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Potential effects of incretin-based therapies on polycystic ovary syndrome in rats: a comparative study of linagliptin versus liraglutide

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ABSTRACT

Low glucagon like peptide1 (GLP-1) level may contribute to the metabolic dysfunction in polycystic ovary syndrome (PCOS). In this study, prospective therapeutic effects of incretin-based drugs; linagliptin versus liraglutide were investigated on letrozole induced PCOS rats. Animals were divided into five groups (control, PCOS, linagliptin, liraglutide and combined). Letrozole was administered for seven weeks (1mg/kg/day,orally). Linagliptin (3mg/kg/day,orally), liraglutide (1.2mg/kg/day,sc) and combined drugs were given for 4 weeks. Measurements of anthropometric, hemodynamics, blood glucose indices, HOMA-IR, serum lipids, TNF-α, NF-kB, and sex hormones were estimated. Antioxidant activities alongside immunohistochemical (PCNA) studies were assessed. The present results proved that both drugs significantly ameliorated most of anthropometric, glycemic, lipid, hormonal, inflammatory and antioxidant indices. Obvious histological improvement was obtained by linagliptin and combined therapy while being questionable by liraglutide . In conclusion, linagliptin caused evident ovarian histological advance rather than liraglutide. Linagliptin may represent a promise in alleviating metabolic, hormonal and unique beneficial histologic effects of PCOS.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders affecting 5- 10% of women at their reproductive age (AlSinan and Shaman, 2017). PCOS is considered as a leading cause for anovulation and infertility, together with significant metabolic alterations including obesity, hyperinsulinism, metabolic syndrome, an increased risk of type 2 diabetes (T2D) and cardiovascular disease (Walters *et al.*, 2012). The incidence of metabolic syndrome is 2 - 3 times higher among

women with PCOS than among women without PCOS (Nestler, 2008). Although the pathogenesis mechanism has not been well defined, PCOS is frequently associated with insulin resistance (IR), chronic inflammation and oxidative stress (OS). Moreover increased secretion of luteinizing hormone (LH) compared to follicle stimulating hormone (FSH) and hyperandrogenism are also classical features of PCOS (Meek *et al.*, 2013).

Many hormonal and inflammatory features of human PCOS are observed among various rodent models including the letrozole-induced rat model (Walters *et al.*, 2012). Letrozole is a potent and selective third-generation aromatase inhibitor, which can selectively block the production of estrogen without disturbing other steroidogenic pathways (Kabel *et al.*, 2017), thus causing increase in the production of androgens developing PCOS. Letrozole induced PCOS had an enhanced level of OS along with hyperglycemia and hyperlipidemia. Subsequently, increased weights of rats and irregular estrous cycle (Jahan *et al.*, 2016). The role of insulin in the pathophysiology of PCOS is very important because it acts in synergy with LH to increase the synthesis of androgens in ovarian cells; in addition the ovaries of women with PCOS appear to be more sensitive to the effect of insulin (Diamanti-Kandarakis and Papavassiliou, 2006). Insulin-sensitizing agents have been used for several years for treatment of PCOS; these agents improve insulin action by increasing insulin sensitivity, thereby decreasing hyperinsulinemia (Pasquali and Gambineri, 2006).

Incretin-based therapy represents a new class of antihyperglycemic drugs for treatment of T2D. The gut-derived incretin hormone glucagon-like peptide1 (GLP-1) enhances glucose-stimulated insulin and lowers glucagon secretions (Janardhan and Sastry, 2014). (Aydin *et al.*, 2014) reported that the metabolic dysfunction in patients with PCOS may include low GLP-1 levels. Liraglutide (LR), a synthetic GLP-1, is approved for treatment of T2D (Neumiller *et al.*, 2010). Previous study reported that LR treatment significantly decreased both body weight and abdominal adiposity in animals with PCOS (Vanessa Hoang *et al.*, 2015). Subcutaneous injection is the main disadvantage of GLP-1 receptor agonists administration. Moreover, rapid degradation by dipeptidyl peptidase-4 (DPP-4) and renal clearance of GLP-1 result in a short half-life of 1 to 2 minutes (Janardhan and Sastry, 2014). Incretin mimetic agents and protease DPP-4 inhibitors (DPP-4i) cause the antihyperglycemic properties of GLP-1 to amplify pancreatic secretion of insulin and inhibit glucagon secretion (Drucker and Nauck, 2006). DPP-4i extends the half-life of endogenous gastrointestinal GLP-1, thereby prolonging its effects (Elkind-Hirsch *et al.*, 2008). It is reported that DPP-4i may improve β - cell function and decrease IR which is the main stay in the pathogenesis of PCOS (Jensterle *et al.*, 2017). Linagliptin (LN) is a selective oral DPP-4i; however, the benefits of DPP-4i alone or in combination with GLP-1 receptor agonists have not been evaluated in the prediabetic PCOS population. The present research is aimed to assess the effects of linagliptin in competition with liraglutide on letrozole - induced PCOS in rats. Also, to determine whether their combination is more beneficial than monotherapy regarding the hormonal, metabolic and ovarian morphology.

MATERIALS AND METHODS

Experimental animals and study procedure

The experimental protocol of the current study was approved by the Ethics Committee of Animal Research of Pharmacy College, Umm Al-Qura University (UQU-COP-EA#143903). The animals were cared for in accordance of the National Institute of Health Guidelines for the care and use of laboratory animals and of the standards of the Convention of Bioethics of the Council of Europe in 1997. Forty female albino Wister rats 6 weeks aged, weighing about 65g to 100g, were purchased from the animal house of King Fahd Medical Research Center, Jeddah, KSA, the animals were kept on free access to water and standard pellets and were allowed one week before starting the experiment for accommodation. All rats were randomly divided into five groups (eight rats for each): Control group were received 0.5ml of 0.5% carboxymethyl cellulose (CMC) once /day orally for the whole period of the study, Letrozole (PCOS) group, linagliptin group received linagliptin (Boehringer Ingelheim, Germany) (3 mg/kg/day, p.o.) (Koibuchi *et al.*, 2014) liraglutide group were given liraglutide (Novo Nordisk, USA) (1.2mg/kg/day, S.C) (Garber *et al.*, 2009) and combined (linagliptin + liraglutide) group. All groups except the control were orally administered letrozole (Axapotex inc, Toronto, Canada) (1mg/kg/day) (Kafali *et al.*, 2004) for 7 weeks. While linagliptin, liraglutide and their combination were initiated in week 4 in addition to letrozole and continued up to the end of the experiment. All agents were suspended in 0.5% CMC solution.

Assessment of estrous cycle

Vaginal smear cytology was done daily to monitor the estrous cycle phases throughout the entire experiment. Smears were collected by vaginal wash-

ing with 0.1ml normal saline using a micropipette, then analyzed with light microscope. The rat estrous cycle usually lasts about 4 days; Only the rats with at least three consecutive 4–5 days regular estrous cycles were considered as regular (Jashni *et al.*, 2016). At the beginning of experiment all rats showed regular cycles.

Anthropometric measurements

Changes in body weight and food intake were recorded every week in the studied groups throughout the experiment. Percentage of weight gain, body length, body mass index (BMI), and Lee index (LI) were determined during the day of dissection (Kabel *et al.*, 2017). Body length was defined as the distance from nose to anus of rats. Lee index reflects the body fat as a parameter [LI= body weight (g) $^{1/3}$ $\times$1000 / body length (cm)] (Beloosesky *et al.*, 2004). Ovary weight, periovarian and mesenteric fat were also evaluated using the usual measurement procedures. The bilateral ovaries of one rat were weighed, in which the mean values were regarded as the ovary weight (Kabel *et al.*, 2017).

Blood pressure measurement

Rat blood pressure and heart rate (HR) were assessed every week by CODA Monitor system, a computerized noninvasive blood pressure monitoring system (Kent Scientific, Torrington, CT, USA) which measures tail blood pressure by means of volume pressure. Recording of the digital value for the systolic blood pressure (SBP) and diastolic blood pressure (DBP) were expressed as millimeters of mercury (mm Hg). While recorded values of HR were expressed as beats per minute (bpm) (Zheng *et al.*, 2008).

Oral glucose tolerance test (OGTT)

One day before the end of experiment, all rats were fasted overnight and infused intragastrically with 2 g glucose per kilogram of body weight. Rat tail blood samples were taken at 0 min, 30 min, 60 min and 120 min to evaluate fasting blood glucose (FBG), 30 min, 1 h postprandial blood glucose (PBG1) and 2 h postprandial blood glucose (PBG2) respectively (Zhang *et al.*, 2008).

Blood Sampling, tissue preparation and histological study

After 7 weeks of the study procedure, the fasted overnight rats were anaesthetized with diethyl ether, blood sampling were performed by intracardiac puncture technique of left ventricle and 5ml of blood was collected. Centrifugation was performed at 2000 rpm for 10 min. Serum was collected and stored at – 18^{o}c for further assessments. Bilateral ovaries, periovarian and mesenteric fats were separated and washed as (Khan *et al.*, 2009). After weighing of the tissues, one ovary was frozen at - 70^{o}c for biochemical assay and the other ovary preserved in 10% neutral buffered formaldehyde and paraffin sections were prepared for histological study. Serial sections of ovaries (5 μm) were stained with haematoxylin and eosin (H&E) for routine histological examination and with Masson's trichrome to detect collagen fibers. The technique of immunohistochemical staining using proliferating cell nuclear antigen (PCNA); an auxiliary protein for DNA polymerase delta activity, was in accordance to (Wood, 1997). The morphometric data were obtained by using "Top view" image analyzer computer system (China).

Assessment of Serum Biochemical measurements

Fasting blood glucose and HbA1c levels were measured according to the instructions of the manufacturer using kits of ERBA Diagnostics (USA). Total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL) levels were analyzed according to the manufacturer's instructions of kits (AMP diagnostics, Austria). Low density lipoprotein cholesterol (LDL) was calculated using formula of (Friedewald *et al.*, 1972).

Enzyme-linked immunosorbent assay (ELISA) Measurements

The following parameters were measured using ELISA kits. Procedures and methods were performed according to the manufacturer's instructions. Determination of serum LH, FSH, estradiol and total testosterone were estimated using kits of Pars Biochem (China), and kits of tumor necrosis factor alpha (TNF-α) and nuclear factor kappa B (NF-kB) were purchased from (Wuhan Fine Biotec, China). While serum insulin was measured using kits of Crystal Chem (USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the following formula,

fasting serum insulin (ng/ml) x fasting serum glucose (mg/dl) / 405 (Deugarte *et al.*, 2005).

Determination of superoxide dismutase (SOD) and catalase in ovarian tissues

The activity of superoxide dismutase (SOD) and catalase in Tissue samples of ovary were measured according to standard protocols by the method of (Ikegami *et al.*, 2002; Aebi, 1984) respectively; (kits were commercially available from (Dijendo Molecular Technologies, USA).

Statistical analysis

Statistical analysis was done using statistical SPSS software Version 18 (SPSS Inc., Chicago, USA). Data

analysis was made using one-way analysis of variance (ANOVA). The comparison among groups was done using post-hoc Tukey test. All data were expressed as means ± standard deviation (SD) and differences were considered significant at p-value < 0.05.

RESULTS AND DISCUSSION

Anthropometric parameters

At the end of experiment, the results of PCOS group showed significant increase in percentage of weight gain, Lee index and weight of ovary, ovarian fat and mesenteric fat compared to control group (P<0.05). Treatment with linagliptin, liraglutide and their combination caused significant reduction in these parameters as compared to PCOS group (P<0.05). However, there was no significant difference between the combined group and each drug individually (P > 0.05). Data represented as mean ± SD; (n=8); BMI: body mass index, BW: body weight, HbA1c : glycosylated haemoglobin A1c, HOMA-IR : Homeostasis assessment of insulin resistance, Differences were considered significant at p < 0.05.(Table 1). Our findings in PCOS demonstrated signs of obesity, mainly visceral adiposity. In accordance it was reported that about 50% of women with PCOS were obese with a predominant abdominal fat distribution alongside increased androgen levels (Escobar-Morreale and Millán, 2007). Also, the results of both linagliptine and liraglutide indicated their impact for obesity control, it could be explained by the effect of liraglutide to suppress appetite and delay gastric emptying resulting in better weight loss (Jensterle *et al.*, 2015). Similarly, linagliptin may induce feelings of satiety and weight loss due to prolonging the GLP-1 half-life (Blech *et al.*, 2010).

Serum levels of glucose in OGTT, HbA1c, insulin, HOMA-IR and reproductive hormones in rats

As compared to control group, the OGTT in PCOS group demonstrated significant (P<0.05) increase in glucose levels of fasting (FBG), 30 min, 1h and 2 hrs post prandial blood glucose (PBG). Alongside significant (P<0.05) increase in fasting HbA1c, insulin and HOMA- IR values; indicating existent hyperinsulinemic insulin resistance. (Table 1). Treatment with linagliptin and liraglutide individually and concurrently caused significant reduction in serum glucose levels throughout the time of OGTT when compared to PCOS (P<0.05). Linagliptin significantly showed the lowest serum glucose level after 1h and 2hrs of OGTT as compared to liraglutide and combined groups. While the combination therapy induced significant reduction after 30 min of PBG level as compared to each drug alone (P<0.05). Furthermore, both drugs caused significant decrease in fasting HbA1c, insulin and HOMA- IR values as compared to PCOS group; with potential improvement in insulin sensitivity. Moreover, the combined group significantly showed the lowest values as compared to either drug alone (P<0.05) (Table 1). Concerning the effect of drugs on hormonal assay, letrozole significantly (P<0.05) increased the serum LH and testosterone levels as compared to control accompanied with decreased FSH and estradiol levels. Linagliptin, liraglutide and their combined treatment caused significant (P<0.05) amelioration in serum levels of testosterone, LH and FSH. However, these groups showed insignificant increase in estradiol levels compared to PCOS group (P>0.05) (Figure 1A). These findings were in accordance with the histological results of H & E stain, it showed significant increase in number of large sized cystic and degenerated follicles with significant(P<0.05) reduction in the mean number of mature follicles and corpus luteum in PCOS group, indicating anovulation. While linagliptin, liraglutide and combined treatment showed significant (P < 0.05) increase in number of developing follicles and corpora lutea alongside decrease cystic and degenerated follicles except for few remaining cysts in liraglutide group. (Figure 2).

The present findings were in agreement with (Shi and Vine, 2012) who stated that the inhibitory effect of letrozole on aromatase activity is one of the pathophysiologic hypotheses of PCOS development. Aromatase is the key enzyme that converts testosterone into estradiol in the ovary, sequentially, suppressed conversion of androgens to estrogens result in increased testosterone and decreased estradiol production. The later can stimulate the hypothalamus and pituitary gland for LH secretion by releasing the negative estrogen feedback response (Jahan *et al.*, 2016). Study of (Holmang *et al.*, 1990) conveyed that the increased testosterone level reduced glucose uptake by skeletal muscle in female rats and induced insulin resistance. Sequentially, hyperinsulinemia and insulin resistance may act indirectly on oocyte competence and quality; increasing the production of ovarian androgens (Palomba *et al.*, 2017). This could elucidate the point that PCOS is usually associated with T2D as well as glucose intolerance (Boudreaux *et al.*, 2006).

Our findings suggested potentially beneficial effects of liraglutide and linagliptin in improving the glycemic control and enhancing insulin sensitivity in polycystic syndrome, either by mimetic the incretin activity (Elkind-Hirsch *et al.*, 2008) or extension of the endogenous GLP-1 half-life (Jensterle *et al.*,

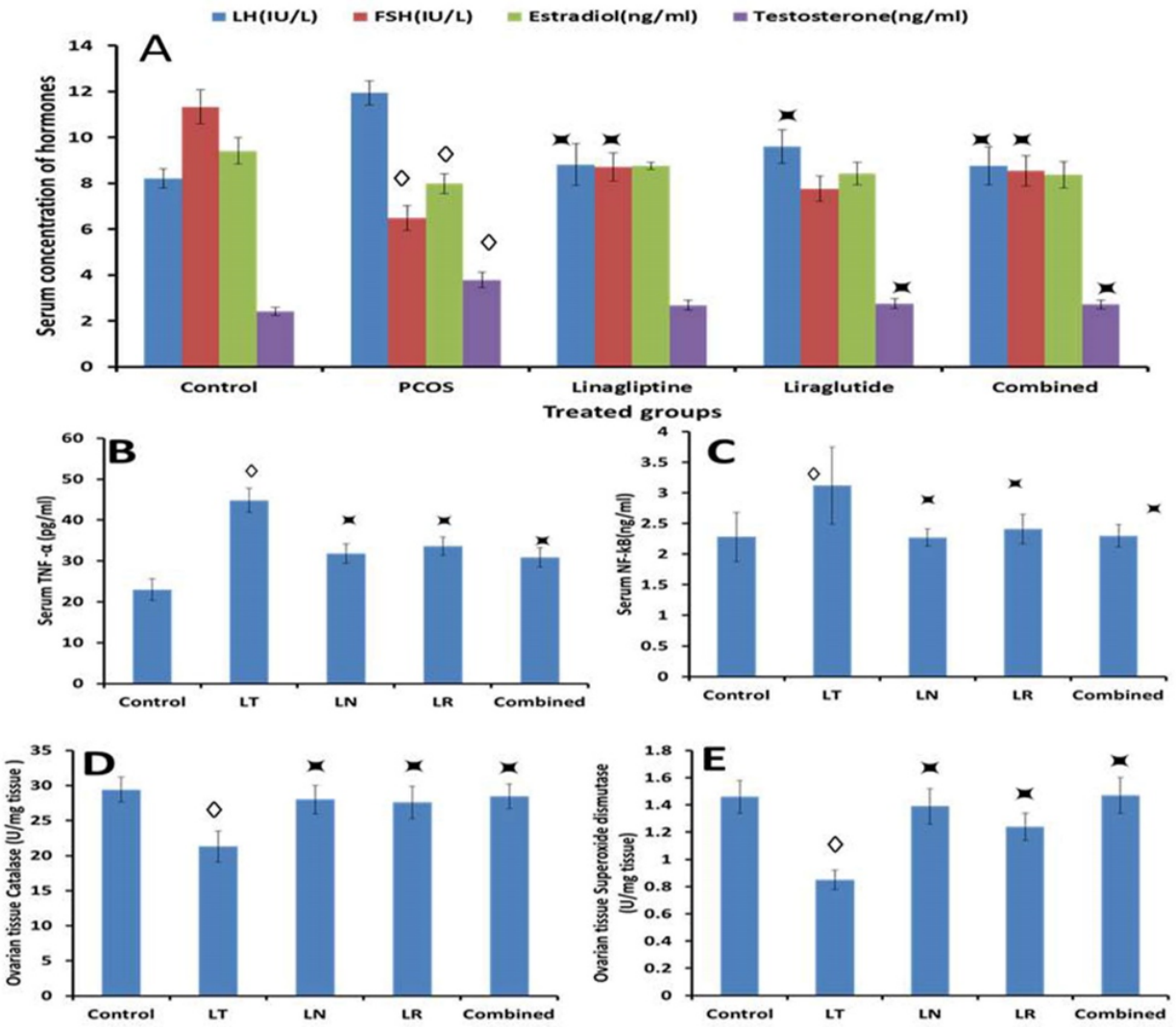

Figure 1: Serum levels of (A) reproductive hormones, (B) TNF –α, (C) NF-kB and tissue concentrations of ovarian antioxidant biomarkers; catalase (D)and superoxide dismutase (E)

2017) respectively. It is suggested that in addition to the insulinotropic action of incretin hormones, GLP-1 also promotes satiety, reduces hepatic glucose production with inhibiting glucagon secretion in a glucose-dependent manner, slows gastric emptying and inhibits gut motility (Blech *et al.*, 2010). The ameliorated effects of both drugs on the reproductive hormones could be mediated indirectly by the significant improvement of the glycemic control and insulin resistance, that is in agreement with the study of (Jakubowicz *et al.*, 1979).

Anti-inflammatory and antioxidant effects of linagliptin and liraglutide in rats

The current results revealed that letrozole administration showed significant elevation in the serum levels of TNF- α and NF-kB compared to control group (P<0.05). Treatment with linagliptin and liraglutide and their combination caused significant improvement of the above parameters compared to untreated PCOS (P<0.05), nevertheless insignificant changes among these groups (P>0.05) (Figure 1B-E). The histological findings confirmed the above results in Masson's trichrome stain that demonstrated significant increase in collagen fibers in PCOS group, indicating persistent chronic inflammation (Nofal *et al.*, 2019). Furthermore, significant low percentage of granulosa cells that expressing PCNA immunoreaction with high percentage of theca and interstitial cells as compared to control group (P<0.05). While linagliptin, liraglutide and their combined treatment showed significant (P < 0.05) decrease in collagen deposition with improvement in PCNA immunoreaction (P < 0.05) (Figures 2 and 3 and Table 2). Data represented as mean ± SD of cell numbers; (n=8); DBP: diastolic blood pressure, HDL: high density lipoprotien, HR: heart rate, LDL: low density lipoprotien, SBP: systolic blood pressure, TC: total cholestrole TG:triglycrides. N: Number of PCNA immunopositive. Differences were

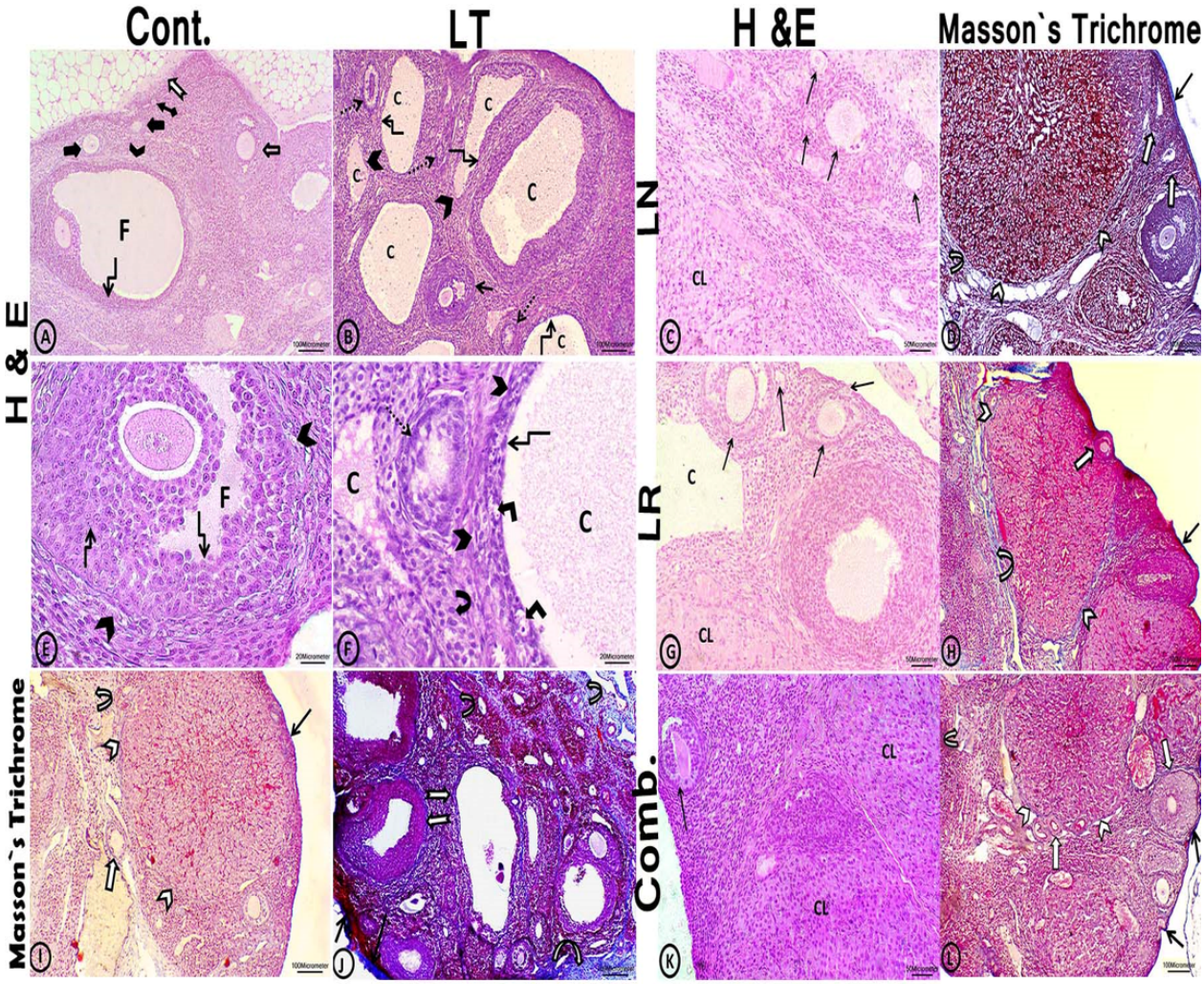

Figure 2: Photomicrographs of rat ovaries from all groups stained with Hematoxylin & Eosin and Masson's trichrome stains

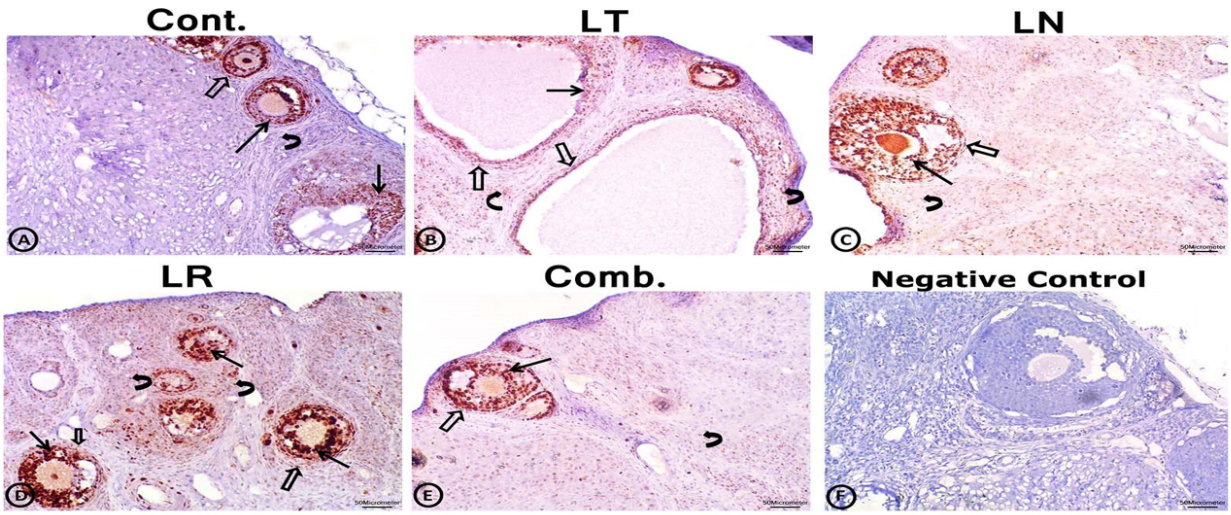

Figure 3: Photomicrographs of rat ovaries from all groups stained with PCNA immunostain

Table 1: Effects of linagliptin, liraglutide and combined therapy on anthropometric measurements and glyceamic indices in rats with PCOS

Parameters	Control	Letrozol	Linaglipten	Liraglutide	Combined
Initial BW (g)	78. 7±6.9	69.2±9.8	69.2±9.9^{a}	88.7±8.5^{b}	79 ±9.3
BW (g) at week3	105.2 ±15.5	101.7±22.7	105±29.7	131.7±11	114±10.4
% weight gain after 3 weeks (%)	33.4±11.5	45.6±13.9	49.8±23.6^{d}	49.1±12.3^{d}	45.6±19
BW (g) at the end of experiment	140.2±18.3	155.3±28.1	127.7±24.6	149.2±9.1	149.7±6
% weight gain at the end of experiment (%)	33.7±9.9	54.1±11.1^{db}	24 ±11.5^{dbc}	15.45±12^{dbc}	32.3±13.9^{bc}
BMI	4.9±0.3	4.9±0.6	4.9±0.3	4.4±0.5	4.3±0.4
Lee index	307.5±9.3	341.2±7.7^{d}	277.7± 8^{db}	290.8±12.4^{db}	289.4±7^{db}
Ovary (g)	0.02±0.01	0.04±0.01d	0.03±0.004^{b}	0.03±0.01^{b}	0.02±0.004^{b}
Ovarian fat (g)	0.09±0.002	0.53±0.03^{d}	0.11±0.01^{b}	0.09±0.04^{b}	0.11±0.02^{b}
Mesenteric fat (g)	0.4±0.02	0.6±0.06^{d}	0.5 ±0.03^{b}	0.31±0.02^{dbc}	0.32±0.02^{dbc}
Fasting serum glucose (mg/dl)	76.4±5.9	117.3±8.6^{d}	86.50±7.0^{b}	99.2±5.4^{dbc}	97.2±6.3^{db}
$\frac{1}{2}$ h postprandial serum glucose (mg/dl)	120.2±8.8	185.33±8.9^{d}	108±8.3^{b}	115±3.5^{b}	99.7±6.1^{dbc}
1h postprandial serum glucose (mg/dl)	116±4.8	236 ±9.6^{d}	103.7±3.3^{db}	121.8±3.1^{bc}	127.3±5.4^{dba}
2 h postprandial serum glucose (mg/dl)	67.6 ±5.4	142.3±5.9^{d}	81±5.89^{db}	94.60±3.97^{dbc}	115.5±4.03^{dbca}
Serum HbA1c (%)	4.7±0.9	9.6±0.7^{d}	6.3±0.9^{db}	7.4±0.7^{db}	5.9±0.6^{ba}
Serum insulin (ng/ml)	3.24±0.28	4.76±0.24^{d}	4.0±0.17^{db}	3.6±0.13^{dbc}	3.1±0.21^{bca}
HOMA-IR	0.61±0.05	1.38±0.07^{d}	0.86±0.1^{db}	0.89±0.06^{db}	0.74±0.05^{dba}

a Significant compared to the control group; b Significant compared to letrozole group; c Significant compared to Linagliptin group; d Significant compared to Liraglutide group.

considered significant at p < 0.05.

The present findings were supported by (González *et al.*, 2012) who reported that OS was significantly associated with obesity, IR, hyperandrogenism, and chronic inflammation. Moreover, the production of reactive oxygen species (ROS) and NF-kB could be triggered by hyperglycemia and elevated free fatty acids. Sequentially NF-kB increases the production of pro-inflammatory cytokines, such as TNF α that facilitates IR (Kauffman *et al.*, 2015). This could explain our results regarding deterioration of the ovarian oxidative scavenging enzymes; SOD and catalase in PCOS group. Furthermore, the deteriorated PCNA immunoreaction verified inhibition of proliferation and promotion of apoptosis in mature antral follicles and granulosa cells. These effects could be due to hyperandrogenism, lowered levels of both FSH and its regulator PCNA (Jahan *et al.*, 2016) as well, impaired antioxidant defense with persistent inflammation. (Rajan *et al.*, 2017).

The improvement effect of linagliptin and liraglutide were close with results of (Arakawa *et al.*, 2010) and (Rezvanfar *et al.*, 2016) who specified that TNFα was significantly reduced by GLP-1 analog exendin-4 through cyclic adenosine monophosphate / Protein kinase A / NF-kB signaling pathway. Also, (Aroor *et al.*, 2017) proposed that linagliptin disrupted the regulation of NF-kB transcription through disturbance of TNF α receptor-associated factor.

The histologic advance in linagliptin group was in accordance with its outcomes of hypoglycemia, decrease IR, constraining the chronic inflammation and conserving the ovarian antioxidant capacity; similarly study of (Jensterle *et al.*, 2017). Although liraglutide has an excellent hypoglycemic outcome but its direct effect on the ovary is questionable owing to incomplete histologic improvement with persistent residual cysts. It is possible that a longer period of treatment is needed to achieve the desired results (Vanessa Hoang *et al.*, 2015). In the current study, the marked improvement of histological structure induced by combined therapy was comparable to the control group, this could be explained by the synergistic beneficial effects of both drugs on

Table 2: Effect of drugs on lipid profile, blood pressure, heart rate and ovarian histological parameters in rats

Parameters	Control	Letrozol	Linagliptin	Liraglutide	Combined
TC (mg/ml)	220±14.4	326.81±13^{d}	280.6±15.6^{db}	262.1±13^{db}	237±13^{bca}
TG (mg/ml)	185.8±14	256.3±9.7^{d}	218.7±1^{db}	202.2±9.5^{b}	194.1±11.2^{bc}
HDL (mg/ml)	83.9±6.8	70.2±7.3^{d}	71.7 ±7^{d}	72.6 ± 6	76.9±6.2
LDL (mg/ml)	98.9±12	205.4±13.7^{d}	165.1±17.8^{db}	149.1±13.4^{db}	121.4±14^{bca}
SBP(Hg)	137.8 ± 11.8	143 ± 24	125.7 ± 10.7	135.8 ± 8	122.2 ± 10.6
DBP(Hg)	97.6 ± 11.5	114.4 ± 32	100.67 ± 14.6	98.9 ± 7	86.6 ± 12.3
HR(beat /min)	433.4 ± 36.2	412.6 ± 36.2	448.67 ± 8.1	408.5 ± 28	441.4 ± 27.6
Area % of collagen	0.74±0.12	1.97±0.12^{d}	1.05±0.36^{b}	1.1±0.24^{b}	0.97±0.15^{b}
Granulosa cells(N)	89.3±3.14	38.5±1.64^{d}	85.17±3.2^{b}	82.5±2.7^{db}	88.7±2.9^{b}
Theca cells(N)	54±5.14	88.17±0.98^{d}	57.5±4.3^{b}	63±2.5^{db}	58.7±4.5^{b}
Interstitial cells(N)	39.7±2.25	78.83±2.04^{d}	43±2.4^{b}	48±2.5^{db}	47.2±5.9^{b}
Primordial follicles	14.3±0.8	1±1.26^{d}	12.5±1.4^{b}	12.7±1.03^{b}	12.7±0.82^{b}
Primary follicles	4.5±0.55	0.33±0.5^{d}	3.3±1.2^{b}	3.5±0.55^{b}	3.3±0.5^{b}
Pre-antral follicles	4.3±0.5	1.17±0.75^{d}	3.5±0.8^{b}	3.7±0.8^{b}	3.6±0.5^{b}
Antral follicles	2.8±0.4	0.5±0.55^{d}	2.7±0.8^{b}	2.5±0.8^{b}	3±0.6^{b}
Degenerated follicles	2.2±0.4	9.2±0.98^{d}	3.2±0.8^{b}	2.8±1.2^{b}	3.2±0.75^{b}
Cystic follicles	0.5±0.8	7.2±0.75^{d}	1.7±1.6^{b}	2±1.09^{b}	0.5±0.83^{b}
Corpus luteum	2.7±0.5	00±00^{d}	2.2±0.4^{b}	1.8±0.75^{db}	2.3±0.5^{b}

a Significant compared to the control group; b Significant compared to letrozole group; c Significant compared to Linagliptin group; d Significant compared to Liraglutide group.

glucose metabolism.

Lipid profile, blood pressure and heart rate measurements in the treated rats

Our findings revealed non-significant differences in measurements of blood pressure and heart rate among all groups (P>0.05). However, changes in lipid profile significantly demonstrated increased serum levels of TC, TG, and LDL with lowering HDL level in letrozole-treated rats compared to control rats (P< 0.05). Both linagliptin and liraglutide treatment, either individual or combined, caused significant reduction in TC, TG and LDL (P < 0.05) and non-significant changes in HDL levels compared to PCOS group (P > 0.05). Moreover, the combined treatment caused significant lowest levels of TC and LDL as compared to either drug alone. (Table 2). In agreement with the present results, (Eckardstein *et al.*, 1998) suggested that dyslipidemia is one of the consequences of PCOS; the significant deterioration of serum lipid profile in letrozole - induced PCOS could be attributed to hyperandrogenemia. On the other hand, (Terawaki *et al.*, 2015) reported that linagliptin improved lipid metabolism by shifting the small dense LDL (oxidized LDL) to larger less atherogenic LDL and by lowering synthesis of insulin-induced free fatty acids. Regarding the present results of hemodynamics, previous studies showed non - significant changes in blood pressure evoked by liraglutide (Frøssing *et al.*, 2018) and linagliptin (Terawaki *et al.*, 2015). While Vanessa Hoang *et al.* (2015) reported amelioration of blood pressure with liraglutide treatment in rats with PCOS, it could be explained by longer treatment period compared to our study.

H&E stain

Control (A, E), PCOS (B&F), linagliptin, liraglutide and combined group (C, G and K respectively). Antral follicle (hollow arrow), Atretic follicles (dotted arrows), Corpora lutea (CL), Follicular cysts (C), Developing follicles (thin arrows), Mature follicle (F), Granulosa cells (broken arrows), Preantral follicles (thick arrows), Primary follicles (diamond ended arrow), Primordial follicles (white notched arrow), and theca cells (arrowheads), Pyknotic nuclei and vacuolations (right-angled arrows). *H&E staining. Scale bar=100 μm(A &B), 50 μm (C, G, K) and 20 μm (E, F).*

Masson's trichrome stain

Control (I), PCOS (J), groups of linagliptin, liraglutide and combined (D, H and L respectively). Collagen fibers deposition in tunica albuginea (thin arrow), ovarian follicles (white arrow) and corpora lutea (white arrowheads), the stroma (curved arrows), cysts (notched white arrows). *Masson's trichrome*

stain. Scale bar=100μm.

PCNA immunostaining

Control (A), PCOS (B), linagliptin, liraglutide and combined (C, D and E respectively) and Negative control (F). Expression of nuclear immunoreactivity for PCNA in granulosa cells (thin arrows), theca cells (hollow arrows) and interstitial (stromal) cells (curved arrows). *PCNA immunostaining. Scale bar=50μm.*

CONCLUSIONS

The present results showed that administration of linagliptin or liraglutide significantly improved the letrozole - induced PCOS components, including metabolic and hormonal disorders together with chronic inflammation and decreased antioxidant defenses. Moreover, linagliptin had more pronounced beneficial effects on glucose excursion during OGTT as well as on the ovarian histopathological changes as compared to liraglutide. So, further studies with longer period of liraglutide therapy are required to attain the target results.

Conflict of interest

The authors declare that they have no conflict of interest for this study.

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