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Original Article

Investigation of Neuronal and Adrenocortical Changes in Metyrapone- An 11 β-*hydroxylase* Inhibitor Induced Adrenal Insufficiency in Wistar Rats

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ABSTRACT

Background: 11*β-hydroxylase* plays a key role in adrenal steroid hormone production, making it crucial in the development of adrenal insufficiency. Recently, there has been increasing interest in targeting this enzyme to induce adrenal insufficiency by using small molecule inhibitors. **Materials and Methods:** Metyrapone was administered to Wistar rats in a dose-dependent manner, ranging from 100 to 200 mg/kg. Levels of cortisol, sodium, potassium, serotonin (5-HT), and Dopamine (DA) were measured. **Results:** Metyrapone significantly reduced cortisol, sodium, and potassium levels, increased dopamine levels, and caused no notable change in serotonin. **Conclusion:** The findings of this study suggest that metyrapone induces Adrenal Insufficiency in Wistar rats by inhibiting adrenal hormones and enzymes involved in steroidogenesis, as well as altering neurotransmitter levels in the brain. Histopathological analysis showed no significant damage or changes in the Paraventricular nucleus of the hypothalamus or the adrenal cortex.

Keywords: Adrenal Insufficiency (AI), Cortisol, Corticosterone, Aldosterone, Metyrapone (mety), Adrenocorticotropic Hormone (ACTH).

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INTRODUCTION

Adrenal Insufficiency (AI) is a serious condition where the adrenal glands do not make enough cortisol, a hormone needed for the body to work properly. If not treated, it can lead to a dangerous condition called adrenal crisis, which can be life-threatening (Kilberg and Vogiatzi, 2024). Currently, adrenal insufficiency affects more than 300 out of every 100,000 people and leads to more illness and a higher risk of death (Peel *et al*., 2024). Despite its clinical significance, there is limited preclinical research on drug-induced AI, particularly involving inhibitors of significant adrenal steroidogenic enzymes such as 11β-hydroxylase, 21-hydroxylase and 17-hydroxylase (Caroste *et al*., 2023; Guengerich *et al*., 2023; Pivonello *et al*., 2023).

Metyrapone is an adrenal steroidogenesis inhibitor that selectively inhibits 11b-hydroxylase (CYP11B1), the last enzyme in cortisol biosynthesis. This effectively prevents the conversion of 11-deoxycortisol into cortisol, as shown in Figure 1. It is also used to study the suppression of the Hypothalamo Pituitary and Adrenal (HPA) axis, although it has not been thoroughly studied (Detomas *et al*., 2022). The lack of adequate preclinical models hinders the development of novel diagnostic approaches and management strategies for drug-induced Adrenal Insufficiency.

This study establishes a reliable and reproducible model of Adrenal Insufficiency using metyrapone, which can be valuable tool for future pharmacological studies. It also helps to understand the pathophysiology of 11 *β-hydroxylase* inhibition and its effect on adrenal hormone production. The findings, which characterize biochemical, physiological, and histological abnormalities, can help to inform techniques for diagnosing and managing metyrapone induced Adrenal Insufficiency.

AI models are crucial for testing safety and efficacy of potential therapies, including hydrocortisone, prednisolone, and dexamethasone replacements (Nowotony *et al*., 2021). AI models help in investigating hormonal dynamics, including interactions between HPA axis and other systems. This model allows to induce the adrenal crisis and adrenal insufficiency, which are very difficult to study directly in humans.

MATERIALS AND METHODS

Animals

Wistar rats ranging 190-210 g were procured from Vaarunya biolabs Pvt. Ltd., Bangalore. Rats were housed in 12:12 hr light and dark, temperature (22±1°C) and humidity (50±5%)

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controlled environment for one week before experimentation. Food (Standard rodent pellets) and water *ad libitum*.

Drugs and chemicals

Metyrapone was acquired from Merck, Bangalore, Sodium chloride, sodium phosphate, potassium phosphate were obtained from the store, Sri Adichunchanagiri College of Pharmacy.

Experimental design and procedures

40 rats were utilized in this study with ten in each group for four weeks. Group 1 serves as normal, Group 2, 3 and 4 are selected to induce adrenal insufficiency in Wistar rats at 100, 150 and 200 mg/kg of metyrapone, an 11 β-*hydroxylase* Inhibitor. The selected doses are well below the toxic level, ensuring safety during the experiment.

The LD_{50} for metyrapone tartrate was 760 mg/kg i.p (Nelson *et al*., 1980). A dose range from 100 mg/kg to 200 mg/kg provides a gradient to study potential dose dependent pharmacological effects. This approach allows to identify both sub-therapeutic and possibly maximal side effects.

Doses between 10 to 30% of the LD_{50} are commonly explored to access therapeutic effects while avoiding the acute toxicity. The highest dose, metyrapone 200 mg/kg is approximately 26% of the LD_{50} making it a rational choice for exploratory studies.

Current study aims to estimate cortisol, sodium, and potassium levels in the blood, as well as serotonin and dopamine levels in the brain.

Biochemical assays

For blood collection, a 23 G needle connected to a syringe was used to draw 1.5 mL via lateral tail vein. Rats were placed in restrainer individually to collect the blood. The blood samples were collected sent to the laboratory, Adichunchanagiri hospital and research centre for the analysis of Cortisol in the blood (Jameel *et al*., 2014).

Enzyme Linked Immunosorbent Assay (ELISA) was performed for the hormonal analysis, which is a sensitive, reliable, replicable, specialized, and speedy method. Sodium, and potassium were measured by spectrophotometer (Farahadi *et al*., 2019; Nassiri *et al*., 2017; Zhou *et al*., 2021).

Neurochemical analysis

Rats brain were dissected, homogenized with 0.1 M hydrochloric acid and butanol, the mixture was centrifuged for 10 min at 2000 rpm. 1 mL of the supernatant was taken, and it was agitated for 10 min before adding 2.5 mL of heptane and 0.31 mL of HCl (0.1 M). After that, the mixture is centrifuged.

Centrifugation was used to separate the two layers. Throughout the process, 0°C is kept as the temperature. After removing the organic layer, 0.2 mL of the aqueous layer was extracted for the measurement of DA and 5-HT (Hira *et al*., 2020; Meissam *et al*., 2021; Saleem *et al*., 2021).

Determination of Serotonin.

0.2 mL of the aqueous phase was mixed with 0.25 mL of O-Phthaldialdehyde (OPT). After that, this combination was cooked for about 10 min at 100°C. The substance was allowed to reach room temperature. In a spectrophotometer, absorbance was measured at 440 nm. 0.25 mL of HCl without OPT served as the blank solution.

Estimation of DA

For the process of oxidation, 0.05 mL of hydrochloric acid (0.4 M) and 0.1 mL of ethylenediaminetetraacetic acid were added to 0.2 mL of aqueous phase. And the mixture was then combined with 0.1 mL of iodine (0.1 M in ethanol) solution.

To stop the oxidation, 0.1 mL of sodium sulfite (Na_2SO_3) was added prior to the addition of 0.1 mL acetic acid. The reaction mixture was allowed to cool after heating for 6 min at 100°C and measured at an absorbance at a wavelength of 350 nm. A blank solution was produced by adding the oxidation process's ingredients in the reverse order.

Histopathology

The Paraventricular Nucleus (PVN) of the hypothalamus, one of the brain's most important autonomic control regions were removed and immediately fixed in buffered neutral formalin, dehydrated and fixed with paraffin, sliced into 5 mm thickness with microtome and stained with eosin and hematoxylin. The sections prepared were visualized under light microscope.

Statistical analysis

All results were expresses as Mean±SD. Two-way ANOVA was used to examine the biochemical changes. Differences between groups were tested by Tukey's multiple comparison test. The statistical significance level (p value) for all analyses was set at 0.05.

RESULTS

Estimation of Cortisol

A crucial steroid hormone produced by the suprarenal glands, has diverse impact on metabolism, immune functions and various physiological processes. Cortisol levels were assessed for every seven days. It was observed that cortisol levels were declined in dose-dependent manner with a p value <0.0001 for mety 100, 150 and 200 mg/kg compared to normal in all the weeks. The results were depicted in Figure 2.

Sodium estimation

Sodium levels were assessed weekly up to 28th day. The values are significant at high doses represented in Table 1.

Potassium estimation

Potassium levels are observed for every seven days up to 28th day. The values were represented in the Table 2.

Estimation of DA and 5-HT

Both DA and 5-HT levels were estimated at the end of the study and there were no significant changes in the serotonin at mety 100 mg/kg. At 150 and 200 mg/kg doses, there is a slight decline with the values of 5-HT represented in Figure 3.

Histopathology

The histopathology of rats' brain focusing Paraventricular nucleus of hypothalamus represented in Figure 4.

DISCUSSION

Throughout the study, the normal rats cortisol levels remain constant at 80-84 ng/mL. Mety 100 mg/kg significantly reduces cortisol levels compared to the normal group while maintaining moderate levels between 59 and 64 ng/mL. Mety 150 mg/kg exhibited further cortisol suppression of 40-54 ng/mL, suggesting dose-dependent adrenal suppression.

With a statistical significance of $p<0.0001$ to normal, Mety 200 mg/kg exhibits the greatest suppression of cortisol during every stage of the research, suggesting acute adrenal insufficiency. The strongest suppression, which happens at 200 mg/kg, indicates severe AI.

Hyponatremia is a common electrolyte imbalance in adrenal insufficiency (Kumar *et al.*, 2021). The sodium levels were constant at 139-141 mmol/L for normal group throughout the study. On days 7 and 21, there is a substantial drop in Mety 100 mg/kg from 133 to 138 mmol/L ($p<0.05$). Mety 150 mg/kg exhibits a steady decrease from 117 to 135 mmol/L, with a significant statistical shift starting on day 14 ($p<0.01$ to $p<0.0001$). By day 21, Mety 200 mg/kg demonstrated significant hyponatraemia (~107-134 mmol/L) with a p-value below 0.0001.

The potassium levels in the normal group remain consistent at 4.7-4.9 mmol/L throughout the research. On days 7 and 28, there is a slight decrease in Mety 100 mg/kg between 4.3 and 4.8 mmol/L that is statistically significant ($p<0.05$ to $p<0.01$). A progressive decrease (~4.4-4.6 mmol/L) was observed for mety 150 mg/kg, with a larger significance beginning on day 7 ($p<0.01$ to $p<0.0001$). Severe hypokalaemia (4.0-4.7 mmol/L) was observed by days 21 and 28, which was very significant ($p<0.0001$) for mety

Table 1: **Sodium levels of four groups on day 1, 7, 14, 21 and 28.**

Groups	Day 1	Day 7	Day 14	Day 21	Day 28
Normal	141.4±7.07	141.7±6.21	140.8±6.71	140.6±4.57	139.6±5.79
mety 100 mg/kg	136.2±3.8^{ns}	133.3±5.22*	138.8±4.18^{ns}	136.1±4.14 ^{ns}	132.9±6.10 ^{ns}
mety 150 mg/kg	133±6.41*	138.8±4.36*	135.6±3.23^{ns}	120.5±8.89****	117±10.45****
mety 200 mg/kg	133.9±4.43*	129.2±6.32***	130±4.66**	117±9.49****	107.7±11.95****

Values were represented in mean±SD (n=10), Two-way ANOVA followed by Tukey's test. **** indicates $p<0.0001$, * indicates $p<0.05$ are significant, ^{ns} indicates $p>0.05$ not significant, compared to Normal Control.

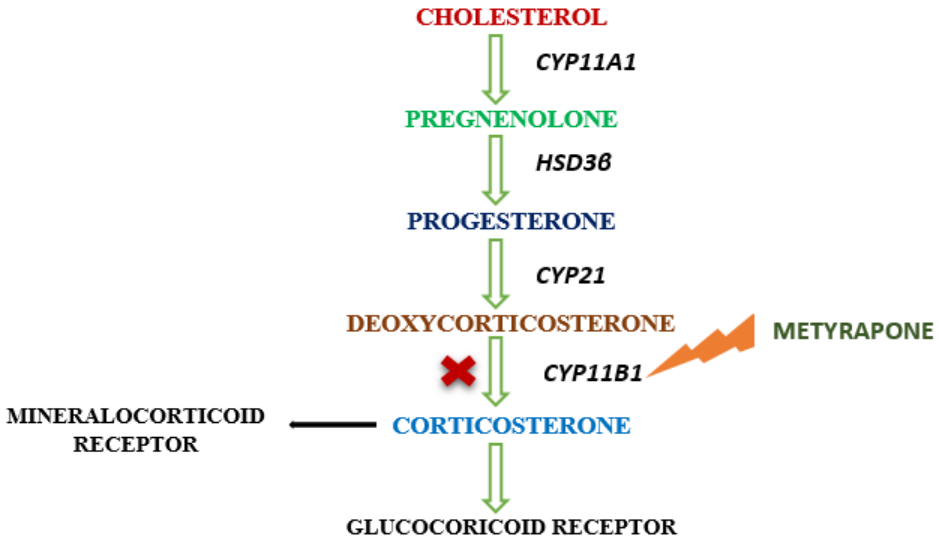

Figure 1: Blocking CYP11B1 by Mety in Adrenal steroidogenesis pathway.

Table 2: Potassium levels of four groups on day 1, 7, 14, 21 and 28.

Groups	Day 1	Day 7	Day 14	Day 21	Day 28
Normal Control	4.882±0.27	4.856±0.32	4.753±0.36	4.768±0.41	4.811±0.30
Mety 100 mg/kg	4.773±0.33^{ns}	4.474±0.39*	4.714±0.29 ^{ns}	4.605±0.29 ^{ns}	4.229±0.31**
Mety 150 mg/kg	4.739±0.34 ^{ns}	4.327±0.34**	4.641±0.26 ^{ns}	4.312±0.34*	4.047±0.32****
Mety 200 mg/kg	4.366±0.43**	4.183±0.30***	4.044±0.45****	3.741±0.44****	424±0.47****

Values were represented in mean±SD (n=10), Two-way ANOVA followed by Tukey's test. **** indicates p<0.0001, ** indicates p<0.005, * indicates p<0.05, are significant, ^{ns} indicates p>0.05 not significant, compared to Normal Control.

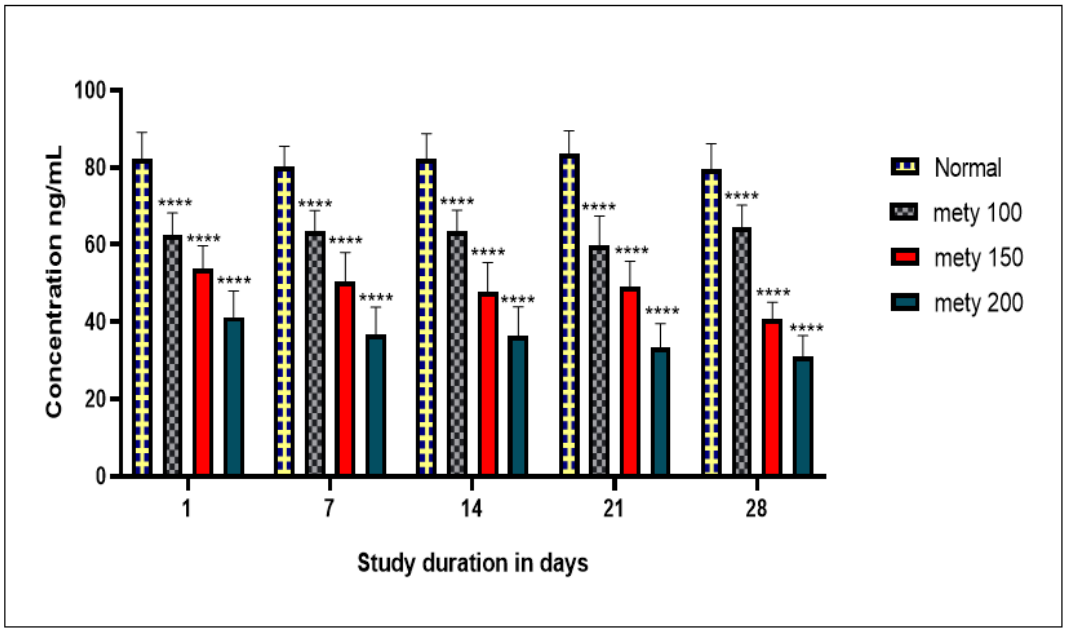

Figure 2: Cortisol levels were observed on day 1, 7, 14, 21 and 28. Data represents mean±SD (n=10), Two-way ANOVA followed by Tukey's test. **** indicates p<0.0001 compared to normal.

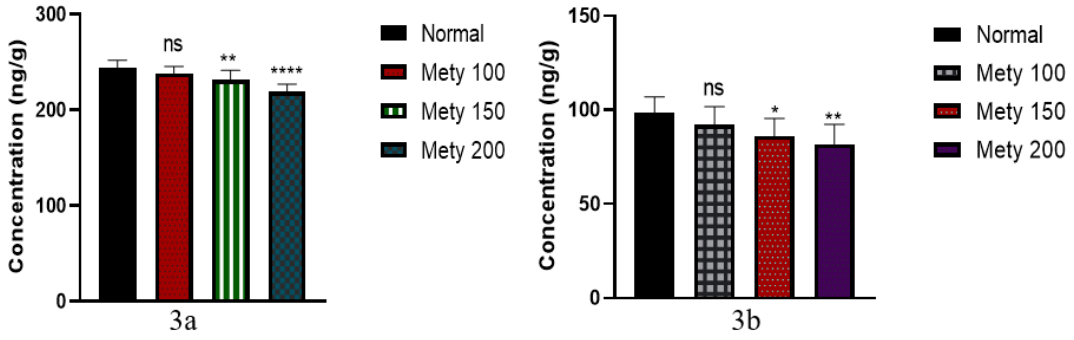

Figure 3: Dopamine and serotonin levels on day 28. Figure a Indicates DA levels on day 28. Values represented as mean±SD (n=10), followed by one-way ANOVA using Tukey's test. Mety 150 and 200 mg/kg showed significant values compared with normal control represented as ** p<0.01 and **** p<0.0001 respectively. Whereas ^{ns} represents p>0.05, not significant compared to normal control. Figure b indicates 5-HT levels on day 28. Values represented as mean±SD (n=10), followed by one-way ANOVA using Tukey's test. Mety 150 and 200 mg/kg showed significant values compared with normal control represented as * p<0.05 and ** p<0.01 respectively. Whereas ^{ns} represents p>0.05, not significant compared to normal control.

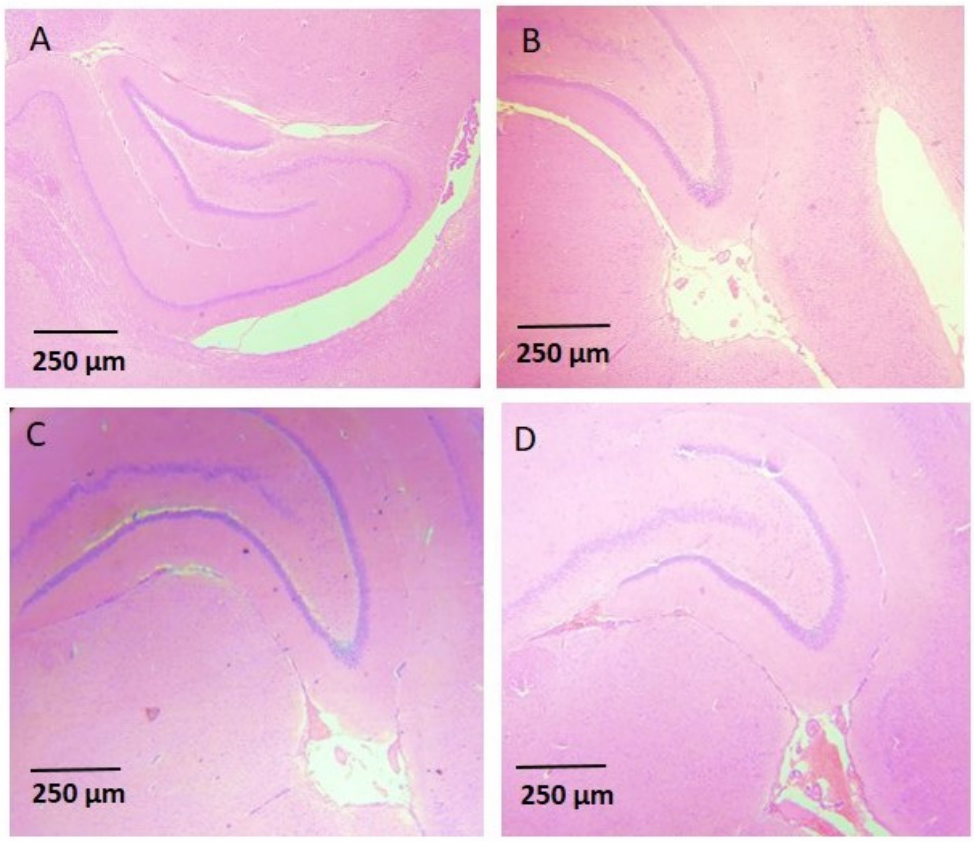

Figure 4: Histological sections of the Paraventricular nucleus of hypothalamus of rats. A Normal rat, B mety 100 mg/kg, C mety 150 mg/kg and D mety 200 mg/kg. No observable changes like neuronal degeneration, vascular changes or gliosis, and inflammation in all the groups.

200 mg/kg. Potassium levels decrease after prolonged use of mety (Kidwara *et al*., 2024).

The DA concentration is higher in the normal group. DA levels are reduced in a dose-dependent manner by Mety 100, 150, and 200 mg/kg. Significant drops ($p<0.01$, $p<0.0001$) are shown in the Mety 150 and Mety 200 groups, suggesting DA depletion.

5-HT is preserved by low dosages of mety, while it is depleted by high concentrations. Serotonin depletion occurs gradually as mety concentrations rise. Serotonin receptor sensitivity is decreased by chronic HPA suppression, which may be a factor in mood disorders.

CONCLUSION

Mety 200 mg/kg shows the highest reduction of cortisol, suggesting severe AI, glucocorticoid medication might be required to restore normalcy. Decreased levels of cortisol or corticosterone can lead to metabolic imbalance, depression-like behavior, and an impaired stress response. Hyponatremia and hypokalemia were observed at higher dosages. There was a dose-dependent decline in the DA and 5-HT. In conclusion, this study may result in acute AI compared to earlier studies, and more comprehensive research should be conducted for chronic conditions.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ABBREVIATIONS

Mety: Metyrapone; **AI:** Adrenal Insufficiency; **ELISA:** Enzyme-Linked Immunosorbent Assay; **HPA:** Hypothalamic Pituitary and Adrenal.

ETHICAL STATEMENT

The study was approved by the institute's animal ethics committee with an approval number SACCP-IAEC/2022-01/57.

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