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Tamoxifen protective role in ovariectomy related- metabolic disorders in rats

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Abstract

Background: postmenopausal women are more susceptible to insulin resistance. Many studies reported that estrogen (E2) contributes in insulin sensitivity improvement. As a result of postmenopausal estrogen replacement therapy (ERT) hazards, tamoxifen (TAM) as an alternative to estrogen are now applicable. There are no published data distinguishing the effects of TAM on insulin sensitivity in non-diabetic post menopause women. Objectives: Test whether TAM acts as E2 in improving insulin sensitivity in post- menopausal period and to what extent the effect would be similar for both, together with exploring the underlying mechanism. **Material & method**: Forty female albino rats were divided into 4 groups (10 rats each): sham (SH), ovariectomized (OV), ovariectomized E2 (OVE) treated and ovariectomized TAM (OVT) treated groups. At the end of experiment, blood samples were taken for estimation of fasting blood glucose (FBG), serum concentration of insulin, homeostasis model assessment of insulin resistance (HOMA-IR), lipid parameters and tumour necrosis factor α (TNFα), then adipose tissues were removed for measurement of MDA and SOD. Also, histopathological and immunohistochemical examination of adipose tissue were done. **Results**: Ovariectomy worsened glycemic state, insulin resistance and lipid profile. TAM was like E2 induced glucose metabolic improvement, decreased insulin resistance alongside with lipid profile improvement. These metabolic changes might assert to oxidative state and inflammatory response improvement accompanying apparent histopathological and immunohistochemical changes on adipose tissue. **Conclusion**: TAM can counterbalance the metabolic disorders in postmenopausal period and this may improve metabolic disorders as E2 with avoiding the undesired effects of ERT.

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INTRODUCTION

Estrogens (E2) have an essential role in female physiology and reproduction. Besides, they regulate multiple physiological and pathological processes (1). E2 deficiency after menopause play a critical role in the pathophysiology of obesity, insulin resistance and type 2 diabetes mellitus (2).

Insulin resistance has been defined as a condition of cells failure to respond adequately to insulin which reduce glucose uptake in muscular and adipose tissues hence promoting metabolic alterations (3). Moreover, insulin resistance is the main clinical attribute of type II diabetes mellitus and importantly plays apart in the development of other conditions such as dyslipidaemia, hypertension and atherosclerosis (4).

Low grade inflammation and oxidative stress usually go along with menopause related disorders (5). Oxidative stress is a state of overproduction of reactive oxygen species (ROS), which exceed the body's anti-oxidant defences (6). ROS are oxidative physiological processes end products (7) that bind with protein, lipid, carbohydrates and DNA within the cells, contributes in cellular damage (1).

Tamoxifen (TAM) is a non-steroidal benzothiophene which behaves as a selective estrogen-receptor modulator (SERM) and used for breast cancer treatment (8). It acts as estrogenic agonist or antagonist, depending on the target tissue. TAM exerts an E2 antagonistic effect in mammary tissue while it mimics the effects of E2 in other tissues, for example the uterus, cardiac and bone tissues, without increasing the undesired risks of E2 such as endometrial cancer (9). However, it is not yet understood whether TAM has estrogenic or antiestrogenic activities regarding insulin resistance and adipose tissues.

MATERIAL AND METHODS

Animals

Forty adult female albino rats were used in the study. The rats were acclimated to their environment for one week and had adlibitum access to tap water and rodent standard diet. All procedures conducted in accordance to the Guide of the Care and Use of Laboratory Animals and approved by the Ethical Committee for Scientific Research at the Menoufia Faculty of Medicine.

Experimental design

Rats were divided into four groups (10 rats per group):

1- Sham operated control group [SH group]: rats were subjected to the same surgical procedure and anesthesia without removal of the ovaries.

2- Ovariectomized group [OV]: ovaries were bilaterally removed under anesthesia.

3- Ovariectomized estrogen treated group [OVE]: after ovariectomy rats were treated with subcutaneous injections of 17β-estradiol valerate (30 mg/kg body weight) dissolved in 0.2 mL sesame oil, three days a week *(Sigma-Aldrich Corp., St. Louis, MO, USA)* (10).

4- Ovariectomized TAM treated group [OVT]: ovariectomized rats were subjected to oral gavage with TAM (2 mg/kg once daily) *(Sigma-Aldrich Corp., St. Louis, MO, USA)* (11).

Drugs treatment started on the second day after ovariectomy and lasted for eight weeks.

Surgical procedure of ovariectomy:

Rats were anesthetized with phentobarbital sodium (60 mg/kg, intraperitoneally (*Sigma–Aldrich Chemie GmbH, Steinheim, Germany)*. bilateral ovariectomy was performed with a single abdominal incision. The abdominal skin and peritoneum were opened, both ovarian arteries were ligated, and both ovaries were removed. The muscle and the skin were sutured. Povidone iodine was applied on the area to disinfect the skin. (12). SH rats were anaesthetized, the skin and muscle layers opened; ovaries manipulated but not removed. Rats allowed to recover in individual cages for one week, during which they received a commercial pelleted diet and water adlibitum and topical antibiotic for five days (13).

Samples collection and biochemical analysis:

At the end of the study period, fasting blood samples were collected from the retro-orbital venous plexus, using a fine heparinized capillary tube introduced into the medial epicanthus of the rat's eye. Two millilitres of blood were collected in a clean graduated centrifuge tube, left for clotting at room temperature in a water bath for 10 minutes, and then centrifuged at 3000 r.p.m (rotation per minute) for 20 minutes. The supernatant serum was collected in a dry clean tube. The collected samples were used for measurement of FBG, lipid profile, serum insulin, homeostasis model assessment of insulin resistance (HOMA IR index) and tumour necrosis factor α (TNFα).

Determination of serum glucose

FBG was determined using enzymatic glucose oxidase method *(Spinreact, Spain)*.

Determination of HOMA-IR

Serum insulin concentration measured using enzyme-linked immunosorbent assay (ELISA) kit *(CAT # RAB0904; Sigma, Germany)* according to the manufacturer's instructions. The insulin resistance index (IRI) assessed by homeostasis model assessment estimate of insulin resistance (HOMA-IR):

HOMA IR = Fasting insulin (MU/ml) X Fasting glucose (mg/dl)/405

Determination of serum lipids

Total cholesterol (TC), triglycerides (TG) and high density Lipoprotein-cholesterol (HDL-C) determined by colorimetric methods *(Spinreact, Spain)*. Low density lipoprotein-cholesterol (LDLC) concentrations estimated according to the formula specified by (14).

LDL-cholesterol = Total cholesterol - (HDL - cholesterol+ TG/5)

Determination of serum TNFα

Serum TNFα was determined by enzyme linked immunosorbent assay (ELIZA) kits *(sunred, Shanghai Biological Technology Co., Ltd, China)*

Isolation of adipose tissues:

Adipose tissues were removed from all groups, then it was homogenized and the resultant supernatant was used for measurement of malondialdahyde (MDA) and superoxide dismutase (SOD) using colorimetric kits *(Biodiagnostic, Egypt)*. Also, adipose tissues were used for histopathological evaluation and immunohistochemical studies for assessment of estrogen receptors (ER).

Statistical analysis:-

All values were expressed as mean ± SEM (standard error of mean). Data analyses were performed using SPSS version 16 for Windows **(SPSS Inc., Chicago, IL, USA)**. All data were

first tested for normality using Shapiro-Wilk test. All data were normally distributed and compared by one-way ANOVA followed by post-hoc Tukey's test. P value of <0.05 is considered to be significant.

RESULTS

Serum FBG level was significantly decreased in OVT group when compared to the OV (86.5±3.4 Vs 145.6 ± 4.5 mg/dl…….. P < 0.01). There was no significant difference in serum FBG levels between OVT, SH and OVE (86.5±3.4 vs 86.7 ±5.4 and 75.6 ± 6.1 mg/dl...P ≥ 0.05) **(Figure 1)**.

Serum insulin level was significantly decreased in OVT when compared to the OV and OVE groups (3.8 ± 0.1 Vs 7.8± 0.4 and 4.2 ± 0.1 Mu/ml …….. P < 0.01). There was no significant difference in serum insulin levels among OVT and SH (3.8 ± 0.1 vs 3.6 ± 0.4 Mu/ml ...P ≥ 0.05) **(Figure 1)**.

HOMA index was significantly decreased in OVT group when compared to the OV (0.6 ± 0.01 Vs 2.8± 0.1, 4.2 ± 0.1 …….. P < 0.01). There was no significant difference in HOMA index levels among OVT, SH and OVE (0.6 ± 0.1 vs 0.8 ±0.06 and 0.7 ± 0.07 ...P ≥ 0.05) **(Figure 1)**.

Serum cholesterol level was significantly decreased in OVT group when compared to the OV and OVE (78.5±4 Vs 132.4 ±5.3 and 97.6 ±1.8 mg/dl…….. P < 0.01). There was no significant difference in serum cholesterol levels between OVT and SH (78.5±4 vs 73.6 ± 2.7 mg/dl...P ≥ 0.05) **(Figure 2)**.

There was no significant difference in Serum TG levels among OVT, SH, OV and OVE (77.6 ± 5.2, 67.9 ± 1.8, 77.2 ± 4.1 and 75.03 ± 4.5 mg/dl...P ≥ 0.05). **(Figure 2)**

Serum HDL level was significantly increased in OVT when compared to OV (31.3 ±3.1 vs 21.1±3.7mg/dl …….. P < 0.01). There was no significant difference in serum HDL levels among OVT, SH and OVE (31.3 ±3.1 vs 40.3±5.2 and 34.01±2.4 Mg/dl ...P ≥ 0.05) **(Figure 2)**.

Serum LDL level was significantly decreased in OVT when compared to OV (52.2 ± 3.4 vs 76.2 ± 2.8 mg/dl …….. P < 0.01). There was no significant difference in serum LDL levels among OVT, SH and OVE (52.2 ± 3.4 vs 45.2±3.1 and 57.4 ±2.1 Mg/dl ...P ≥ 0.05) **(Figure 2)**.

Serum MDA level was significantly decreased in OVT group when compared to OV and OVE (78.1±2.1 vs, 160.8 ± 2.9 and 86.9 ± 2.1 nmole/ml …….. P< 0.01). There was no significant difference in serum MDA levels between OVT and SH (78.1±2.1 vs 74.8 ± 1.6 … nmole/ml … P ≥ 0.05) **(Figure 3)**.

Adipose tissues SOD level was significantly elevated in OVT when compared to OV (50.7 ± 3.5 Vs 12.5 ± 1.5 U/ml…….. P < 0.01). There was no significant difference in adipose tissues SOD levels among OVT, SH and OVE (50.7 ± 3.5 vs 74.7 ±3 and 40.2 ±3.3 U/ml …… P ≥ 0.05) **(Figure 3)**.

Serum TNFα level was significantly decreased in OVT when compared to OV (104.8 ± 3.2 Vs 154.4 ± 5.9 pg/ml …….. P < 0.01). There was no significant difference in serum TNFα levels among OVT, SH and OVE (104.8 ± 3.2 vs 86.9 ± 5.9 and 99.5 ±1.4 pg/ml…… P ≥ 0.05) **(Figure 3)**.

By immunohistochemical staining, ER intensity was significantly increased in OVT when compared to OV group (2.38 ± 0.18 vs 1.25 ± 0.16…….. P<0.001). There was no significant difference in ER intensity among OVT, SH and

OVE (2.38 ± 0.18 vs 2.6 ± 0.18 and 2.12 ± 0.13…… P ≥ 0.05) **(Figure 4, 5)**.

ER percentage was significantly increased in OVT when compared to OV (4.4 ± 2.9 vs 26.3±1.6…….. P<0.0001). There was no significant difference in ER percentage among OVT, SH and OVE group (74.4 ± 2.9 vs 83.8 ± 2.8 and 66.3 ± 2.6…… P ≥ 0.05) **(Figure 4, 5)**.

H score for ER expression was significantly increased in OVT group when compared to OV and OVE (197.5±8.8 vs 41.3±5.2 and 127.5±11.5…….. P <0.001). There was no significant difference in ER H score between OVT and SH (197.5±8.8 vs 216±8.9…… P ≥ 0.05) **(Figure 4, 5)**.

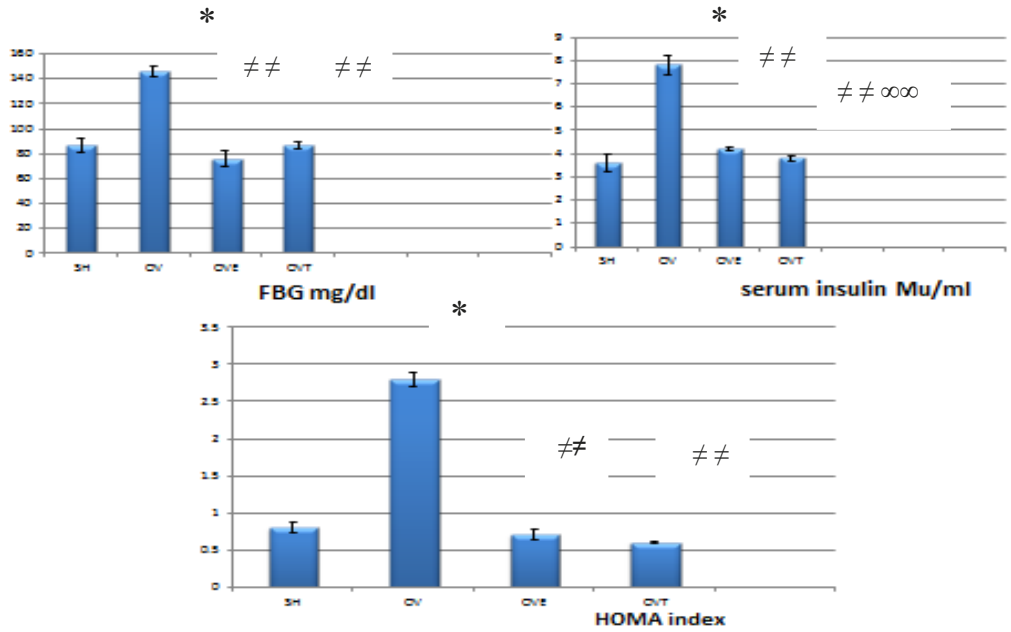

Figure 1: The mean values of FBG, serum insulin and HOMA index in the studied group (n= 10 rats per group)
***significant to SH group p value< .01, ## significant to OV group p value< .01 , ∞∞ significant to OVE p value< .01*

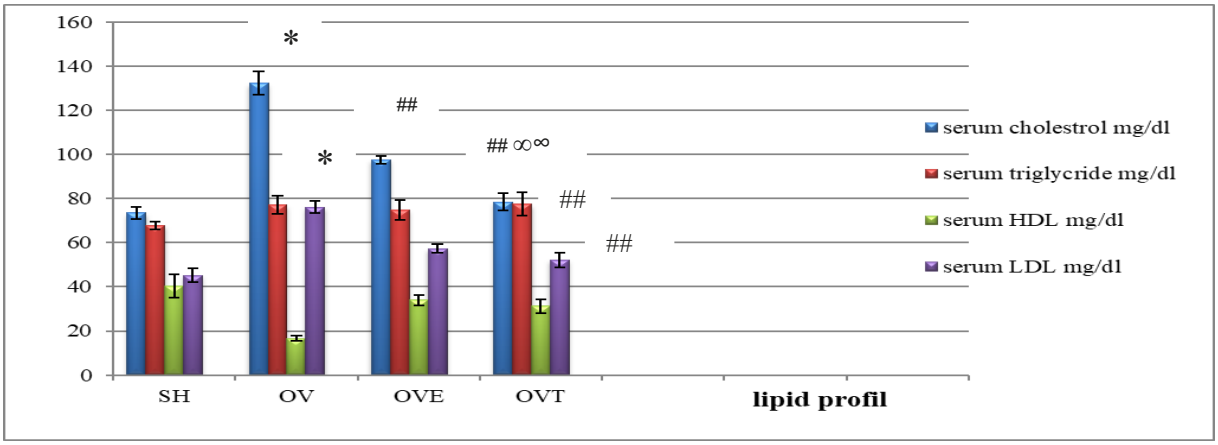

Figure 2: **The mean values of lipid profils in the studied groups (n= 10 rats per group)**
***significant to SH group p value< .01, ## significant to OV group p value< .01 , ∞∞ significant to OVE p value< .01.*

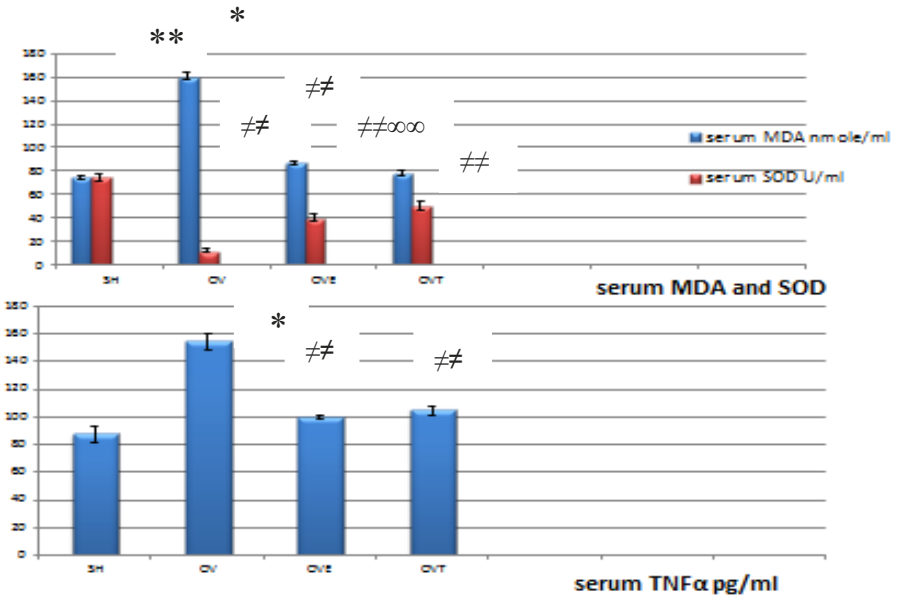

Figure 3: The mean values of MDA, SOD and TNFα in the studied groups (n= 10 rats per group)

***significant to SH group p value< .01,## significant to OV group p value< .01 , ∞∞ significant to OVE p value< .01.*

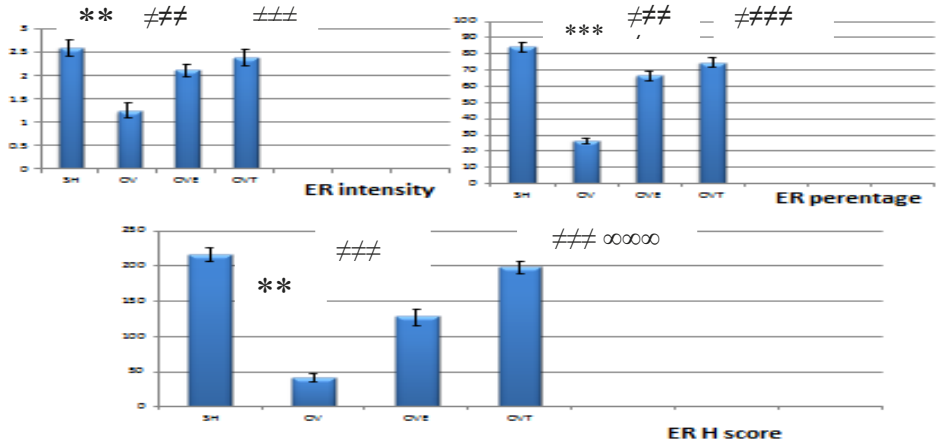

Figure 4: The mean values of ER intensity,percentage and H score in the studied groups (n= 10 rats per group)

****significant to SH group p value< .001, ****significant to SH group p value< .0001,## ≠significant to OV group p value< .001,## ##significant to OV group p value< 0.0001 , ∞∞∞ significant to OVE p value< .001, ∞∞∞∞∞ significant to OVE p value< .0001,.*

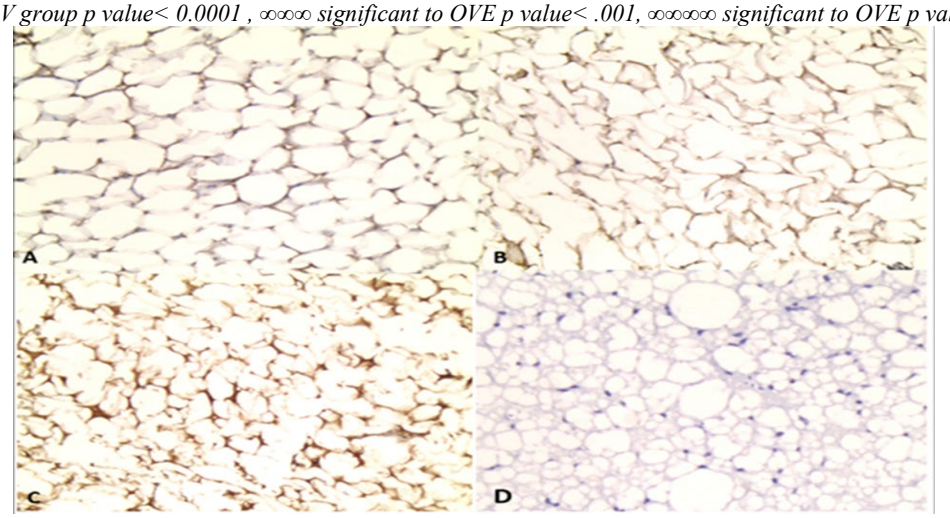

Figure 5: { A} Adipose tissue of OVE, showed scattered moderate nuclear staining of ER, {B} OV rats showed few mild ER positivity, {C} OVT showed scattered strong nuclear staining of ER in comparison with OV group. {D} sham group [IHCx200].

DISCUSSION

Postmenopausal women develop more fat in their intra-abdominal depot than premenopausal ones do and therefore have a greater possibility of developing metabolic disorders accompanying trunk obesity. For example, the prevalence of diabetes, dyslipidemia, hypertension, and coronary heart disease increases more rapidly in aging women than in premenopausal ones (15). OV rats imitate menopause and E2 deficiency in rats causes obesity and its associated complications including adipose inflammation, hyperglycemia. Based on the previous studies of TAM as SERM, we investigated whether TAM counterbalance metabolic disorders and adipose tissues inflammation in OV rats and examined the feasible underlying mechanisms of the action.

This study was conducted on the effect of TAM on OV animal (menopause model), concerning glucose homeostasis, lipid profiles, insulin resistance and ER.

The main findings include;

a) Ovariectomy according to the current study, resulted in disturbance of FBG due to insulin resistance with lipid profile disorders and elevation of oxidative stress markers and inflammatory markers with reduced ER expression.
b) TAM treatment to OV rats resulted in improvement in FBG, insulin resistance, lipid profile, oxidative- antioxidative state and ER expression.

The present study revealed that ovariectomy resulted in elevation of FBG. This may be due to impairment of glucose metabolism as a result of increased insulin resistance (as indicated in this study by increased insulin concentration and elevated HOMA-IR index). This was in agreement with the results obtained by Fahmy et al. (1), they reported that ovariectomy worsened glucose metabolism and impaired insulin tolerance test. This suggested that loss of ovarian hormones resulted in insulin resistance. Sankar et al. (2015) explained the mechanism of glucose intolerance (GI) and IR in OV rats by reduced expression of adipose and hepatic insulin receptor beta (IRβ) and adipose tissue GLUT4 (5). Moreover, ovariectomy decreased phosphorylation of activated mitogen protein kinase (AMPK) and its substrate acetyl-CoA carboxylase in adipose tissue. Several genes in the maintenance of glucose homeostasis are also affected which are involved in decreased adiponectin, uncoupling protein 2 (UCP2) and increased resistin (16).

Compatible with the current study, it has been declared that, there was an improvement in insulin secretion, metabolism, and also the peripheral insulin sensitivity in a selected postmenopausal population treated with E2 (17). Reduction in GLUT-4 protein level induced by ovariectomy could be countered by E2 supplementation (18). E2 also, prevents building up of visceral fat, increases central sensitivity to leptin, increases the insulin receptors expression in adipocytes, and decreases the lipogenic activity of lipoprotein lipase in adipose tissue, preventing obesity and insulin resistance (19). In addition, E2 supplements restore phosphorylation of AMPK, increases the expression of adiponectin, PGC-1α, and UCP2; and decreases the expression of resistin (16).

Nevertheless, a minority of postmenopausal women currently take E2 or HRT for long period to reveal its maximum benefits, because of its side

effects such as vaginal bleeding and breast pain and uterine, breast cancer risks (20).

Current results put on view that TAM reduce FBG, insulin level and HOMA-IR, this was similar to results of Lampert et al. (11). This improvement may be due to restoration of peripheral insulin sensitivity and its hepatic clearance (17). Of course, there are reports that contradict the results of this study, since TAM is associated with increased insulin resistance and decreased insulin secretion, which may be caused by differences in the duration of diabetes and dosage of TAM.

It has been clarified that diabetes is considering as a lipid metabolism disturbance more than carbohydrate (4). This was confirmed by our result, which revealed that lipid profiles were disturbed in OV group when compared to sham operated group, this was partly in agreement with the results of Hoseini et al. (21). But TG did not differ significantly, which was similar to results of Camara et al. (22). This proposed to enhance lipolysis in insulin-resistant adipocytes (23). Increased adiposity is associated with systemic loss of E2 at menopause. However, Antunes et al. reported no significant changes in total cholesterol (TC) and LDL-C levels in OV rats (24).

Furthermore, E2 replacement therapy is conventional to reduce body weight and fat deposition and resulted in favorable changes in plasma lipid and this was further confirmed by this current study. Several clinical studies have demonstrated that E2 can directly inhibit adiposity by decreasing lipogenesis. These studies clarify that E2 is a major suppressor of fasting lipoprotein lipase (LPL) (16), an enzyme that regulates lipid uptake by adipocytes, and that E2 also represses LPL gene expression at the transcriptional level *via* an estrogenic response element (ERE) found on the LPL promoter (25). Moreover, E2 therapy in postmenopausal women decreases the expression of genes involved in lipogenesis including acetyl-CoA carboxylate-α and -β, sterol regulatory element binding protein 1c, stearoyl-CoA desaturase, LPL, fatty acid synthase and desaturase (26).

The decrease in plasma LDL by estradiol may be due to increased hepatic LDL receptor expression which increases plasma LDL clearance and secretion of cholesterol into the bile (27). In addition, LDL receptor transcription activation occurs via ERα (28). Estrogen is known to increase plasma levels of HDL cholesterol (29). This may be due to an increase of hepatic apo A-I expression (29) and by a modulation of the proteins involved in the metabolism of HDL expression, such as HDL receptor SR-BI (29).

It is well known that brown adipose tissue (BAT) is absent in adult humans, but recently, studies have elucidated that BAT may be restorative in adults and might play a role in the treatment of obesity (30). Experimental studies have shown that E2 can reinforce brown adipocytes thermogenic property, by an increase of UCP1 mRNA expression (31). ERα is expressed in BAT tissue and mainly localized in mitochondria, which indicated that BAT mitochondria could be targeted by E2 and demonstrated the possible role of ERα in synthesis of mitochondria (31).

The results demonstrated that the serum TC and LDL-C levels were reduced and HDL level was elevated by TAM, in agreement with the results of Lampert et al. (11). However, the results of this

study were in contrast with those of a Japanese study: Hozumi et al. reported that the serum TC levels remained unchanged after TAM treatment (32).

TAM may act by similar mechanism of E2, increasing hepatic LDL receptor expression with a consequent decrease in LDL levels. With regard to HDL levels, the effect of TAM is less conclusive. TAM also reduces the LPL activity in humans and rats and induces hepatic triacylglycerol accumulation, probably due to an increase in the synthesis or a fatty acid oxidation blockade (33).

One of the most important finding in this work is that ovariectomy resulted in elevated MDA (an indicator of oxidative stress) and reduced SOD (antioxidant enzyme) in adipose tissues, when compared to sham operated group. This was in agreement with results of Lamas and his colleagues (34), they revealed an increased production of ROS in OV rats compared with the sham rats. The exacerbation of oxidative stress could be explained by loss of E2 which are efficient anti-oxidants, while ovariectomy increased the expression of pro-oxidative enzyme NADPH oxidase (NOX4) mitogen activated protein (MAP) kinases ERK 1/2 and p38 at different tissues (5). Oxidative stress has a major role in the development of insulin resistance (35). Also, Matsuda and Shimomura, 2013 suggested that both oxidative stress and decreased antioxidant defense mechanisms had a part in increasing insulin resistance in diabetic rats. E2 treatment significantly reduced reactive oxygen species (ROS) production at the level of adipose tissues in OV rats (36). The same results were reported by Faulds et al. (37). This supports the idea that E2 has a direct antioxidative effect due to the presence of a phenolic group in the steroid structure, which refines glucose metabolism and insulin resistance. However, the mechanisms of the antioxidant effect of E2 are incompletely understood and may vary in different tissues. E2 might play an inhibitory role either in production and/or scavenging of ROS. An ER-dependent inhibition of superoxide anion production has been linked to the activation of mitogen activated protein kinase (MAPK) (36).

In addition, it is also known that inflammation is a secondary to impaired oxidative metabolism. This explains presence of an inflammatory state in OV rats as indicated by high pro-inflammatory cytokine (TNF-α). Minihane et al. (38) proposed that pro-inflammatory mediators released from adipose tissue impaired the insulin signaling and induced insulin resistance.

It was shown recently that heat shock protein72 (HSP72) overexpression prevents fat-induced inflammation and insulin resistance in male mice (39). HSP72 levels are modulated by E2 and or ERα. HSP72 expression was found to be absent in ERα knock out (40).

Treatment with TAM or E2 leads to decrease in the level of inflammatory marker TNFα. In line with the results of the present study, TAM was able to reduce the levels of interferon γ and TNF-α in the study performed by Behjati and Frank (41) and therefore, improved insulin signaling. Currently, little information is available about the TAM anti-inflammatory mechanism (s), and possibly TAM reduces inflammation by having an impact on the soluble TNF-α receptors expression (42).

From the previous studies, it was a strong relationship between oxidative stress, inflammation and insulin resistance in OV rats. And this, explained the mechanisms by which E2 and TAM ameliorated insulin resistance in OV rats by improving oxidative state and its associated inflammatory condition.

Although adipose tissue is considered a non-classical target of E2 stimulation, in vivo and in vitro studies have shown that ER are included in the modulation and distribution of body fat mass, and these receptors, expressed on adipose tissue, appears to mediate the lipolytic effect of E2. Two forms of the ER have been identified, ERα and ERβ, respectively. Both receptors are expressed in a variety of cell types; however, the α isoform is more highly expressed than the β isoform in insulin-responsive tissues such as adipose tissues (43). Altered fat distribution and weight gain are sequel of menopausal hypoestrogenism, but the mechanisms responsible are not completely known.

Immunohistochemistry of this study showed that TAM over expressed ER in adipose tissue more than E2 suggesting that TAM acts as an agonistic effect on ER. Because E2 deficiency causes metabolic dysfunction, obesity, metabolic syndrome and type 2 diabetes, hence, there is a great potential for the treatment of these pathologies with appropriate ER-activating agents.

This study has several potential limitations. It tested a single dose of E2 and TAM for a short-term, 8-week, duration. It is unclear what the dose equivalency between TAM and E2. These doses were chosen because they are the standard ones used in clinical practice for prevention or treatment of osteoporosis in postmenopausal women. Because the study was terminated after 8 weeks of treatment, the effect of the longer use of TAM or E2 would have on insulin sensitivity and metabolic parameters cannot be predicted. Also, because only the effect of unopposed E2 was studied, the results cannot be extended to the more common clinical use of estrogen in combination with progesterone therapy. Further studies would be helpful to confirm these results and clarify questions about dosing, duration, and long-term effects of these therapies.

In summary, after 8 weeks of drug therapy, TAM was able to reduce insulin resistance and improved lipid profil in OV rats mostly, by reducing oxidative stress and inflammatory marker and overexpression of ER in adipose tissues.

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