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Down Regulation of Plasma and Tissue Biomarkers by Homocastasterone

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Abstract

Homocastasterone is a ketosteroid and a member of the brassinosteroid family of plant hormones. Earlier studies with 28-homobrasslinolide, an aldosteroid, had indicated that rat blood and tissue biochemical parameters studied were affected by this compound, resulting in altered homeostasis and cellular phosphorylation status, rendering this plant oxysterol inappropriate for high energy related work activities. The use of the ketosteroid in this study presents evidence for renormalization of elevated plasma lipid content in diabetic rat, antiglycemic potency, increase in liver glycogen and glucose level and diminished ALT and AST enzyme activities. A role for this ketosteroid in rat liver gluconeogenesis and in lipid homeostasis is suggested while the aldo and keto forms regulated glucose homeostasis in the rat. The observed differences in the effects of homobrasslinolide and homocastasterone as exogenous oxysterols on normal and diabetic rat plasma lipid level suggests the possibility of differential influence by endogenous aldo and keto oxycholesterol forms on glucose and lipid homeostasis in mammalian physiology.

Keywords: Homocastasterone, ketosteroid, plasma lipid, blood glucose, marker enzyme.

Introduction

Homocastasterone is a steroid plant hormone, structurally a keto-isoform, which is an active plant growth regulator among the brassinosteroid (Bs) family members. Homocastasterone and 28-homobrassinolide (aldo-isoform) are actively synthesized in plants by CYP72B1 enzyme and contributes to growth regulation, seed germination, stress response and photomorphogenesis through modulation of cellular signalling processes. Bs role in plants is understood based on the outcome of their specific applications in agriculture [1]. Bs isoforms have structural similarity with insect growth regulators (IGR's), and have been shown to affect their egg laying capacity, fecundity and longevity among different insects groups [2]. Humans and animals are exposed to these hormones, through consumption of food. Assimilation of these compounds differentially into tissues may induce metabolic stress in animal cells. Certain Bs isoforms exhibit structural similarity with endogenous oxycholesterols, 7-ketocholesterol, 24-hydroxycholesterol and 27-hydroxycholesterol, being oxidation products of cholesterol through enzymatic (Cyt P450) and non-enzymatic (ROS) processes in animal tissues. Oxysterols can be endogenous (tissue based) and exogenous origins (of plant and insects) [3, 4]. Their levels are very low in tissues, and they exhibits biological potency by acting through nuclear orphan receptors, namely the liver x receptor (LxR). LxR are considered as potentially regulating cholesterol, triglyceride and glucose homeostasis, through nuclear signaling and transcriptional regulation of cellular catalytic activities. Recently LxR has been recognised as drug target for hyperglycaemic and atherosclerosis conditions [5]. Yet till date, plant oxysterol effects on LxRs remain little studied while identification of potent LxR ligands from natural sources remains an exciting possibility.

We have previously reported the effect of the plant aldosteroid 28-homobrassinolide, in diabetic rat model, as exhibiting altered tissue enzyme activity and hexokinase gene expression. Improvement in tissue architecture and reduced lipid peroxidation concomitant with improved antioxidant status was noted in the study [6-9]. However, these studies indicated elevation of blood cholesterol and triglyceride in the experimental animal though blood glucose level was found to be regulated. Recently, a steroidogenesis promoting effect of 28-HB through elevation of StAR protein and ABP in rat testis was reported [10]. However, 28-HB did not reduce blood/plasma levels of triglyceride and cholesterol. Nevertheless, 28-HB caused changes in tissue histology and tissue marker enzyme activity in the rat. The anabolic effect of brassinosteroids, its anticancer activity in human cancer cell line, beneficial effects on high fat-induced diabetic mice, improved insulin sensitivity and reduced gluconeogenesis have also been reported earlier [11].

Search for a natural product that did not raise blood lipid level when administered for countering hyperglycaemia, therefore led us to investigate the biopotency of the ketosteroid- homocastasterone (HC), as an alternate compound for the investigation, employing normal and STZ induced diabetic male rat to understand its impact on lipid and carbohydrate metabolism while monitoring plasma and tissue bio markers to assess specific biological responses.

Material and Methods

Chemicals, Reagents and Experimental rat

All chemical used in this study were analytical grade purchased from Sigma Aldrich (USA). Homocastasterone was gifted by Dr. V.S. Pori, NCL, Pune, India. Blood diagnostic Kits were purchased from Agape diagnostics, Kerala, India. Rats were purchased from the authorised dealer Sri Ragavendra Enterprises, Bangalore, India, and were maintained at 22˚C with 12hr light/dark cycle and were given normal standard chow and water *ad libitum,* throughout the experimental period. Following one week of acclimatisation, 24 healthy, normal, 6-8 week old rats weighing 150-180gm were selected for the study. Rats were divided into four groups and each group contained 6 rats. Animal use and care was strictly followed as per CPCSEA regulations and Institutional Animal Ethics Committee (IAEC) guidelines. The animals were segregated into four groups. Such as,

Group I: Normal control
Group II: Normal + Homocastasterone treated
Group III: Diabetic Control
Group IV: Diabetic + Homocastasterone treated

Induction of diabetes

Diabetes was induced by a single intraperitoneal injection of 60 mg/kg bwtstreptozotocin in citrate buffer (0.1 M, pH 4.5) to overnight fasted rat. A 5% glucose solution was given to the STZ injected rats to prevent hypoglycemic death due to use of STZ. After 48hr, tail vein blood was collected from the animal and the glucose level was measured with the help of a glucometer. Glucose content greater than 250 mg/dL was considered diabetic, for use in the experiments.

Homocastasterone treatment

Groups II and IV received 666 mg/ kg bwt HC in 50% ethanol by oral gavage for 15 consecutive days and Control groups I and III received 50% ethanol alone.

Blood and organ collection for assay

At the end of 15 days, rats were euthanized and blood collected by cardiac puncture into heparinised tubes, centrifuged at 1000 rpm for 10 minutes in a clinical centrifuge and plasma collected and stored at -20˚C until analysis. Tissues were removed immediately, washed with phosphate buffer (0.1M pH 7.4) thoroughly and stored in phosphate buffer at -80˚C for further analysis.

Determination of plasma biomarkers

Glucose, cholesterol, triglycerides, HDL, urea, creatinine, and uric acid, ALT, AST and LDH were analysed employing the kit method as per manufacturer's instruction.

Analysis of tissue biomarkers

Sliced portion of tissues were weighed (1g) and washed with ice cold saline (0.9% NaCl), and homogenized (10% w/v) in 10ml of 0.1M phosphate buffer pH 7.4 with help of a Potter-Elvehjem Teflon homogenizer followed by centrifugation at 3000x g for 30min at 10ºC in a refrigerated eppendorf centrifuge. The supernatant was collected and was used for the determination of biochemical parameters such as cholesterol, triglycerides and urea, by the kit method. Total protein content in the homogenate was estimated by the method of Lowry [12].

Tissues glycogen and glucose estimation

200 mg of tissue was taken in 20 ml 5% TCA solution and homogenized with the help of a Potter-Elvehjem Teflon homogenizer. After centrifugation at 6000 rpm for 10 minutes in a refrigerated eppendorf centrifuge, the clear supernatant was collected for glycogen assay. 2 ml of supernatant and 3 ml the lugol's reagent were mixed thoroughly and the colour developed was measured for glycogen content at 650 nm [13]. Values were expressed as mg/gm tissue. Liver glucose was assayed by the O-toludine method [14].

Histological of rat liver for glycogen content

5 µm sections of rat liver were prepared as paraffin embedded liver blocks with the help of a digitalized microtome and standard PAS staining procedure was adopted to stain these sections. Photomicrographs of the stained sections (100x) were taken with the aid of camera and attached to a binocular microscope [15].

Statistical analysis

Results were expressed as mean ± SD, and the data was analysed by one way ANOVA. Following this, Post doc test was used employing SPSS software 16.0 version. A value of $p < 0.05$ was considered significant.

Result

The specific biological effect observed in rat plasma and in selected rat tissues due to the plant ketosteroid homocastasterone is detailed below.

On plasma studies

Administration of rat by oral gavage with homocastasterone for 15 days reduced rat plasma glucose level from 277 mg/ dL to 165 mg/dL in the diabetic animal indicating 40% reduction plasma glucose in the treated group.

Plasma cholesterol level reduced from 96 mg/dL to 64mg/dL in the HC treated group registering a 31.5% reduction. A decrease (110 mg/dL to 74 mg/dL) in plasma TG level was noted in diabetic treated rat group corresponding to 32.7% reduction while 9.8% in TG level in the normal rat treated group was noted.

Elevated LDL and VLDL levels noted in the diabetic rat group reversed after oral treatment with HC. In diabetic treated group the LDL level reduced from 33 mg/dl to 18 mg/dL, exhibiting 44.8% reduction. VLDL level decreased from 22mg/dL to 14mg/dL corresponding to 32.7% reduction. There was no change in the plasma HDL level.

Table 1. Plasma glucose and lipid in normal and diabetic HC treated rats.

GROUP	Glucose mg/dL	Cholesterol mg/dL	Triglyceride mg/dL	HDL mg/dL	LDL mg/dL	VLDL mg/dL
Normal control	92 ± 2.98	111 ± 2.88	123 ± 2.50	38 ± 0.81	50.4 ± 0.30	24.6 ± 0.18
Normal + HC	88 ± 3.16*	77 ± 3.41*	112 ± 1.86*	36 ± 0.89	16.6 ± 0.36*	22.4 ± 0.19
Diabetic control	277 ± 4.37	95 ± 2.36	110 ± 2.03	40 ± 1.16	33.0 ± 1.47	22.0 ± 1.41
Diabetic + HC	165 ± 4.72*	64 ± 2.81*	74 ± 3.16*	31 ± 0.81	18.2 ±0.30*	14.8 ±0.17*

These biomarkers were analysed by the kit method. Values are expressed ± SD mg/dL and mean of three determinations. Statistical significance is indicated where p<0.05*.

Elevated level of ALT (165U/dL) in diabetic group, after treatment with homocastasterone, was restored to near normal (32 U/dL) control, a reduction of 80.6%. Normal treated group also showed an 80% reduction in ALT enzyme level compared to the controls. Increase in AST level in STZ treated diabetic group, was restored to near normal level the 17.8% to the following HC treatment. In normal treated group, a 3% in the enzyme activity was seen compared to control rat group.

Table 2. Tissues marker enzyme ALT, AST and LDH level in plasma in normal and diabetic HC treated rats.

Groups	ALT IU/l	AST IU/l	LDH IU/l
Normal control	10 ± 2.41	96 ± 3.16	199 ± 4.89
Normal + HC	2.0 ± 3.51*	93 ± 2.09	115 ± 5.26*
Diabetic Control	165± 2.71	95 ± 4.75	277 ± 3.89
Diabetic + HC	32 ± 2.33*	78 ± 3.36*	243 ± 4.75*

Plasma enzymes were analysed by kit method. Values are expressed ± SD IU/ l and mean of a three determinations. Statistical significance is indicated where p<0.05*.

Plasma urea level in diabetic rat increased three fold compared to that of normal rat (46 into 113 mg/dL) but reduced 24.7% for the HC treated group. There was no significant change in plasma urea level in homocastasterone treated normal group. Significant changes were not seen in uric acid level in normal and diabetic homocastasterone treated groups. Increased creatinine level in diabetic rat was restored to near normal following HC treatment, yielding a 28.5% reduction. There is no change seen in normal homocastasterone treated group. Albumin decreased 3.5 to 2.8 mg/dL in the diabetic group compared to control. No significant change was observed in normal and diabetic rat groups treated with HC.

Table 3. Plasma renal biomarker parameters in normal and diabetic HC treated rats.

Groups	Urea mg/dL	Uric Acid mg/dL	Creatinine mg/dL	Albumin mg/dL	Total protein mg/dL
Normal control	46 ± 2.03	11.1 ± 2.20	0.5 ± 0.12	3.5 ± 0.52	8.0 ± 2.09
Normal + HC	52 ± 4.19	12.6 ± 3.14	0.5 ± 0.11	3.3 ± 0.49	7.5 ± 3.13
Diabetic Control	113± 3.06	2.25 ± 1.27	0.7 ± 0.21	2.8 ± 0.82	5.6 ± 1.07
Diabetic + HC	85 ± 2.72*	3.15 ± 1.13	0.5 ± 0.31	2.6 ± 0.58	5.9 ± 1.15

Renal markers were analysed by kit method. Values are expressed ± SD IU/l and mean of three determinations. Statistical significance is indicated where p<0.05*.

On tissues studies

Reduction in cholesterol level (Table 4) was seem in the liver, heart and kidney of diabetic treated rat group by 35.5%, 65% and 55.54% respectively. In the normal treated group, a reduction of 15% in liver, 51% in heart and 23% in kidney of cholesterol was noted. In testis of diabetic treated rat group, cholesterol level increased (7.65 to 9.22 mg/dL) 20.5% compared to the control group. There was no change in cholesterol level of normal treated rat group. In brain however, cholesterol level increased 60% in normal treated group compared to control (9.56 to 15.31 mg/dL). There was no significant change in brain cholesterol in diabetic treated group.

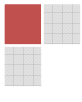

Table 4. Tissue lipids, total protein and urea in diabetic and normal HC treated rats.

Groups	Normal control	Normal + HC	Diabetic Control	Diabetic + HC
Liver				
Cholesterol	19.13 ± 2.40	16.26 ± 1.83*	14.83 ± 2.90	9.56 ± 2.65*
Triglycerides	132.93 ± 4.53	122.15 ± 2.75*	160.47 ± 3.62	129.34 ± 2.34*
Total protein	100 ± 3.15	125 ± 4.18*	125 ± 3.10	130 ± 4.23*
Urea	10 ± 2.27	11.8 ± 3.54	14.0 ± 2.14	11.4 ± 2.72*
Heart				
Cholesterol	37.32 ± 2.58	18.18 ± 3.52*	16.43 ± 4.32	5.74 ± 2.50*
Triglycerides	59.88 ± 2.80	20.35 ± 3.72*	70.18 ± 4.75	38.43 ± 3.90*
Total protein	86.0 ± 2.30	82.6 ± 3.14	43.0 ± 2.57	43.0 ± 2.52
Urea	6.6 ± 1.45	6.6 ± 2.16	5.37 ± 1.39	3.37 ± 1.26*
Kidney				
Cholesterol	24.88 ± 2.32	18.18 ± 2.50*	34.44 ± 3.02	15.31 ± 2.60*
Triglycerides	52.17 ± 2.02	59.18 ± 3.24	98.13 ± 2.85	47.26 ± 3.83*
Total protein	78 ± 3.59	89.5 ± 2.23*	90.0 ± 2.83	60 ± 3.69
Urea	31.0 ± 3.63	18.0 ± 2.44*	25.6 ± 2.44	14.30 ± 2.30*
Testis				
Cholesterol	5.74 ± 3.02	5.74 ± 2.03	7.65 ± 2.02	9.22 ± 2.01*
Triglycerides	35.92 ± 4.02	69.46 ± 3.03	34.73 ± 2.02	16.76 ± 3.01*
Total protein	43.70 ± 2.14	50.50 ± 2.20*	49.0 ± 3.58	75.90 ± 2.63*
Urea	5.0 ± 0.64	6.8 ± 0.35	8.2 ± 0.63	6.63 ± 0.29*
Brain				
Cholesterol	9.56 ± 2.94	15.31 ± 3.67	12.44 ± 2.24	12.44 ± 2.15
Triglycerides	33.75 ± 3.47	22.75 ± 2.94*	23.95 ± 4.27	11.97 ± 3.01*
Total protein	18.5 ± 2.47	30 ± 2.06*	10.0 ± 1.80	11.5 ± 1.16
Urea	5.59 ± 0.13	4.35 ± 0.10	7.25 ± 0.13	7.25 ± 0.13

Table 4.Liver, heart, kidney, testis, brain tissues cholesterol, triglyceride, total protein and urea levels were analysed in diabetic and normal HC treated rats by kit method. Total protein was estimated by Lowry method. Values are expressed ± SD mg/dL and mean of three determinations. Statistical significance is indicated where p<0.05*.

Similarly the triglyceride level in liver, heart, kidney, testis and brain of the diabetic rat increased compared to control. However triglyceride level reduced in diabetic treated group compared to diabetic control liver (20%), heart (45%), kidney (34%), testis (51%), and brain (50%). In normal HC treated group, triglyceride level in liver, heart and brain reduced by 8%, 66%, and 32% respectively. However, twofold increase in triglyceride level was seen in testis of normal treated group compared to control. In normal HC treated rat group, significant increase in total protein level was noticed in the liver (25%), kidney (14%), testis (15%) and brain (62%) compared to normal control.

In the diabetic treated rat, protein level increased in testis (61.55%) than liver (4%) and brain (15%) compared to diabetic control. In the diabetic treated rat tissue urea level was noted to be reduced, in liver, heart, kidney and testis registered are 18, 37, 44, 19% compared to diabetic control. There was no significant change in brain urea level in the diabetic treated and diabetic control rat group. Normal treated rat tissue urea level reduced 18% in liver, 41% in kidney, 36% in testis and 22% in brain compared to control.

A 50% reduction (Table 5) in glycogen level in diabetic rat group was also noted compared to normal control. However in homocastasterone treated diabetic rat group, the liver glycogen level increased by 233% compared to control. In normal treated rat, liver glycogen level increased 133% compared to control. Liver glucose content reduced significantly in diabetic rat. In diabetic treated rat however, liver glucose level increased 120% compared to control. In normal treated group, liver glucose level increased 26% (160 mg/dL to 202 mg/dL) compared to control.

PAS stained sections of diabetic control rat liver showed few PAS positive glycogen granules in hepatocytes compared to normal control (Figure 1c). For the diabetic treated group, however many of the hepatocytes were filled with PAS positive glycogen granules. Homocastasterone treated normal rat liver showed increased hepatic lobules packed with glycogen granules compared to control (Figure 1b).

Table 5. Liver glycogen and glucose content in normal and diabetic HC treated rats.

Groups	Glycogen mg/g tissue	Glucose mg/g tissue
Normal control	1.000 ± 0. 0.27	160.0 ± 8.41
Normal + HC	2.333 ±0.25 *	202.8 ± 6.57*
Diabetic Control	0.500 ± 0.18	42.81 ± 5.47
Diabetic + HC	1.666 ±0.35 *	94.24 ± 6.73*

Liver biomarkers were analysed as described in methods. Values are expressed ± SD mg/g tissue and mean of six determinations. Statistical significance is indicated where p<0.05*.

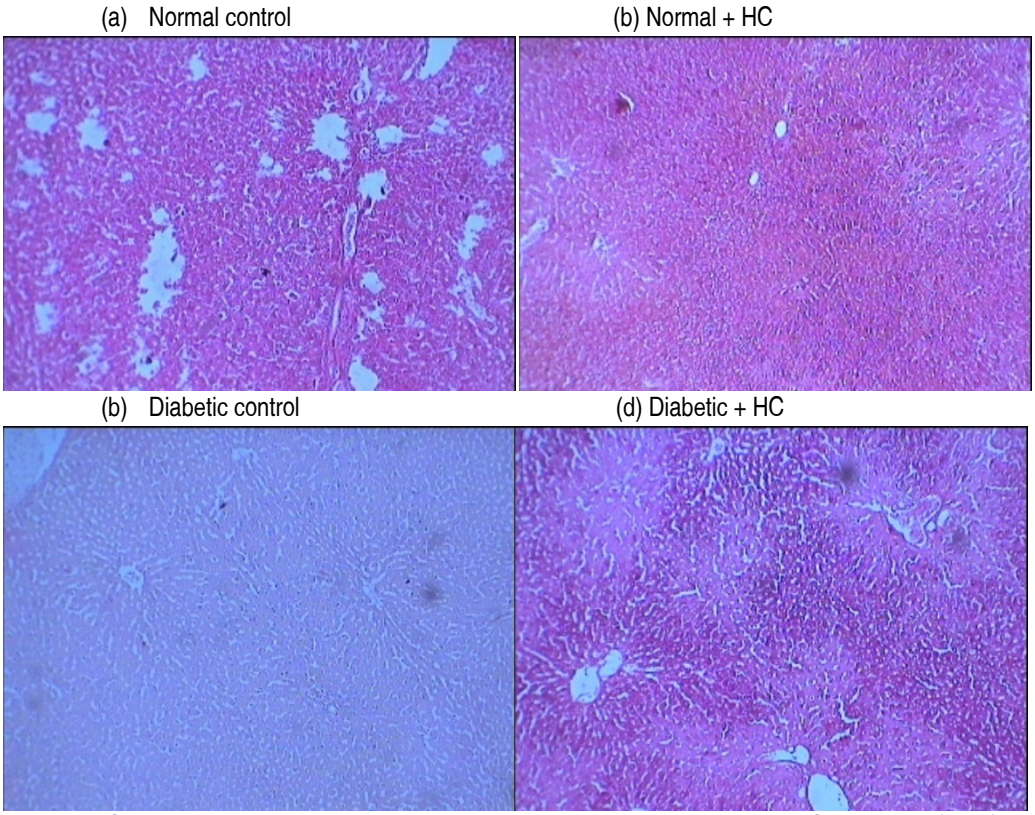

Figure 1. PAS staining of liver section for glycogen granules in diabetic and normal HC treated rat (100x).

(a) Normal control shows hepatocytes filled with PAS positive glycogen, (b) Normal + HC treated groups shows most of the hepatic lobules packed with glycogen, (c) Diabetic control very few hepatocytes shows, few granules of PAS positive glycogen granules in cytoplasm, (d) Diabetic + HC treated group shows majority of hepatocytes are filled with PAS positive glycogen granules.

Discussion

Diabetes was established in the experimental rat blood employing streptozotocin at 60mg/kg bwt (blood glucose <250 mg/dL). Both normal as well as the diabetic rats were orally fed homocastasterone for 15 consecutive days thereafter at 666 µg/kg bwt. Following administration of this ketosteroid, blood glucose level determined was found to be different for the control and treated animals. HC treated animals yielded lower blood glucose level than their control counterpart. Similar results were noted earlier from this lab using 28-homobrassinolide through the work of Muthuraman and Srikumar [6,7,9]. In the present study, it was noted that liver content of glycogen increased following HC treatment of the diabetic animal while concomitantly observing reduction in blood glucose level, indicative of increasing liver stores of this sugar. Histological section of the HC treated diabetic rat liver confirmed this finding, indicating increased liver glycogen synthesis.

The use of 28-HB in earlier studies had noted increased plasma cholesterol and triglyceride in the experimental normal and diabetic rat. Such physiological responses promote the use of 28-HB as a potent antihyperglycemic factor. Surprisingly, the use of the plant ketosteroid HC resulted in renormalizing the elevated lipid content of diabetic rat plasma. The observed difference in the potential to increase plasma lipid content by 28-HB in normal and diabetic rat versus the potential to renormalize the elevated lipid content of diabetic rat plasma by HC leads us to suggest that lipid homeostasis in this mammalian model is under regulation by aldo-keto isomeric states of endogenous oxysterols, whereas the potential to regulate glucose homeostasis remained common to both chemical forms.

Liver marker enzyme ALT and AST levels increased in the blood of STZ induced diabetic rat. These enzymes are present in high amounts in the liver and have a role in supplying intermediates for gluconeogenesis through transamination reactions. ALT and AST are there for diagnostically important in liver damage. In diabetic animals increased ROS production caused hepatocyte membrane damage and release of the enzymes into circulating blood [16]. Alteration in liver function in diabetes causes significant increase in these enzymes in the plasma of the diabetic subject. Oral

administration of homocastasterone for 15 consecutive days to normal and diabetic rat yielded dramatically reduced ALT and AST enzyme levels in rat plasma compared to their respective controls. The observed reduction in plasma ALT and AST levels by HC is indicative of reduced release of these marker proteins from hepatocytes simultaneous with protection of membrane injury[17].Increase in the ALT enzyme level noted in diabetic control rat was renormalized by treatment of the animal with this phytohormone.

In STZ induced diabetic control rat plasma urea and creatinine levels were (Table 3) significantly increased compared to that in normal control. This effect in the diabetic rat is a consequence of increased protein breakdown and impaired renal function. In the 15 day ketosteroid treated diabetic group, significant reduction of blood urea level were noted compared to that in control group, while there was no change observed in the normal treated group. Esposito *et al* (2012) had reported antihyperglycemic effect of 28-HB in high fat diet-induced obese mice. Esposito *et al* (2011) also reported that plant 28-HB had anabolic effect in mammalian system [11,18].Improvement in renal structure and function therefore contributed to decreased urea and creatinine levels. The observed decrease in plasma level of these compounds therefor suggested that the administered plant ketosteroid improved rat renal architecture and function.

Plasma albumin and total protein presented in table 3, shows reduced level of these markers in diabetic rat compared to control. Diabetic rat treated with homocastasterone showed no significant changes.

This study attempts to understand the keto-steroid effect on lipid profile in rat plasma and in selected rat tissues such as the liver, testis, heart, brain and kidney (Table 4). Cholesterol and triglyceride levels increased significantly in the diabetic control rat compared to the normal control rat. This observation is similar to those of the others [19,20].Diabetes induced lipolysis and lipid mobilization from adipose tissues. Abnormal synthesis of cholesterol and triglycerides is also reported in diabetes and contributes to enhanced lipid profile in diabetic tissues. Following oral administration of homocastasterone to normal and diabetic rat, statistically significant decrease in the level of cholesterol and triglycerides in the liver, heart, kidney and testis were experimentally observed in our study.

In the STZ induced diabetic rat (Table 4) elevated urea level and decreased protein level in liver and testis were noted compared to normal control. These marker changes may due to the increased tissue protein breakdown and impaired renal function in diabetic rat [20-22]. In HC treated rat tissues, however urea level reduced significantly compared to the control and increased protein level was noted in liver, kidney and testis after oral administration of HC to the normal and diabetic rat. Increase in protein suggests anabolic effect by homocastasterone.

Conclusion

Several studies carried out in the past on the effect of the brasslinolide family of phytohormones in normal and different diabetic mammalian models have shown beneficial effects in terms of antihyperglycemic, antihyperlpidemic and anabolic effects. This lab has previously reported that 28-homobrasslinolid an aldo-form of brasslinolide has antihyperglycemic potency, but caused elevation of cholesterol and triglycerides level in plasma. The present study demonstrates that homocastasterone, a keto-form of the brasslinolide, caused significant reduction in plasma and tissue cholesterol and triglyceride level, but retained the antihyperglycemic potency in diabetic rats on 15 days treatment. Further work is underway to address the mechanism of the action of this phytohormone on lipid lowering effect.

Conflict Of Interest

The authors declare that there are no conflicts with respect to the authorships or publication of this article.

Author's Contribution

The concept and design of this work is contribution from the corresponding author.The experimental work and preliminary draft preparation was carried out by the first author. Work related scientific discussions and manuscript proof reading, editing and formatting was carried out by the second author.

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