Section A -Research paper

New insights in targeting 11β-hydroxylase of Rat Adrenal Cortex using Metyrapone

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Abstract: Adrenal insufficiency is a rare disorder of adrenal glands that are unable to produce enough cortisol (glucocorticoid) from the adrenal cortex. Cholesterol is converting into cortisol during the adrenal steroidogenesis process. Deficiency or lack of enzymes required to synthesize the cortisol leads to adrenal insufficiency and its main cause is congenital adrenal hyperplasia. Transgenic, knockout mice and rat models like (targeted disruption of the mouse gene encoding StAR protein provides insights into congenital lipoid adrenal hyperplasia, Cyp11b1 Null Mouse, a Model of Congenital Adrenal Hyperplasia, inducing Congenital Lipoidal Adrenal Hyperplasia by Inhibition of cholesterol side chain cleavage, inhibition of 3 β-Hydroxysteroid dehydrogenase by Estradiol-17 β and inducing congenital adrenocortical hyperplasia in rats, Chronic stress induces adrenal hyperplasia and hypertrophy in rats and constitutive β-catenin activation induces adrenal hyperplasia) are expensive. This study focused on inducing the adrenal insufficiency in the Wistar rats by metyrapone, which blocks the enzyme 11β- hydroxylase essential for cortisol synthesis. Metyrapone at three different doses were selected (100, 150, 200 mg/kg) and administered for 28 days and assessed the cortisol and locomotor scores on day 0, 7, 14, 21 and 28 respectively. Results indicates decreased cortisol and reduced locomotor scores. Results indicates adrenal insufficiency was developed in Wistar albino rats. Hence it is simple and cost effective model to induce adrenal insufficiency for day to day screening of animal activity.

Keywords: *Adrenal insufficiency, Glucocorticoid, Cortisol, Transgenic, Metyrapone.*

Introduction

Adrenal insufficiency is the clinical manifestation of deficient production or action of glucocorticoids, mineralocorticoids and increased adrenal androgens [1]. Adrenal insufficiency is subdivided into congenital and acquired form. The defect is in the production of cortisol, and often aldosterone, in the adrenal cortex. In infancy, the most common cause of congenital adrenal insufficiency is congenital adrenal hyperplasia (CAH) [2]. CAH is a group of autosomal recessive

disorders characterized by varying degrees of enzyme deficiencies affecting adrenal steroidogenesis. The most common form of CAH is 21-hydroxylase deficiency (21OHD) [3]. This enzyme, converts 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol and progesterone to deoxycorticosterone, which are precursors of cortisol, regulates body's stress and aldosterone regulates sodium and potassium. The blockage of cortisol synthesis activates a negative feedback pathway, resulting in increased Adrenocorticotropic hormone (ACTH) release, which promotes over secretion of 17-hydroxyprogesterone (17OHP), progesterone, and adrenal androgens [4]. 11 β-Hydroxylase (CYP11B1) deficiency is the next most common form of CAH. In the cortisol pathway, it is the final step from 11-deoxycortisol to cortisol. In the aldosterone pathway, it is the next step from 11-deoxycorticosterone into the final steps towards aldosterone formation [5]. The disease presentation may be classical (salt-wasting, simple virilizing) and non-classical [6]. CAH affects approximately 1:10,000 to 1:15,000 people in the United States and Europe. Among the Yupik Eskimos, the occurrence of the salt-wasting form of this disorder may be as high as 1 in 282 individuals. Other forms of CAH are much rarer. In contrast, non-classical CAH affects approximately 1 in 100 to 1 in 200 individuals in the general population [7]. In a multicentric study of the 104,066 newborns screened for CAH, 18 infants, 16 Salt wasting, 2 simple virilizing were confirmed to have CAH, suggesting a collective incidence of 1 in 5762. The study showed marked regional differences, with the prevalence being 1 in 2036 in Chennai, 1 in 7608 in Delhi and 1 in 9983 in Mumbai. Other recent studies from India, found the incidence of CAH to be 1 in 2800 in South India and 1:6334 in North India [6].Till today there is no alternative therapy for congenital adrenal hyperplasia other than replacement therapy with Glucocorticoids and Mineralocorticoids to replace cortisol and aldosterone respectively. Glucocorticoids have a range of common metabolic side effects including hypertension, osteoporosis and diabetes [8]. Since, synthetic steroids are having few side effects, we are moving towards plant sources and planning for extraction and isolation of cortisol related biomolecules for this study. Transgenic or Knock-out mice/ rat models, inhibition of cholesterol side chain cleavage models and stress induced models are available for inducing CAH. Because of few disadvantages in the above mentioned two models, we are going for developing an alternative model. Here, the drugs used to treat Cushing's syndrome might be helpful in inducing the adrenal hyperplasia in rats/ mice. In this study Metyrapone (an inhibitor of 11beta-hydroxylase, inhibits the synthesis of cortisol from 11-deoxycortisol and aldosterone from 11-deoxycorticosterone), may be used to induce CAH like condition. Cortisol has been measured using enzyme-linked immunosorbent assay (ELISA), one of the most suitable immunoassay techniques owing to high sensitivity and specificity. In addition, this technique does not require expensive instrumentation or advanced technical expertise. Perhaps, the most significant advantage on others immunoassays such as radioimmunoassay is that ELISA does not need radioisotopes [9]. Our exploration was restricted to preclinical studies using metyrapone, 11β-hydroxylase inhibitor on rat adrenal steroidogenesis.

Materials and Methods

Animals

Both Male and female rats with a mean weight of 250 g were selected for this study and maintained under specific conditions. Temperature within the range of 18-29°C, humidity 30-70 %, light and dark cycles and standard pellet diet, purified drinking water *ad libitum.* All the animals and protocols described in this study were approved by IAEC of Sri Adichunchanagiri College of Pharmacy, B.G.Nagar, Karnataka, India.

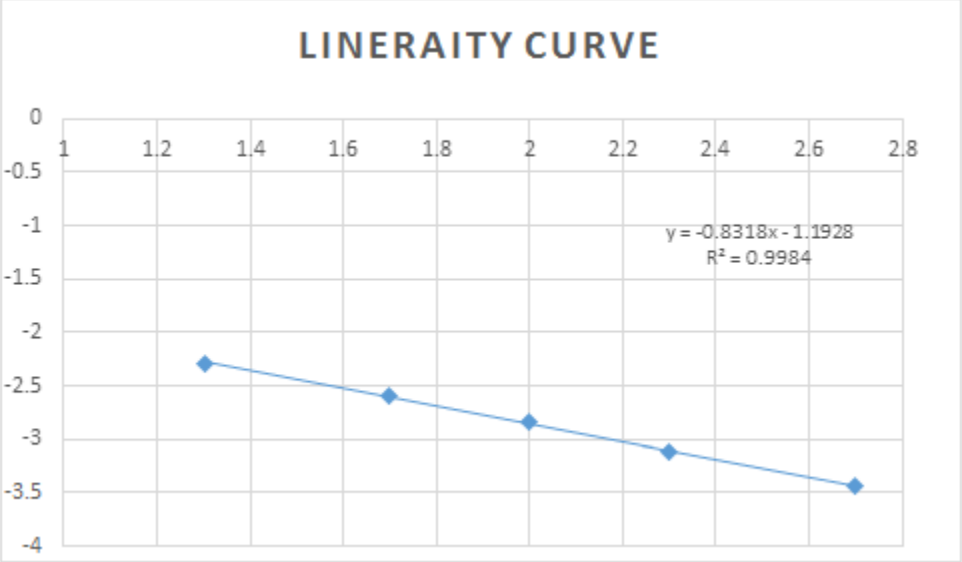

Fig 1: Linearity curve represents absorbance for Cortisol standards (Y axis) versus Cortisol standard concentrations (X axis). Coefficient of determination R^2 = 0.9984.

Chemicals

Metyrapone was purchased from Merck, Bangalore. Other chemicals and kits used in the study were of analytical grade, Cortisol ELISA kit from Calbiotech. Distilled or deionized water, Precision pipettes, Disposable pipette tips, Absorbance paper or paper towel and Graph paper provided by Sri Adichunchanagiri College of Pharmacy.

Study design:

Four groups of six rats in each were selected for this study over a period of 28 days. Metyrapone was selected as inducing agent at various concentrations (100, 150 & 200 mg/kg) to induce adrenal insufficiency. Group I serves as normal control, Group II, III & IV considered as metyrapone in 100, 150 and 200 mg/ kg respectively. The cortisol and locomotor activity of all the rats were assessed on day 0, 7, 14, 21 and 28 individually.

Pipetting calibration.

Pipetting volume of 20, 50, 100 and 200 µl were weighed in analytical balance in order to guaranty the accuracy of the pipetting procedure during the ELISA assay. Each volume taken was weighed in a plastic container with a previous zeroing of the balance.

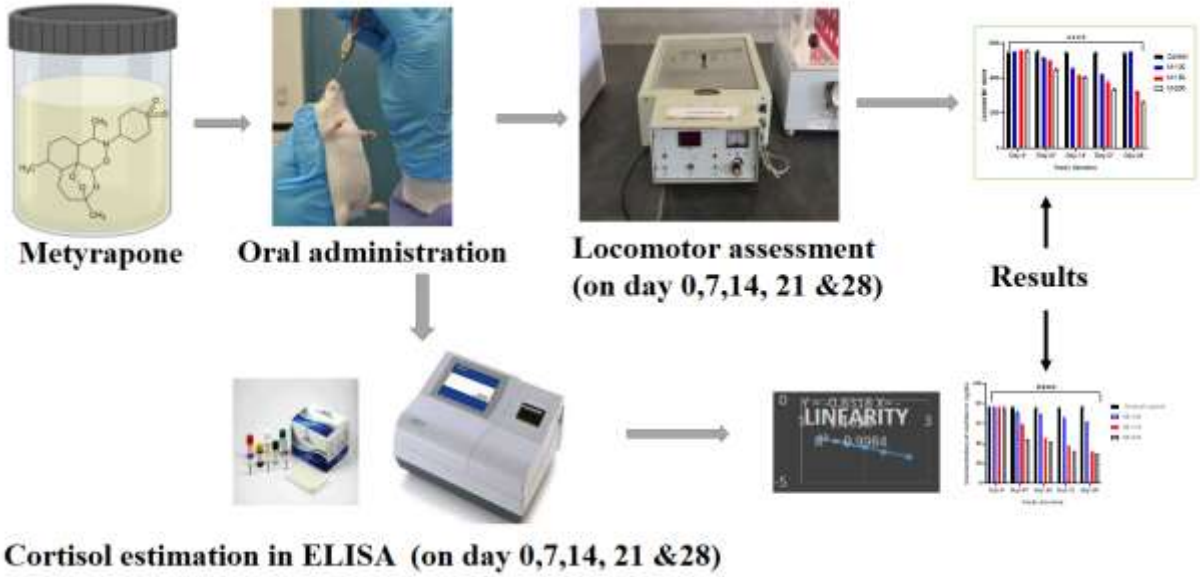

Fig 2: Determination of Rat Serum Cortisol.

Cortisol assay in Rat Serum

Cortisol assay was performed using Calbiotech Cortisol kit. Prior to assay, all the reagents were allowed to stand at room temperature and mixed well. The desired number of coated strips were placed into the holder and 25 µl of Cortisol standards, control and rat's sera were pipetted out. To it 50 µl of Biotin reagent and 100 µl of Cortisol enzyme conjugate were added to all the wells and mixed for 10 seconds, incubated for 60 minutes at room temperature (20-25°C). Removed liquid from all the wells and washed thrice with 300 µl of 1x wash buffer, blotted on absorbent paper towels.100 µl of TMB substrate added to all wells and incubated for 15 minutes at room temperature (20-25°C). Finally 50 µl of stop solution is added to all wells and the plate was shaken gently to mix the solution. Finally, absorbance on ELISA Reader measured at 450 nm.

Principle of the test

Cortisol test kit is a solid phase competitive ELISA. The samples, working Cortisol-HRP Conjugate and anti-cortisol-biotin solution are added to the wells coated with streptavidin. Cortisol in the rat serum competes with the cortisol enzyme (HRP) conjugate for binding sites. Unbound cortisol and cortisol enzyme conjugate is washed off by washing buffer. Upon the addition of the substrate, the intensity of color is inversely proportional to the concentration of

Cortisol in the samples. A standard curve is prepared relating color intensity to the concentration of the cortisol. The standard curve is constructed between the absorbance for Cortisol standards (vertical axis) versus Cortisol standard concentrations (horizontal axis) on a linear graph paper.

Results

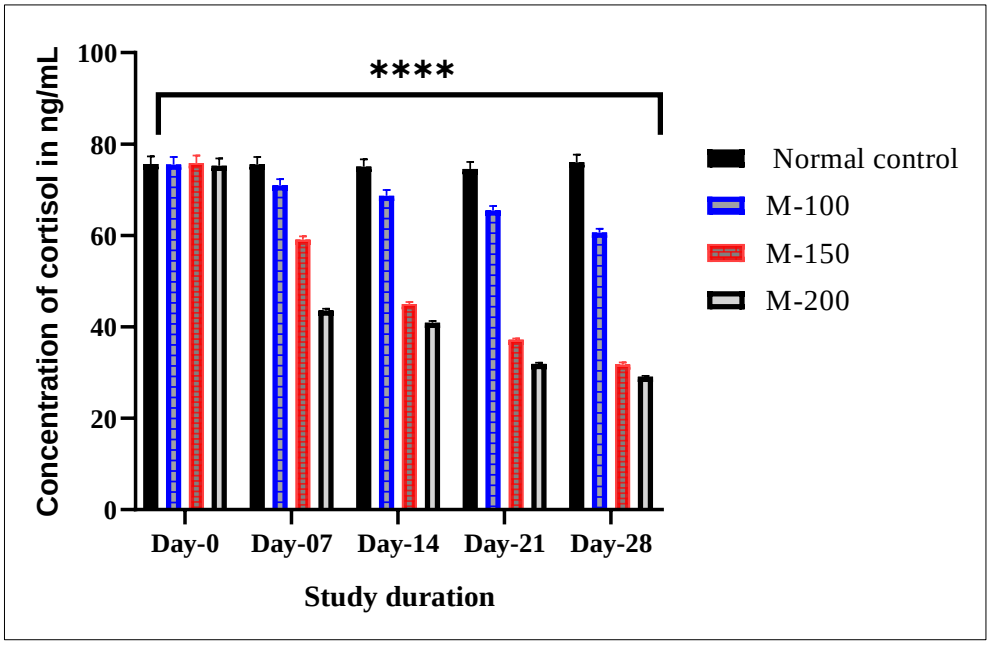

Fig 3: Serum cortisol levels in ng/ml on day 0, 7, 14, 21 and 28.

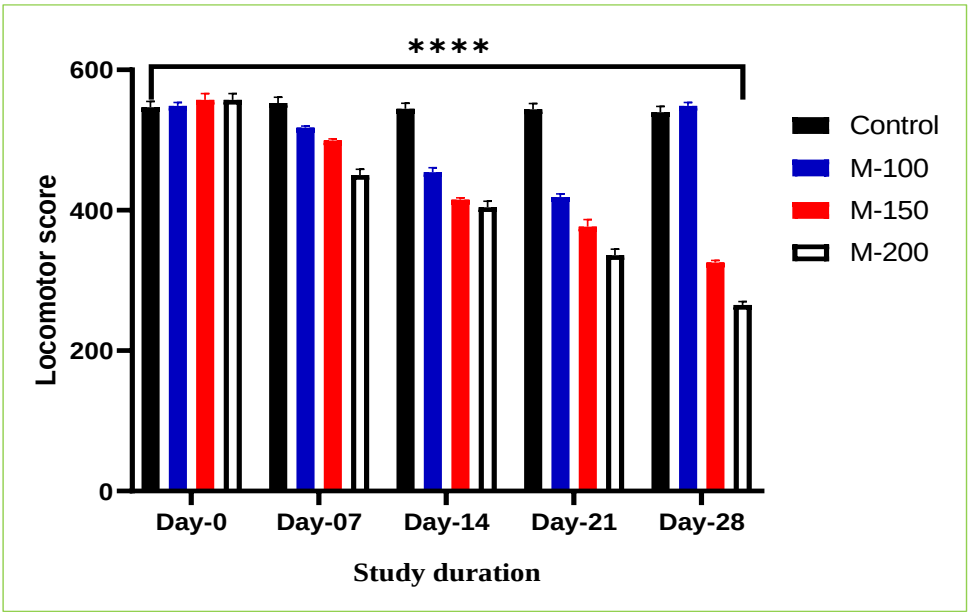

Fig 4: Locomotor scores on day 0, 7, 14, 21 and 28.

Discussion

This study is conducted to induce adrenal insufficiency in the Wistar rats. Figure 3 represents decreased cortisol (75.666 ± 1.635, 75.568 ± 1.627, 75.086 ± 1.589, 74.523 ± 1.546 & 76.011 ± 1.663 for normal control group. 75.519 ± 1.623, 70.971 ± 1.323, 68.676 ± 1.253, 65.474 ± 0.995 & 60.653 ± 0.788 for Metyrapone 100mg/kg group. 75.813 ± 1.647, 59.070 ± 0.744, 45.008 ± 0.394, 37.193 ± 0.246 & 31.739 ± 0.476 for Metyrapone 150mg/kg group and 75.278 ± 1.604, 43.586 ± 0.361, 40.918 ± 0.368, 31.866 ± 0.271 & 29.075 ± 0.193 for Metyrapone 200mg/kg group). Figure 4 indicates reduced locomotor scores (546.80 ± 8.18, 552.80 ± 8.18, 544.60 ± 7.65, 543.80 ± 8.18 & 539.80 ± 8.18 for normal control group. 548.70 ± 4.72, 517.70 ± 2.39, 454.30 ± 6.46, 418.70 ± 4.72 & 548.70 ± 4.72 for Metyrapone 100mg/kg group. 557.10 ± 9.29, 499.50 ± 2.34, 415.20 ± 2.59, 376.67 ± 9.94 & 325.57 ± 3.06 for Metyrapone 150mg/kg group and 557.20 ± 8.96, 450.20 ± 8.32, 404.44 ± 8.45, 336.14 ± 8.57 & 265.00 ± 5.12 for Metyrapone 200mg/kg group respectively). With the obtained results, adrenal insufficiency was developed in Wistar albino rats. Hence it is simple and cost effective model to induce adrenal insufficiency for day to day screening of animal activity.

Conclusion

The cytochrome P450 (CYP) enzymes are membrane-bound hemoproteins that play a pivotal role in the detoxification of xenobiotics, cellular metabolism and homeostasis. Induction or inhibition of CYP enzymes is a major mechanism that underlies drug-drug interactions. CYP enzymes can be transcriptionally activated by various xenobiotics and endogenous substrates through receptor-dependent mechanisms. CYP enzyme inhibition is a principal mechanism for metabolism- based drug-drug interactions. Many chemotherapeutic drugs can cause drug interactions due to their ability to either inhibit or induce the CYP enzyme system [10]. Metyrapone is an inhibitor of enzymes of the cytochrome P45011B and P4502B family, which includes 11beta-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2), enzymes required for the synthesis of cortisol from 11-deoxycortisol and of aldosterone from 11-deoxycorticosterone. Thus, administration of metyrapone inhibits the synthesis of these adrenal steroids. The resultant fall in serum cortisol decreases negative feedback on the hypothalamus and adenohypophysis, thereby increasing the secretion of corticotrophin. This results in increased secretion of 11-deoxycorticosterone and an increased urinary excretion of 11-deoxycorticosterone and 17-hydroxycorticosteroids. Metyrapone decreased the serum cortisol levels and locomotor activity in rats by blocking the 11beta-hydroxylase in adrenal steroidogenesis pathway. Hence this can be considered as a model for inducing adrenal insufficiency in Wistar rats.

Conflict of interest

Authors declare no conflict of interest.

Source of funding

Sri Adichunchanagiri College of Pharmacy, B.G Nagara, Karnataka.

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