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Gamma Oryzanol: A natural compound with potential for treating polycystic ovary syndrome

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ABSTRACT

Introduction: Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age. Current management strategies only provide symptomatic relief, and there is a need for better treatments. Gamma-oryzanol, a dietary component found in rice bran oil, has been shown to be effective in increasing insulin sensitivity, reducing weight, and alleviating oxidative stress. In modern Chinese medicine, 香格里拉谷维素胶囊, 纯净谷维素软胶囊, and 诺特博谷维素片 are some of the medicines marketed in China that contain gamma-oryzanol. This study aims to evaluate the effects of gamma-oryzanol on clinical hallmarks of PCOS. Also to compare the efficacy of gamma-oryzanol alone versus in combination with clomifene citrate.

Methods: In this study, PCOS was induced in a rat model using estradiol valerate. Gamma-oryzanol was then administered both alone and in combination with clomifene citrate.

Results: The effects of gamma-oryzanol on estrous cyclicity, fertility, histopathological alterations, estradiol and testosterone levels, and oxidative parameters were evaluated. The results showed that gamma-oryzanol, both alone and in combination with clomifene citrate, had protective effects against the estradiol valerate-induced PCOS rat model. The combination of gamma-oryzanol and clomifene citrate was particularly effective, showing significantly better results than the standard treatment group (clomifene citrate alone).

Discussion: These results suggest that gamma-oryzanol could be used in combination with existing standard treatments to enhance the management of PCOS. However, further molecular and clinical studies are needed to confirm these findings.

Introduction

PCOS is considered the most common endocrinological disorder among women of reproductive age, with a prevalence of 4 to 4.7 % in United States [1]. In Europe, some studies have reported that the prevalence of PCOS is between 420 and 440 cases per 100,000 women [2,3].

PCOS is a metabolic syndrome and complete cure from PCOS is yet not available. PCOS management can be achieved through lifestyle modifications and medications, including diet, exercise, and pharmacological treatments. (ex: Clomifene Citrate, metformin (Glucophage) (Glucophage), Oral contraceptive pills, spironolactone (Aldactone) etc) [4–7] and Dietary supplements (ex: Chromium, Selenium, Omega 3 fish oils, Vitamin D, Inositol etc.) [8–12].

Animal models are essential tools for studying the underlying mechanisms of diseases. Researchers have conducted various animal studies to better understand the pathophysiology and etiology of PCOS. This complex disorder is characterized by insulin resistance, elevated androgen levels, infertility, and menstrual irregul arities. These metabolic complications can lead to obesity and other features of metabolic syndrome. Animal models have provided valuable insights into the roles of insulin resistance, androgens, and other factors in the development of PCOS. Additionally, these studies have helped identify potential targets for new treatments [13].

Numerous animal studies have shown that PCOS-like symptoms can be induced by interventions involving testosterone, estrogen, and androgens, as well as by environmental disruptors [14–25]. DHEA, letrozole (Femara), d-galactose, Bisphenol A, Monosodium-l-glutamate are also found to be causative chemical compound of PCOS [26–29].

PCOS does not have any direct treatment approach. Symptomatic relief and improvement of fertility are considered as PCOS management strategies. However, various current researches tried to combat with

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Table 1
Experimental design of approved treatment protocol.

Group	Treatment/Dose/Route	No of animals
Group 1: Vehicle control	PBS 1 ml/kg	6
Group 2: Gamma-Oryzanol *per se* treated group	Gamma-Oryzanol 200 mg kg^{-1} p.o.	6
Group 3: Disease control group	Estradiol Valerate 4 mg kg^{-1}	7
Group 4: Clomifene citrate treated group	Clomifene citrate 1 mg kg^{-1} in 0.5 % CMC p.o.	6
Group 5: Gamma-Oryzanol low dose treated group	Gamma-Oryzanol 100 mg kg^{-1} p.o.	7
Group 6: Gamma-Oryzanol high dose treated group	Gamma-Oryzanol 200 mg kg^{-1} p.o.	7
Group 7: Gamma-Oryzanol + Clomifene citrate treated group	Gamma-Oryzanol 100 mg kg^{-1} + Clomifene citrate (1 mg kg^{-1} in 0.5 % CMC p.o.)	7
Group 8: Fertility study*	–	10

Table 2
Histopathological findings in all the groups.

Groups	Histopathological findings
Group 1: Vehicle control	Normal architecture of ovary is observed. A healthy advanced follicle is present.
Group 2: Gamma-Oryzanol *per se* treated group	Normal architecture of ovary is observed. A healthy advanced follicle is present.
Group 3: Disease control group	Normal architecture of ovary is not preserved. No specific healthy follicle is observed. Multiple cystic structure is observed, which are ceased in different maturation stage. Disruption of overall cellular structure is observed. The central and peripheral stroma both are disrupted.
Group 4: Clomifene citrate treated group	The disruption is central and peripheral stroma is much better than G3. Multiple cysts are also present. One healthy mature follicle is present and one follicle in early maturation stage can be observed. A corpus luteum is also present.
Group 5: Gamma-Oryzanol low dose treated group	The disruption is central and peripheral stroma is not significantly improved as compared to G3. Multiple cysts are still present. One follicle in early maturation stage can be observed.
Group 6: Gamma-Oryzanol high dose treated group	The disruption is central and peripheral stroma is improved as compared to G3. Multiple cysts are not present. No healthy mature follicle is present.
Group 7: Gamma-Oryzanol + Clomifene citrate treated group	The disruption is central and peripheral stroma is significantly improved as compared to G3. Multiple cysts are not visible. Two follicles in early maturation stage can be observed.

underlying cause of PCOS by identifying different component. Melatonin, a neurohormone and metformin (Glucophage) found to be effective in the PCOS condition as it not only declined testosterone level, ovarian and body weight, promoted regularity of estrous cycle but also restored the numbers of the ovarian follicles (increased corpus luteum and primordial and Graafian follicles and decreased the primary, secondary, atretic, and cystic follicles) and increased the endometrial vessels length and volume [30]. Another study showed restoration of stereopathological parameters of ovary and uterus with the help of N-acetylcysteine and metformin (Glucophage). Both N-acetylcysteine and metformin (Glucophage) found to be helpful to reduce the number of primary follicles, secondary follicles, and ovarian cysts in PCOS rat [31]. Thymoquinone an antioxidant naturally found in black cumin also exhibit beneficial in PCOS condition. It improved folliculogenesis by significantly reduction of increased number of atretic follicles, altered the sexual hormone levels as it decreased the level of LH (leutinizing hormone), decreased LH/FSH ratio and increased FSH level, regulated the Bax/Bcl2 ratio gene expression [32]. Extract of natural components are also now a days a target of PCOS management. The hydroalcoholic extract of *Mentha Spicata* showed beneficial in PCOS condition. It decreased the testosterone level, restore ovarian morphology by reduction of the ovarian cysts, decreased atretic, primordial, primary and secondary follicles and increased corpus luteum [33].

Table 2

Gamma-oryzanol is extracted from *Oryza sativa* (rice). Chemically, it is a mixture of three lipids: esters of ferulic acid with phytosterols and triterpenoids, 24-methylenecycloartanyl ferulate, and campesteryl ferulate. [34,35]. As a dietary supplement, Gamma oryzanol is found to be very beneficial for various diseases and disorders. Several studies have proved the efficacy of Gamma oryzanol in increasing insulin sensitivity [36,37], reducing oxidative stress [38], modifying steroidogenesis, decrease xenoestrogen effect [39] along with these, Gamma oryzanol can also control lipid profile, reduce weight, reduce Basal Metabolic Rate (BMI), control diastolic blood pressure, lowers cardiovascular risk factors, minimize inflammatory marker level like hs-CRP (C reactive Proteins) [40]. Most importantly, it also has an anti-carcinogenic effect [34]. Gamma oryzanol also has oestrogen receptors modulating potential by which oestrogen level is found to be increase in rat uterus [41]. In another study, gamma oryzanol effectively improved the endometriosis condition by altering proteins like ER-α [42].

Therapeutic efficacy of Gamma-oryzanol

In traditional Chinese medicine, rice bran oil is valued for its various health benefits, as below:

A. **Digestive Health**: Rice bran oil is often used to support the digestive system. It is believed to have a mild, soothing effect on the stomach and intestines, potentially aiding in digestion and reducing symptoms of indigestion.
B. **Nourishing the Blood**: In traditional Chinese Medicine, rice bran oil is considered beneficial for nourishing the blood and improving circulation. This can help support overall vitality and energy levels.
C. **Skin Health**: The oil is used to promote healthy skin, thanks to its high content of vitamin E and antioxidants. It is thought to help moisturize and protect the skin, potentially reducing signs of aging and dryness.
D. **Liver Health**: Rice bran oil is sometimes used to support liver function and detoxification. Its antioxidant properties can help protect liver cells from damage and support overall liver health.
E. **Anti-inflammatory Properties**: It is believed to have anti-inflammatory effects, which can help alleviate conditions related to inflammation, such as joint pain or skin irritation.

Along with the traditional Chinese medicine in modern Chinese medicine and complementary health practices, gamma oryzanol is appreciated for its potential therapeutic benefits. Below are some marketed product of Gamma oryzanol used as modern Chinese medicines:

1. 香格里拉谷维素胶囊：Shangri-La Gamma Oryzanol Capsules, a dietary supplement that combines gamma oryzanol with other beneficial compounds for overall health.
2. 纯净谷维素软胶囊: Pureness Gamma Oryzanol Softgel capsules offering gamma oryzanol for its antioxidant and anti-inflammatory benefits.
3. 诺特博谷维素片: Gamma Oryzanol Tablets from NutraBio marketed for its cardiovascular and hormonal health benefits.
4. 方中谷维素胶囊: Fangzhong Gamma Oryzanol Capsules aimed at supporting general wellness.
5. 同仁堂谷维素: Tong Ren Tang Gamma Oryzanol marketed for overall health benefits.

Gamma-oryzanol has been shown to be effective in reducing insulin resistance, weight, and oxidative stress, and in increasing estrogen levels. These effects suggest that gamma-oryzanol could be a potential candidate for managing PCOS, as it addresses some of the underlying causes of the condition.

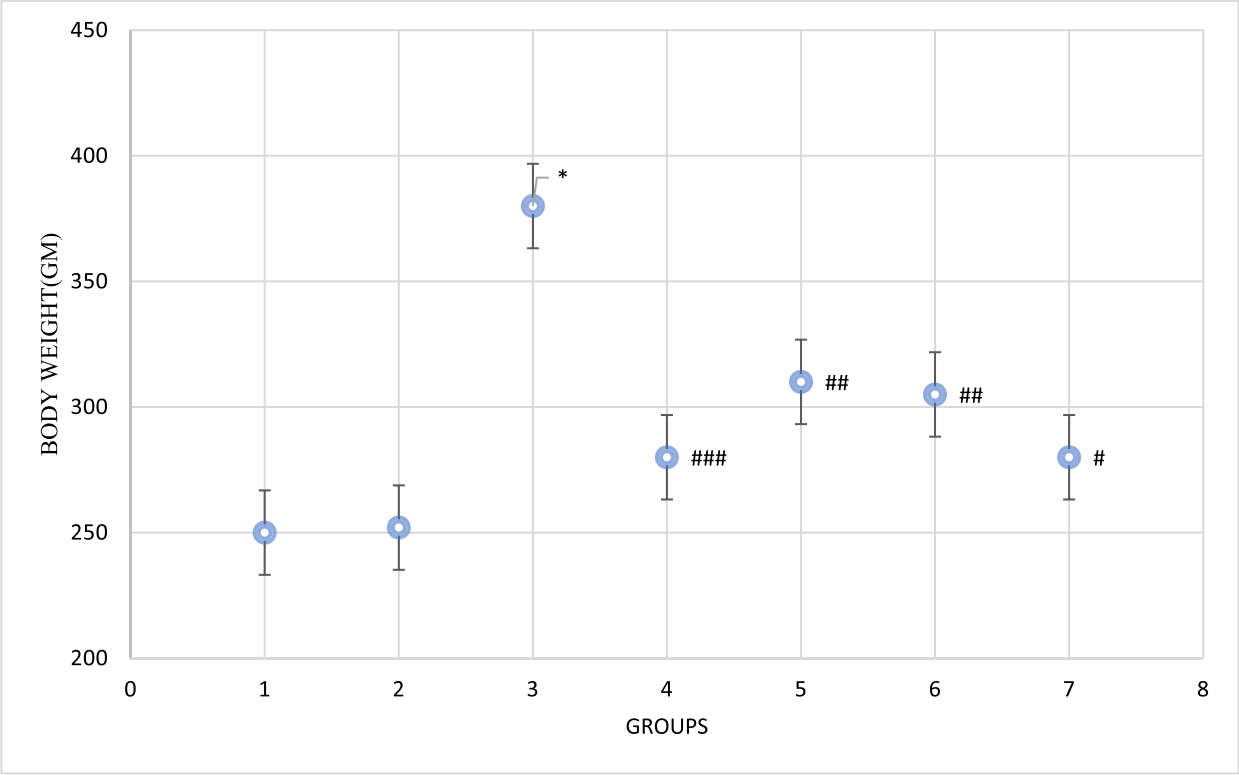

Fig. 1. Body weight in groups: Data are presented as means ± SEM. Statistical significance at the level of $p < 0.05$, $p < 0.01$ and $p < 0.001$. *** represents $p < 0.001$ significant difference, when compared to vehicle control group; $^{\#,\ \#\#}$ and $^{\#\#}$ represent $p < 0.05$, $p < 0.01$ and $p < 0.001$ significant difference, respectively, when compared to disease control group. Group numbers are same as Table 1.

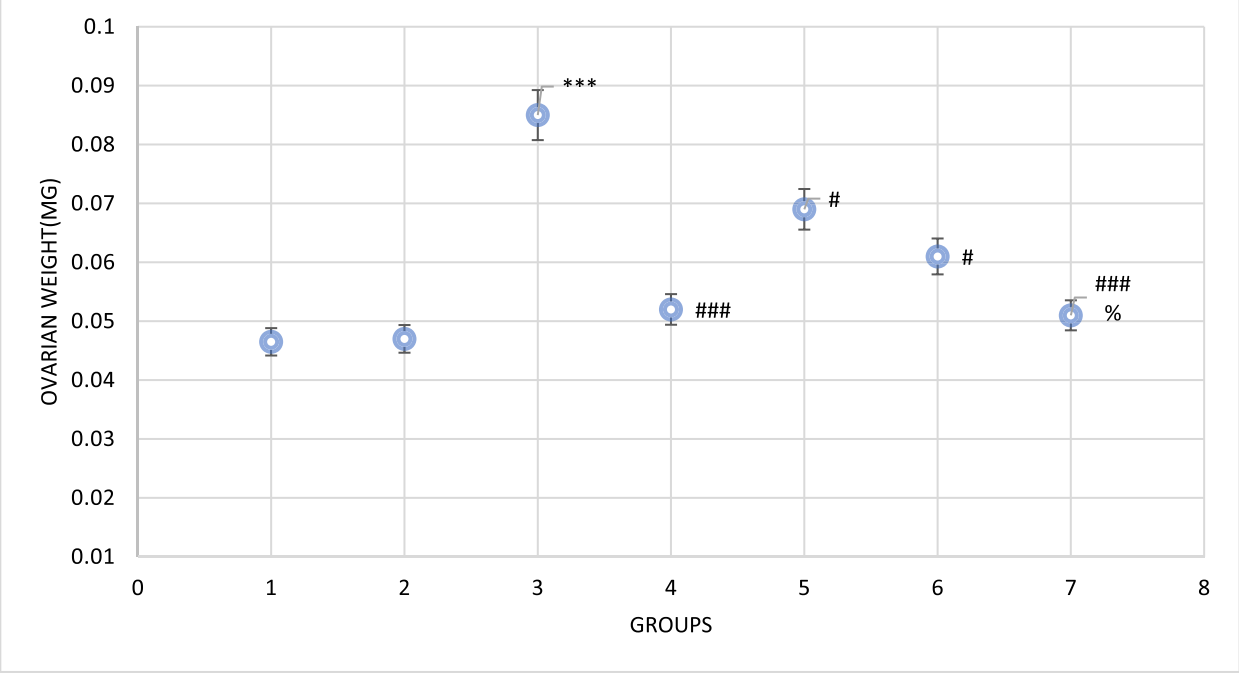

Fig. 2. Ovarian weight in groups: Data are presented as means ± SEM. Statistical significance at the level of $p < 0.05$, $p < 0.01$ and $p < 0.001$. *** represents $p < 0.001$ significant difference, when compared to vehicle control group; $^{\#}$ and $^{\#\#\#}$ represent $p < 0.05$ and $p < 0.001$ significant difference, respectively, when compared to disease control group; $^{\%}$ represents $p < 0.05$ significant difference, when compared to gamma-orazanol low dose treated group. Group numbers are same as Table 1.

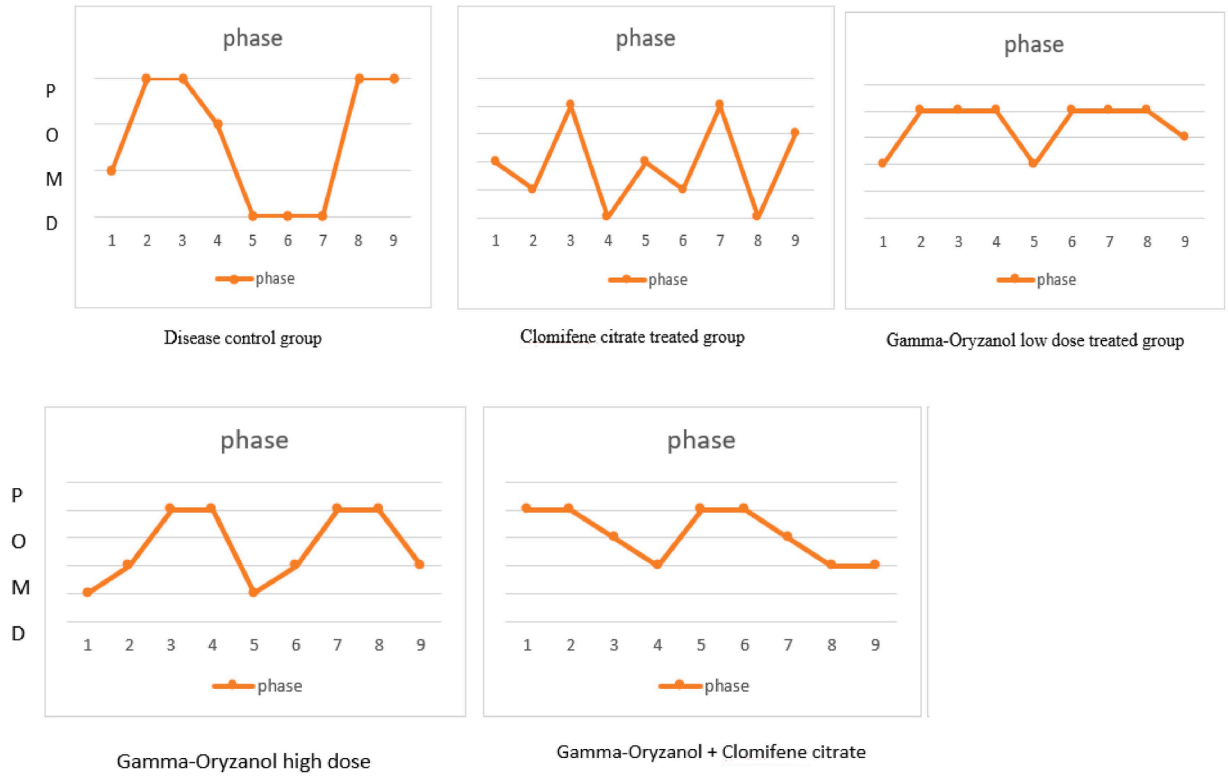

Fig. 3a. Disruption of estrous cyclicity of the groups.

In the condition of PCOS, alteration of lipid profile caused obesity, insulin sensitivity and oxidative stress increased, most importantly oogenesis; steroidogenesis; folliculogenesis is disrupted due to lack of oestrogen which leads to menstrual irregularities and finally infertility.

This study aims to assess the effects of gamma-oryzanol on estrous cyclicity and fertility, evaluate histopathological alterations in ovarian tissues, measure changes in estradiol and testosterone levels, and analyze oxidative stress parameters. Additionally, it seeks to compare the efficacy of gamma-oryzanol alone versus its combination with clomifene citrate. By determining the overall effectiveness of gamma-oryzanol as a potential adjunct therapy to standard PCOS treatments, the study aims to provide insights into the benefits of incorporating gamma-oryzanol into existing PCOS management strategies and to lay the groundwork for future molecular and clinical investigations.

Material and methods

Animals

Forty-six female and ten male Sprague Dawley rats, aged 3–5 months and weighing between 200 and 250 gs, were procured from the National Institute of Pharmaceutical Education and Research (NIPER) in S.A.S. Nagar, Punjab. The research proposal was approved under protocol number IAEC/LPU/2020/78 by the Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) in New Delhi.

The experiment was conducted at the central animal house facility at Lovely Professional University (Phagwara, Punjab), which is registered with CPCSEA under registration number 954/PO/*Re*/S/06/CPCSEA. The rats were transported by road in an institutional van to minimize stress. Upon arrival, they were housed in appropriately sized polypropylene cages to ensure their comfort and allow for free movement. The animals were provided with adequate food and water in suitable containers during transportation. After arrival at the central animal house facility, the rats were kept under a 12-hour light/12-hour dark cycle at controlled ambient temperature and humidity conditions and were acclimatized for 7 days.

All experiments conducted in this study were performed in compliance with the World Medical Association (WMA) Statement on Animal Use in Biomedical Research and the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The study also adhered to the guidelines of CPCSEA to ensure ethical practices in experimental design and analysis.

Below is the table for the experimental design of approved treatment protocol

Chemical and equipment

The chemicals and equipment are enlisted below which were required to conduct the study.

Equipment	Manufacturer
Centrifuge	Remi Instruments, India
Deep freezer	Blue Star Ltd., India
Digital weighing balance	Contech Instruments Ltd., India
Refrigerator	Kelvinator International, India
Microscope	Remi Instruments, India
Rat testosterone, T ELISA Kit	EVERON life sciences
Rat Estrogen, E ELISA Kit	EVERON life sciences
Elisa reader	iMarkTM Microplate Reader, Bio-Red
Chemical	**Manufacturer**
Clomifene citrate (gm)	Shanghai Huirui Chemical Technology Co., Ltd.
Oestradiol valerate (gm)	BB Chemicals
Gamma-Oryzanol (gm)	Excolla Pharma Inc

Treatment protocol

In the vehicle control group, olive oil was administered at a dose of 1 ml/kg. Estradiol valerate was given as a single dose of 4 mg kg^{-1}, and PCOS was induced after 28 days [43,44]. Gamma-oryzanol was

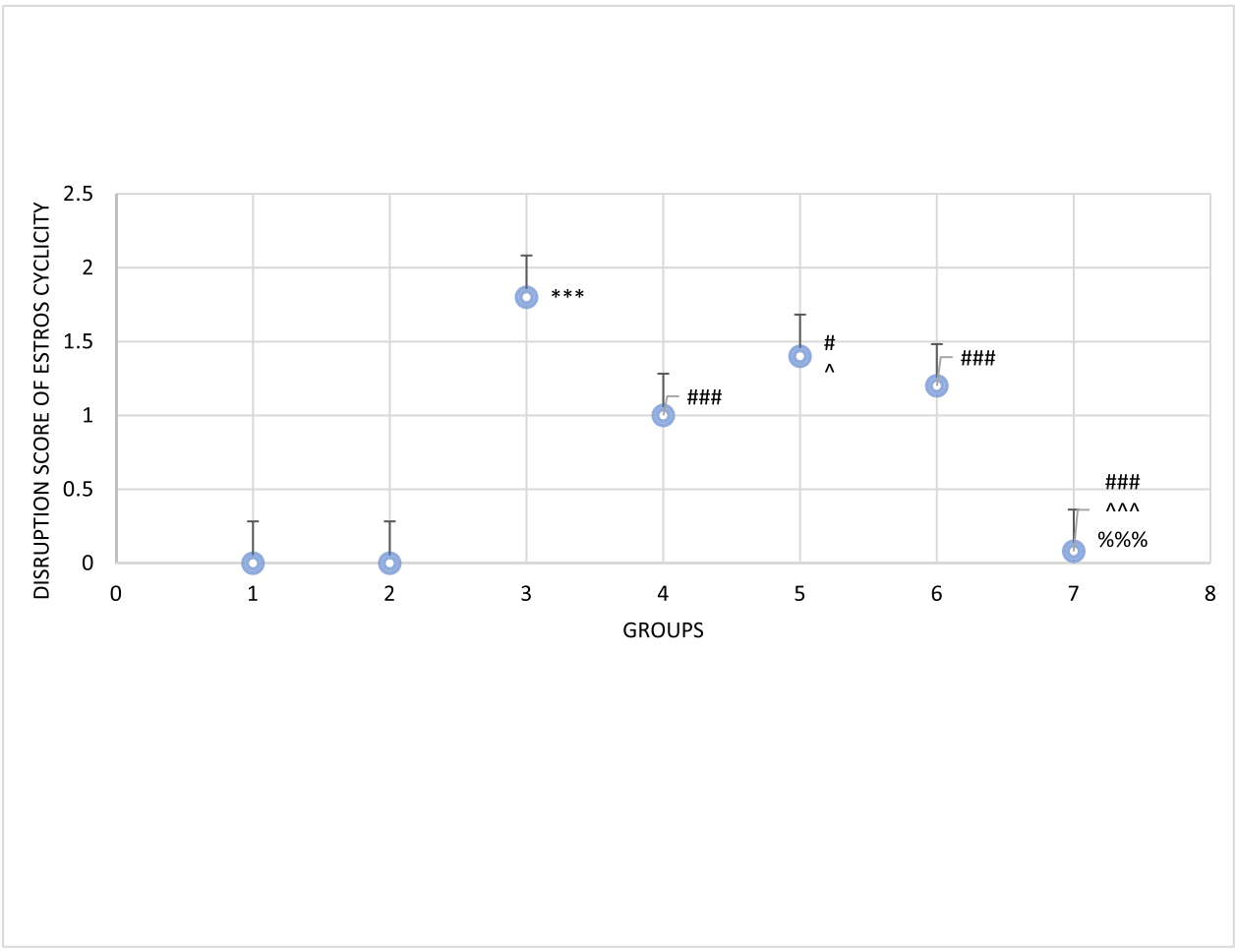

Fig. 3b. Estrous cyclicity in groups: Data are presented as means ± SEM. Statistical significance at the level of $p < 0.05$, $p < 0.01$ and $p < 0.001$. *** represents $p < 0.001$ significant difference, when compared to vehicle control group; # and ### represent $p < 0.05$ and $p < 0.001$ significant difference, respectively, when compared to disease control group; ^^^ represents $p < 0.001$ significant difference, when compared to clomifene citrate standard treated group; %%% represents $p < 0.001$ significant difference, when compared to gamma-orazanol low dose treated group. Group numbers are same as Table 1.

administered orally at doses of 100 mg kg^{-1}/day and 200 mg kg^{-1}/day [45,46]. Clomifene citrate was also administered orally at a dose of 0.1 mg kg^{-1}/day in 0.5 % carboxymethyl cellulose (CMC). For the combination treatment, a dose of 100 mg kg^{-1}/day of gamma-oryzanol was used. All treatments with gamma-oryzanol and clomifene citrate continued for 14 days. The various parameters were evaluated on the 14th day of treatment.

Estrous cyclicity

PCOS is a condition that can disrupt regular menstrual cyclicity. To study estrous cyclicity in rats, researchers used a technique called vaginal smear cytology. This technique involves collecting cells from the vaginal lining and examining them under a microscope to determine the phase of the estrous cycle.

The estrous cycle in rats is a 4-day cycle, which can be tracked by collecting vaginal smears throughout the study period. Each rat was monitored for 28 days of PCOS induction followed by 14 days of treatment. The estrous cycle consists of four phases: proestrus (P), estrus (E), metestrus (M), and diestrus (D).

The pipette smear technique is preferred over the cotton swab technique because it is less likely to induce pseudo-pregnancy. Additionally, the pipette technique is easier to perform and provides clearer images of the cells.

To perform a pipette smear, the rat was held with its thorax facing upwards. Approximately 0.2 ml of normal saline (0.9 % w/v NaCl) was drawn into the pipette tip. The end of the pipette was then gently inserted into the vaginal cavity, and the saline was flushed out and drawn back into the pipette. The collected vaginal smear was placed on a glass slide (2–3 drops), covered with a coverslip, and observed under a microscope with a 10× objective to identify the phase of the estrous cycle [47]. The disruption of estrous cyclicity in the different groups is depicted in Fig 3a in graphical form.

Fertility study

The vaginal smears of all the female rats were observed daily. The rats in the pre-estrous phase were isolated and kept with fertile male rats overnight. The following morning, the female rats were inspected for signs of copulation using a vaginal lavage test. Copulation was confirmed by the presence of sperm in the vaginal smear. The fertility rate of each animal was assessed after the animals were sacrificed. A laparotomy was performed to determine the number of implantation sites in both uterine horns [48].

Body weight and ovarian weight

According to the procedure described by [43], ovarian and body weights were evaluated. Animals were selected to weigh 200 to 250 gs each, and the standard deviation was applied to group them in order to

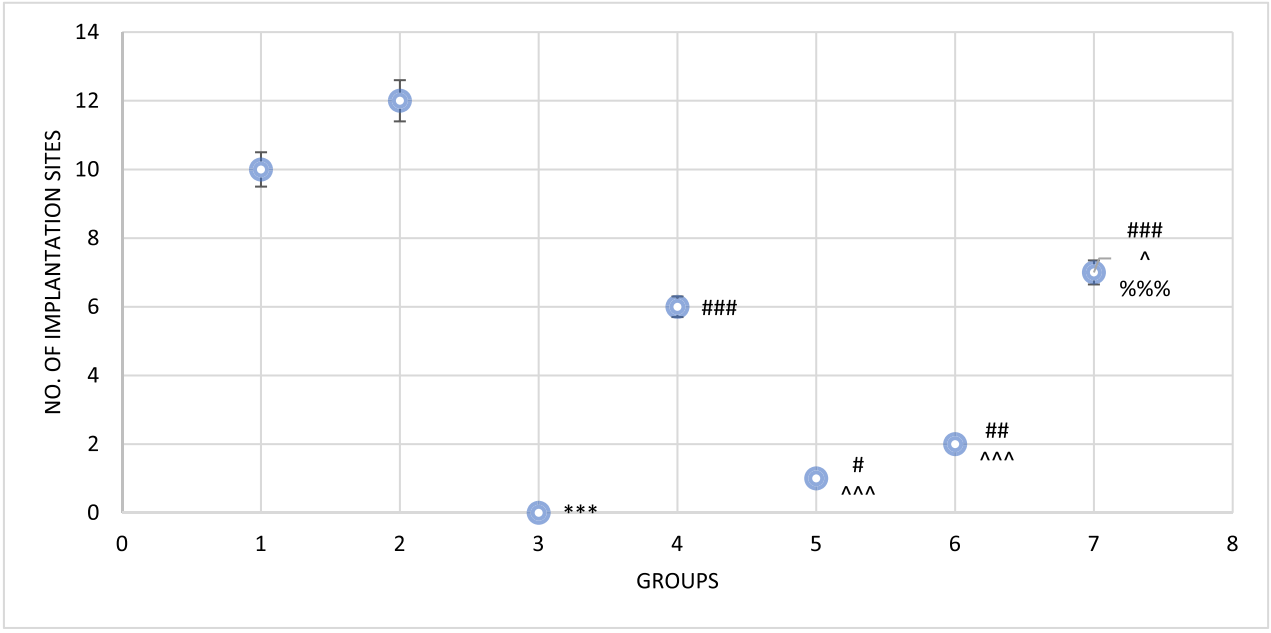

Fig. 4. Fertility study in groups: Data are presented as means ± SEM. Statistical significance at the level of $p < 0.05$, $p < 0.01$ and $p < 0.001$. *** represents $p < 0.001$ significant difference, when compared to vehicle control group; $^{\#}$, $^{\#\#}$ and $^{\#\#\#}$ represent $p < 0.05$, $p < 0.01$ and $p < 0.001$ significant difference, respectively, when compared to disease control group; ^ represents $p < 0.05$ significant difference, when compared to clomifene citrate standard treated group; $^{\%\%\%}$ represents $p < 0.001$ significant difference, when compared to gamma-orazanol low dose treated group. Group numbers are same as Table 1.

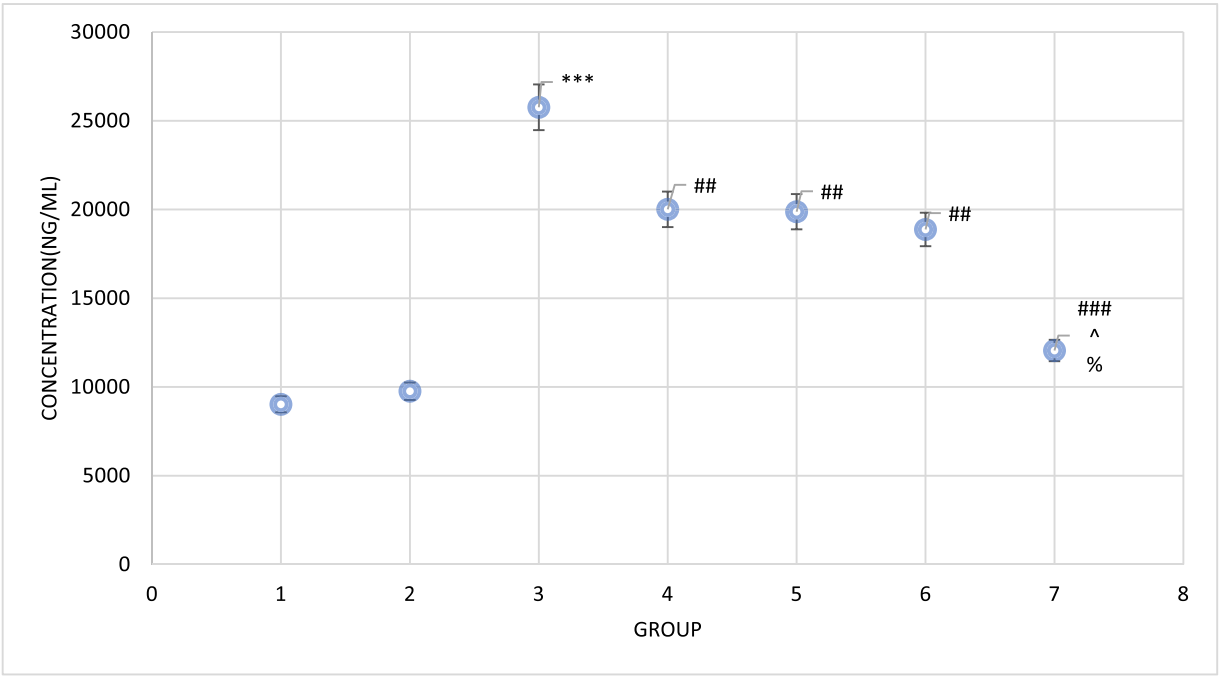

Fig. 5. Serum concentrations of estrogen in groups: Data are presented as means ± SEM. Statistical significance at the level of $p < 0.05$, $p < 0.01$ and $p < 0.001$. *** represents $p < 0.001$ significant difference, when compared to vehicle control group; $^{\#\#}$ and $^{\#\#}$ represent $p < 0.01$ and $p < 0.001$ significant difference, respectively, when compared to disease control group; ^ represents $p < 0.05$ significant difference, when compared to clomifene citrate standard treated group; $^{\%}$ represents $p < 0.05$ significant difference, when compared to gamma-orazanol low dose treated group. Group numbers are same as Table 1.

account for weight variation across different groups. Body weight was recorded before the experiment began, and again on the 28th day and the 42nd day (28+14).

Post-mortem evaluations

Histopathological study

A histopathological assessment of the ovaries was performed. After the animals were sacrificed, the ovaries were fixed in 10 % formaldehyde for at least 24 h. The tissue was then processed according to standard protocols. The ovaries were embedded in paraffin

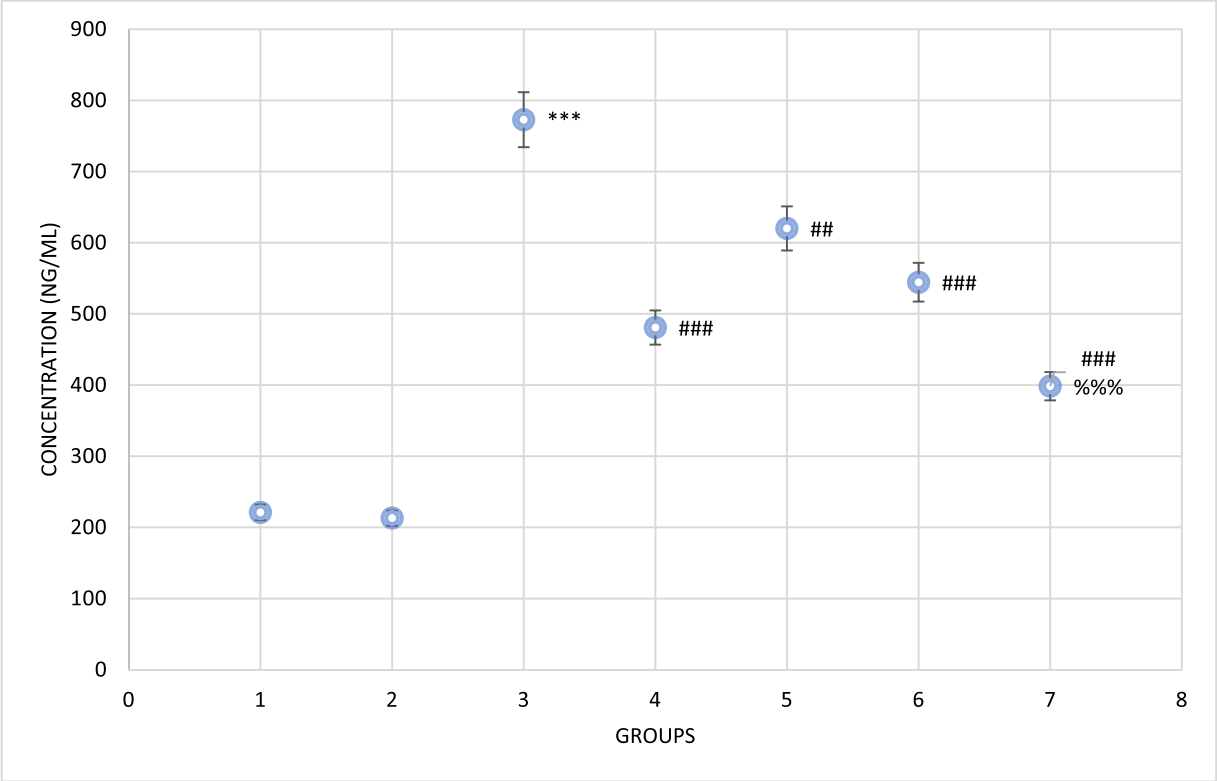

Fig. 6. Serum concentrations of testosterone in groups: Data are presented as means ± SEM. Statistical significance at the level of $p < 0.05$, $p < 0.01$ and $p < 0.001$. *** represents $p < 0.001$ significant difference, when compared to vehicle control group; ## and ## represent $p < 0.01$ and $p < 0.001$ significant difference, respectively, when compared to disease control group; % represents $p < 0.05$ significant difference, when compared to gamma-orazanol low dose treated group. Group numbers are same as Table 1.

longitudinally and cut into 5-micrometer sections. The sections were stained with hematoxylin and eosin. The presence of follicles in the ovaries was observed. All slides were examined using a light microscope at 200× magnification to observe the structure of the follicles [43].

Biochemical studies

The animals were sacrificed using the cervical dislocation method. Blood was collected from each animal via cardiac puncture and allowed to clot for 1 hour at room temperature. The serum was then obtained by centrifugation at 14,000 × *g* for 10 min and immediately frozen at −20°C. Serum concentrations of estradiol and testosterone were measured using a commercially available kit. The immunoenzymatically process was performed on an ELISA plate with horseradish peroxidase as the enzyme-labeled antigen. Absorbance was detected at 450 nm [49].

Enzymatic assay

A 10 % homogenate of ovarian tissue was prepared in Tris–HCl buffer (0.1 M) at pH 7.8. The homogenate was then centrifuged at 8000× *g* for 1 h. The supernatant was collected and used to determine the enzymatic parameters listed below [50].

a. Superoxide dismutase (SOD) activity

The activity of SOD was evaluated using the method described by [51] In this method, epinephrine auto-oxidizes to adrenochrome. The color reaction was observed at 480 nm. At 26°C, the amount of enzyme that inhibits 50 % of auto-oxidation was defined as one enzymatic unit (1 UI).

b. Glutathione S-transferase (GSH) activity

According to the method described by (Habig et al., 1974), GSH activity was measured using a spectrophotometer at 340 nm. A mixture of homogenized tissue, 100 mM GSH, 0.1 M potassium phosphate buffer (pH 7.4), and 100 mM CDNB was used as the substrate. Enzyme activity was reported as nmol CDNB conjugated per minute per mg of protein..

c. Catalase (CAT) activity

The activity of catalase (CAT) was measured using a spectrophotometer at 240 nm by monitoring the disappearance of hydrogen peroxide (H_2O_2). An aliquot of 50 mM potassium phosphate buffer (pH 7.0) was added to the sample, followed by H_2O_2 to initiate the enzymatic reaction. The amount of enzyme required to decompose 1 micromole of H_2O_2 per minute was expressed as Units (U) per milligram of protein [52].

Statistical analysis

All results were expressed as mean ± SD. One-way ANOVA was performed, followed by the Tukey test (SigmaPlot 14.0). P-values of <0.05, <0.01, and <0.001 were considered statistically significant for all comparisons.

Results

Effect on body weight

The body weight decreased significantly in all gamma oryzanol treatment groups compared to the estradiol valerate-treated group ($p < 0.01$). The clomifene citrate group also showed a significant decrease in body weight ($p < 0.001$). The gamma oryzanol + clomifene citrate group exhibited a significantly greater decrease in body weight ($p < 0.001$). The combination treatment group of gamma oryzanol and

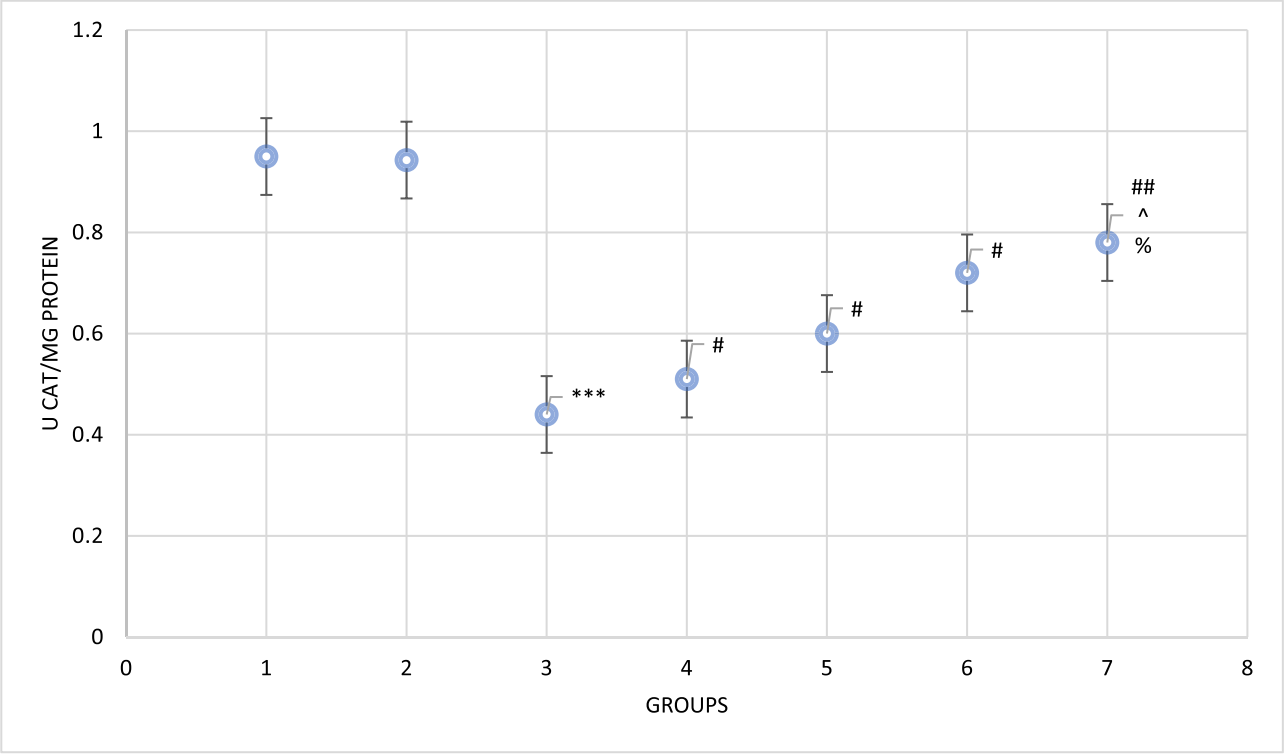

Fig. 7. levels of CAT in groups: Data are presented as means ± SEM. Statistical significance at the level of $p < 0.05$, $p < 0.01$ and $p < 0.001$. *** represents $p < 0.001$ significant difference, when compared to vehicle control group; $^{\#}$ and $^{\#\#}$ represent $p < 0.05$ and $p < 0.01$ significant difference, respectively, when compared to disease control group; $^{\wedge}$ represents $p < 0.05$ significant difference, when compared to clomifene citrate standard treated group; $^{\%}$ represents $p < 0.05$ significant difference, when compared to gamma-orazanol low dose treated group. Group numbers are same as Table 1.

clomifene citrate showed a decrease in body weight compared to both the standard drug (clomifene citrate)-treated group and the gamma oryzanol-treated groups, but the difference was not statistically significant (Fig. 1).

Effect on ovarian weight

The weight of the ovaries decreased significantly in all gamma oryzanol treatment groups compared to the estradiol valerate-treated group ($p < 0.01$). The clomifene citrate group also showed a significant decrease in ovarian weight ($p < 0.001$), and the gamma oryzanol + clomifene citrate group exhibited an even greater decrease ($p < 0.001$). The combination treatment group of gamma oryzanol and clomifene citrate showed a decrease in ovarian weight compared to the standard drug (clomifene citrate)-treated group, but this difference was not statistically significant. However, the gamma oryzanol and clomifene citrate combination treatment group showed a statistically significant decrease in ovarian weight compared to the gamma oryzanol-treated groups ($p < 0.01$) (Fig 2).

Effect on estrous cycle

The irregularity of estrous cyclicity decreased significantly in all treatment groups compared to the estradiol valerate-treated group ($p < 0.001$). The gamma oryzanol low-dose group also showed a significant decrease in the irregularity of estrous cyclicity ($p < 0.05$). The gamma oryzanol and clomifene citrate combination treatment group exhibited a significantly greater decrease in estrous cyclicity irregularity compared to both the standard drug (clomifene citrate)-treated group and the gamma oryzanol-treated group ($p < 0.001$). The results of this study suggest that the combination treatment of gamma oryzanol and clomifene citrate is more effective than either the standard drug (clomifene citrate) or gamma oryzanol alone in reducing the irregularity of estrous cyclicity (Fig 3b).

Effect on fertility

The number of implantation sites increased significantly in all gamma oryzanol treatment groups compared to the estradiol valerate-treated group. The gamma oryzanol low-dose group showed a significant increase ($p < 0.05$), the gamma oryzanol high-dose group showed a significant increase ($p < 0.01$), and the gamma oryzanol + clomifene citrate group showed a significant increase ($p < 0.001$). The combination treatment group of gamma oryzanol and clomifene citrate demonstrated a greater increase in the number of implantation sites compared to both the standard drug treatment group and the gamma oryzanol-only treatment groups, with the difference being statistically significant (Fig 4).

Effect on oestrogen level

The level of estrogen decreased significantly in all treatment groups compared to the estradiol valerate-treated group ($p < 0.01$). The combination treatment group (gamma oryzanol and clomifene citrate) showed a significantly greater decrease in estrogen levels ($p < 0.001$). This group also exhibited a significantly greater decrease in estrogen levels compared to both the standard drug (clomifene citrate)-treated group ($p < 0.05$) and the gamma oryzanol-treated group ($p < 0.05$). The results of this study suggest that the combination treatment of gamma oryzanol and clomifene citrate is more effective than either the standard drug (clomifene citrate) or gamma oryzanol alone in reducing estrogen levels (Fig. 5).

Effect on testosterone level

The level of testosterone decreased significantly in all treatment

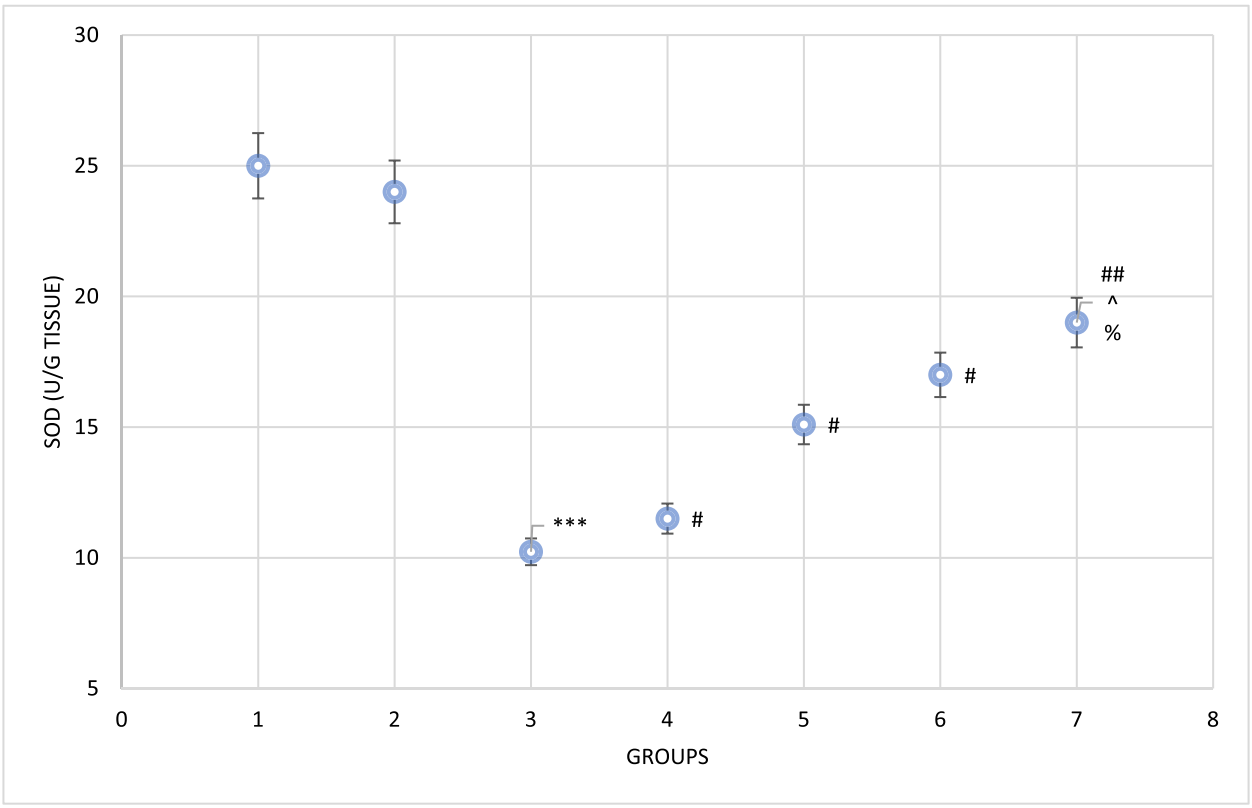

Fig. 8. levels of SOD in groups: Data are presented as means ± SEM. Statistical significance at the level of $p < 0.05$, $p < 0.01$ and $p < 0.001$. *** represents $p < 0.001$ significant difference, when compared to vehicle control group; # and ## represent $p < 0.05$ and $p < 0.01$ significant difference, respectively, when compared to disease control group; ^ represents $p < 0.05$ significant difference, when compared to clomifene citrate standard treated group; % represents $p < 0.05$ significant difference, when compared to gamma-orazanol low dose treated group. Group numbers are same as Table 1.

groups compared to the estradiol valerate-treated group ($p < 0.001$). The gamma oryzanol low-dose treatment group also showed a significant decrease in testosterone levels ($p < 0.01$). The gamma oryzanol and clomifene citrate combination treatment group showed a decrease in testosterone levels compared to the standard drug (clomifene citrate)-treated group, but this difference was not statistically significant. However, the combination treatment group showed a significant decrease in testosterone levels compared to the gamma oryzanol-only treatment groups ($p < 0.001$). The results of this study suggest that the combination treatment of gamma oryzanol and clomifene citrate is more effective than gamma oryzanol alone in reducing testosterone levels. The difference between the combination treatment group and the standard drug (clomifene citrate)-treated group was not statistically significant (Fig 6).

Effect on catalase activity

The level of catalase increased significantly in all treatment groups compared to the estradiol valerate-treated group ($p < 0.05$). The gamma oryzanol + clomifene citrate group showed a significantly greater increase in catalase levels ($p < 0.01$). This combination treatment also demonstrated a significantly greater increase in catalase levels compared to both the standard drug (clomifene citrate)-treated group and the gamma oryzanol-treated groups ($p < 0.05$). The results of this study suggest that the combination treatment of gamma oryzanol and clomifene citrate is more effective than either the standard drug (clomifene citrate) or gamma oryzanol alone in increasing catalase levels (Fig 7).

Effect on superoxide dismutase activity

The level of SOD increased significantly in all treatment groups compared to the estradiol valerate-treated group ($p < 0.05$). The gamma oryzanol + clomifene citrate group showed a significantly greater increase in SOD levels ($p < 0.01$). This combination treatment also resulted in a significantly greater increase in SOD levels compared to both the standard drug (clomifene citrate)-treated group and the gamma oryzanol-treated groups ($p < 0.05$). The results of this study suggest that the combination treatment of gamma oryzanol and clomifene citrate is more effective than either the standard drug (clomifene citrate) or gamma oryzanol alone in increasing SOD levels (Fig 8).

Effect on reduced glutathione activity

The level of GSH increased significantly in all treatment groups compared to the estradiol valerate-treated group ($p < 0.01$). The clomifene citrate group also showed a significant increase in GSH levels ($p < 0.05$). The gamma oryzanol + clomifene citrate combination treatment group exhibited a significantly greater increase in GSH levels compared to the standard drug (clomifene citrate)-treated group ($p < 0.05$). This combination treatment also showed a slight decrease in GSH levels compared to the gamma oryzanol-treated groups, although this difference was not statistically significant. The results of this study suggest that the combination treatment of gamma oryzanol and clomifene citrate is more effective than either the standard drug (clomifene citrate) or gamma oryzanol alone in increasing GSH levels. However, the difference between the combination treatment group and the gamma oryzanol-treated groups was not statistically significant (Fig 9).

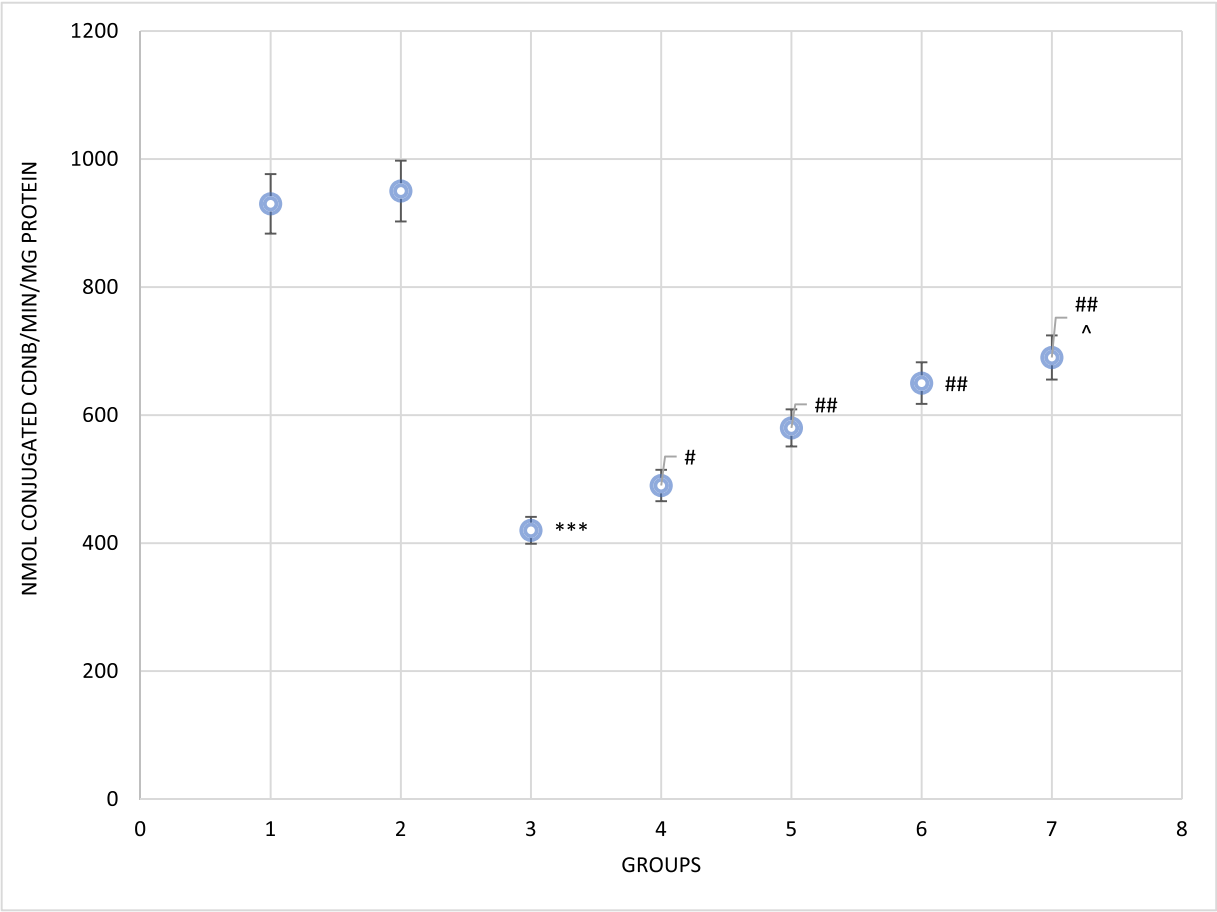

Fig. 9. levels of GSH in groups: Data are presented as means ± SEM. Statistical significance at the level of $p < 0.05$, $p < 0.01$ and $p < 0.001$. *** represents $p < 0.001$ significant difference, when compared to vehicle control group; # and ## represent $p < 0.05$ and $p < 0.01$ significant difference, respectively, when compared to disease control group; ^ represents $p < 0.05$ significant difference, when compared to clomifene citrate standard treated group. Group numbers are same as Table 1.

Histopathological study

In a histopathological analysis of the estradiol valerate-treated groups, multiple cystic structures were found inside the ovaries. The maturation of follicles was arrested at various stages, leading to polycystic ovarian morphological changes. These disruptions were reversed in the treatment groups. In the gamma oryzanol and clomifene citrate-treated groups, the number of follicles was found to be decreased (Fig. 10). Below are the details of the histopathological findings in all the groups.

Discussion

PCOS is characterized by various hormonal imbalances and is marked by symptoms of hyperandrogenism. Key clinical features of PCOS include menstrual disorders, multiple ovarian cysts, increased ovarian weight, decreased fertility, elevated levels of androgen and testosterone, and metabolic disorders such as obesity and insulin resistance [13].

In the present study, we evaluated the ameliorative effect of gamma oryzanol on PCOS. Gamma oryzanol treatment significantly increased the activities of SOD, CAT, and GSH, which contributed to a significant reduction in oxidative stress. When combined with clomifene citrate, the increase in these enzyme levels was more pronounced than with gamma oryzanol alone.

Obesity is a major clinical feature of PCOS. In our study, body weight increased in PCOS-induced groups, but a significant reduction in body weight was noted in the gamma oryzanol treatment group. The combination with clomifene citrate led to an even greater reduction in body weight compared to gamma oryzanol alone.

Ovarian weight also decreased significantly in the gamma oryzanol-treated group, as evidenced by the decreased number of follicles observed in the histopathological study. The combination with clomifene citrate resulted in a more significant reduction in ovarian weight compared to gamma oryzanol alone.

In PCOS, estrous cyclicity is disrupted, with irregularities in the four phases of the cycle. This study demonstrated that gamma oryzanol can significantly reverse these irregularities. The combination with clomifene citrate provided better management of estrous cyclicity than gamma oryzanol alone.

Infertility is a primary outcome of PCOS. To assess fertility, we calculated the number of implantation sites in the rat uterus. Gamma oryzanol significantly increased the number of implantation sites in PCOS conditions. The combination with clomifene citrate resulted in a more significant increase in fertility compared to gamma oryzanol alone.

Hormonal imbalance is another critical aspect of PCOS. In our study, estrogen and testosterone levels were evaluated. Gamma oryzanol was found to significantly reduce these hormone levels, reversing the imbalance. The combination with clomifene citrate resulted in a more pronounced reduction in estrogen and testosterone levels compared to gamma oryzanol alone.

Current management strategies for PCOS are limited and typically start with lifestyle modifications, including changes in diet, sleep, and exercise, and can extend to surgical interventions. Laparoscopic ovarian drilling is used when medications like clomifene citrate are ineffective

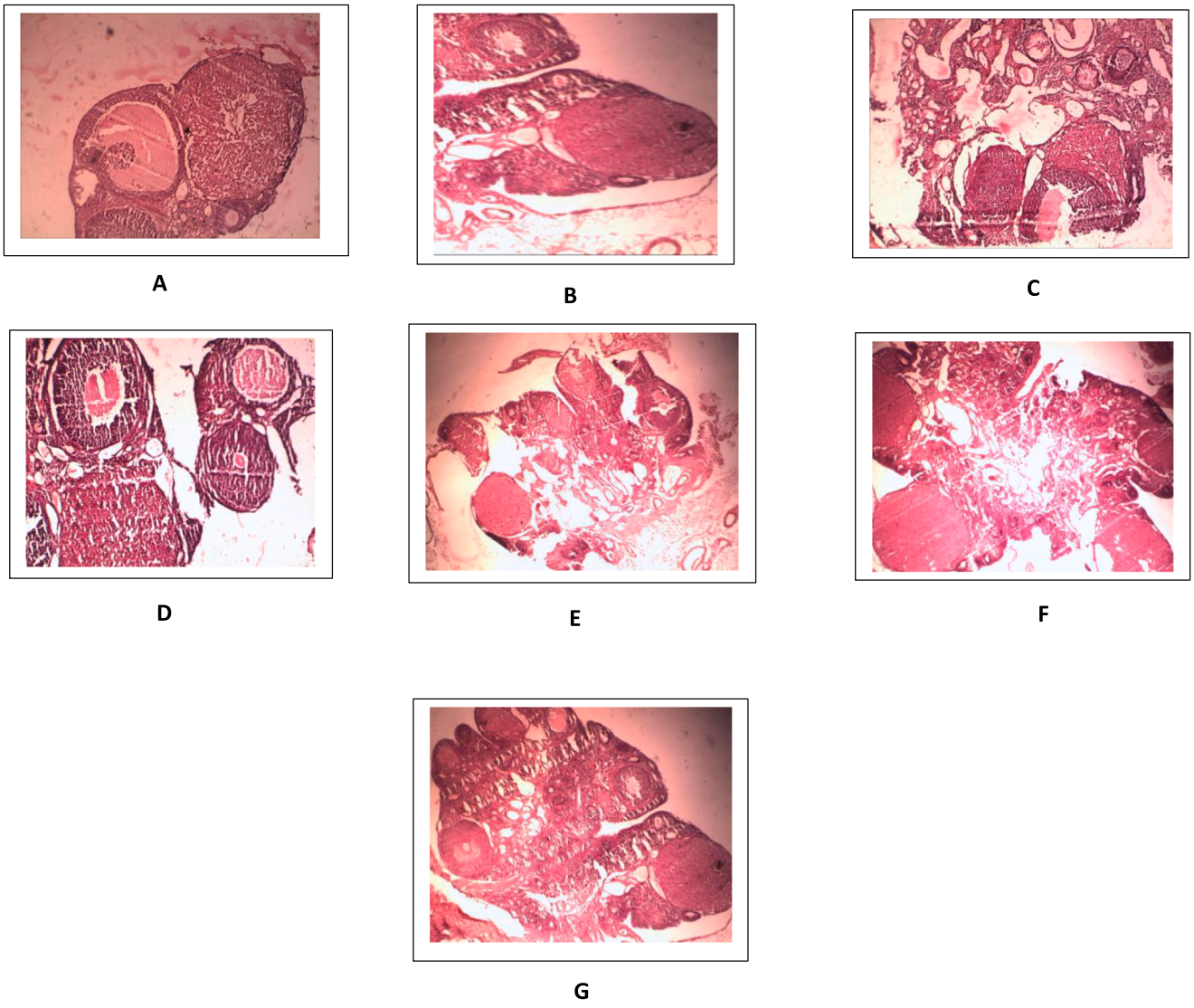

Fig. 10. Histology of ovaries of different groups. A: Control group, B: Gamma-Oryzanol per se, C: Disease control, D: Clomifene citrate, E: Gamma-Oryzanol low dose, F: Gamma-Oryzanol high dose, G: Gamma-Oryzanol + Clomifene citrate.

(Farquhar et al., 2012). Oral contraceptive pills can regulate menstrual cycles but may increase the risk of endometrial cancer with long-term use [53].

This study aimed to investigate major clinical aspects of PCOS with gamma oryzanol alone and in combination with clomifene citrate. The results indicate that gamma oryzanol has the potential to offer beneficial effects in PCOS, and its efficacy is enhanced when combined with clomifene citrate.

Limitation

This study has some limitations. Although we aimed to identify the ameliorative effect of gamma oryzanol on PCOS and evaluate all clinical outcomes of this syndrome, the underlying molecular mechanisms by which gamma oryzanol reverses PCOS were not explored. Additionally, the study results may not be sufficient to draw definitive conclusions applicable to human populations, as they do not account for variations related to race, age, or ethnicity.

Conclusion

In this experiment, we found that gamma oryzanol significantly decreased levels of estrogen and testosterone, reduced oxidative stress, decreased body and ovarian weight, reversed estrous cycle irregularity, and promoted fertilization. These effects were more pronounced when gamma oryzanol was combined with clomifene citrate, suggesting that gamma oryzanol has a beneficial effect on PCOS. Further research is needed to elucidate the underlying molecular mechanisms of gamma oryzanol's effects on PCOS.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Google Bard in order to improve language and readability of certain sections of the manuscript. After using this tool, all the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare no conflict of interest.

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