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Original Article

EFFICACY OF AN ACTIVE COMPOUND OF THE HERB, ASHWAGANDHA IN PREVENTION OF STRESS INDUCED HYPERGLYCEMIA

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ABSTRACT

Objective: To find out whether an isolated compound (IC) from the ethanolic extract of roots of ashwagandha prevents stress-induced hyperglycemia by direct interference with the action of increased concentration of corticosterone on hepatocytes or by preventing hyper-secretion of corticosterone or both.

Methods: A group of rats served as controls, and those in another group were subjected to restraint (1 h) and forced swimming exercise (15 min), after a gap of 4 h daily for 4 w. The third group of rats received orally IC (5 mg/kg bw/rat) 1 h prior to exposure to stressors. After the last treatment period, a blood sample was collected and serum was separated for the estimation of corticosterone and glucose. In *in vitro* experiment, hepatocytes were treated with different concentrations of corticosterone (100, 200, 300, 400 and 500 ng/ml). In another set of experiment, hepatocytes were treated with different doses of IC (1, 10, 100, 1000 and 10 000 µg/ml of medium) along with corticosterone (400ng/ml). The concentration of glucose and activities of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were determined after the treatment.

Results: Stress exposure caused a significant increase in serum concentration of corticosterone and glucose whereas, administration of IC did not result in similar changes. Further, treatment of corticosterone in *in vitro* significantly increased the activities of PEPCK and G6Pase and concentration of glucose in a dose-dependent manner in hepatocytes. However, treatment with IC did not interfere with the corticosterone-induced an increase in the activities of PEPCK and G6Pase as well as the concentration of glucose in hepatocytes.

Conclusion: The *in vivo* and *in vitro* results put together reveal that IC does not directly interfere with the action of corticosterone on hepatocytes. However, it prevents stress-induced hyperglycemia by suppressing hyper-secretion of corticosterone.

Keywords: Ashwagandha, Corticosterone, G6Pase, Gluconeogenesis, PEPCK

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INTRODUCTION

Stress disturbs the homeostatic equilibrium of the body and makes individuals susceptible to diseases. Stress affects physiological energy balance via the activation of hypothalamo-pituitary-adrenocortical (HPA) and sympathetic-adreno-medullary (SAM) axes by producing an excess amount of glucocorticoids (GC) and catecholamines respectively [1]. The Liver is a central organ of energy metabolism and regulates carbohydrate metabolism in all vertebrates. A variety of environmental factors viz., fasting, hypoxia, temperature, stress and seasonality alter the liver metabolism [2]. The hepatocytes are the sites of endogenous glucose synthesis (gluconeogenesis) from non-carbohydrate sources. The neuroendocrine response to stress is characterized by excessive gluconeogenesis, glycogenolysis and insulin resistance. Chronic stress results in hyperglycemia affecting all the pathways of carbohydrate metabolism viz. glycolysis, tricaboxylic acid cycle, gluconeogenesis, hexose monophosphate shunt, glycogenolysis and glycogenesis [3-5]. Numerous studies reveal stress-induced alterations in the enzyme activities and concentrations of substrates of different pathways of carbohydrate metabolism [4, 6-8]. Stress increases gluconeogenesis by increasing the activities of key gluconeogenic enzymes viz. phosphoenolpyruvate carboxykinase (PEPCK), fructose-1, 6-bisphosphatase and glucose-6-phosphatase (G6Pase) and serves as a mechanism by which the availability of glucose is maintained. An increase in activities of hepatic G6Pase and PEPCK under different stressful conditions has been reported [9-12]. Hyperglycemia due to increased gluconeogenesis has a negative impact on the body as it results in insulin resistance [13].

Glucocorticoids have long been known to regulate glucose homeostasis and have a vital role in gluconeogenesis. Metabolic effects of GC include an increase in blood glucose concentration through the activation of key enzymes involved in hepatic gluconeogenesis and inhibition of glucose uptake in peripheral tissues such as skeletal muscles [14]. Studies have shown that the primary effect of GC is exerted on existing enzymes to increase their activities. Numerous *in vivo* studies have shown the effects of a higher level of corticosterone on the activities of hepatic gluconeogenic enzymes in animal models under stressful condition [4, 10, 12, 15]. However, there are no reports on the effect of physiological concentration as well as stress level concentration of corticosterone on the activities of gluconeogenic enzymes of hepatocytes in *in vitro.*

It is reported that stress-induced higher levels of GC affect different pathways of carbohydrate metabolism which might lead to metabolic syndrome like diabetes [16]. Since stress cannot be avoidable in the modern day society, the effects of stress can be controlled or prevented with suitable remedies. Though, numerous anti-stress synthetic compounds are available to prevent stress effects, because of their undesirable side effects there is a need for better compounds that can prevent stress effects and maintain normoglycemia despite experiencing stressful conditions. The herb ashwagandha (*Withania somnifera*), also knew as Indian Ginseng or Winter cherry has been well documented in the Ayruveda the traditional Indian medicine system and has multiple biological properties viz. antioxidant, adaptogenic, aphrodisiac, astringent and antiulcer [17-20]. Root extracts of ashwagandha are known to prevent stress-induced hyperglycemia, alterations in hepatic enzyme activities and glucose intolerance in *in vivo* [21-23]. It is also known that a root extract of ashwagandha prevents a rise in stress-induced glucocorticoid levels [22, 23] in *in vivo.* However, whether the normoglycemia maintained by ashwagandha under stress

exposure is due to the prevention of excess secretion of GC alone or due to direct interference with gluconeogenic enzymes in hepatocytes or both is not known. Hence, in the present study effects of an isolated compound (IC) from root extract of ashwagandha on corticosterone-induced alterations in activities of gluconeogenic enzymes in hepatocytes *in vitro* were studied to understand whether or not IC acts directly on hepatocytes.

MATERIALS AND METHODS

Chemicals

Corticosterone, Krebs-Ringer-Hepes (KRH), glucose 6 phosphate, dithiothreitol, adenosine diphosphate (ADP), 3-phosphoglycerate, phosphoenolpyruvate, 3-phosphoglycerate phosphokinase, and glyceraldehyde 3-phosphate dehydrogenase were purchased from Sigma Aldrich (United Kingdom). Tris-hydrochloric acid (HCL), trichloroacetic acid, sodium bicarbonate (NaHCO3), magnesium chloride (MgCl2) and BIS-TRIS buffer were obtained from Merks specialties, Pvt. Ltd (Mumbai, India). Serum glucose was measured by using the kit supplied by Span Diagnostics Ltd. (Gujarat, India). The ELISA kit for the estimation of serum concentration of corticosterone was supplied by Demeditec Diagnostics GmbH, Germany). All the other reagents and chemicals used were of analytical grade.

Animals

Adult male albino Wistar rats weighing 200-220 g were obtained from the inbred colony of the central animal facility of University of Mysore. The rats were provided a standard rat chow and water *ad libitum* and were kept in temperature 27±2˚C, under 12 h: 12 h light: dark cycle (light on 07:00-19:00 h). The experimental design was approved by Institutional Animal Ethics Committee of University of Mysore, India (Reference number UOM/IAEC/ 07/2016) and guidelines of the committee was followed for care and treatment of the rats.

Isolation of active compound from ethanolic extract (EE) of ashwagandha root

Earlier *in vivo* and *in vitro* studies showed the potential anti-stress activity of EE in roots of ashwagandha [22]. Hence, active compound was isolated from the EE as per the procedure of Nirupama *et al.* [23]. The EE was subjected to thin layer chromatography and was further fractionated in column chromatography. The extract was first run on thin layer chromatography plate to detect the number of compounds. The solvent system used contained chloroform and methanol. The same solvent was used for column chromatography to isolate a single compound. The compound isolated was subjected to nuclear magnetic resonance, infrared spectroscopy and liquid chromatography-mass spectrometry studies for the characterization of its chemical nature.

In vivo experiment

Adult male rats were divided into three groups of 5 animals each (n=5). First group rats were treated as controls and were maintained in a normal condition without any disturbance. Animals in the second group were exposed to two stressors viz., restraint (1 h) followed by a gap of 4 h to force swimming exercise (15 min) for 4 w. Each rat in the third group was orally treated with IC (5 mg/kg bw/rat) 1 h prior to exposure to stressors similar to rats in the second group. The fasting blood glucose concentration was measured in animals of all groups at weekly intervals. After the treatment period, the serum concentration of corticosterone was estimated by ELISA using kit and methods of the manufacturer (Demeditec Diagnostics GmbH, Germany).

In vitro experiment

Two sets of *in vitro* experiments were conducted using the hepatocytes. In the first experiment dose-dependent effect of corticosterone, if any on the activity of hepatic G6Pase and PEPCK were studied. The effect of IC on the action of corticosterone on the activities of gluconeogenic enzymes in hepatocytes was studied in the second experiment. Three replicates were used for each dose.

Preparation of the liver tissue slice system

The liver for these studies was collected from control rats used for *in vivo* experiment. The liver lobes were processed following the procedure of Wormser *et al.* [24]. Briefly, the liver lobes were placed on a glass surface and sliced into small pieces of about 0.5 x 0.5 x 0.5 mm. Slices were incubated with KRH medium for 1 h and washed every 10 min with this medium. The slices were then divided into small portions (100-120 mg wet weight each) and were incubated in glass tubes containing 2 ml KRH medium at 37 ˚C for l h before experimentation.

Effect of corticosterone on the activities of key enzymes of gluconeogenesis

The liver slices were incubated with different concentrations of corticosterone (100, 200, 300, 400 and 500 ng/ml of medium) at 37 ˚C for 2.5 h. At the end of incubation, the tissue homogenate was used for the estimation of the activities of key gluconeogenic enzymes, G6Pase and PEPCK and concentration of glucose.

Effect of IC on corticosterone-induced alterations in the activities of key enzymes of gluconeogenesis

The liver slices were incubated with the different concentrations of IC (1, 10, 100, 1000 and 10 000 µg/ml of medium) and 400 ng corticosterone at 37 ˚C for 2.5 h. At the end of incubation, the tissue homogenate was used for the estimation of activities of G6Pase and PEPCK and concentration of glucose.

Activity of G6Pase

The activity of G6Pase was estimated following the method of Zhu *et al.* [25]. Liver slices were homogenized in 0.25 M sucrose solution and centrifuged at 3000 rpm for 15 min. The supernatant containing enzyme source (1 µl) was mixed with 100 mmol BIS-TRIS buffer and 200 mmol of glucose 6 phosphate (substrate). The reaction mixture was incubated at 37 ˚C for 5 min. The reaction was stopped by adding 20 % trichloroacetic acid, incubated for 5 min at 25 ˚C and centrifuged at 4000 rpm for 10 min to remove the precipitate. The supernatant (1 µl) was mixed with Taussky shorr color reagent for the development of color. The mixture was incubated at 25 ˚C for 5 min and the optical density was measured at 660 nm. The specific activity was expressed as µmol/mg/min.

Activity of PEPCK

The activity of PEPCK was estimated following the method of Kin *et al.* [26]. The enzyme sample was prepared in 5 ml of 100 mmol tris-HCL at 4 ˚C. The sample (200 µl) was added to the reaction medium containing 500 mmol tris-HCl (pH 6.6), 350 mmol NaHCO3, 160 mmol MgCl2, 6 mmol NADH, 20 mmol dithiothreitols, 0.2 M ADP, 36 mmol 3-phosphoglycerate, 50 mmol phosphoenolpyruvate, 3-phosphoglycerate phosphokinase and glyceraldehyde 3-phosphate dehydrogenase. The absorbance was read at 340 nm for 60 seconds at room temperature using a UV-Visible spectrophotometer.

Concentration of glucose

The concentration of glucose was estimated by glucose oxidase and peroxidase method using a kit manufactured by Arkray Healthcare, India and the procedure of the manufacturer was adopted. The sample (10 µl) was added to 1 ml reagent and incubated at 37 ˚C for 10 min. Optical density was read at 505 nm and the concentration of glucose was expressed as mg/dl.

Statistical analysis

Mean±SE of each parameter was computed considering the data on at least 5 rats per group (N=5) and 3 replicates for each dose of *in vivo* and *in vitro* experiments respectively. The mean values of each parameter of different groups were compared using one way ANOVA followed by Duncan's multiple range test and judged significant if $P<0.05$.

RESULTS

In vivo experiment

A significant increase in the serum concentration of corticosterone was observed in stressed rats compared to controls, whereas that of IC pretreated rats exposed to stressors was similar to controls (fig. 1).

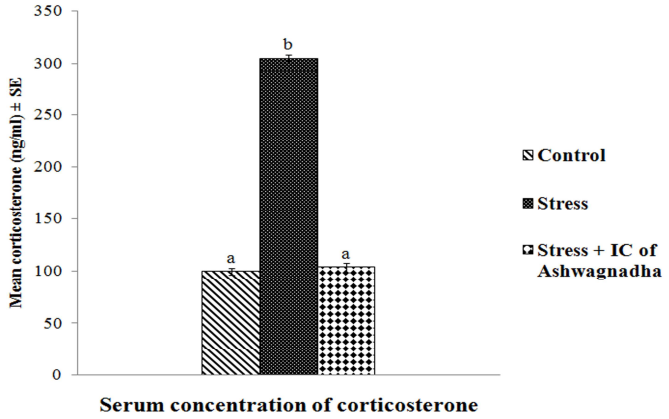

Fig. 1: All values are mean±SE. Vertical bars to show serum concentration of corticosterone in control, stress and IC pretreated rats exposed to stress. Note the higher level of corticosterone in stressed rats compared with controls and IC pretreated rats exposed to stress. Bars with the same superscript letters do not significantly differ among themselves whereas those with different superscript letters significantly differ. IC-isolated compound from ashwagandha (n=5)

There was a significant increase in the fasting blood glucose concentration in stress group rats from 1st w through 4th w, compared to controls. The IC pretreated rats exposed to stress did not differ from controls (fig. 2).

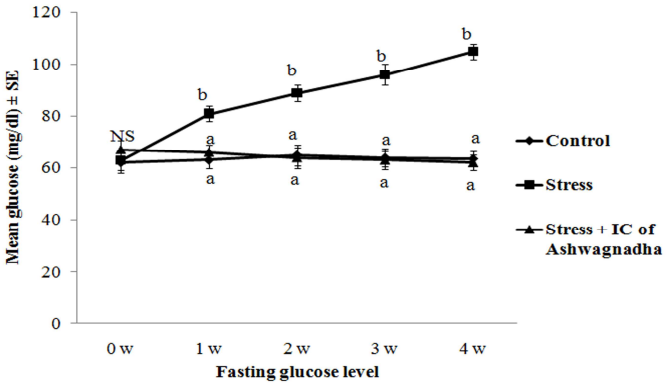

Fig. 2: All values are mean±SE. Fasting blood glucose concentrations in control, stress and IC pretreated rats exposed to stress. Note the higher level of glucose in stressed rats compared with controls and IC pretreated rats exposed to stress. Groups with the same superscript letters at each weekly interval do not significantly differ among themselves whereas those with different superscript letters significantly differ. IC-isolated compound from ashwagandha (n=5)

***In vitro* experiment**

Activities of G6Pase and PEPCK and concentration of glucose in hepatocytes

There was a significant dose-dependent increase in the activities of G6Pase and PEPCK following treatment with increasing doses of corticosterone (100, 200, 300, 400 and 500 ng/ml) compared to control and vehicle control. However, G6Pase activity after the treatment of 300 ng did not significantly differ either from that of 200 ng or 400 ng and that of PEPCK after the treatment of 400 ng did not significantly differ either from that of 300 ng or 500 ng (table 1).

The concentration of glucose in the hepatocytes showed a dose-dependent significant increase following treatment with increasing doses of corticosterone (100, 200, 400 and 500 ng/ml) compared to control and vehicle controls.

However, glucose concentration after treatment of 300 ng did not significantly differ either from that of 200 ng or 400 ng (table 1).

Table 1: Effect of corticosterone on the activities of key enzymes of gluconeogenesis and concentration of glucose in hepatocytes *in vitro*

Groups	Glucose 6 phosphatase (µmol/mg/min)	Phosphoenolpyruvate carboxykinase (U/mg protein)	Glucose mg/dl
Control	2.74±0.04^{a}	2.29±0.08^{a}	29.35±0.87^{a}
Control+0.1 % DMSO	2.72±0.08^{a}	2.25±0.05^{a}	29.43±0.62^{a}
100 ng corticosterone	3.30±0.09^{b}	4.40±0.10^{b}	33.32±0.73^{b}
200 ng corticosterone	3.76±0.21^{c}	4.86±0.04^{c}	38.17±0.76^{c}
300 ng corticosterone	4.07±0.15^{cd}	5.50±0.06^{d}	40.32±1.69^{cd}
400 ng corticosterone	4.43±0.12^{d}	5.88±0.03^{de}	42.13±1.47^{d}
500 ng corticosterone	5.47±0.20^{e}	6.06±0.14^{e}	46.16±1.74^{e}
ANOVA F Value	25.56	312.52	21.77
(df = 6, 28)	P<0.001	P<0.001	P<0.001

Note: All values are mean±SE, Groups with the same superscript letters do not significantly differ among themselves, whereas groups with different superscript letters significantly (P<0.05) differ as judged by ANOVA followed by Duncan's test. df: degree of freedom. DMSO-dimethyl sulfoxide (n=3).

The activities of hepatic G6Pase and PEPCK and concentration of glucose were significantly increased in hepatocytes following treatment with corticosterone alone as well as corticosterone+different concentrations of IC (1, 10, 100, 1000 and 10 000 µg/ml) compared to controls and vehicle controls (1 % carboxy methyl cellulose) (table 2).

Table 2: Effect of a compound isolated from ashwagandha root on corticosterone-induced alterations in the activities of key enzymes of gluconeogenesis and concentration of glucose in hepatocytes *in vitro*

Groups	Glucose 6 phosphatase (µmol/mg/min)	Phosphoenol pyruvate carboxy kinase (U/mg protein)	Glucose mg/dl
Control	2.80±0.08[a]	2.44±0.05[a]	29.83±0.71[a]
Control+1 % CMC	2.73±0.08[a]	2.41±0.07[a]	29.91±0.52[a]
400 ng corticosterone	4.12±0.09[b]	5.61±0.06[b]	41.66±0.65[b]
400 ng corticosterone+1 µg IC	4.01±0.11[b]	5.53±0.09[b]	40.14±0.42[b]
400 ng corticosterone+10 µg IC	4.09±0.23[b]	5.60±0.10[b]	39.49±0.90[b]
400 ng corticosterone+100 µg IC	4.07±0.08[b]	5.47±0.05[b]	40.69±1.25[b]
400 ng corticosterone+1000 µg IC	4.41±0.09[b]	5.46±0.06[b]	40.65±1.26[b]
400 ng corticosterone+10 000 µg IC	4.20±0.13[b]	5.51±0.05 [b]	40.77±0.48[b]
ANOVA F Value	26.65	406.92	34.51
(df=7, 32)	P<0.001	P<0.001	P<0.001

Note: All values are mean±SE, Groups with the same superscript letters do not significantly differ among themselves, whereas groups with different superscript letters significantly (P<0.05) differ as judged by ANOVA followed by Duncan's test. df: degree of freedom. CMC-carboxy methylcellulose, IC-isolated compound from ashwagandha (n=3).

DISCUSSION

Metabolic stress responses are mediated by hormones of the adrenal gland [27] and GC have long been known to regulate glucose homeostasis [28]. In the present study, increased concentration of corticosterone in stressed rats accompanied with higher blood glucose level indicates that stress-induced excess production of GC affected glucose metabolism. Blood glucose level is an important indicator of stress responses in animal [29] and a higher level of glucose in stressed rats is due to enhanced hepatic gluconeogenesis [30]. This was further supported by the present *in vitro* study wherein treatment of different doses of corticosterone significantly increased the activities of gluconeogenic enzymes, G6Pase and PEPCK of hepatocytes in a dose-dependent manner. Although most steps in gluconeogenesis are the reverse of those found in glycolysis, PEPCK and G6Pase regulate the rate process of gluconeogenesis by converting oxaloacetate to phosphoenolpyruvate and glucose-6-phosphate to glucose respectively [31]. The over-expression of these enzymes in hepatocytes results in a reduction of glycogen synthesis and an increase in the production of glucose [32]. Hence, studies related to carbohydrate metabolism mainly focus on changes in the activities of these hepatic enzymes.

An increase in the activities of gluconeogenic enzymes due to higher level of endogenous GC under stressful condition has been observed in *in vivo* system [10, 12, 15]. GC increase the synthesis of PEPCK and G6Pase by increasing the expression of their transcription factors [33, 34]. In addition to transcriptional regulation, studies have shown that GC directly enhance the activities of gluconeogenic enzymes under stressful condition [35-37]. Stress-induced increased gluconeogenesis results in hyperglycemia. Present *in vivo* and *in vitro* studies support this view as there was a significant increase in serum glucose concentration in *in vivo* as well as in hepatocytes in *in vitro* system accompanied with increased activities of PEPCK and G6Pase of hepatocytes *in vitro* following corticosterone treatment.

The physiological concentration of corticosterone varies from 50 to100 ng/ml and during stressful condition it rises from 120 to 425 ng/ml. In the present study, corticosterone at the dose in the range of stress-induced alterations (100-500 ng/ml) significantly increased the activities of key enzymes of gluconeogenesis and glucose concentration in a dose-dependent manner. Thus action resembled the action of endogenous GC on glucose metabolism during stress. The severe implication of GC induced chronic hyperglycemia is insulin resistance and glucose intolerance [38] which may lead to type 2 diabetes mellitus [29]. Since stress induced higher level of GC is the main cause of hyperglycemia, it is logical that, either suppression of hypersecretion of GC or interference with action of corticosterone on hepatocytes under stressful conditions could prevent hyperglycemia and insulin resistance and consequent pathological implications.

Interestingly, pretreatment of IC in rats exposed to stressors maintained normoglycemia and normal blood corticosterone levels as blood glucose level and corticosterone level of IC pretreated rats exposed to stressors were similar to controls in contrast to a hyperglycemic condition in rats exposed to stressors alone. In contrast, in *in vitro* system, treatment of IC did not prevent corticosterone induced enhanced activity of G6Pase and PEPCK as well as the concentration of glucose in hepatocytes as there was no difference in the activities of these enzymes and glucose concentration in corticosterone alone and corticosterone+IC treated hepatocytes. The fact that IC maintained normal blood concentration of glucose and corticosterone in stressed rats in *in vivo* and it did not prevent corticosterone induced increase in activities of gluconeogenic enzymes and glucose concentration in hepatocytes in *in vitro,* indicates that normoglycemic effect of IC during stressful condition *in vivo* is by suppressing the HPA activation and subsequent excess production of GC, whereas it does not directly interfere with the action of corticosterone on hepatocytes. This view is further supported by a report of Nirupama *et al.* [23] wherein treatment of IC from EE of ashwagandha root normalized the adrenal activity as well as the activities of gluconeogenic enzymes under stressful condition. Ashwagandha is known to stimulate the central nervous system (CNS) [17, 39] by modulating different neurotransmitter receptors particularly gamma-aminobutyric acid (GABA) [40]. The GABAergic neurons inhibit HPA activity by reducing the secretion of adrenocorticotropic hormone [41, 42]. However, under stressful condition there is depletion of GABA receptor binding in the CNS [43, 44] which leads to the activation of the HPA axis. Studies have shown that extracts and active compounds of ashwagandha have GABA-like activity and also increase the production of GABA in brain [45-47]. Therefore, it is suggested that IC might induce higher levels of GABA or exert GABA like action in brain under the stressful condition and thereby suppress the activation of the HPA axis and the subsequent increase in serum level of corticosterone which is to be substantiated by future studies.

CONCLUSION

The *in vivo* and *in vitro* studies put together reveal that IC does not interfere with the action of corticosterone on hepatocytes, whereas, it prevents stress-induced hyperglycemia by preventing hyper-secretion of corticosterone.

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AUTHORS CONTRIBUTIONS

H N Sarjan performed the experiments and Dr. H N Yajurvedi designed the study and wrote the paper.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest to disclose.

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