



Pharmacological Evaluation of Combination Effect of Ketoconazole and Metformin with N-Acetylcysteine in Letrozole Induced PCOS in Female Wistar Rats

Sweta Kumari*, Rahul Singh Dhariyal, Dr. Sanjay Singh

Siddhartha Institute of Pharmacy, Sahastradhara Road, Near IT Park, Do Bachi Road, Dehradun, Uttarakhand, India

(Received: 16 May 2025

Revised: 20 June 2025

Accepted: 24 July 2025)

KEYWORDS

PCOS treatment, Ketoconazole, Polycystic Ovary, Hyperandrogenism.

ABSTRACT:

Polycystic Ovary Syndrome (PCOS) is a complex endocrine disorder with hyperandrogenism, insulin resistance and chronic anovulation as its primary characteristics that affects a significant number of females in their reproductive age. The letrozole induced animal study on female Wistar rats makes it easier to study the pathophysiology and therapeutic effects on PCOS similar to that on humans that makes it a suitable experimental method for the evaluation of pharmacological effects of different drugs. This animal study evaluates the efficacy of ketoconazole, metformin and N-acetylcysteine (NAC) in combination and also compare its effect with respect to the individual drugs against PCOS, to reverse the hormonal and metabolic effects that were induced in the rats by letrozole.

Ketoconazole, primarily used for its antifungal effect also shows anti-androgenic properties. It inhibits the steroidogenic enzymes, that results in reducing the synthesis of androgens. Metformin helps to manage insulin sensitivity which is now commonly being used in PCOS patients. It improves ovulatory function along with reducing insulin resistance to lower the blood glucose level in females having polycystic ovary. NAC is a precursor of glutathione that acts as an antioxidant to manage oxidative stress and also provide cytoprotective effect. The combined therapy has shown a significant improvement in PCOS by suppressing the level of testosterone, luteinizing hormone and blood glucose level. Histopathological examination of ovaries of different groups have also shown the reduction in cystic follicles along with restoration of healthy corpus luteum. Thus, this study provides a potential treatment option for PCOS using the synergistic effect of ketoconazole, metformin and NAC.

INTRODUCTION

Polycystic Ovary syndrome (PCOS) is one of the common disorders in this era that impacts around 5-10% of females worldwide in their fertile age. PCOS is a reproductive, endocrine and metabolic disorder and so it shows signs and symptoms related to the same (1). Some common signs symptoms of polycystic ovarian morphology that helps in PCOS diagnosis includes hyperandrogenism (HA), insulin resistance, gonadotropin abnormalities, ovulatory dysfunction (OD), as well as compensatory hyperinsulinism. PCOS is regarded as complicated genetic disorder with great inheritance factor. The Polycystic ovary disease is more prominent in females who are in their reproductive phase

or we can say in-between 18 - 44 years old (2). In this condition the female hormones are in imbalanced range that causes several complications in which the most prominent complication is formation of cysts in the ovaries and irregular menstrual cycle. This eventually leads the female to be incapable to conceive.

In the current scenario PCOS is one of the most complicated conditions in females due to such a high level of androgen or testosterone, formation of multiple cysts in ovaries and irregular menstrual cycles (3). Several studies have provided evidences that links PCOS to different metabolic disorders including insulin resistance, obesity, increased blood insulin levels and type 2 diabetes. PCOD females are also been clinically



diagnosed with associated infertility issued like dysfunctional haemorrhage, obesity, carcinoma, dyslipidaemia, polygenic disorder, and also cardiovascular disease (4).

Mechanism of PCOS

All these changes in the female body causes hormonal imbalance, hyperandrogenism and insulin resistance (5). The increased luteinising hormone secretion from pituitary and decreased follicular stimulating hormone secretion can be seen in such cases which causes the increased androgen production from theca cells. Due to increased androgen level hyperandrogenism occur that disrupts the maturation of follicles. As a result, anovulation and oligomenorrhea occurs in the females. It develops the formation of multiple small follicles or cysts and causes irregular metabolism that also affects the lipid profiles like increase in triglycerides, decrease in high density lipoprotein and increased body mass index can be seen (6). The patients also have high risk to develop type 2 diabetes, cardiovascular diseases, and even infertility.

Pathophysiology

The Pathophysiology of PCOS is multifactorial and complex having several different factors including insulin resistance, ovarian steroidogenesis, imbalance of hormones like AMH and LH due to dysfunction of hypothalamic pituitary ovarian axis (7). Luteinizing hormone and androgen level increases in PCOS. Due to the Gonadotropin releasing hormone (GnRH) is also disrupted which results in the access release of luteinising hormone (LH). In the result the development of oocytes is impacted along with the synthesis of ovarian androgen hormones.

PCOS is an endocrine disorder primarily dependent upon the elevated level of androgens, altered hormones and development of insulin resistance that causes metabolic disturbances. These multiple factors often result into absence or irregular ovulation or menstrual cycle and multiple small cysts are formed on the ovaries.

MATERIAL AND METHODS

Hypothesis

Primary Hypothesis

Ketoconazole and Metformin with N-Acetylcysteine will exhibit a synergistic effect by reducing the androgen levels and thereby managing the symptoms associated with PCOS while improving the insulin sensitivity. It will also show the hepatoprotective effect of N-Acetylcysteine against ketoconazole induced hepatotoxicity.

Sub-Hypothesis

Ketoconazole will help in the reduction of androgen levels and will help to manage the morphology of ovaries in PCOS induced rats. Metformin will manage the insulin sensitivity to reduce blood glucose level, body weight, while improving the body metabolism. N-Acetylcysteine will show the antioxidant property and also help to avoid the hepatotoxicity induced by ketoconazole. The combination therapy of these drugs will help in management of symptoms of the PCOS (8).

Methodology

Females Wistar albino rats are used for this preclinical experimental study to evaluate the pharmacological effects of the combination drugs of Ketoconazole, Metformin and N-Acetylcysteine (NAC) against PCOS that is induced by letrozole. A randomized and controlled study will be conducted for a non-biased result. Different groups are prepared for Normal control, Diseased control, Test groups, and Standard group that are later compared to see the final effect of the drug therapy of the Test group animals.

Test Group Drugs:

Ketoconazole: Ketoconazole drug is used as the test drug for PCOS at a dose of 25 mg/kg/day to be administered orally. To prepare the stock solution, 250 mg of ketoconazole API was dissolved in 100 ml of distilled water.

Metformin: Metformin drug is used as complimentary test drug for PCOS at a dose of 100 mg/kg/day to be administered orally. To prepare the stock solution, 1500 mg of metformin API was dissolved in 150 ml of distilled water.



N-Acetylcysteine: NAC drug is used as the test drug for PCOS with ketoconazole at a dose of 100 mg/kg/day to be administered orally. To prepare the stock solution, 1500 mg of ketoconazole API was dissolved in 150 ml of distilled water.

Dosing Protocol

Induction of PCOS: After the acclimatation period, letrozole is orally administered in dose of 1 mg/kg/day daily for 21 days (9) (10). The induction of disease is the most essential part to conduct any preclinical study. Letrozole is administered to diseased control group, test group I, test group II, test group III, and standard group to check and compare the results that the treatment drug are efficient to treat the disease or not. Normal control group does not receive letrozole because the results are also compared with non-diseased group kept in the same environment and conditions for same number of days.

Treatment of PCOS: The treatment protocol is conducted after the 21 days of PCOS induction when the androgen levels were found to be elevated in the confirmatory test. For the next 28 days the combination therapy is administered to the rats according to the dose protocol in test groups as well as standard group. The therapy continued from the day 22 to the 49th day of the animal study.

Female Wistar rats were divided into six groups having 6 rats in each group that were classified according to their dosing protocol as follows:

Group I: Normal control group

Groups	Testosterone level in (ng/ml)
Normal Control group	0.7850 ± 0.01871
Diseased Control Group	3.150 ± 0.1871
Test Group I	2.350 ± 0.1871
Test Group II	2.900 ± 0.1414
Test Group III	1.050 ± 0.1871
Standard group	0.9333 ± 0.1633

This group were only given normal saline and pellet food for 21 days.

Group II: Diseased control group

This group were given Letrozole for 21 days for the induction of PCOS at a dose of 1mg/kg/day through oral route.

Group III: Test Group 1

Letrozole for 21 days (1mg/kg/day), and ketoconazole was administered at 25mg/kg/day oral dose with NAC administered at 100mg/kg/day oral dose for the next 28 days.

Group IV: Test Group 2

Letrozole for 21 days (1mg/kg/day), and Metformin was administered at 100mg/kg/day oral dose for the next 28 days.

Group V: Test Group 3

Letrozole for 21 days (1mg/kg/day), and ketoconazole was administered at 25mg/kg/day oral dose with Metformin at 100mg/kg/day and NAC administered at 100mg/kg/day oral dose for the next 28 days.

Group VI: standard group

Letrozole for 21 days (1mg/kg/day), and Clomiphene Citrate was administered at 1mg/kg/day oral dose for the next 28 days.

During the study, the body weight of the rats was monitored on a regular basis to check the inappropriate fluctuation in their weight. On the 50th day of the study, after the completion of the protocol, the animals were sacrificed under the effect of appropriate anaesthesia. The blood samples were collected first and then histopathological examination is done after the dissection of the rats. The ovaries are collected in the formalin solution and sent to the pathology lab for the biochemical tests and histopathology study where the slides and blocks were prepared to study about the tissues of ovary.

These physical parameters were examined on a regular basis. The biochemical parameters include blood glucose level that were checked using glucometer, while the testosterone level and luteinizing hormone level were tested by sending the blood to a pathology lab.

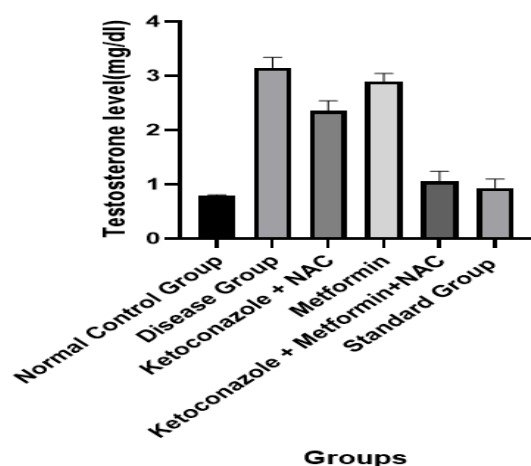


RESULTS

BLOOD TESTOSTERONE LEVEL

Table no.01: showing the testosterone level of rats

Sl. No	Tukey's HSD multiple comparison tests	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
1.	Normal vs. Disease	-2.365	-2.645 to -2.085	Yes	<.001
2.	Disease Group vs. Ketoconazole + NAC	0.8000	0.5205 to 1.080	Yes	<.001
3.	Disease Group vs. Metformin	0.2500	-0.02952 to 0.529	No	.101
4.	Disease Group vs. Ketoconazole + Metformin+NAC	2.100	1.820 to 2.380	Yes	<.001
5.	Disease Group vs. Standard Group	2.217	1.937 to 2.496	Yes	<.001
6.	Ketoconazole + Metformin+NAC vs. Standard Group	0.1167	-0.1629 to 0.3962	No	.799



Graph no.01: showing the difference in testosterone level among various groups.

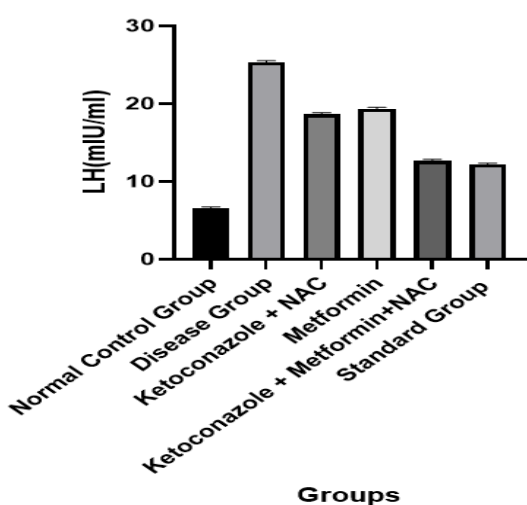
BLOOD LUTEINIZING HORMONE LEVEL

Groups	LH in (mIU/ml)
Normal Control group	6.550 ± 0.1871
Diseased Control Group	25.35 ± 0.1871
Test Group I	18.68 ± 0.1472
Test Group II	19.35 ± 0.1871
Test Group III	12.65 ± 0.1871
Standard group	12.22 ± 0.1472

Table no.02: showing the LH level of rats



Sl. No	Tukey's HSD multiple comparison tests	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
1.	Normal vs. Disease	18.80	-19.11 to -18.49	Yes	<.001
2.	Disease Group vs. Ketoconazole + NAC	6.667	6.360 to 6.974	Yes	<.001
3.	Disease Group vs. Metformin	6.000	5.693 to 6.307	Yes	<.001
4.	Disease Group vs. Ketoconazole + Metformin+NAC	12.70	12.39 to 13.01	Yes	<.001
5.	Disease Group vs. Standard Group	13.13	12.83 to 13.44	Yes	<.001
6.	Ketoconazole + Metformin+NAC vs. Standard Group	0.4333	0.1264 to 0.7403	Yes	.002



Graph no.03: showing the difference in LH level among various groups.

BLOOD GLUCOSE LEVEL

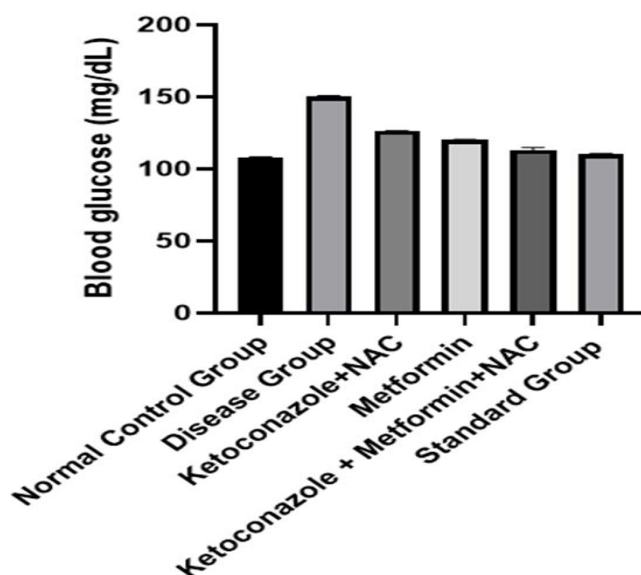
Groups	Glucose level in (mg/dl)
Normal Control group	108.0 ± 0.3578
Diseased Control Group	150.5 ± 0.4446
Test Group I	126.5 ± 0.1871
Test Group II	120.3 ± 0.1871
Test Group III	113.0 ± 1.871
Standard group	110.4 ± 0.1871

Table no.03: showing the glucose level of rats

Sl. No	Tukey's HSD multiple comparison tests	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
1.	Normal vs. Disease	42.48	43.90 to 41.06	Yes	<.001



2.	Disease Group vs. Ketoconazole + NAC	24.03	22.61 to 25.45	Yes	<.001
3.	Disease Group vs. Metformin	30.23	28.81 to 31.65	Yes	<.001
4.	Disease Group vs. Ketoconazole + Metformin+NAC	37.48	36.06 to 38.90	Yes	<.001
5.	Disease Group vs. Standard Group	40.13	38.71 to 41.55	Yes	<.001
6.	Ketoconazole + Metformin+NAC vs. Standard Group	2.650	1.229 to 4.071	Yes	.002



Graph no.03: Showing the difference in blood glucose level among various group

EVALUATED BODY WEIGHT DIFFERENCES AMONG DIFFERENT GROUPS

Groups	No of Rats	Day 1 (gm)	Day 21 (gm)	Day 35 (gm)	Day 49 (gm)
Group 1: Normal Control (NC)	Rat 1	75	100	100	125
	Rat 2	75	100	100	125
	Rat 3	100	100	125	150
	Rat 4	175	175	175	175
	Rat 5	100	100	125	125
	Rat 6	125	125	125	150



Group 2: Diseased Control (DC)	Rat 1	75	100	100	75
	Rat 2	125	150	150	125
	Rat 3	75	100	100	75
	Rat 4	100	125	125	100
	Rat 5	100	125	125	100
	Rat 6	75	100	100	75
Group 3: Test Group 1 (TG1)	Rat 1	75	100	125	75
	Rat 2	100	125	125	100
	Rat 3	75	125	100	75
	Rat 4	75	100	100	75
	Rat 5	75	100	100	75
	Rat 6	100	125	125	100
Group 4: Test Group 2 (TG 2)	Rat 1	150	175	175	150
	Rat 2	125	150	150	125
	Rat 3	100	125	125	100
	Rat 4	75	100	100	75
	Rat 5	100	125	125	100
	Rat 6	100	125	125	100
Group 5: Test Group 3 (TG 3)	Rat 1	75	100	100	75
	Rat 2	75	125	125	75
	Rat 3	75	100	100	100
	Rat 4	75	125	100	75
	Rat 5	75	100	100	75
	Rat 6	75	100	100	100
Group 6: Standard Group (SG)	Rat 1	50	100	75	75
	Rat 2	125	150	150	125
	Rat 3	75	100	100	75
	Rat 4	75	100	100	75
	Rat 5	125	150	150	125
	Rat 6	100	125	125	100



HISTOPATHOLOGY OF OVARIES OF THE WISTAR RATS

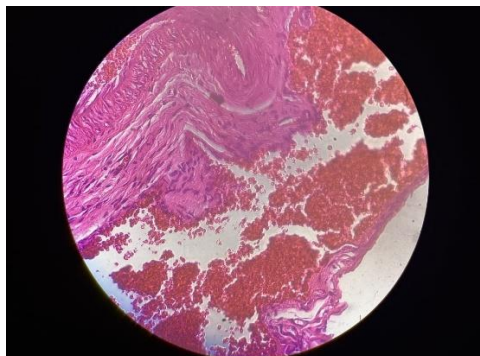


Fig.no. 1- (a) Normal Control

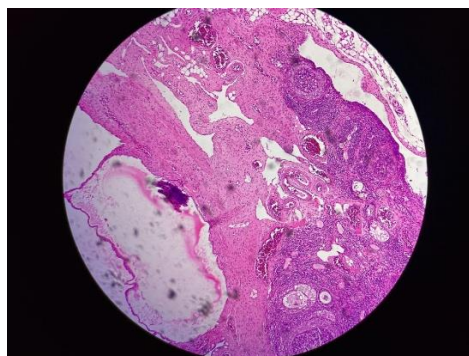


Fig.no. 1- (b)Diseased Control

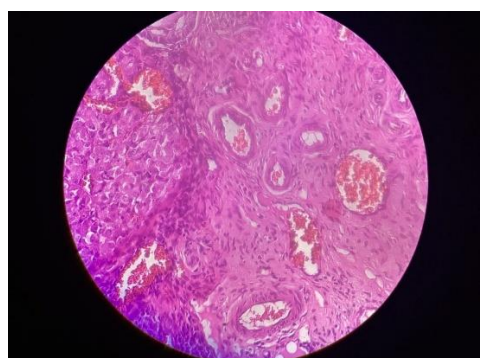


Fig.no. 1- (c) Test Group I

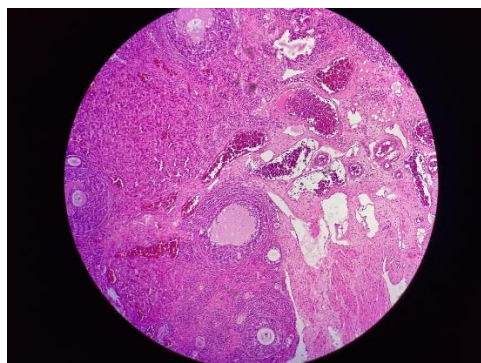


Fig.no. 1- (d) Test Group II



Fig.no. 1- (e) Test Group III

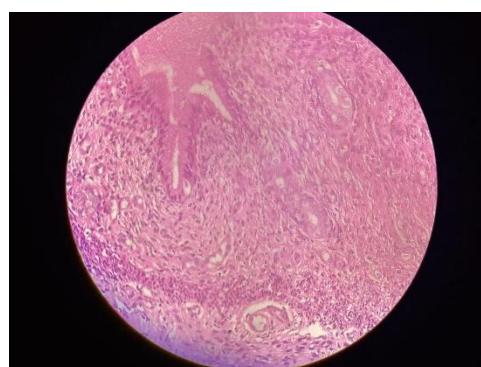


Fig.no. 1- (f) Standard Group

Figure no. 1- Photomicrograph of histopathological examination of ovaries of different groups (magnification 100X).

(Fig.a) Ovaries were found to be healthy normal with no significant histopathological findings.

(Fig.b) Ovaries were showing morphological features of polycystic ovaries with multiple cysts, no corpora lutea, and stromal hyperplasia.

(Fig.c) Ovaries show partial regression of polycystic ovary associated changes. Reduction of cystic follicles and emergence of Corpora Lutea suggests that ovulatory activity is getting better.



(Fig.d) Ovaries show very mild regression of polycystic ovary associated changes. Reduction of cystic follicles and emergence of Corpora Lutea suggests that ovulatory activity is getting better.

(Fig.e) Ovaries show consistently regressing phase of polycystic ovary associated changes that indicates a therapeutic intervention. Reduction of cystic follicles and emergence of Corpora lutea suggests that the recovery of ovarian cycle in the female rats.

(Fig.f) Ovaries histopathological examination shows partial regression of polycystic ovary associated changes. Healthy developing follicles and emergence of Corpora Lutea suggests that the ovulatory activity have been restored and ovulation cycle is back to normal.

DISCUSSION

Ketoconazole was used to evaluate its protective effect in Letrozole induced PCOS in female Wistar rats. The induction of PCOS was done by administering Letrozole for 21 days. Every animal's body weight was recorded at the start of the trial and then every week after that. Serum glucose and testosterone were measured on days 21st and 49th day of the study. Hormone levels were assessed and the ovary was removed for histopathological analysis on the last day.

Ketoconazole with Metformin and NAC can be a ray of hope where it normalizing the LH/FSH ration, decreasing testosterone level, decreasing blood glucose level and cholesterol level. Testosterone levels were normalized by this therapy showing similar effect like Clomiphene citrate. In PCOS-induced group, levels of male hormones in serum were decreased and the normal level of female hormone were maintained successfully. It was also found that the body weight and body mass index of the animals, increased significantly in all groups when Letrozole was administered. Standard drug Clomiphene citrate was administered to the standard group which shows a reduction in body weight and BMI. Test groups also shown decreased body weight and BMI similar to the standard group after treatment and compare at day 0, 21 and 49. Also, the histopathological report shows that the cyst formed in PCOS induced Wistar rats are recovering when treated with standard and Test drugs. Histopathology report show that ovary of Test group III was treated with mild recovery.

CONCLUSION

This study concluded that Ketoconazole has a potential benefit in the treatment of PCOS by lowering the testosterone level and also be lowering the luteinizing hormone. It helped in suppressing other symptoms related to increased androgen level also like hirsutism,

increased body weight, etc. On the other hand, Metformin which is already being used for PCOS has supported the effect of ketoconazole by managing the insulin sensitivity and lowering blood glucose level. N-Acetylcysteine when complimented with both the former drugs help to avoid any hepatic damage and also shows the synergistic property through its antioxidant effect. The combination therapy in the female Wistar rats have shown a numeral pharmacological effect in their body, which was determined through various tests. The test group was seen to have lower testosterone level, low blood glucose, decreased LH level, the body weight was also seen to be decreasing at the end of the treatment in the rats.

Thus, we can conclude that ketoconazole with metformin and NAC can also be a good option as a potential drug for the treatment therapy of PCOS to manage the symptoms this disorder and even treating this disease.

Conflict of Interest: The author has no conflict of interest.

Acknowledgement:

The author is thankful to Dr. Sanjay Singh (Principal) and her guide, Mr. Rahul Singh Dhariyal (Associate Professor), for their scientific advice and for providing all necessary facilities during the research protocol.

Author Contribution

SK- Writing original

Draft RSD- Original concept

SS- Supervision

Ethical Approval: The research study was conducted at Siddhartha institute of pharmacy, Near IT park, Dehradun 248001. The animal house is CPCSEA



approval. And the registration no. of the animal house 1435/PO/RE/S/11/CPCSEA.

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