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Original Article

HOMOCASTASTERONE: A NOVEL PLANT KETOSTEROID INDUCING HAEMATOLOGICAL CHANGES IN NORMAL AND DIABETIC MALE RAT

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ABSTRACT

Objective: To study the effect of brassinosteroid keto isoform homocastasterone, in diabetic male wistar rat as an antihyperglycemic factor and to evaluate its effects on the hemodynamic parameters in rat blood.

Methods: Diabetes was induced in a group (n=6) of rats with a single peritoneal injection of streptozotocin at 60 mg/kg. bw. With a treatment schedule of 15 consecutive days, control (n=6) and diabetic rats received 666µg/kg bw, of homocastasterone. Circulating blood glucose, cell count, cell indices, and MDA level was assessed.

Results: Significant reduction ($p<0.05$) in blood glucose level and increase in RBCs, WBCs, granulocytes, lymphocytes, monocytes and platelets count($p<0.05$) along with improved functional indices for HCT, PCV, MCV, MCH, MCHC, MPV, PDW, PCT in homocastasterone treated diabetic group was noted. A significant reduction in RBC-MDA level ($p<0.001$) in the treated group was noted.

Conclusion: It is suggested that brassinosteroid keto isoform homocastasterone exhibits antiglycemic effect in diabetic rat, and improves RBC, WBC, Platelet counts, haemoglobin level, and cell indices, while reducing per oxidative cell damage in RBCs.

Keyword: Homocastasterone, Oxysterol, Haematological parameters, Blood glucose, Lipid peroxidation.

INTRODUCTION

Plant growth regulators (PGRs) abscissic acid, indole acidic acid (IAA), gibberrellic acid (GA3), jasmonic acid and brassinosteroids (BS) are facilitate growth, flowering, seedling and immune responses in plants. Brassinosteroids also show significant effect on insect egg laying capacity, insect growth regulation, mosquito larval growth, fecundity, viability and longevity. Structurally, they are similar to mammalian steroids and oxycholesterol. These compounds elicit biological response inhuman and animals when exposed through diet and herbal folk medicine [1, 2]. Specific biological effect of plant BS on mammalian system is not fully understood. Studies by Muthuraman *et al.*[4] and Nirmal kumar *et al.* [5] recognized that 28-homobrassinolide, as an aldo isoform yielded antiglycemic effect and increased RBC, WBC, lymphocyte, monocyte, granulocyte and haemoglobin (Hb) level in male adult wistar rat. PGRs such as indole acidic acid (IAA) had no significant effect on haematological parameters while gibberrellic acid (GA3) caused the significant increase in RBC and Hb level, in a dose dependent manner Muthu *et al.* [3-7].

Diabetic anaemia due to non-enzymatic glycation of RBCs and oxy-free radical induced cell membrane damages results in decreased RBC count and Hb level in Diabetes Mellitus (DM). Abnormal platelet count and seen in DM result in the formation of thrombotic plugin the blood vessels causing vascular defect. In recent years, attention is sought in improving the platelet indices for the treatment of DM in addition to improving the RBC and Hb level [7, 8]. As blood is a connective tissue comprised of a number of formed elements such as RBC, WBC, and platelets, it has a significant role in maintaining homeostasis. Factors that modulated their circulating levels were considered as holding promise to act as potential therapeutic agents or drug candidates.

In this study the keto phyto steroid homocastasterone (HC) was therefore used to investigate its antihyperglycemic effect on normal and diabetic rat[9, 10], on their blood elements, and on platelet indices (mean platelet volume, platelet distribution width, and platelet–large cell ratio) considered as major determinants of atherosclerosis.

MATERIALS AND METHODS

Chemical and experimental rat

All chemicals used in the study were of analytical grade purchased from Sigma Aldrich USA, Homocastasterone was courtesy of Dr. V. S. Pori. NCL, Pune, India. Experimental male wistar albino rats (8-10 wks) were purchased from Sri Ragavendra Enterprises, Bengaluru, India.

Experimental design

A group of 24rats (150-200 gm) was used for the study. Animal use and care were in compliance of the CPCSEA regulations and Institutional Animal Ethics Committee (IAEC) guidelines, (IAEC/Approval No. 2013-14/01). Rats were divided into four groups of 6 animals each, Group I: Normal rat control, Group II: Normal rat treated with HC. Group III: Diabetic rat control and Group IV: Diabetic rat treated with HC. Diabetes was induced in the experimental animals by use of a single intraperitoneal injection of 60 mg/kg bw streptozotocin in citrate buffer (0.1 M, pH 4.5), overnight fasted experimental rats. 48 hrs later, circulating blood glucose level in the animals was measured using a glucometer (One Touch Horizon, Accuva check). Rats exhibiting blood glucose content>250 mg/dl were considered diabetic for use in the experiment. Control groups I and III received 50% ethanol alone. Groups II and IV were administered 666 mg/kg bw HC in 50% ethanol by oral gavage for 15 consecutive days.

Blood glucose analysis

Glucose level in circulating rat blood was measured with a glucometer (One Touch Horizon, Auccva check) at 5 day interval.

Blood cell count

Rat blood cell count was determined every fifth day. RBC, WBC, granulocytes, monocytes, lymphocytes, platelets, HCT, PCV, MCV, MCHC and MPV, PWD, and PCT were analysed by fully automated blood cell counter (RC-210 Fully Automatic Blood Cell Counter, Rohan Consortium Pvt. Ltd). Hb level was also determined by the same instrument.

Lipid peroxidation assay

Heparinised blood was centrifuged at 3000 rpm in a refrigerated table top eppendorf centrifuge for 15 min to remove plasma and the buffy coat. The sedimented RBC was washed with physiological saline and re-centrifuged at 3000 rpm, three consecutive times, for 15 min each. Packed RBCs was then collected and stored at-20^{o}C until further use. Malondialdehyde (MDA) level of RBC was measured by the method of okhava *et al.*[10].

Statistical analysis

Results of the investigations were expressed as mean±SD, and the data were analysed by one way ANOVA employing SPSS software 16.0 versions. Value of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Effect on blood glucose

The effect of homocastasterone on blood glucose level was assessed every 5 days from day one of treatment (table 1). HC treated diabetic rat blood glucose level reduced 45% on day 5, 50% on day 10and 55% on day 15, compared to the diabetic control ($p<0.05$).

Effect on RBC count and haemoglobin level

RBC and Hb level increased significantly in HC treated diabetic group compared to diabetic control. RBC count increased 5.2% by day 5, 21.25% by day 10, and 5.6% by day 15, respectively. RBC count of HC treated normal rat only 2.5% by day 5 and 2% by day 15. Hb level was 4.76%, 0.16%, and 5.66% on days 5, 10and 15 of diabetic treated rat when compared to that of diabetic control.

Effect on the RBC indices

Values of RBCs indices, haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), and mean corpuscular haemoglobin (MCH) are as presented (table 4). A decrease in the indices was noted in HC treated rat blood compared to the respective control. HCT and MCV levels decreased significantly in diabetic treated group by 5th and 10th day. MCH and MCHC levels also decreased significantly in the diabetic treated.

Table 1: Effect of homocastasterone on blood glucose level

Group	0 Day glucose mg/dl	5 Day glucose mg/dl	10 Day glucose mg/dl	15 Day glucose mg/dl
Control	103.0±6	110.0±5	105.0±2	92.0±3
Control+HC	106.0±4	97.0±6$^{\#}$	96.0±4$^{\#}$	88.0±2$^{\#}$
Diabetic control	280.0±7	300.0±8	300.0±8	315.0±6
Diabetic+HC	282.0±6	206.0±3*	187.0±7*	172.0±4*

Values are expressed±SD. Group n = 6, #Indicates statistical significance against normal control ($p<0.05$), *Indicates statistical significance against diabetic control ($p<0.05$).

Table 2: Effect of homocastasterone on RBC, Hb, WBC and platelet count

Group	RBC 10×6/µl			Hb g/µl			TWBC10×3/µl			Platelets 10×3/µl		
	5 Day	10 Day	15 Day	5 Day	10 Day	15 Day	5 Day	10 Day	15 Day	5 Day	10 Day	15 Day
Control	7.17±1.02	7.65±1.02	7.83±1.02	14.7±1.08	16.3±0.73	15.9±0.85	25.4±0.86	36.3±0.86	11.5±0.86	424±12	444±12	334±12
Control+HC	7.35±0.95	7.99±0.95	7.86±0.95	15.±1.08	16.4±0.73	16.8±0.85$^{\#}$	15.6±0.42$^{\#}$	22.4±42$^{\#}$	9.4±.42$^{\#}$	366±16$^{\#}$	334±16$^{\#}$	163±16$^{\#}$
Diabetic control	7.97±0.84	5.69±0.84	6.42±0.84	16.0±1.08	13.7±0.73	12.6±0.85*	16.4±0.26	20.8±0.26	3.5±0.26	268±9	164±9	179±9
Diabetic+HC	8.39±0.72	6.90±0.72	6.78±0.72*	16.6±1.08	14.4±0.73	14.9±0.85*	13.1±0.37*	26.5±0.37*	4.9±0.37*	298±14*	226±14*	266±14*

Values are expressed±SD. Group n = 6, #Indicates statistical significance against normal control ($p<0.05$), *Indicates statistical significance against diabetic control ($p<0.05$).

Table 3: Effect of homocastasterone on lymphocyte, monocyte and granulocyte count

Group	Lymphocytes 10×3/µl			Monocytes 10×3/µl			Granulocytes 10×3/µl		
	5 Day	10 Day	15 Day	5 Day	10 Day	15Day	5 Day	10 Day	15 Day
Control	20.1±0.5	30.1±0.5	10.6±0.5	2.3±0.07	2.5±0.07	0.4±0.07	3.0±0.03	2.7±0.03	0.6±0.03
Control+HC	14.8±0.52	19.6±0.52	8.6±0.52	0.4±0.02	1.1±0.02	0.2±0.02	0.4±0.04	1.7±0.04	0.6±0.04
Diabetic control	16.8±0.23	18.7±0.23	3.2±0.23	1.3±0.02	0.9±0.02	0.1±0.02	0.4±0.03	1.2±0.03	0.2±0.03
Diabetic+HC	12.3±0.28	23.0±0.28*	4.1±0.28*	0.4±0.02	1.3±0.02	0.1±0.02	0.7±0.04*	2.2±0.04*	0.7±0.04*

Values are expressed±SD. Group n = 6, #Indicates statistical significance against normal control ($p<0.05$), *Indicates statistical significance against diabetic control ($p<0.05$).

Effect on WBCs count

A 45 % reduction of total WBC count was noted for normal HC treated group compared to control. Diabetic the WBC count was 27.4% on day 10 and 33.33% on day 15 in HC treated rat showing a significant increase compared to the diabetic control.

Effect on WBC differential count

A significant reduction in lymphocyte, monocyte, and granulocyte counts was noted in normal HC treated rat. In the diabetic treated group, the differential count showed significant elevation compared to diabetic control ($p<0.05$). Lymphocytes increased 22.99% and 28.12% on only 10th and on 15th day. Monocyte count elevated by 44.44% at day 10. No significant change in this cell count was observed on 5th and 15th day of treatment. Granulocyte count reduced 75 to 83.3%, through 5th to 15th day of treatment.

Effect on platelets

In HC treated normal rat, platelet count reduced 13.67% on day 5th (table 2) when compared to normal, 32.93% on day 10, and 51.19%by day 15. In HC treated diabetic group, the platelet count increased 18.29% on day 5th, 37.80% on day 10th, and 48.60% on day 15th ($p<0.05$) compared to diabetic control.

Effect on platelet indices

MPV, PDW and PCT values decreased in HC treated groups compared to control ($p<0.05$). Diabetic control group showed abnormal platelet indices compared to normal control.

Effect on the lipid peroxidation

Lipid peroxidation (LPO) status in RBC is indicated in table 6 ($p<0.001$). In diabetic control rat, in packed RBC was noted as significantly elevated compared to the normal controls. Following HC treatment LPO status in rat blood RBCs indicated significant reduction compared to respective control groups.

The control blood glucose estimate in the male wistar rats employed for the study was 5.11-6.11 mmol/dl, and falls within the range of 4-10 mmol/dl reported in literature[11]. Streptozotocin induced diabetes elevated the rat blood glucose level to greater than 16.0 mmol/dl, whereas oral administration of homocastasterone through 15 day regimen to diabetic rats diminished the blood glucose level to 9.55 m

mol/dl, returning the circulating glucose level to within the reported range. Use of ethanol in rat is known to depress hematopoiesis and contribute significantly to changes in RBC, Platelet and Hb count, PCV, MCV, and MCH. Ethanol was however used in this study as a vehicle for oral administration of HC to the experimental rat. Diabetes is known to cause anemia in humans and reduced RBC count in diabetic rat has been reported [12].

In our study, elevation in RBC count in the normal rat blood post HC administration was only 2.5% whereas 20% decrease in RBC count was noted in HC administered diabetic rat blood. The observed increase in RBC may be due to: (1) RBC generation to compensate low tissue oxygen level caused by deficient heart or lung function, (2) erythropoietin synthesis and release causing marrow induction of red blood cell production, and (3) change in blood plasma volume through sodium and water depletion resulting in higher RBC count. Increase in red blood cell count can also be due to the anabolic steroid potency associated with this ketosteroid similar to that noted for 28-homobrassinolide reported [3, 4] earlier from this laboratory. Whether the observed decrease in RBC number is due to increase *in vivo* hemolysis or greater spleenic clearance of this cell type is not clear however.

Table 4: Effect of homocastasterone on RBC indices

Group	HCT %			MCV fL			MCH%			MCHC%		
	5 Day	10Day	15 Day	5 Day	10Day	15 Day	5 Day	10 Day	15 Day	5 Day	10 Day	15 Day
Control	44.8±3.04	46.0±3.04	47±3.04	61±7.53	60.2±7.53	60.1±7.53	20.9±2.86	21.3±2.86	21.4±2.86	34.3±2.35	35.4±2.35	35.7±2.35
Control+HC	43.7±3.09	47.7±3.09	46.7±3.09	61±6.38	59.8±6.38	59.6±6.38	20.5±4.35	20.5±4.35	20.2±4.35	33.6±2.48	34.3±2.48	34.0±2.48
Diabetic control	50.6±2.38	37.7±2.38	37.4±2.38	63.5±4.84	66.3±4.84	58.1±4.84	21.5±3.15	24±3.15	21.2±3.15	33.9±1.38	36.3±1.38	36.6±1.38
Diabetic+HC	47.7±2.47*	24.4±2.47*	38.9±2.47	56.9±5.92*	54.8±5.92*	62.1±5.92	19.7±3.45	18.6±3.45	20.0±3.45	34.3±1.94	34.0±1.94	32.3±1.94

Values are expressed±SD. Group n = 6, #Indicates statistical significance against normal control (p<0.05), *Indicates statistical significance against diabetic control (p<0.05).

Table 5: Effect of homocastasterone on Platelet indices

Group	MPV %			PDW %			PCT fL		
	5 Day	10 Day	15 Day	5 Day	10 Day	15 Day	5 Day	10 Day	15 Day
Control	7.5± 1.20	7.4± 1.20	7.1± 1.20	9.7± 1.21	9.2± 1.21	8.4± 1.21	0.31± 0.05	0.32± 0.05	0.23± 0.05
Control+HC	7.2± 1.58#	6.7± 1.58#	6.9± 1.58#	9.5± 1.20#	7.9± 1.20#	7.9± 1.20#	0.26± 0.08#	0.22± 0.08#	0.15± 0.08#
Diabetic control	7.4± 1.25	8.6± 1.25	9.7± 1.25	8.4± 1.39	10.5± 1.39	10.0± 1.39	0.19± 0.06	0.14± 0.06	0.12± 0.06
Diabetic+HC	7.3± 0.93*	7.2± 0.93*	7.0± 0.93*	7.9± 1.24*	9.5± 1.24*	8.7± 1.24*	0.21± 0.08	0.16± 0.08	0.12± 0.08

Values are expressed±SD. Group n = 6, #Indicates statistical significance against normal control (p<0.05), *Indicates statistical significance against diabetic control (p<0.05).

Table 6: Effect of homocastasterone on RBC peroxidation status

Group	RBC MDA nmmol/mg protein/min
Control	0.166±0.03
Control+HC	0.170±0.05
Diabetic control	1.024±0.07
Diabetic+HC	0.820±0.04*

Values are expressed±SD. Group n = 6, #Indicates statistical significance against normal control (p<0.05), *Indicates statistical significance against diabetic control (p<0.001).

The raise in hemoglobin level in the range 0.16–5.66% noted for the diabetic rat through the 15 day period of HC administration correlates with reported values in humans and could be used as a predictive biomarker for reduced mortality of these animals. It can be a Prognostic [13] for effective decongestion and possibly weight loss in these species.

In case of normal rats, the increase in Hb content was not in excess of 2%, although RBC count reduced by 20%, suggestive of the insignificant effect of homocastasterone on rat blood Hb level. The hematocrit value of 44-47% noted for control rat through the 15 day study did not change effectively following HC administration. For the diabetic control however, nearly 26% decline in the hematocrit value was noted by day 15, while 18.4% decline only was observed following HC fed through the same period. The diabetic control hematocrit was nearly 13% higher than normal rat hematocrit at day 5. MCV is a measure of the morphological status of RBC and is predictive of microcytic, normocytic and macrocytic RB cell population in circulating blood. Control rat MCV was without significant change through the experimental period. Diabetic control MCV decreased 8.5% during the 15 day test period. HC fed diabetic rat registered MCV value 10% below diabetic control at day 5 and 7% above diabetic control at day 15. Where MCV value was high, pernicious anemia, alcoholism, vitamin B_{12}/Folic acid deficiency and macrocytic anemia is indicated for man. With low MCV, microcytic, thalassemia sidero blastic, chronic and iron deficiency anemia are suggested. Whether the indicated changes in rat blood MCV due to HC is indicative of anemia in the experimental rat is to be established. Estimate of Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) did not yield meaningful differences between normal and diabetic, control and treated rats. In rat and mice, the normal WBC counts are reported to be in the range 2000–10,000/µl. Changes in white blood cell count is known to facilitate diagnosis of bacterial/viral or fungal infections, inflammatory disorders such as rheumatoid arthritis, vasculitis, inflammatory bowel disease, allergic reactions/asthma and leukemia or myeloproliferative neoplasms in humans. In the present study, WBC count varied between 25400–26,300/µl for the normal rat through 5-15 days. In the diabetic rat, the WBC count was in the range 16,400-20,800/µl for the same duration. Oral administration of HC diminished the WBC counts 15-39% in the normal rat, whereas in diabetic rat an initial reduction in WBC count by 20% at day 5 was followed with an increase of 27% in these cell count by day 10. However, by day 15, reduction in WBC cell count was noted to be 64% in normal and 76% in diabetic rat following HC consumption [13].

Physiological responses as a consequence to WBC count variation in the murine species remain to be clearly understood. It is reported that sampling site and intrinsic variability due to factors affecting

the rodent can cause wide variations in WBC count in rat. Collection of blood from the rat tail vein initially and from the heart later (day15) during this study, can be considered a possible reason for the observed differences noted. The observed increase in rat WBC count as opposed to the normal reported range can be a result of stress influences experienced by the caged animal. Differential counts of lymphocytes, monocytes and granulocytes yielded reduced cell count for each of these types following HC administration. Lymphocyte count diminished up to 35 % in normal rat whereas this cell type reduced only by 25% in diabetic rat following HC intake through the 15 day duration. Monocytes reduced greater than 50 % during the 15 day study while granulocytes reduced > 80 % in normal and diabetic rat [14].

Blood platelet count registered values of 424-334 x 10^{-3} per µl for the normal rat and 268-179 x 10^{-3} per µl for the diabetic control. Following HC administration, platelet count reduced in the normal rat blood by 14-51% whereas in diabetic rat blood, the platelet count increased by 12-38 %. Similar to WBCs, variations in platelet count are due to many factors affecting the animal. Specific reasons cannot therefore be assigned for the observed change in rat platelet count. Since platelets affected the hemodynamic status in an animal, it may be construed that administration

of HC to diabetic rats possibly enhanced their blood platelet profile and improved the hemodynamic status in the animal. Reduction in platelet count due to HC in the normal rat probably affected the hemostasis potential of the animal contributing possibly to the acquired clotting disease in the species [15].

Platelet indices such as MPV, PDW and PCT measured in normal, diabetic control and HC administered rat blood yielded varying results. In humans, an inverse relationship between platelet count and MPV values had been suggested[16]. In wistar rat, changes in MPV, PDW and P-LCR (platelet-larger cell ratio) had been studied earlier as a follow-up of di chlorovinyl dimethyl phosphate pesticide administration [17]. Although platelet indices had been studied in several investigations, clinical correlations supporting their use have not found significant approval. In this study, 15 day oral administration of HC resulted in significantly reduced MPV, PDW and PCT values in the normal control rat. MPV values reduced 3-9%, PDW decreased 6-16% and PCT decreased 16-35% post HC intake during the study period. In the diabetic rat, MPV value decreased 16-28%, PDW reduced 6-13% whereas PCT increased 10-14% during the same period. Decrease in PDW and MPV in rats suggest thrombocytopenia due to homocastasterone consumption [18-20].

Peroxidative damage in the RBC population evaluated indicated 84% greater damage in diabetic control rat cells. Even though administered level of homocastasterone reduced peroxidative damage in the diabetic rat RBCs by 20%, a 2.5% elevation in peroxidation was noted in normal rat RBC following administration of an identical level of HC. While peroxidative damage remains a focal theme when considering factors causing cell dysfunction, homocastasterone's potency to reduce peroxidative damage in diabetic rat RBC by 20% should outweigh the observed increase of 2.5% in the normal rat RBC. The specific causes contributing to the contrasting observations shall need further investigation [4, 5].

CONCLUSION

The present study confirms that the plant ketosteroid homocastasterone, exhibits anti-hyperglycemic effect. When administered, this compound improves platelet indices in diabetic rat blood and increased RBC, Hb and WBC levels significantly. Peroxidative damage to lipids studied using packed red blood cells showed that HC prevented lipid peroxidation in treated rat. The molecular mechanism underlying alteration in blood cells and its indices requires further study.

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CONFLICT OF INTERESTS

Declared None

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