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Research article

A combination of gliclazide and metformin attenuates obesity-induced polycystic ovary syndrome in female Wistar rats

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ABSTRACT

Presently, it is known that the progression of obesity concomitantly leads to polycystic ovary syndrome and infertility. This study aimed to evaluate the potential effects of metformin (M; insulin secretagogues) and gliclazide (G; insulin sensitizer) alone and their combination at different doses to treat obesity-induced PCOS. High high-fat diet was given to all female Wistar rats for nine weeks to induce obesity except for the normal control group which received a normal chow diet. Estradiol valerate (0.8 mg/kg) was also given to all obese rats to induce polycystic ovarian syndrome. After the induction, M (100, 300 mg/kg) and G (5, 10 mg/kg) were given orally either individually or in combination for 28 days. The notable ($p < 0.0001$) reduction in body weight and blood glucose level was observed in treatment groups in contrast to disease control (DCG). The marked ($p < 0.05$–0.0001) decrease in hemocylated hemoglobin, serum insulin, cholesterol, triglycerides, and testosterone was observed in treated groups, notably in combination groups (M100+G10 mg/kg) in contrast to DCG. There was a considerable ($p < 0.01$–0.0001) increase in progesterone E2, estradiol, luteinizing, and follicle-stimulating hormones in treated groups as compared to DCG. Treatment with M and G treated groups also exhibited marked ($p < 0.05$–0.0001) increases in SOD, CAT, and GSH while decreased in NO and MDA levels in ovary tissue as evidenced by the histological study of the ovary. Treatment with M and G alone and in combination significantly ($p < 0.0001$) restored the serum IL-6, NrF2, and NF-κB levels as compared to DCG. The results inveterate that the M and G combination (M100+G10, and M300+G10) was useful in treating obesity-induced infertility due to antioxidant properties, hypolipidemic effects, and modulation of inflammatory markers.

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1. Introduction

Obesity is a disproportionate and abnormal fat accumulation that impairs health [1]. The prevalence of obese population has significantly increased from 30.5% in 1999 to 39.6% in 2016 and it is estimated to cross 2.1 billion globally in 2030 [2]. Obesity is considered the fifth most frequent cause of non-communicable diseases [3], such as impaired cardiovascular function, type 2 diabetes mellitus (T2DM), cerebral ischemia, and reproductive disorders [4]. It is characterized based on body mass index (BMI). Although the normal BMI is 18.5–24.9 kg/m^2 while value more than 30 kg/m^2 considered as obese [1]. The prevalence of obesity among females older than 20 years is 38.3% [5].

Excessive consumption of a caloric-rich diet and low physical activities cause obesity due to the deposition of fats in adipose and non-adipose tissues. However, environmental factors, genetics, neurologic abnormalities, endocrine disorders, and various physiological factors, such as stress, eating disorders, and depression) play their role in obesity. Numerous drugs for instance, beta-blockers, glitazones, anticonvulsants, tricyclic antidepressants, and glucocorticoids also cause obesity. Genetic causes of obesity include monogenic disorders linked to leptin deficiency, melanocortin-4 receptor mutation, Agouti-Related Protein (AgRP), and obesity-associated genes (FTO) [6].

According to WHO, 50–80 million females are affected by infertility [7]. Severe reproductive defects are associated with obesity, including menstrual irregularities, polycystic ovarian syndrome (PCOS), and infertility [8]. Among them, PCOS is the most common cause of ovulation failure. PCOS is associated with excessive release of androgen by ovarian theca cells that leads to hyperprolactinemia [9].

Though, insulin together with leptin acts on the hypothalamus to promote hunger and satiety [10], however, increased levels of leptin and insulin resistance substantially contribute to obesity [11]. Reduced insulin sensitivity promotes the production of androgen which results in increased release of estrogen that in turn forms unwarranted adipose tissues, harming the hypothalamus-pituitary axis (HPA) and disturbing the gonadotropin production [12,13]. The hypothalamic-pituitary gonad axis is involved in regulating reproductive functions through the production of various hormones such as gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), testosterone, estradiol and follicle-stimulating hormone (FSH) [14]. Moreover, in obesity, levels of sex hormone-binding Globulin (SHBG), growth hormone (GH), and Insulin-like growth factor binding protein (IGFBP) are decreased which increase leptin levels [15,16]. Consequently, abnormal menstrual cycle, insulin resistance, ovulatory irregularities, oligomenorrhea, and hyperandrogenism appear as clinical manifestations of PCOS [11,17]. Furthermore, there is overwhelming evidence that obese females are at a three-fold higher risk of being infertile than non-obese and require a longer time to conceive mostly due to insulin resistance [18].

The prevalence of dyslipidemia and glucose intolerance in females associated with PCOS increases remarkably with obesity. Hence, obesity must be the first preference to treat when treating PCOS and infertility [19]. Metabolic dysfunction, deregulated ovarian functions, tubal infection, endometriosis, cervical factors, uterine causes, hormonal imbalance, dysregulation of inflammatory factors, or changes in metabolism all of these pathologies ultimately lead to infertility [20].

Gliclazide is an oral anti-diabetic drug that belongs to the sulfonylureas class and acts as an insulin secretagogue to stimulate insulin from β cells of the pancreas. Gliclazide has antioxidant properties that improve the pathological changes caused by type 2 diabetes mellitus (T2DM) [21]. Metformin belongs to the class of biguanides which is an insulin sensitizer and a potent oral antihyperglycemic drug used in T2DM. It could restore ovulation by decreasing body weight [22]. It is also found that metformin lowers the synthesis of androgen from ovarian theca cells and suppresses ovarian steroidogenesis. Metformin also possesses antioxidant properties that protect the body from oxidative stress and insulin-mediated oxidative damage [23]. As insulin resistance and oxidative stress are predisposing factors of PCOS leading to infertility, therefore, gliclazide and metformin both may reduce the insulin resistance and normalize oxidative stress evident in PCOS.

Epidemiological studies have demonstrated a strong link between obesity, PCOS, and infertility as 35–85% of PCOS patients are either overweight or suffer from obesity [24]. There is an intricate relationship between obesity and PCOS because the release of adipokines, insulin resistance, and obstructive sleep apnea in obese patients contribute to PCOS through metabolic and ovarian dysfunction and increased ovarian production of androgens. However, PCOS, in return, contributes to obesity by androgen-mediated lipolysis, altering bioenergetics, and adversely affecting emotional and mental wellbeing [25]. Obese individuals have lower levels of sex hormone-binding Globulin (SHBG), growth hormone (GH), and Insulin-like growth factor binding protein (IGFBP) and thus an increased level of leptin [15,16]. These clinical manifestations lead to the abnormal menstrual cycle, insulin resistance, and ovulatory irregularities majorly leading to PCOS [11,17]. Being obese is related to a decrease in insulin sensitivity that also contributes to PCOS symptoms [18]. Most studies have suggested that obese females have a three-fold higher risk of being infertile than the non-obese, and require a longer time to conceive. Therefore, the objectives of the proposed study were to find out the curative effect of gliclazide and metformin against high-fat diet (HFD)-induced obesity, to evaluate the comparative effect of metformin and gliclazide in treating PCOS and the mechanism of action of individual and combination treatment in obese-PCOS rats.

2. Materials and methods

All the chemicals are reagents were of analytical grade. Gliclazide (Dimicron 60 mg) (Servier Pharma, Pakistan), Metformin (Glucophage 500 mg) by (Martin- Dow, Pakistan), and Estradiol valerate (EV) (Progynova) by (Bayer Schering Pharma, Pakistan). Carboxymethylcellulose (CMC), Chloroform, 10 % solution of formalin, Methylene blue, Methanol, Pyrogallol, Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Sodium phosphate buffer solution, Potassium phosphate, Potassium hydroxide, Hydrogen peroxide solution, Monosodium phosphate, Disodium phosphate, Dithiobis nitrobenzoic acid (DTNB) reagent were purchased from Sigma Aldrich, UK. The Diagnostic kits for FSH (Cat no. EEL125), LH (Cat no. EEL122), Nrf2 (Cat no. MBS752046), IL-6 (Cat no. BMS625) and

NF-KB (EEL138) (Thermo Fischer Scientific, United States) were also purchased. Progesterone (Cat no. 80558), estrogen (Cat no. 80548), testosterone (Cat no. 80500) were obtained from Crystal Chem,USA.

2.1. Experimental animals

The sixty Female Wistar rats (approximately 7–8 weeks of age) were acquired from the University of Veterinary and Animal Sciences Lahore, kept in steel cages, and acclimatized in the animal house of the Riphah Institute of Pharmaceutical Sciences (RIPS) and provided with free access to water and chow diet. All rats were provided with standard laboratory conditions (temperature 23 ± 2 °C; humidity 55 ± 5, and light and dark cycles of 12 h. All animals were weighed at the beginning of the experiment. The treatment groups were provided a high-fat diet (Rodent diet D12492) that provided energy from fats 60%, carbohydrates 20%, and proteins 20%) [13,26,27]. While the normal control group was given a standard pallet diet during an experiment. The experimental study was approved (REC/RIPS-LHR/011) by the Research Ethical Committee of Riphah International University, Lahore [28]. Guidelines of the National Research Council were adopted for working with animals. Undue harm was avoided.

2.2. Induction of obesity and PCOS

Female Wistar rats (54) were given HFD for 9 weeks to induce obesity except the normal control group which was given a normal chow diet. After exposure to HFD, all animals were weekly weighed until the end of the experiment. Obesity was confirmed by the Lee obesity index which was calculated as a cube root of animal body weight (g) multiplied by 1000 and divided by naso-anal distance in centimeters. All animals that showed a Lee index of more than 320 were considered obese and included in the study [29]. EV dissolved in olive oil was given to all animals as a single oral dose of 4 mg/kg except normal control for the induction of PCOS at the second week of HFD [30]. Vaginal swabs from animals were observed under a light microscope to identify the estrus cycle which confirmed PCOS.

2.3. Experimental design

All obese animals were divided into 9 groups, each consisting of six animals. Nonobese animals were kept as normal control. Treatment was started on the 2nd day (start of therapy) after the successful induction of PCOS and lasted for a further 28 days (end of therapy). Dosing was done orally by oral gavage between 9 and 11 a.m. daily. Metformin and gliclazide were dissolved in a carboxymethylcellulose solution (CMC). The scheme of the present study is presented in Fig. 1.

Animals were divided into groups as given below.

- Group I (normal control without obesity): received a normal chow diet.
- Group II (disease control or Obese): HFD and EV
- Group III (obese + PCOS rats): metformin 100 mg/kg/day [31].
- Group IV (obese + PCOS rats): metformin 300 mg/kg/day.
- Group V (obese + PCOS rats): gliclazide 5 mg/kg/day.
- Group VI (obese + PCOS rats): gliclazide 10 mg/kg/day [32].
- Group VII (obese + PCOS rats): 100 mg/kg metformin + gliclazide 5 mg/kg/day.
- Group VIII (obese + PCOS rats): 100 mg/kg metformin + gliclazide 10 mg/kg/day.

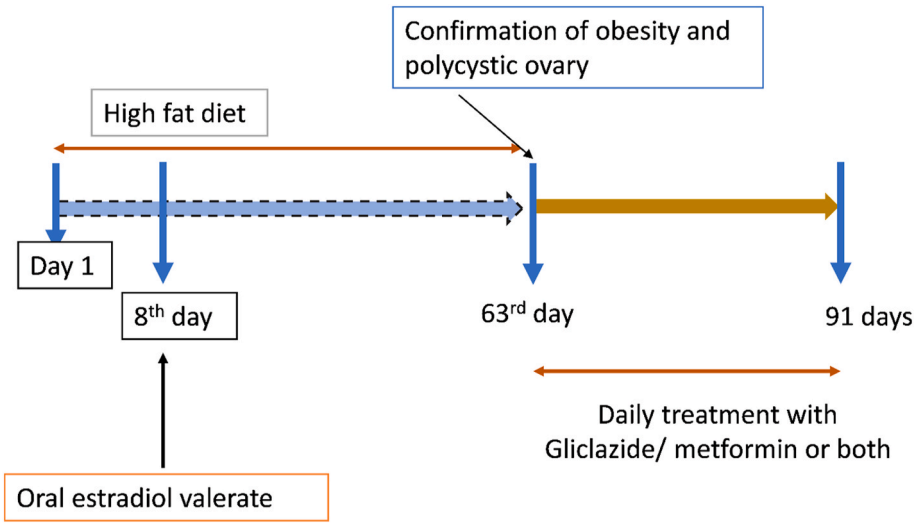

Fig. 1. Scheme of study for the evaluation of anti-obesity and anti-infertility effects of gliclazide and metformin.

- Group IX (obese + PCOS rats): 300 mg/kg metformin + gliclazide 5 mg/kg/day.
- Group X (obese + PCOS rats): 300 mg/kg metformin + gliclazide 10 mg/kg/day.

2.4. Assessment of bodyweight

The body weight was measured in grams before administering HFD, at the start of treatment, and after treatment. Effects of metformin and gliclazide on body weight reduction were calculated [33].

2.5. Oral glucose tolerance test (OGTT)

After 21 days of drug treatment, the basal fasting blood glucose level was measured prior to OGTT. Before OGTT, the rats were fasted overnight. After half an hour of therapy administration described earlier, the rats were orally given a 2 g/kg glucose solution. Blood samples were taken by tail vein using On-call plus glucometer to take readings at 0, 60, 120, and 180 min post-glucose administration [34].

Estimation of Lipid, hormonal and inflammatory profiles, and other biochemical parameters.

After 5 h of food withdrawal, the body weight of all animals was measured [6]. All animals were anesthetized with pentobarbital 40 mg/kg i. p. and blood samples were collected via cardiac puncture to measure total cholesterol (TC), triglycerides (TG), HbA1c, serum insulin, testosterone, FSH, LH, estradiol, progesterone, and IL-6, NF-κB, and Nrf2 with standard diagnostic and ELISA kits by following kit maker protocols. Serum was separated from the blood by centrifugation at 2500 rpm for 15 min at 4 °C which was used in the study [32].

2.6. Estimation of oxidative stress biomarkers

A 10 % w/v ovary tissue homogenate was prepared in 0.1 M phosphate buffer (pH; 7.4) and homogenized using a tissue homogenizer. After homogenization, the tissue mixtures were centrifuged at 1000 rpm to isolate supernatants that were subsequently used to evaluate oxidative parameters such as reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), nitric oxide (NO), malondialdehyde (MDA) by following earlier procedures [35]. The estimation of protein content in tissue homogenate was performed by the Lowry method using bovine serum albumin to draw the standard curve. The absorbance of the reactant mixtures was recorded at 660 nm wavelength [36].

2.7. Estimation of GSH

GSH content in the ovary was estimated by Elman's reagent method. In this method, 1 ml of ovary tissue homogenate was precipitated with 1 ml of TCA (10 %) reagent followed by the addition of 4 ml PBS (pH 7.4) already containing 0.5 ml of DTNB reagent to the supernatant aliquot. The absorbance of the reaction mixture was checked at 412 nm with a UV–Vis spectrophotometer and GSH content was measured in μM/mg [37].

2.8. Determination of catalase activity

CAT activity in the ovary tissue homogenate was estimated by the hydrogen peroxide degradation method. For assessment of CAT activity, the reaction mixture containing 0.05 ml of ovaries tissue homogenate and 1.95 ml of 50 mM phosphate buffer solution maintained at 7.0 pH was prepared in the cuvette. Afterward, 1 ml of 30 mM H_2O_2 was added and absorbance was recorded at 240 nm for 1 min at 15-s intervals and the CAT activity was expressed as U/mg of protein [38].

2.9. Determination of SOD activity

The activity of SOD was determined by pyrogallol reagent method. For estimating SOD activity, the reaction mixture was prepared with 3 ml of tissue homogenate, 2.8 ml PBS and 0.1 ml pyrogallol solution. The absorbance of the reaction mixture was recorded at 325 nm for 2 min at 15 s intervals and the activity of SOD was expressed as U/mg of protein [33].

2.10. Estimation of nitrite content

The nitrite level in the ovary tissues was determined with the Griess reagent method. In this method, equal volumes of Griess reagent (0.1 % w/v N- 1 naphthyl ethylene amine dihydrochloride, 2.5 % v/v phosphoric acid, and 1 % w/v sulphanilamide) and sample solution were mixed, shaken thoroughly and incubated for 10 min. The absorbance of the reaction mixture was determined at 546 nm and the nitrite content was estimated as μg/ml [39].

2.10.1. MDA content in the ovary

An elevated level of MDA depicts increased lipid peroxidation. For estimation of MDA level, TBA reagent was used which comprised of TBA 0.38 % w/w, 0.25 M HCl, and 15 % w/v TCA. In this procedure, 1 ml of tissue homogenate was taken in the test tube and 3 ml of TBA reagent was added. The sample solution was shaken thoroughly and heated at 90 °C in a water bath for 15 min and then cooled to

room temperature over an ice bath. Afterward, the sample solution was centrifuged for 10 min at 3500 rpm to separate the upper layer the absorbance of which was determined at 532 nm. The amount of MDA was expressed as μg/mg [39].

2.11. Histopathology of ovary

The anesthetized animals were euthanized by cervical dislocation, and the ovaries of all animals were removed and washed with ice-cold normal saline. Then these ovaries were placed in 10 % formaldehyde solution. Ovaries of about 5 μm thickness were cut using a Rotary microtome and embedded in paraffin wax. Right and left ovaries were kept on a glass slide and stained with hematoxylin-eosin dye (BDH chemicals England, UK) to evaluate changes including the number and size of follicles and corpus luteum using a light microscope (Meiji Techno, Japan) at 40X and 100X [40].

2.12. Statistical analysis

Results were presented as mean ± S.D. For statistical analysis, one-way and two-way analysis of variance (ANOVA) were applied followed by Tukey's multiple comparison test by using Graph pad prism software version 7.0. These results were considered moderately significant when $p < 0.01$ and highly significant when $p < 0.0001$.

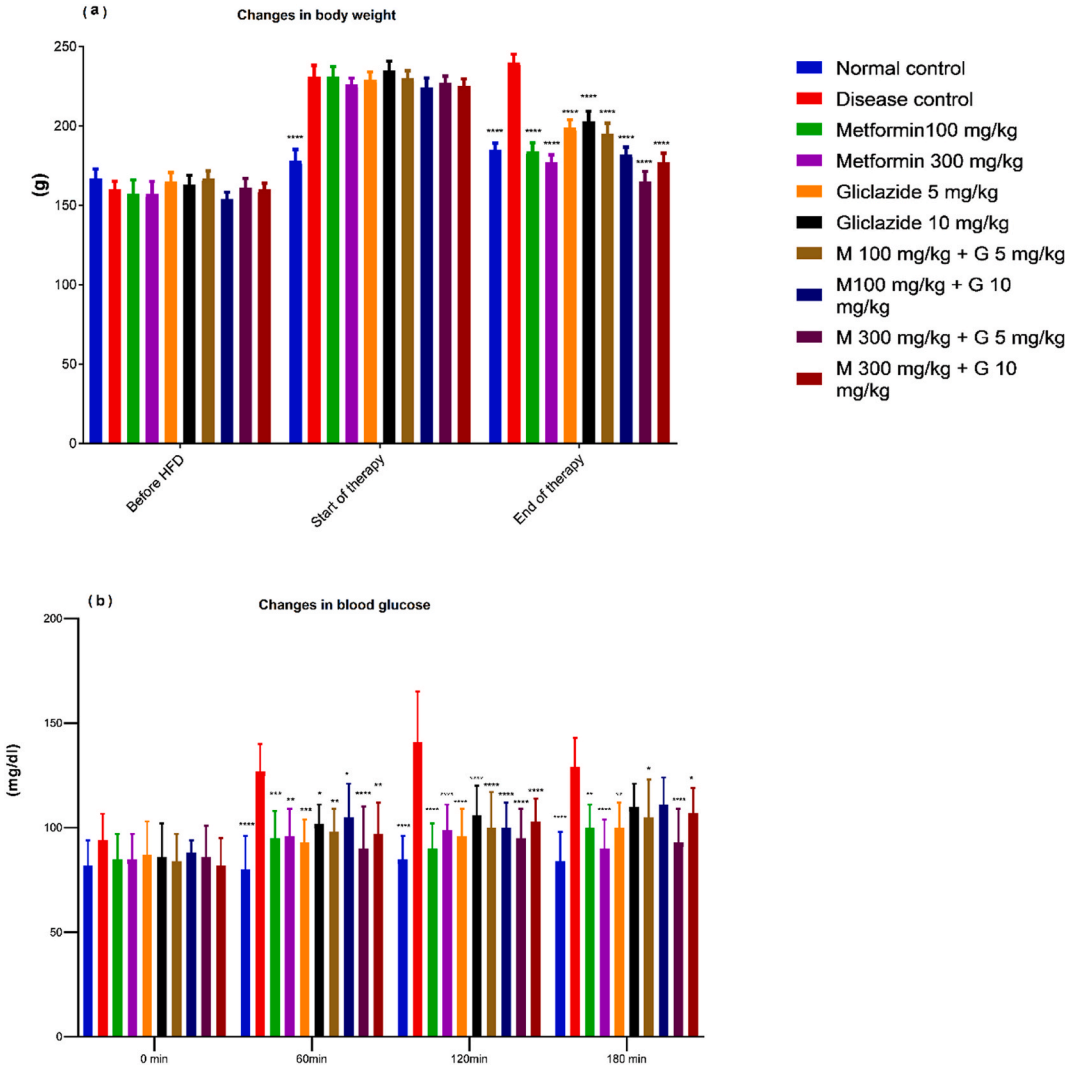

Fig. 2. Effect of metformin and gliclazide (a) on body weight and (b) on blood glucose level in female infertile rats. Results were shown as mean ± S. D (n = 6). Two-way ANOVA followed by Tukey's test. * and **** indicated significant variation at $p < 0.05$ and 0.0001 respectively in contrast to disease control at the same time intervals.

3. Results

3.1. Effect on body weight

There was an insignificant difference in body weight in all groups in contrast to normal control before administering HFD. Body weight was noticeably elevated in all groups in contrast to normal control at the start of therapy. The treatment with metformin and gliclazide individually and also in combination doses showed a significant reduction ($p < 0.0001$) in body weight in all treatment groups as compared to the disease control group (240 ± 5.14 g). At the end of therapy, the body weight of rats in metformin 100, 300, M100+G5, M100+G10, and M300+G10 mg/kg groups are insignificantly different from normal control. Additionally, the fall in body weight in M100+G10 is insignificantly varied from the M300+G10 mg/kg treated group. The effect of metformin and gliclazide alone and in combination on body weight in PCOS rats is shown in Fig. 2 (a).

3.2. Effect on blood glucose level in OGTT

For the performance of blood glucose levels, rats were given oral glucose after overnight fasting. Blood glucose concentrations were determined at 0, 60, 120, and 180 min by using a glucometer. The blood glucose level in all groups significantly ($p < 0.0001$) varied from normal control at 60,120 and 180 min. The blood glucose level was notably ($p < 0.0001$) reduced in all treatment groups in contrast to disease control as shown in Fig. 2 (b).

3.3. Effect on lipid profile

A lipid profile was performed to analyze the protective effect of metformin and gliclazide alone and in combination against obesity-induced infertility. Serum triglycerides (101.66 ± 3.06 mg/dl) and total cholesterol (97.66 ± 2.52 mg/dl) levels were significantly increased in the disease control group which was fed on HFD and EV in comparison to a normal control group as displayed in Fig. 3a and 3b. The treatment with G 10 mg/kg, M 100 mg/kg + G 5 mg/kg, and M 300 mg/kg + G 10 mg/kg notably ($p < 0.0001$) reduced TG and TC levels in comparison to the disease control group. Treatment with M 300 mg/kg reduced TG with a significant difference ($P < 0.001$) in comparison to the disease control group.

Triglyceride level was significantly ($p < 0.01$–0.0001) different in all groups in contrast to normal control except gliclazide 10, M

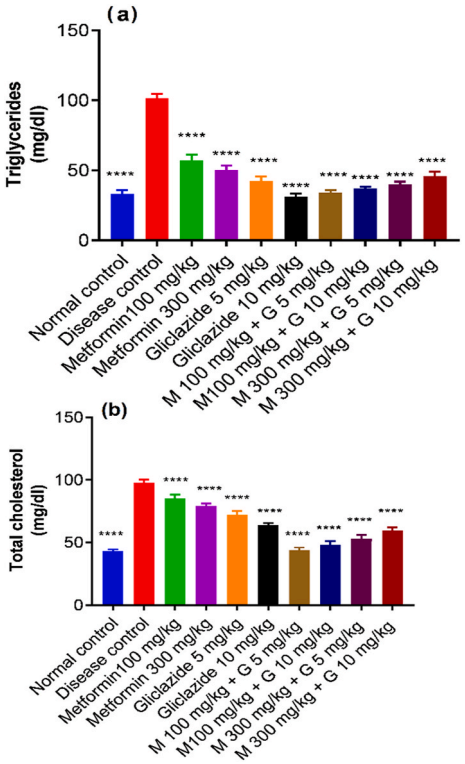

Fig. 3. Effect of metformin and gliclazide on lipid profile in obese-PCOS rats.
Results were expressed as mean ± S. D (n = 6) and analyzed by one-way ANOVA followed by Tukey's test. Where, ****, ***, **, and * showed significance at $p < 0.0001$, 0.001, 0.01, and 0.05 respectively in contrast to disease control.

100 + G 5, and M100+G10 treated groups. Cholesterol levels in all the treatment groups were significantly ($p < 0.0001$) varied from normal control except it was insignificantly varied in M300+G5 (53 ± 3.01) and M 300 + G 10 (59.66 ± 2.52) treated rats in contrast to normal control.

3.4. Effect on insulin and HbA1c

The insulin resistance increased in disease control so more insulin secreted and insulin level rise while there is less insulin resistance and so less insulin level was observed in treatment groups in contrast to disease control. The insulin resistance notably enhanced in disease control as serum insulin level (0.15 ± 0.02 μIU/ml) elevated in disease control in contrast to normal control. Different treatment groups exhibited notable ($p < 0.05$–0.0001) decreases in serum insulin levels in contrast to disease control rats. Serum insulin levels were insignificantly different in all groups in contrast to normal control except in metformin 300 mg/kg treated rats. The combination groups are insignificantly varied from each other (Fig. 4a).

The percentage of glycated hemoglobin (HbA1c) was notably ($p < 0.0001$) increased in disease disease-treated group (13.2 ± 0.9 %) In contrast to the normal control. The HbA1c was noticeably ($p < 0.0001$) decreased in all treatment groups when treated with metformin and gliclazide alone and in combinational doses in contrast to diseased control rats. HBAIC level varied significantly ($p < 0.0001$) in all groups in contrast to normal control except M100+G10 (5.3 ± 0.2) and M 300 + G 10 (6.1 ± 0.2). Additionally, combination treatment groups were significantly varied from each other (Fig. 4b).

3.5. Effect on reproductive hormones

The noticeable ($p < 0.001$) effect on LH was seen in M 300 + G 10 (14.12 ± 0.37 μIU/ml) treated groups in contrast to the disease group (10.93 ± 0.69 μIU/ml) as presented in Fig. 3a. The LH level profoundly declined in disease control in contrast to normal rats that

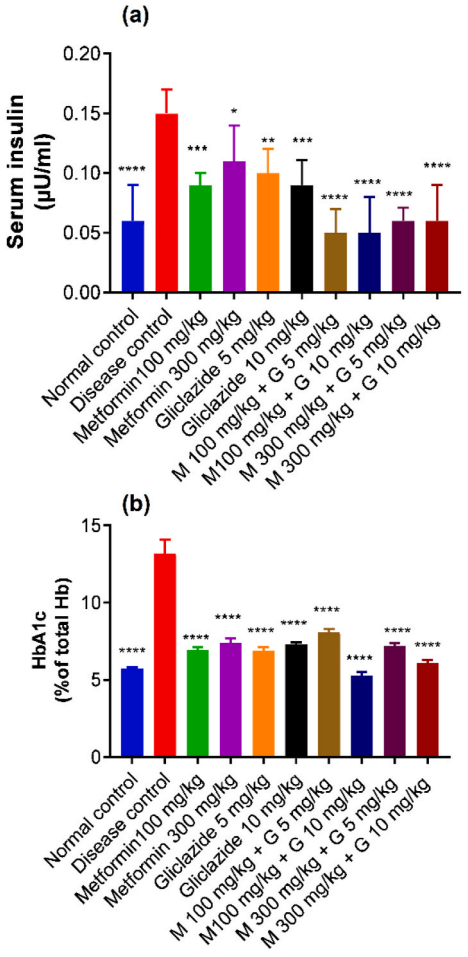

Fig. 4. Effect of metformin and gliclazide on insulin and HbA1c in obese-PCOS rats.
Results were expressed as mean ± S. D (n = 6) and analyzed by one-way ANOVA followed by Tukey's test. Where, ****, ***, **, and * showed significance at $p < 0.0001$, 0.001, 0.01, and 0.05 respectively in contrast to disease control.

were prominently ($p < 0.01–0.0001$) elevated in treatment groups. The LH level was significantly ($p < 0.01–0.0001$) varied in all treatment groups in contrast to normal control except Metformin 100 (13.73 ± 0.17) and M 300 + G 10 (14.12 ± 0.37) treated groups (Fig. 5a).

The estradiol level was noticeably raised in disease control in contrast to normal control which was significantly restored ($p < 0.01–0.0001$) with treatment (Fig. 5b). The estradiol level in all treatment groups was profoundly ($p < 0.1–0.0001$) varied from normal control except in G10 and M 300+G5 mg/kg treated groups. The combination groups are notably varied from each other except M100+G5 from the M100+G10 mg/kg treated group (Fig. 5b).

The serum level of testosterone was measured after treatment with metformin and gliclazide to treat obesity-induced infertility. The level of testosterone was notably ($p < 0.0001$) decreased in all treatment groups when compared with the disease control group (0.17 ± 0.03 ng/ml). The testosterone level in all treatment groups was insignificantly varied from the normal control. The combination groups are insignificantly varied from each other as presented in Fig. 5c.

A profound decrease ($p < 0.0001$) in the level of progesterone was found in the disease control group as compared to normal control. Treatment groups showed a significant increase ($p < 0.0001$) in progesterone levels in female infertile rats when compared to a disease-control group (Fig. 5d). The restoration of PG in treatment groups was notably varied from normal control except in Metformin 100 and M 100 + 10 mg/kg treated groups. The effect of the combination groups was significantly different from each other except the PG level in M 100 mg/kg + G 5 mg/kg was insignificantly varied from M 300 mg/kg + G 5 mg/kg treated group.

The FSH level was notably reduced in disease control in contrast to normal which was notably ($p < 0.0001$) restored in treatment groups in contrast to disease control rats. FSH level was insignificantly varied in all treatment groups in contrast to normal control except in Metformin 100 and M 300 + G 5 treated groups (Fig. 5e).

3.6. Effect on inflammatory markers

The levels of IL-6 (1320.35 ± 91.15 pg/ml), Nrf2 (194.61 ± 5.27 pg/ml) and NF-κB (569.49 ± 16.81 pg/ml) were found to be elevated ($P < 0.0001$) in the serum of disease control group in contrast to normal control that were notably ($P < 0.0001$) restored with treatment either alone or in combination as mentioned in Fig. 6 a, b and c. The maximum reduction of inflammatory markers was

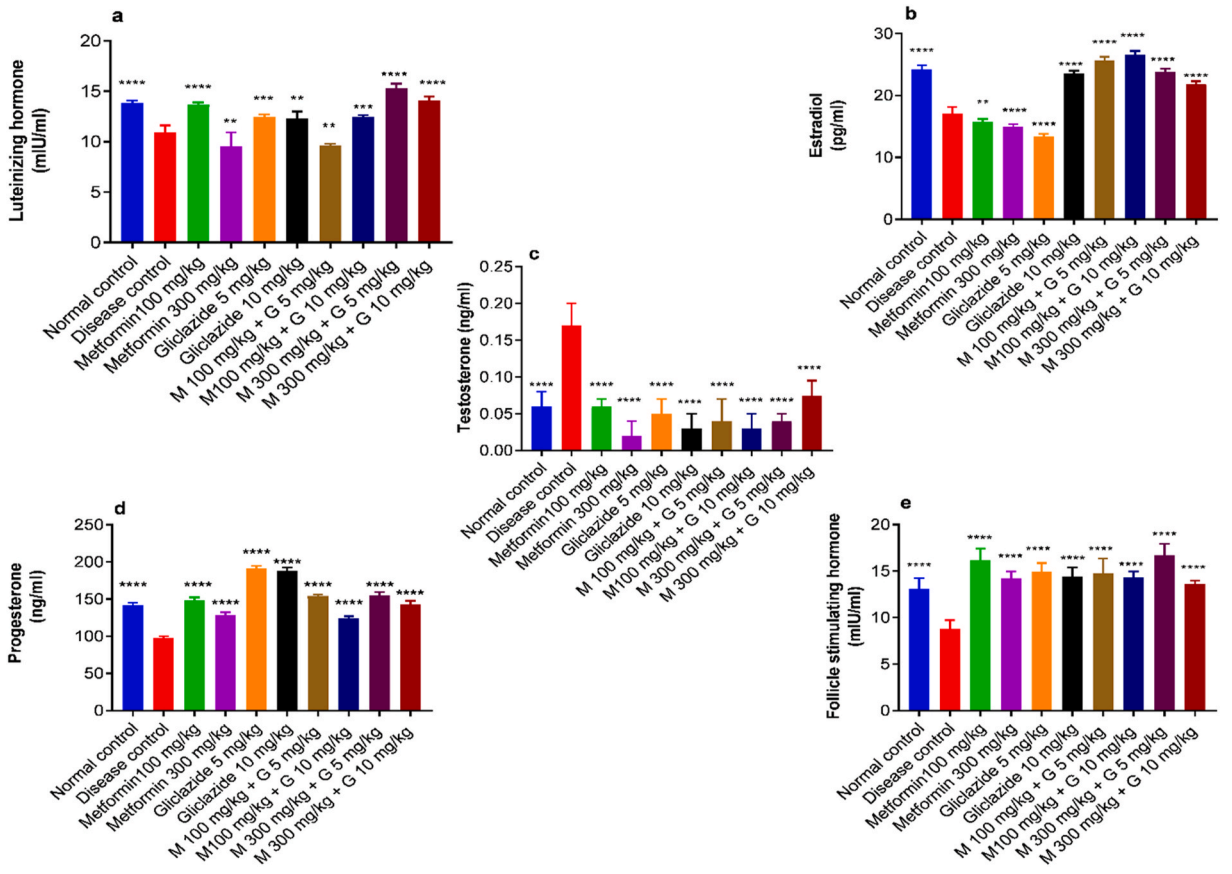

Fig. 5. Effect of metformin and gliclazide alone and in combination on reproductive hormones in obese-PCOS rats.
Data were presented as mean ± S. D (n = 6) and analyzed by one-way ANOVA followed by Tukey's Where, **, ***, and **** showed significance at $p < 0.01$,0.001, and 0.0001 respectively in contrast to disease control.

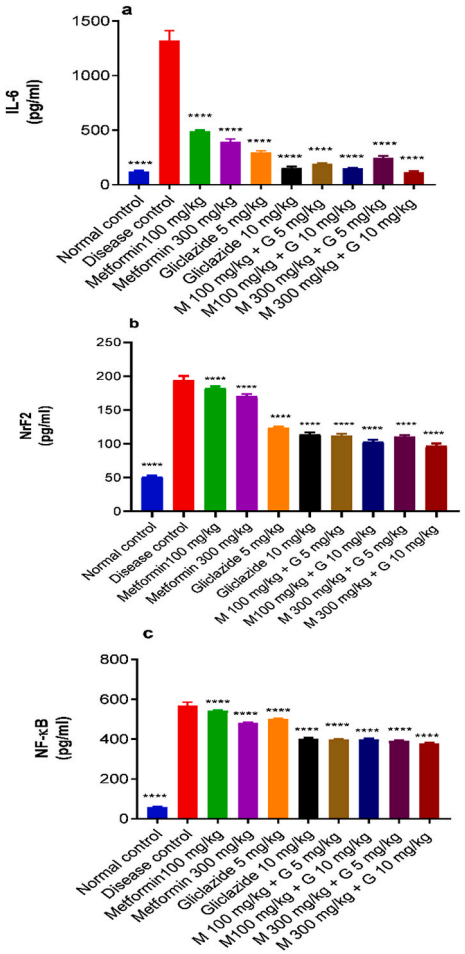

Fig. 6. Effect of metformin and gliclazide on various biomarkers in female obese-PCOS rats.
Results were shown as mean ± S. D (n = 6). One-way ANOVA followed by Tukey's multiple comparison test. Where **** showed statistically significant at $p < 0.0001$ in comparison to disease control.

noticed in M 300 + G 10 (IL:116.78 ± 8.29; Nrf2: 96.89 ± 3.28; NF-κB:379.43 ± 4.24) treated groups in contrast to other treatment groups as shown in Fig. 6. However, IL6 level in all treatment groups was notably higher than the normal control except in gliclazide 10, M100+ G10 mg/kg treated groups. The NF-κB and Nrf2 levels in treatment groups were notably ($P < 0.0001$) higher than normal control (Fig. 6b and c).

3.7. Effect of metformin and gliclazide against oxidative stress

The levels of GSH, CAT, and SOD were found to be significantly ($p < 0.0001$) decreased in the disease control group as compared to the normal control. The treatment with metformin at 100, and 300 and gliclazide at 5 and 10 mg/kg alone or in combination significantly ($p < 0.0001$) restored their levels in contrast to the disease control group (Fig. 6 b, c, d). The GSH level in all treatment groups was notably ($p < 0.0001$) varied from normal control except for metformin 100, gliclazide 5, and M100 + G 10 mg/kg treated obese-PCOS groups (Fig. 7b).

The CAT activity in all the treatment groups was notably varied from normal control except M300 and M100+G5 mg/kg treated groups. Additionally, CAT activity in all combination groups was significantly different from each other except M300+G5 from M300+G10 (Fig. 6c). The SOD activity in all treatment groups was notably ($p < 0.001$–0.0001) different from the normal control except metformin 300, gliclazide 5, 10, M100 + G 10, and M 300 + G mg/kg treated PCOS rats (Fig. 7d).

Whereas, NO and MDA levels were noticeably ($p < 0.0001$) increased in disease control as compared to the normal control group that was notably ($p < 0.05$–0.0001) restored in treatment groups alone and in combination except MDA level was insignificantly varied in M100+G10 and M300+G5 mg/kg treated PCOS groups in contrast to the disease control as mentioned in Fig. 7e. The MDA level in all treatment groups was notably varied from the normal control. The MDA level in M 100 mg/kg + G 5 mg/kg was notably varied ($p <$ 0.001–0.0001) from M 100+G10, M 300 + G5, and M 300 + G 10 mg/kg treated PCOS rats. The NO level was notably reduced in all treatment groups in contrast to normal control except metformin 100, gliclazide 5, 10, and M100 + G 10 mg/kg treated obese- PCOS

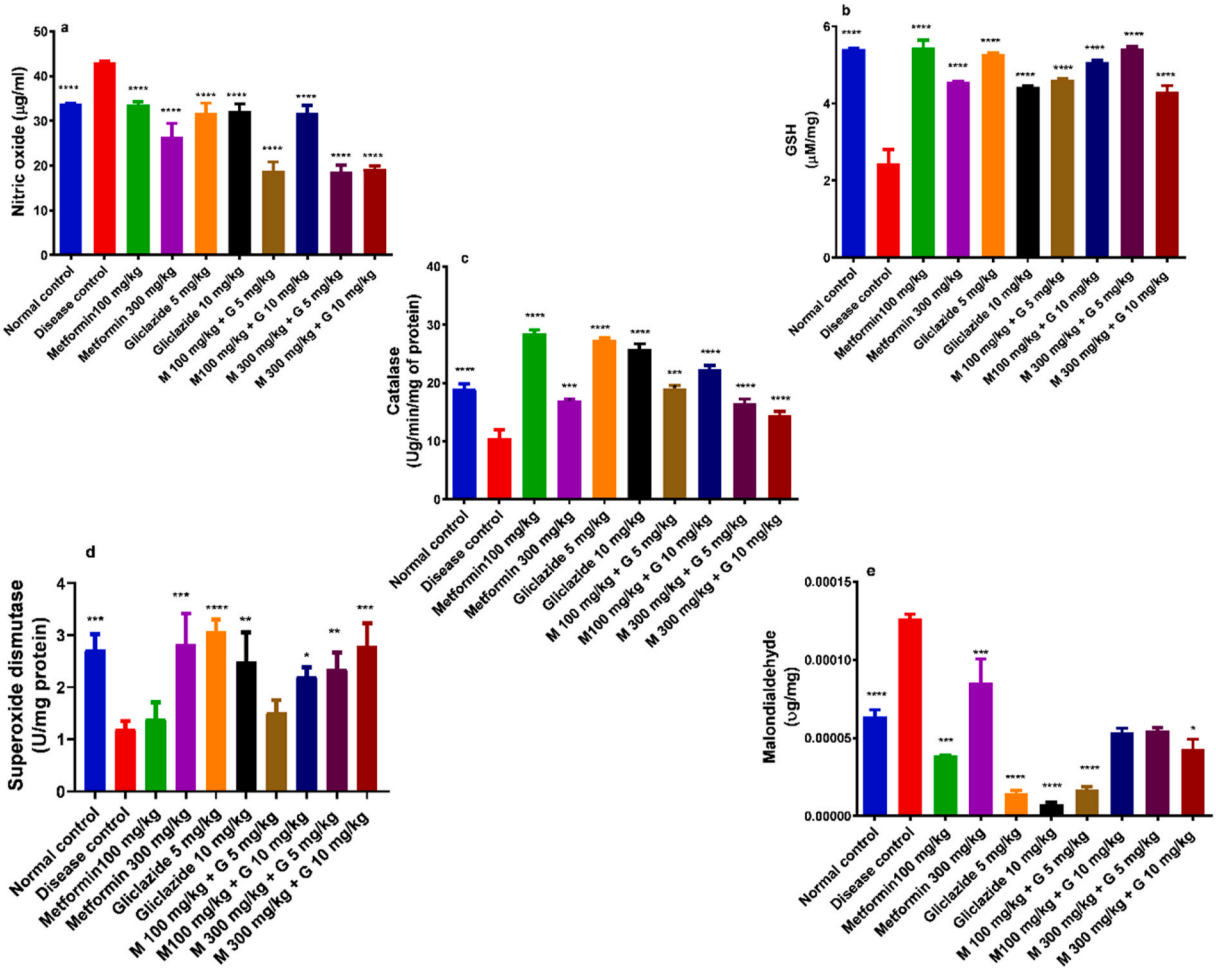

Fig. 7. Effect of gliclazide and metformin alone and in combination against oxidative stress biomarkers in obese-PCOS rats.
Results were shown as mean ± S. D (n = 6). One-way ANOVA followed by Tukey's multiple comparison test. Where *, **, ***, and **** showed statistical significance at $p < 0.05$, 0.01, 0.001, and 0.0001 respectively in comparison to disease control.

rats (Fig. 7a).

3.8. Histopathology of the ovary

Histopathological slides of the ovary of all PCOS rats treated with individual and combined therapy of metformin and gliclazide were examined to estimate the number and size of follicles and the effect of corpus luteum by comparing it with the normal control group (Fig. 8a). All the slides were identified under a light microscope at 40X, 100X and 400X. In the normal control group, normal corpus luteum and the maximum number of primary follicles were also observed. Changes in the number of antral follicles were also observed in treated groups (Fig. 8 c-j). There was a marked decrease in the number of oocytes thus causing changes in the structural and morphological parameters of ovaries in disease-control rats (Fig. 8b). In obese-PCOS rats treated with metformin 300 mg/kg + gliclazide 5 mg/kg shrinkage of the ovary tissues was seen with decreased corpus luteum. Primary and secondary follicles were also seen. However, the main prominent change was observed in obese- PCOS rats treated with high-dose combinational group metformin 300 mg/kg + gliclazide 10 mg/kg with many follicles at different stages of development (Fig. 8j). Corpus luteum was also observed with decreased numbers of secondary follicles thus restoring fertility.

4. Discussion

The present study was designed to attenuate obesity and PCOS by administering metformin, gliclazide, and their combination in the animal disease model. Increase of oxidative stress, decrease in insulin sensitivity, and hyperlipidemia are the predisposing causes of obesity which are further associated with PCOS. To determine the ameliorating effects of metformin, gliclazide, and their combination against obesity-induced ovarian damage, various biochemical parameters, reproductive hormones, oxidative stress, inflammatory

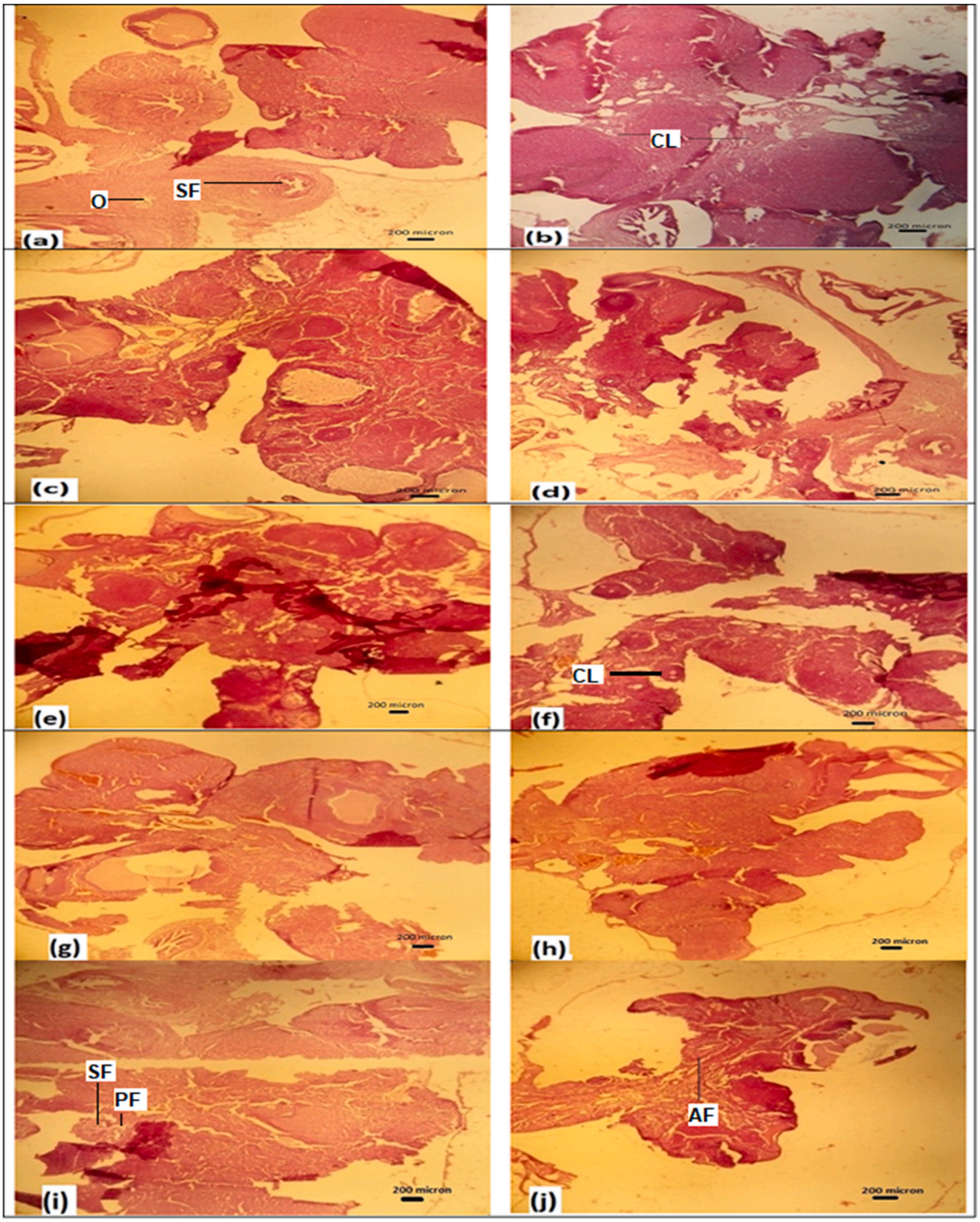

Fig. 8. Effect of metformin and gliclazide alone and in combination on the histopathology of the ovary in obesity-induced infertile rats at 40X **a;** normal control group of rats showed maximum no. of follicles**;** diseased control group showed reduction in no. of follicles **c-d;** groups given 100, 300 mg/kg doses of metformin showed changes in number of antral follicles **e-f;** groups given 5, 10 mg/kg doses of gliclazide showed relatively few numbers of antral follicles **g-h-i-j;** groups given high and low combinational doses of gliclazide and metformin showed maximum numbers of primary and secondary follicle

CL: Corpus luteum, CF: Cystic follicle, AF: Antral Follicle, O: oocytes, PF: primary follicle, SF: secondary follicle.

biomarkers, and histological examinations were performed.

The HFD leads to excessive fat deposition in both adipose and non-adipose tissues which results in lipotoxicity, causing disturbances in cellular function leading to cell death [41]. The HFD and EV were given to female rats except normal control to induce obesity and PCOS. As in previous research, data have shown that EV was directly related to polycystic ovarian damage and changes in ovarian morphology when it was orally administered at 2 mg/kg/day and vaginal swabs were prepared [42]. Whereas, HFD induced various metabolic dysfunctions. In the literature, it is obvious that administration of HFD along with EV in animals was associated with irregularity in the estrous cycle, obesity, insulin resistance, increased concentration in serum insulin levels, and hyperandrogenism [43]. Gareeb et al., 2016 reported that gliclazide, as an add-on therapy to metformin in diabetic patients, resulted in reduced blood glucose level, HbA1c, insulin resistance, and improved glycemic control that coobserved in the current study also [44].

Weight loss is associated with higher chances of spontaneous ovulation and the ability to conceive. According to previous research, the fertility rate can be improved by losing 5–10% of body weight. Approximately, 5% weight loss meaningfully improved endocrine parameters to restore fertility. Thus in the current study, weight loss observed in therapy groups in contrast to the disease control group may demonstrate the restoration of reproductive hormones in treatment groups [45].

Several studies acknowledged that metformin alone or in combination played an important role in reducing body weight as well as ovary weight in PCOS. The valuable effect of metformin in reducing weight loss is attributed to the intracellular adenosine monophosphate protein kinase (AMPK) which decreases the activity of acetyl CoA carboxylase. In addition, as the acetyl CoA activity decreased, the biosynthesis of fatty acids was reduced while increased β-oxidation of lipids further promoted weight loss [46]. Similar findings regarding metformin were also observed in current study also. Wang et al., 2013 reported that gliclazide, acarbose, and metformin as monotherapy resulted in the loss of fat mass, improved blood glucose, glycosylated Hb, and lipid profile in subjects as observed in present findings [47].

The gliclazide increased insulin secretion by inhibiting the ATP-dependent potassium channels and exerting an effect on both pulsatile and basal insulin stimulation. According to previous studies, gliclazide also has an extra-pancreatic effect which is favorable in reducing insulin resistance [28]. In the current study, gliclazide reduced insulin resistance by a similar mechanism. The present study showed that the level of HbA1c and serum insulin were considerably reduced in the treatment group, especially in G + M combination groups in contrast to the disease control group which might be due to improved glucose sensitivity in β cells and decreased insulin resistance as evidenced from a previous study involving monotherapy with G and M [47,48].

The PCOS is commonly associated with dyslipidemia. In the present findings, the increased serum triglycerides and total cholesterol level in obese-PCOS rats as compared to the normal control might be due to insulin resistance which decreased the activity of lipoprotein lipase causing hyperlipidemia. Present results indicated that metformin and gliclazide alone and in combination improved antilipidemic effects that proved useful in treating obesity. The therapeutic effect of metformin may be due to the activation of AMPK which has a direct effect on lipid metabolism by inhibiting HMC-CoA reductase (a cytosolic enzyme of cholesterol biosynthesis) and activating insulin signaling which promotes lipolysis [49]. The combination treatment of gliclazide with 300 mg of metformin showed marked improvement in lipid profile as compared to other treatments. A previous study reported the effect on lipid profile exhibited by Gliclazide and metformin in a previous study [44,49].

The histology of HFD diet-induced obese rats' ovaries showed a marked decrease in the number of oocytes thus causing changes in structural and morphological parameters of ovaries. The previous studies suggested that a decrease in follicular growth was due to the negative interaction of testosterone, FSH, LH, estradiol, and progesterone causing follicular apoptosis which further impaired the estrous cycle and led to obesity-induced ovarian damage [50] as observed in present study. The serum testosterone and estradiol levels were elevated in the disease control group while FSH, LH, and progesterone were lowered in the diseased group. Metformin and gliclazide alone or in combination decreased testosterone levels and also restored the estradiol levels as compared to the disease control group.

Previous research studies confirmed that increased serum levels of pro-inflammatory cytokines dysregulate the menstrual cycle causing PCOS in females. NF-kB is the most important transcriptional factor in promoting gene regulation. Disruption in NF-kB levels causes inflammatory diseases like asthma, rheumatoid arthritis, and Cushing syndrome. Several studies concluded that ovulation results in response to inhibition of NF-kB activity [51]. NF-kB is sensitive to reactive oxygen species production and imparts a crucial role in oxidative stress [52]The pathogenicity of obesity is linked with the upregulation of NF-kB that contributes towards inflammation and oxidative stress that cumulatively triggers insulin resistance as well as decreased lipid metabolism as noticed in disease control rats in current research. The metformin and gliclazide-treated rats alone or in combination therapy showed a marked decrease in NrF2, IL-6, and NF-kB in contrast with the disease control group, which might be due to the activation of the AMPK pathway in metformin-treated PCOS rats [53]. The maximum synergistic therapeutic efficacy of IL-6 was seen in the M 300 mg + G 10 mg treated group when compared to the disease-control obese group. According to previous research, metformin, and gliclazide serve as a powerful free radical scavenger by reducing pro-inflammatory cytokines levels as well as oxidative stress biomarkers and thus promoting ovulation [28] as noticed in the current study.

An oxidative stress causes irregularities in cellular function leading to many pathologic conditions such as PCOS, and impaired fertility in females because it is directly related to decreasing insulin sensitivity and impaired glucose metabolism in muscle and adipose tissues [33]. The GSH is a non-enzymatic antioxidant parameter that protects the body from the unfavorable effects of ROS. As in PCOS, lipid peroxidation increased, and GSH played an important role in protecting the peroxidation of lipid membranes by conjugating with an electrophile such as 4-hydroxy 3-neonatal HNE [54]. In the current study, metformin, gliclazide, and their combined therapy showed a marked increase in GSH levels and activity of SOD and CAT in ovary tissue homogenate as reported in previous studies that both gliclazide and metformin possess strong antioxidant properties [55]. Signorin et al., 2002 in a previous study reported that G and M have a positive impact on oxidation–reduction system in diabetic patients [49]. The raised level of NO is

associated with oxidation-induced ovarian damage as it directly reacts with superoxide anion to oxidize and produce peroxy nitrite-free radicals from DNA causing cell injury [56].

Metformin and gliclazide at different and combinational dosages showed their ameliorative effect by reducing serum NO and MDA levels and thus retaining their antioxidant activities to protect the biological system from oxidative stress. The ovary of PCOS rats showed an increase in the size of oocytes, in secondary and atretic follicles which were probably due to increased AMH levels and linked to the activity of metformin and gliclazide as evidenced by previous study that exhibited the positive role of M and G on oocytes [42]. This study lacks a comprehensive and detailed molecular mechanism regarding the effect of gliclazide alone or in combination with metformin against obesity and PCOS especially concerning peroxisome proliferated activated receptor (PPARγ).

5. Conclusion

Findings of the current study assured that individual therapy of metformin, gliclazide, and their combinations especially M100+G10, M300+ G5, and M300+G10 effectively reduced weight gain, improved lipid profile, HbA1c, insulin level, hormonal profile (PGE2, LH, FSH, and estradiol) and follicular cell architecture through antioxidant action in PCOS rats as both gliclazide and metformin exhibited antioxidant properties. The gliclazide and metformin efficiently treated obesity-induced infertility by reducing inflammatory (i.e. NrF2, IL-6, and NF-kB) and modulating oxidative stress biomarkers (i.e. GSH, SOD, CAT, NO, and MDA) whereas, more significant activity was observed in combination groups (M100+G10, M300+ G5, and M300+G10). Gliclazide as an add-on to metformin might be valuable in treating the issues of obesity and PCOS that should be evaluated in clinical settings. This study has also revealed that gliclazide in high doses may be used as an alternative to metformin in PCOS treatment. There is an immense need for further study to determine the dose-dependent experiments in which the gliclazide dose stabilized by reducing metformin.

Data availability statement

Authors declare that all the data supporting the findings of this study are included in the article.

CRediT authorship contribution statement

Anam Moazzam: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ammara Saleem:** Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation. **Shahid Shah:** Writing – review & editing, Visualization, Validation. **Liaqat Hussain:** Writing – review & editing, Resources. **Mirza Muhammad Faran Ashraf Baig:** Writing – review & editing, Resources. **Abdulrahman Alshammari:** Writing – review & editing, Funding acquisition, Formal analysis. **Norah A. Albekairi:** Writing – review & editing, Funding acquisition. **Muhammad Furqan Akhtar:** Writing – review & editing, Visualization, Validation, Conceptualization, Data curation, Formal analysis, Project administration, Resources, Supervision.

Declaration of competing interest

The authors declare that they have no conflict of interest.
https://www2.cloud.editorialmanager.com/heliyon/default2.aspx.

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