control was clearly demonstrated after estradiol treatment where renal gluconeogenesis was significantly decreased and skeletal glucose uptake was significantly improved and thereby blood glucose level was significantly reduced compared to untreated group, yet, their improvement was not to such an extent that their values were back to normal. This finding is consistent with results obtained from ovariectomized diabetic group that showed tendency to hyperglycemia. All these positive changes towards better glycemic control could be attributed to the roles played by estradiol both at β-cells of the pancreas as well as peripheral insulin-sensitive tissues. It was found that 17β-estradiol at physiological concentrations protects pancreatic β-cells against lipotoxicity, oxidative stress, and apoptosis [29]. Estrogens and their receptors (ER) have direct effects on islet biology. The estrogen receptor ERα, ERβ, and the G-protein coupled ER are present in β-cells and enhance islet survival. They, also, improve islet lipid homeostasis and insulin biosynthesis [29]. *In vivo*, estradiol treatment rescued streptozotocin-induced β-cell apoptosis, helped sustain insulin production, and prevented diabetes. *In vitro*, in mouse pancreatic islets and β-cells exposed to oxidative stress, estradiol prevented apoptosis and protected insulin secretion. Estradiol protection was through activation of ERα as it was partially lost in β-cells and islets treated with an ERα antagonist [30].

At the peripheral insulin-sensitive tissues, estradiol is known to modulate insulin sensitivity and, consequently, glucose homeostasis. Estradiol was found to counteract the effects of hyperglycemia-induced downstream of the insulin receptors, as well as modulating insulin receptors tyrosine phosphorylation [31]. Some data, also, revealed a surprising role for estradiol in regulating energy metabolism and opened new insights into the role of the two estrogen receptors, ERα and ERβ, in this context. New findings on gene modulation by ERα and ERβ of insulin-sensitive tissues indicate that estradiol participates in glucose homeostasis by modulating the expression of genes that are involved in insulin sensitivity and glucose uptake [32]. Therefore, drugs that can selectively modulate the activity of either ERα or ERβ in their interactions with target genes represent a promising frontier in diabetes mellitus coadjuvant therapy.

Skeletal muscle glucose uptake is maintained by one of the isoforms of the glucose transporter family, GLUT4 [33]. The rate of glucose transport into muscle cells is limited by the concentration of GLUT4 at the cell surface. The enhancement of diaphragmatic glucose uptake following estradiol administration shown in this study could partly be attributed to the elevated glucose transporter–4 protein expression. It was discovered that estradiol acts on ERα and not ERβ to enhance glucose transporter-4 expression [34].

The antiatherogenic cardiovascular protective properties of estrogen emerge from its ability to direct the lipoprotein metabolism towards higher HDL-c and lower LDL-c. It is obvious from our results that treatment with estradiol gave the ovariectomized diabetic females the opportunity of lowering their plasma lipids, and this effect was prominent by the significant improvement of all the measured lipid parameters as compared to non treated group of rats.

The mechanisms of such effects are mediated through the ability of estradiol to stimulate the expression of LDL-receptor gene and increasing the number of LDL receptors. This effect was confirmed by Parini *et al*. [35] who found that treatment of rats with ethyl estradiol for 7 days increased the hepatic LDL receptor protein and mRNA level from 3 to 4 folds. Also, Distefano *et al*. [36] reported that the expression of LDL-receptor gene is stimulated by

estrogen *in vivo*. Also, our results came in accordance with Granfone *et al*. [37] and Walsh *et al*. [38] who reported that estrogen replacement is effective in decreasing LDL-c and apo B concentrations and increasing HDL-c and apo A concentrations in dyslipidemic postmenopausal women. LDL-c internalizes into the cells through the process of LDL-receptor mediated endocytosis accelerating LDL catabolism. The expression of LDL-receptor on the cell surface is a function of various hormone regulated transcription of the receptor gene; β-estradiol is considered the prime hormonal regulator of LDL-receptor expression [39].

Another protective mechanism offered by estradiol in lowering LDL-c and increasing HDL-c is through depression of hepatic lipase enzyme activity [40], thereby decreasing HDL-c catabolism. The elevated levels of HDL-c following estradiol treatment seen in our study is in agreement with Walsh *et al*. [41] who demonstrated that HDL elevation following oral estradiol treatment in postmenopausal women is dose dependent. Estradiol fatty acyl esters incorporate into HDL and enhance the atheroprotective properties of HDL by mediating the initial steps of reverse cholesterol transport [42].

Another benefit offered by estrogen replacement therapy in ovariectomized diabetic rats was the significant decrease in plasma MDA, adding extra evidences that estrogen is more than a sex hormone and that its loss after menopause requires therapeutic intervention. It was found that lipid peroxidation is most often induced by reactive oxygen species, $\cdot O_2^-$ and H_2O_2, and this damage is inhibited by superoxide dismutase and catalase. The remaining amount of damage appears to be caused by peroxyl radicals. It was documented that estradiol (E_2) alone collectively blocks 70% of such damage [43].

This indicates that estradiol is acting as a chain-breaking antioxidant, inhibiting the effect of H_2O_2, $\cdot O_2^-$ and hydroperoxyl radicals. E_2 action in inhibiting DNA damage supports this view. E_2 prevented DNA strand breaks in a manner similar to the free radical scavengers; catalase and superoxide dismutase. E_2 might be preventing oxidative DNA damage to some extent by inhibiting the formation of superoxides [43]. Our results came in accordance with Kii *et al*. [44] who demonstrated that acute treatment with 17beta-estradiol showed a protective effect against ischemia-reperfusion injury through its antioxidant effects. Also, Hernández *et al*. [45] showed that the lower plasma total antioxidant status, reduced thiol groups and the increase in plasma lipoperoxides presented in ovariectomized animals were reestablished with the estrogen treatment. It is to be noted here that estradiol treatment although successfully lowered plasma MDA yet it was still higher than control values, this is attributed to that these rats are diabetic which represents other cause of oxidative stress.

Combination of estradiol and insulin therapy was the last line of treatment investigated in ovariectomized diabetic rats. Rats received combined treatment of estradiol and insulin showed normal pattern of gaining weight with lower BMI; this proves that normal hormonal state is essential to direct metabolism towards optimal balance between opposite metabolic pathways as lipolysis versus lipogenesis, glycolysis versus gluconeogenesis and positive versus negative nitrogen balance. It seems that the lowered adiposity seen in these rats with normal weight gain reflects the anabolic action of insulin on protein metabolism. The additive action of estradiol and insulin supplementation on leptin hormone was manifested by higher levels of this hormone in combined hormone-treated than in estradiol-treated alone or insulin-treated alone ovariectomized diabetic rats. Estradiol enhanced leptin gene expression [25] and also insulin stimulated leptin mRNA and protein expression [22]. The resulted increase in leptin values ultimately contributes in optimization of body weight.

Combined estradiol and insulin treatment successfully normalized blood glucose through optimization of skeletal muscle uptake of glucose as well as renal gluconeogenesis. It is believed that estradiol not only increases insulin secretion from pancreatic β-cells but also enhanced insulin sensitivity in target organs [4], an effect that is most obviously seen in our study through increasing diaphragmatic glucose uptake. Also, the significant positive correlation between plasma estradiol and insulin hormones seen in untreated ovariectomized diabetic rats showed less positivity following combined estradiol and insulin treatment implying an effect of estradiol on insulin hormone and thereby glycemic control.

Insulin and estradiol teamed up to shift lipid profile towards more protective healthy picture, actually triglycerides, HDL-c and atherogenic index values were completely normalized. In addition, significant negative correlations existed between both estradiol and insulin and each of total cholesterol, LDL-c and atherogenic index, whereas, the significant positive correlation between both levels of plasma estradiol and insulin with HDL-c were abolished. These results, therefore, encourages postmenopausal diabetic women not only to control their blood glucose but also to start hormonal replacement therapy.

Moreover, marked reduction of oxidative stress was recorded, following estradiol and insulin therapy, as the lipid peroxide marker, MDA, was significantly reduced to control values. Also, the significant

negative correlations between plasma levels of each of estradiol and insulin and levels of MDA, found in the ovariectomized STZ-diabetic group were abolished by combined treatment.

In explanation of these results, it could be suggested that estradiol by its antioxidant effect reduces reactive oxygen species and insulin by its hypoglycemic effect reduces blood glucose and thereby glycosylation and autooxidation of glycation products [46].

In view of the aforementioned data, insulin therapy alone which induced euglycemia, reduced dyslipidemia and oxidative stress yet their values were still higher than controls; while estrogen therapy in ovariectomized diabetic rats succeeded to some extent in reducing hyperglycemia, dyslipidemia and oxidative stress yet not completely normalized. Thus, from the above discussion it is clear that postmenopausal diabetic women suffer the consequences of both estradiol and insulin deficiency, and trials to reverse any of them although to some extent improved the condition yet they were not optimally successful. Combination of both estradiol and insulin therapies in ovariectomized diabetic rats showed synergistic effects and was superior in terms of optimization of blood glucose, peripheral glucose uptake and oxidative marker, plasma malondialdehyde together with alleviation of dyslipidemia. This denotes that insulin therapy together with hormonal replacement therapy as a coadjuvant might be the most advisable line of treatment in postmenopausal diabetic women. Therefore, we may advocate the use of estrogen replacement therapy side by side with insulin in postmenopausal diabetic women to achieve better glycemic control and thereby improving the general metabolic state.

Corresponding author
Mona A. Ahmed
Department of Physiology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
drmona_agha70@yahoo.com

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