Oral hormonal therapy with ethinylestradiol/levonorgestrel improves insulin resistance, obesity and glycogen synthase kinase-3 independent of circulating mineralocorticoid in estrogen-deficient rats

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Oral hormonal therapy with ethinylestradiol/levonorgestrel improves insulin resistance, obesity and glycogen synthase kinase-3 independent of circulating mineralocorticoid in estrogen-deficient rats

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Running title: Oral HRT improves cardiometabolic disorder via suppressed GSK-3 independent of aldosterone

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Abstract

Estrogen deficiency has been associated with increased cardiovascular diseases (CVD) and recent clinical trials of standard formulations of hormonal therapies have not demonstrated consistent beneficial effects. Estrogen-progestin therapy has been used as exogenous estrogen to normalise depressed estrogen level during menopause. Ovariectomized rodents mimic estrogen-deficient state in that they develop **cardiometabolic** dysfunction, including insulin resistance (IR). We therefore hypothesized that hormonal therapy with combined oral contraceptive steroids, ethinylestradiol/levonorgestrel (EEL) improves IR, obesity and glycogen synthase kinase-3 (GSK-3) through reduction in circulating mineralocorticoid level in ovariectomized rats. Twelve week-old female Wistar rats were divided into four groups; sham-operated (**SHM**) and ovariectomized (OVX) rats treated with or without EEL (1.0µg ethinylestradiol and 5.0µg levonorgestrel) daily for 8 weeks. Results showed that OVX **or SHM+EEL-treated rats** had increased HOMA-IR, 1-h postload glucose, HOMA-β, triglycerides (TG), total cholesterol (TC), TC/HDL-cholesterol, TG/HDL-cholesterol, plasma insulin, GSK-3, corticosterone and aldosterone. On the other hand, **OVX+EEL treatment** ameliorated all these effects except aldosterone. Taken together, the results demonstrate that oral hormonal replacement with EEL improves IR, pancreatic β-cell function, suppresses GSK-3 and glucocorticoid independent of circulating aldosterone, suggesting a positive cardiometabolic effect of oral EEL **therapy** in estrogen-deficient state.

Key words: Cardiometabolic syndrome, oral contraceptive, hormone replacement therapy, GSK-3, aldosterone

Introduction

Sexual dimorphisms in the development of cardiovascular diseases (CVDs) are well documented in both human and animal studies. The incidence of CVDs among women increases sharply **after menopause** (Mendelsohn and Karas 1999) which is thought to result, in part, from the deficient endogenous estrogen **with** its associated cardioprotective effects (Ren and Kelley 2009). **The cessation of ovarian function after menopause or ovariectomy** is associated with increased fat, along with metabolic pathologies including insulin resistance (IR) and type 2 diabetes (T2DM) (Carr et al. 2004). The fact that increasing numbers of postmenopausal women (**Post-MW**) expect longer life spans requires an understanding of the pathologies of metabolic diseases associated with **the** estrogen deficiency observed in them. Ovariectomy in rodent is considered to resemble human **postmenopausal** estrogen **deficient** state (Ko et al. 2013) as **it causes** increase fat mass (Rogers et al. 2009; Kanaya and Chen 2010) and chronic inflammation accompanied by IR (Abu-taha et al. 2009; Abbas andElsamanoudy 2011; Choi et al. 2012; Stubbins et al. 2012), implying a connection between **the cardiometabolic** complications observed **during** estrogen deficiency and CVDs. More so, metabolic syndrome afflicts both men and women and increases the risk of heart disease in both sexes, although it appears to impose a greater burden in women.

Insulin resistance (IR) is a condition whereby there is reduced sensitivity of organs to insulin-initiated physiologic processes **which results** in metabolic defects. IR occurs in 20%–25% of the human population (Holland et al. 2007) **and** is rapidly becoming one of the major public health challenges, signifying an enormous global economic burden (Wang et al. 2010). IR is a hallmark of both the prediabetic state and overt T2DM, and also a critical metabolic link among hypertension, dyslipidemia, T2DM, obesity and atherosclerotic cardiovascular disease (CVD)

(Psaty and Furberg 2007; Fonseca 2010; DeFronzo 2010; Bodmer et al. 2010; Jiang et al. 2010; Faulds et al. 2012).

GSK-3, a serine/threonine kinase which can be regulated by insulin, can phosphorylate and inactivate glycogen synthase (Parker et al. 1983; Roach 1990; Zhang et al. 1993). **GSK-3 can serve as a negative modulator of the insulin action on glycogen synthesis and, potentially, glucose transport activity** (Cross et al. 1995; Eldar-Finkelman and Krebs 1997). Hence, GSK-3 has been implicated in the development of IR, pancreatic β -cell dysfunction and diabetes (Eldar-Finkelman et al. 1996; Nikoulina et al. 2000; Liu et al. 2008; Tanabe et al. 2008; Liu et al. 2010).

Combined oral contraceptives containing a combination of ethinylestradiol and levonorgestrel are used by over 100 million women around the world (Hillard 2005). Since its introduction, the impact of COC on cardiometabolic morbidity remains unresolved (Wei et al. 2011; Olatunji et al. 2016). Surprisingly, estrogen plus progesterone therapy has been used as exogenous estrogen to normalize the physiologic drop in estrogen level seen **in Post-MW** (Rossouw et al. 2002) but with conflicting results.

A **major** issue faced by most **Post-MW** is the potential impact of estrogen replacement therapy on the prevalence of CVDs **among them. Despite the sufficient experimental evidence of vascular benefits of estrogen, randomized clinical trials such as the Heart and Estrogen/progestin Replacement Study (HERS) and Women's Health Initiative (WHI) have suggested that, contrary to expectations, hormone replacement therapy (HRT) in Post-MW may increase the risk of atherothrombotic CVD events** (Rossouw et al. 2002; Manson et al. 2003), hence innovative alternative strategies for prevention of CVD in **Post-MW** are necessary. We therefore designed the present study to investigate whether oral hormonal therapy with EEL will improve IR,

obesity and GSK-3 in OVX rats. We also aimed at testing the hypothesis that the beneficial effects of the EEL in OVX rats would be through mineralocorticoid-dependent pathway.

2. Materials and methods

2.1. Animals

The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (The National Academies Press, 2011) and every effort was made to minimize both the number of animals used and their suffering. The experimental protocol was reviewed and approved by the University Ethical Review Committee, University of Ilorin. Twenty female Wistar rats (10-12 weeks) were obtained from the animal house of the College of Health Sciences, University of Ilorin (Ilorin, Nigeria) and kept under standard environmental conditions of temperature, relative humidity, and dark/light cycle with unrestricted access to standard rat chow and tap water.

2.2. Surgical procedure

After 1 week of acclimatization, animals were anesthetized (ketamine, 50 mg/kg i.p.) under aseptic conditions and underwent ovariectomy (OVX group) or sham surgery (SHM group). The ovariectomy was performed by a ventral abdominal midline incision to access the abdomen. Ovaries were bilaterally clamped and removed. The uterine horns were clamped and the uterus was left intact. Then, the abdominal wall was sutured. After surgery, rats were maintained under good conditions to recover. In the sham procedure, animals were anesthetized and the abdominal wall was opened like the OVX rats, the ovaries were exteriorized to create similar stress, but they were not removed (Marques et al. 2006).

After a 1-wk resting period, each group (SHM and OVX) was randomized into four new groups (n=5/group) based on the treatment: sham-operated control (SHM), ovariectomized rats (OVX), sham-treated with EEL (SHM+EEL) and OVX+EEL groups. Food and water intakes were monitored daily while body weight was weekly.

2.3. Treatment

SHM and OVX groups received distilled water (vehicle; *po*) while the EEL-treated groups (SHM+EEL and OVX+EEL) received (*po*) a combination of 1.0 µg ethinylestradiol and 5.0 µg levonorgestrel (Wyeth–Ayerst, Inc., Montreal, Canada) daily. The treatment lasted for 8 weeks.

2.4. Sample preparation

At the end of treatment, the rats were anesthetized with pentobarbital sodium. Blood was collected by cardiac puncture into heparinized bottle and was centrifuged at 3000 rpm for 5 min. Plasma was stored frozen until needed for biochemical assay. Liver and pancreas were excised, blotted and weighed immediately.

2.5. Oral glucose tolerance test (OGTT), insulin resistance (IR) and pancreatic β -cell function

Glucose challenge test was performed 24 h before the end of the experiment. The rats had 12 h overnight fast. Glucose (2 g/kg bw) was given (*po*). Blood sample was obtained from the tail vein before glucose load and then sequentially after 30, 60, 90 and 120 min and blood glucose was determined immediately after each sample was obtained with a glucometer (ONETOUCH®-LifeScan, Inc., Milpitas, CA, USA). Glucose tolerance was expressed as a

function of the area under the curve (AUC) for oral glucose tolerance test (OGTT) as previously described (Olatunji et al. 2012) and postload glucose responses. The IR was determined using the homeostasis model assessment for insulin resistance ($\text{HOMA-IR} = \text{fasting glucose (mmol/l)} * \text{fasting insulin (}\mu\text{IU/l)} / 22.5$) where as $\text{HOMA-}\beta$ ($20 * \text{fasting insulin (}\mu\text{IU/l)} / \text{fasting glucose minus } 3.5$) and 1-h postload glucose level were used to determine pancreatic β -cell function. Elevated 1-h postload glucose level is a reliable predictor of IR, pancreatic β -cell function, atherosclerotic CVD and renal dysfunction (Succurro et al. 2010; Sciacqua et al. 2011; Marini et al. 2012; Bianchi et al. 2013). Triglyceride-glucose index was also used to determine IR ($\text{TyG-index} = \text{Ln} [\text{TG (mg/dl)} * \text{FPG (mg/dl)} / 2]$) (Simental-Mendia et al. 2008; Guerrero-Romero et al. 2010).

2.6. Biochemical assays

Corticosterone, aldosterone, 17β -estradiol (E2) and GSK-3, were determined using ELISA kits (Elabscience Biotechnology Co., Ltd., Wuhan, China). Insulin was determined using ELISA kit (Ray Biotechnology, Georgia, USA). Total cholesterol (TC) and triglyceride (TG) were measured by standardized enzymatic colorimetric methods using assay kit obtained from Fortress Diagnostics Ltd. Antrim, UK. High-density lipoprotein cholesterol (HDL-C) was measured by enzymatic clearance assay (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) whereas low-density lipoprotein cholesterol (LDL-C) was estimated using Ananandaraja formula (Ananandaraja et al. 2005). TC/HDL-C and TG/HDL-C ratios were estimated as marker of lipid atherogenic index.

2.7. Statistical analysis

All data were expressed as means $\pm$ standard deviation (SD). Statistical group analysis was performed with SPSS statistical software. One-way analysis of variance (ANOVA) was used to compare the mean values of variables among the groups. Bonferroni's test was used to identify the significance of pair wise comparison of mean values among the groups. Statistically significant differences were accepted at $p < 0.05$.

3. Results

3.1. Effects of EEL on body weight gain, food intake, visceral adiposity and 17β -estradiol level in OVX rats

After ovariectomy and 8 weeks of experimentation, OVX rats gained more weight than the SHM rats (**Table 1 and Figure 1A**), and they also had increased visceral adiposity (**Figure 1B**) suggesting that estrogen deficiency leads to obesity in rats. SHM+EEL rats also had increased visceral adiposity compared with SHM rats (**Figure 1B**) despite no significant difference in body weight (**Table 1 and Figure 1A**). EEL treatment in OVX rats prevented both the increases in body weight and visceral adiposity (**Table 1 and Figure 1B**). OVX rats and SHM rats treated with or without EEL had significantly increased food intake (**Table 1**). As expected, the OVX and SHM+EEL rats had significantly reduced plasma E2 levels compared with OVX+EEL and SHM rats. (**Table 1**). OVX+EEL rats displayed modestly elevated plasma E2 level compared with SHM (**Table 1**).

3.2. Effects of EEL on fasting and postload glycemia in OVX rats

Despite the observed normoglycemia in all the experimental groups (Table 2), OVX rats had elevated 1-h postload glycemia when compared with the SHM rats (Figure 2A). The SHM+EEL-treated rats also had increased 1-h postload glycemia compared with the SHM rats (Figure 2A). EEL treatment in OVX rats attenuated the elevated 1-h postload glycemia. Area under glucose tolerance curve was increased in the SHM+EEL rats and OVX rats compared with SHM rats (Figure 2B). Treatment with EEL in OVX rats attenuated the area under glucose tolerance curve when compared with the OVX rats (Figure 2B).

3.3 Effects of EEL on fasting insulinemia, IR, pancreatic β -cell function and TyG in OVX rats

There was increased fasting insulinemia in the SHM+EEL and OVX rats compared with SHM rats. EEL treatment in OVX rats attenuated the fasting insulinemia compared with OVX rats (Figure 3A). HOMA- β , HOMA-IR and TyG values were increased in the SHM+EEL and OVX rats respectively, compared with the SHM rats (Figure 3B-D). However, HOMA- β , HOMA-IR and TyG were significantly attenuated respectively, in the OVX+EEL rats compared with the OVX rats (Figure 3B-D).

3.4. Effects of EEL on atherogenic lipids in OVX rats

Triglyceride (TG), total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) were elevated in SHM+EEL and OVX rats compared with SHM rats (Table 2). The TG, TC and LDL-C were however reduced in OVX+EEL rats compared with OVX rats (Table 2). High density lipoprotein cholesterol (HDL-C) was significantly decreased in all the experimental

groups compared with SHM rats (**Table 2**). Atherogenic lipid indices (TG/HDL-C and TC/HDL-C) were elevated in SHM+EEL and OVX rats (**Figure 4A & B**) whereas OVX+EEL treatment led to attenuation of the atherogenic lipid indices compared with OVX rats.

3.5. Effects of EEL on pro-inflammatory markers in OVX rats

Plasma uric and PLT/LYM ratio were increased in SHM+EEL rats compared with the SHM rats (**Figure 5A & B**). However, uric acid level in OVX and OVX+EEL rats were not significantly different from SHM rats. PLT/LYM ratio on the other hand, was increased in OVX rats but was attenuated in OVX+EEL rats.

3.6 Effects of EEL on GSK-3, circulating glucocorticoid and mineralocorticoid in OVX rats

GSK-3 and corticosterone were increased in SHM+EEL and OVX rats whereas in the OVX+EEL-treated rats there was significant decrease in GSK-3 and corticosterone compared with the OVX rats (**Figure 6 & 7A**). However, circulating aldosterone was increased in all the experimental groups compared with SHM rats (**Figure 7B**).

Discussion

In the present study we sought to investigate whether oral hormonal therapy with ethinylestradiol/levonorgestrel (EEL) will improve IR, obesity and GSK-3 in ovariectomized (OVX) rats through reduction in circulating aldosterone. Our results show that OVX rats developed IR that is accompanied by elevated 1-h postload glucose level and pancreatic β -cell dysfunction, hyperinsulinemia, atherogenic dyslipidemia, increased circulating corticosterone and aldosterone and depleted estradiol (E2) level. The results also indicate that oral therapy with

EEL replenished the E2 level in OVX rats, thereby improving IR, obesity, pancreatic β -cell function and GSK-3. In addition, the beneficial effect of the EEL in OVX rats is independent of circulating aldosterone. To the best of our knowledge, our study is the first to make use of this EEL as a source of E2 repletion in OVX rats.

Epidemiological, clinical and molecular studies have shown that estrogen plays an important role in metabolic homeostasis (Barros and Gustafsson 2011) as well as nutritional state (Gao et al. 2007) and the loss of estrogen may have profound effects on glucose homeostasis and body composition in both **Post-MW** (Lovejoy et al. 2008) and rodents (Jones et al. 2000; Rogers et al. 2009). In the present study, OVX rats had increased body weight gain which was accompanied by increased visceral fat deposition (**Table 1 and Figure 1B**). EEL treatment in OVX rats however, led to reduced body weight gain and visceral fat content. This finding is consistent with previous studies (Mueller and Korach 2001; Ren and Kelley 2009) in which repletion of estrogen was found to decrease body weight in obese animals.

Postmenopausal women tend to have a higher risk in developing obesity and thus IR because estrogens play an important role in the control of energy homeostasis in females (Park et al. 2003; Simpson et al. 2005a). The estrogen receptor (ER) contributes to the modulation and distribution of body fat mass, and its expression in adipose tissue appears to be responsible for the lipolytic effect of estrogen (Mueller and Korach 2001). It is well established that estrogen deficiency seems to have an unfavorable impact on mobilization of fatty acids, body fat distribution and glucose-absorbing capacity of different tissues (Tremollieres and Pouilles 1996; Toth et al. 2000; Jones et al. 2007). Total body fat mass in **Post-MW**, for example, is around 28% higher compared to premenopausal women (Ley et al. 1992; Toth et al. 2000). **Similarly**

ovariectomized rats with depleted estrogen level had increased body weight gain and fat mass whereas estrogen treatment antagonizes these effects in a positive manner (Richard et al. 1987; Hertrampf et al. 2007; Zoth et al. 2010).

In consonance with our finding, reports exist that endogenous estrogen favors body weight homeostasis by increasing energy expenditure. Likewise exogenous estrogens also may promote energy balance by influencing both energy intake and energy expenditure (Rogers et al. 2009). Furthermore, estrogen deficiency through ovariectomy or menopause results in a decrease in energy expenditure causing increased body weight, adiposity and obesity whereas increased estrogen (ER α and ER β) signaling resulting from elevating serum E2 concentrations suppresses energy intake and increases energy expenditure. The effects of E2 on energy balance are primarily mediated by ER α . Therefore, Heine and co-workers reported that female mice ER α gene mutations result in obesity (Heine et al 2000). Similarly, deletion of ER α in mice blocks the antiobesity effect of estrogen replacement (Ohlsson et al. 2000; Geary et al. 2001).

The present study showed that the OVX and SHM+EEL treatment induced disturbed metabolism that is associated with dysregulated glucose homeostasis despite unaltered fasting glycemia. Furthermore, our finding that OVX and SHM+EEL rats showed significantly elevated insulin levels compared to SHM control and OVX+EEL rats may perhaps be explained by the fact that deficiency of female sex hormones results in declined insulin-stimulated glucose disposal (Livingstone and Collison 2002). Likewise, increased body weight gain and visceral fat observed in OVX rats is accompanied with IR, impaired glucose tolerance and pancreatic β -cell

dysfunction. This is in consonance with the metabolic dysfunction associated with estrogen deficiency in Post-MW.

Postmenopausal estrogen deficiency in human and experimental studies has been reported to enhance overall IR (Bailey and Ahmed-Sorour 1980; Ho and Mosca 2002; Ko et al. 2013) while the use of HRT for treating estrogen deficiency symptoms has been conflicting (Rossouw et al. 2002; Manson et al. 2003). Furthermore, due to remarkable increases in life expectancy in developed countries, many women will spend the second half of their lives in a state of estrogen deficiency. Apart from degenerative diseases of the cardiovascular, skeletal, and central nervous systems, estrogen deficiency enhances metabolic derangement predisposing to obesity, T2DM and metabolic syndrome (Carr, 2003; Mauvais-Jarvis, 2011). Thus, the involvement of estrogen deficiency in the pathogenesis of multiple chronic diseases in women is emerging as a new global therapeutic challenge. Hence, factors or intervention that could promote or prevent the development of IR, pancreatic β -cell dysfunction and its associated atherosclerotic CVD risk factors will be of great public health importance, particularly in Post-MW.

Our finding that OVX led to increased body weight gain and visceral adiposity was associated with IR, impaired glucose tolerance and pancreatic β -cell dysfunction is consistent with a previous study (Riant et al. 2009) in OVX mice. An elevated 1-h postload glycemia has been shown to be a reliable independent determinant of impaired pancreatic β -cell function and IR that are accompanied by increased atherosclerotic CVD and renal dysfunction even in individuals otherwise regarded as having normal glucose tolerance (Manco et al. 2010; Succurro et al. 2010; Sciacqua et al. 2011; Marini et al. 2012; Bianchi et al. 2013). The protection of the OVX+EEL

rats from IR may be explained by the increase in E2 level (Table 1). In this context, EEL use might be a suitable alternative for HRT, especially in the treatment of IR and hence, **metabolic dysfunction associated with estrogen deficiency or menopause**. This is because oral hormonal therapy with EEL used in this experiment led to repletion of the E2 level in OVX rats which was accompanied by improvements in IR and pancreatic β -cell dysfunction. Although still physiological (Bryzgalova et al. 2008; Rogers et al. 2009), repletion of estrogen with EEL results in slightly higher plasma concentrations than were measured in the SHM group.

Interestingly, EEL treatment in sham-operated (SHM) rats resulted in IR that is accompanied by reduced body weight gain and increased visceral adiposity suggesting that EEL-induced IR is associated with accumulated visceral adiposity regardless of body weight gain. These findings further imply that IR/cardiometabolic disorder induced by EEL is associated with similar effect on visceral adiposity. Accumulation of visceral fat is located upstream of metabolic syndrome and fat distribution is important in metabolic disorders (Matsuzawa et al. 2004). The observation that EEL induces IR in non-OVX rats is consistent with previous studies in our laboratory (Olatunji et al. 2016).

Studies have revealed that in Post-MW, when the ovaries fail to produce E2, E2 does not function as a circulating hormone; rather, it is synthesized in extragonadal sites such as breast, brain, muscle, bone, and adipose tissue where it acts locally as a paracrine or intracrine factor (Simpson et al. 2005b). Therefore in Post-MW, the determinant of E2 action is not circulating estrogens; rather, E2 function depends on estrogen biosynthesis from a circulating source of androgens. Consequently, in these individuals, a major driver of E2 action is the aromatization of androgens to estrogens (Simpson et al. 2005b). Hence, the presence of estrogen in OVX rats,

though significantly reduced compared to the ovary-intact SHM rats and estrogen repleted OVX+EEL rats in the present study.

The finding that IR induced by OVX and EEL treatment in SHM rats is accompanied by increased atherogenic dyslipidemia characterised by elevations of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG) concentrations, decrease in high-density lipoprotein cholesterol (HDL-C) and TG/HDL-C ratio which is a useful marker in identifying individuals at risk for developing atherosclerotic CVD is noteworthy (Musso et al. 2011; Kim and Choi 2013; Phan and Toth 2014). These disturbances in OVX rats are consistent with previous studies in animals (Hong et al. 2009) and humans (Mooradian 2009; Yazdani et al. 2014) while that noticed in SHM+EEL-treated rats is consistent with previous study in our laboratory (Olatunji et al. 2016). The observed derangements in serum lipids may be a cause or a consequence of IR and depleted E2 levels (Matthews et al. 2009). Interestingly, EEL treatment in OVX rats ameliorated these atherogenic dyslipidemia except HDL-C. This is consistent with a previous study in humans in which repletion of estrogen reduced plasma TC and LDL-C with no change in HDL-C (Xu et al. 2014).

It is interesting to note that our findings in the current study that EEL treatment in SHM rats caused increased pro-inflammatory biomarkers (plasma uric acid and platelet-lymphocyte ratio; PLT/LYM ratio) is in consonance with some studies among women using EEL (Woodward et al. 1999). PLT/LYM ratio has been shown to be an inflammatory marker (Smith et al. 2008; Wang et al. 2013) and this ratio was recently assessed in relation to coronary artery disease (Temiz et al. 2014). Evidences exists that increased pro-inflammatory markers (Tzoulaki et al. 2007) play considerable pathogenic role in the development of IR. Surprisingly, OVX caused increase in

only PLT/LYM. Treatment with EEL in OVX rats ameliorates the PLT/LYM ratio. Increased visceral fat and pro-inflammatory activity have been observed in **Post-MW** (Pfeilschifter et al. 2002; Lee et al. 2009).

There is a growing interest in the role of corticosteroids, aldosterone and glucocorticoid, in the pathogenesis of IR (Sherajee *et al.* 2012). Aldosterone plays a critical role in CVD as well as in metabolic disorder including IR, T2DM and obesity (Sowers et al. 2009; Briet and Schiffrin, 2011). Aldosterone exerts negative effects on structural and functional integrity of the pancreatic β -cell by favouring inflammatory and oxidative stress conditions, which lead to decreased insulin release and actions, including actions in the vasculature (**Urbanet *et al.* 2010**). Glucocorticoids (GCs), like cortisol in humans and corticosterone in rodents, are stress and catabolic hormones primarily responsible for mobilizing glucose to the circulation from the liver and also by inhibiting the uptake and utilization of glucose in the skeletal muscle and adipose tissue. Hence, IR, glucose intolerance and pancreatic β -cell dysfunction as well as other features observed in cardiometabolic disorder are concerns in conditions with elevated circulating GCs or during GC-based therapy (Schacke et al. 2002; Gulliford et al. 2006; Fransson et al. 2013).

Since glucocorticoids have also been reported to cause impaired insulin action/dysmetabolism through glycogen synthase kinase-3 (GSK-3)-dependent pathway (Ruzzin et al. 2005). Therefore, the results from the present study that estrogen deficiency through OVX induces IR that is accompanied by elevated circulating GC (Boisseau et al. 2013), **aldosterone**, and GSK-3 is suggestive of the possibility that OVX-induced IR might be attributed to GC/GSK-3 or **aldosterone-mediated mechanisms**. **The elevated GC and GSK-3 in SHM+EEL group is consistent with previous finding in our laboratory where amelioration of cardiometabolic**

disorder was associated with decreased GSK-3 (Michael and Olatunji, 2017). EEL treatment in OVX rats ameliorates the GC and GSK-3, whereas the elevated aldosterone was not affected by the treatment. Hence the beneficial effects of EEL treatment in postmenopausal estrogen-deficient state may be independent of circulating mineralocorticoids.

Conclusions

Abrupt reduction in circulating E2 triggers complex changes resulting in adverse cardiometabolic events. Our study demonstrates that replacement of E2 with oral estrogen-progestin in OVX rats improves IR and obesity, at least in part, by suppression of GSK-3 and glucocorticoid regardless of circulating aldosterone. Therefore, usage of oral estrogen-progestin with EEL might be a suitable alternative for HRT in postmenopausal women.

Conflict of Interest

The authors declare no conflict of interest.

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Table 1: Effect of oral hormonal therapy on body weight, food and water intake and 17 β -estradiol level in estrogen-deficient rats

	SHM	SHM+EEL	OVX	OVX+EEL
Final body weight (g)	218.2 $\pm$ 28.2	221.8 $\pm$ 19.0	242.3 $\pm$ 23.3*	220.9 $\pm$ 22.6 [#]
Food intake (g/kg. bw)	67.2 $\pm$ 6.7	85.3 $\pm$ 12.7*	76.5 $\pm$ 15.4*	80.8 $\pm$ 8.7*
Water intake (ml/kg. bw)	105.4 $\pm$ 3.6	113.9 $\pm$ 12.7*	98.6 $\pm$ 7.4	122.9 $\pm$ 4.9* [#]
17 β -estradiol (pg/ml)	320.2 $\pm$ 16.8	281.9 $\pm$ 21.7*	226.4 $\pm$ 15.2*	352.3 $\pm$ 19.5 [#]

Data are expressed as mean $\pm$ SD of 5 rats per group. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test (* p <0.05 vs SHM; [#] p <0.05 vs OVX). SHM; sham, SHM+EEL; sham + ethinylestradiol/levonorgestrel, OVX; ovariectomy, OVX+EEL; ovariectomy+ ethinylestradiol/levonorgestrel

Table 2: Effect of oral hormonal therapy on fasting blood glucose and lipid parameters in estrogen-deficient rats

	SHM	SHM+EEL	OVX	OVX+EEL
Blood glucose (mmol/l)	3.4 $\pm$ 0.2	3.7 $\pm$ 0.4	3.7 $\pm$ 0.4	3.6 $\pm$ 0.4
Triglyceride (mg/dl)	108.2 $\pm$ 16.1	149.0 $\pm$ 7.8*	155.7 $\pm$ 15.4*	123.6 $\pm$ 8.7* [#]
Total cholesterol (mg/dl)	62.9 $\pm$ 11.6	84.8 $\pm$ 8.9*	100.1 $\pm$ 9.6*	84.4 $\pm$ 10.1* [#]
HDL-cholesterol (mg/dl)	70.6 $\pm$ 9.4	31.8 $\pm$ 14.8*	23.3 $\pm$ 3.8*	22.3 $\pm$ 3.8*
LDL- cholesterol(mg/dl)	40.5 $\pm$ 6.5	56.8 $\pm$ 8.7*	59.3 $\pm$ 10.5*	44.2 $\pm$ 12.5 [#]

Data are expressed as mean $\pm$ SD of 5 rats per group. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test (* p <0.05 vs SHM; [#] p <0.05 vs OVX). SHM; sham, SHM+EEL; sham + ethinylestradiol/levonorgestrel, OVX; ovariectomy, OVX+EEL; ovariectomy+ ethinylestradiol/levonorgestrel

FIGURE LEGENDS

Figure 1. Effect oral hormonal therapy on body weight gain (A) and visceral adiposity (B). There was significant increase in body weight gain and visceral adiposity in OVX rats. However, EEL therapy attenuated the body weight gain and visceral adiposity in OVX+EEL–treated group. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 5 rats per group (* p <0.05 vs SHM; # p <0.05 vs OVX).

Figure 2. Effect oral hormonal therapy on oral glucose tolerance test (OGTT; A) and area under curve (AUC) of OGTT (B).OVX led to increase in 1hr postload glucose and AUC respectively.The increase in 1hr postload glucose and AUC was attenuated in OVX+EEL–treated.Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 5 rats per group (* p <0.05 vs SHM; # p <0.05 vs OVX).

Figure 3. Effect oral hormonal therapy on fasting insulinemia (A) HOMA- β (B) insulin resistance (IR; C) and triglyceride index (TyG; D).OVX led to increased fasting insulinaemia, HOMA-BETA, IR and TyG which were attenuated in the OVX+EEL– treated rats. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 5 rats per group (* p <0.05 vs SHM; # p <0.05 vs OVX).

Figure 4. Effect oral hormonal therapy on atherogenic indices (A&B). OVX led to an increase in atherogenic dyslipidemia which was attenuated the OVX+EEL– treated rats. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 5 rats per group (* p <0.05 vs SHM; # p <0.05 vs OVX).

Figure 5. Effect oral hormonal therapy on circulating pro-inflammatory markers (A&B). COC treatment caused an increase in PLT/LYM ratio when compared with the SHM. OVX+EEL treatment attenuated the increased PLT/LYM ratio observed in the OVX animals. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 5 rats per group (* p <0.05 vs SHM; # p <0.05 vs OVX).

Figure 6. Effect oral hormonal therapy on circulating glycogen synthase kinase-3 (GSK-3).OVX led to increase in circulating GSK-3 compared with the SHM which was attenuated in the OVX+EEL rats.Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 5 rats per group (* p <0.05 vs SHM; # p <0.05 vs OVX).

Figure7. Effect oral hormonal therapy on circulating glucocorticoids and mineralocorticoids.OVX led to increase in circulating glucocorticoid and mineralocorticoid compared with the SHM. However, while the increased glucocorticoid was attenuated in the OVX+EEL rats, the mineralocorticoid remained unchanged. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SD of 5 rats per group (* p <0.05 vs SHM; # p <0.05 vs OVX).

Figure 8. Schematic diagram of the possible mechanism by which oral hormonal therapy improves insulin resistance, obesity and GSK-3. EEL; ethinylestradiol/levonorgestrel, GSK-3; glycogen synthase kinase-3.

Draft

Figure 1

Figure 2

A

B

Figure 3

Figure 4

Figure 5

A

B

Figure 6

Figure 7

Figure 8