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Insulinotropic Effect of *S*-Allyl Cysteine in Rat Pups

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ABSTRACT: *S*-Allyl cysteine (SAC) is found in garlic and has been reported to exert antidiabetic and antiobesity properties in drug-induced adult experimental models of metabolic dysfunction, but its potential beneficial effects in high-fructose diet neonatal rat models have not been determined. This study investigated the potential prophylactic effects of SAC in high-fructose diet fed suckling rat pups modelling human neonates fed a high-fructose diet. Four-day-old male (n=32) and female (n=32) Wistar rat pups, were randomly assigned to and administered the following treatment regimens daily for 15 days: group I, distilled water; group II, 20% fructose solution (FS); group III, SAC; group IV, SAC+FS. The pups' blood glucose, triglyceride, cholesterol, plasma leptin and insulin concentration, liver lipid content, and liver histology were determined at termination. In female rat pups, orally administered SAC prevented FS-induced hypoinsulinemia but significantly increased ($P \leq 0.05$) liver lipid content. Oral administration of SAC significantly increased ($P \leq 0.05$) plasma insulin concentration and homeostasis model assessment for insulin resistance in the male pups. The potential sexually dimorphic effects of SAC (insulinotropic effects in male pups and protection of female pups against fructose-induced hypoinsulinemia) suggest that SAC could be potentially exploited as an antidiabetic and insulinotropic agent. Caution should, however, be exercised in the use of SAC during suckling as it could result in excessive liver lipid accumulation and insulin resistance.

Keywords: *S*-allyl cysteine, neonatal, fructose, insulinotropic

INTRODUCTION

Globally, approximately 42 million children under the age of 5 are classified as being overweight or obese (1). Childhood obesity is associated with the development of metabolic derangements such as type II diabetes mellitus (DM II), metabolic syndrome (MetS), and non-alcoholic fatty liver disease (NAFLD) in childhood or later in adulthood (2,3). Sedentary lifestyles and poor dietary habits have been identified as the main contributors to the global childhood obesity epidemic. The 'developmental origins of health and disease' theory states that environmental and dietary events in perinatal life can temporarily or permanently alter an organism's physiology (4). The physiological alterations can program resistance or susceptibility to disease, and thus dietary events during perinatal life may also contribute to the increase in obesity and MetS in childhood and adulthood (5).

Rats are altricial rodents who depend exclusively on the dam's milk during early postnatal (neonatal) development, which is equivalent to the last trimester of pregnancy in humans (6). The neonatal rat model is thus well suited for investigating the effects of early life dietary manipulations on the programming of adverse metabolic outcomes in early life and later life. Furthermore, during suckling (early postnatal), the majority of the physiological systems including the endocrine system are immature (7). Studies using neonatal rat models have shown that dietary manipulations during suckling can cause temporary or permanent alterations to physiological functions and increased susceptibility to disease (4,8,9). Importantly the rat's early postnatal (neonatal) period is a feasible target period for investigating prophylactic intervention (pharmacological and ethnomedicinal substances) against the diet-induced programming of adverse metabolic outcomes in perinatal life (4,9). Although there are numerous conventional antidiabetic and antiobesity pharmacological agents that could be used to prevent diet-induced (i.e. high-fructose diet) adverse short- and long-term metabolic programming outcomes, an estimated 80% of the global population prefers using plant-derived ethnomedicines to treat ailments (10,11). Plant-

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derived ethnomedicines are natural and thus are perceived to have fewer or reduced side effects and are considered safer than conventional pharmacological agents (10).

Garlic, *Allium sativum*, is globally used as a spice and as an ethnomedicine. It contains health beneficial phytochemicals such as alliin, diallyl disulphide, and *S*-allyl cysteine (SAC) (12). SAC, a water-soluble organosulphur peptide found in abundance in aged garlic preparations, has been shown *in vitro* and *in vivo* to possess health beneficial properties (13,14). The administration of SAC at 50～150 mg/kg body weight was shown to attenuate hyperglycaemia, hypoinsulinaemia, hyperlipidaemia, and oxidative stress in adult STZ-diabetic rats (14-16). Takemura et al. (17) reported that *in vivo* SAC alleviated hypercholesterolemia and hypertriglyceridemia in non-alcoholic fatty liver diseased Otsuka Long-Evans Tokushima male rats. *In vitro*, SAC has been shown to inhibit lipogenesis, promote lipid hydrolysis, and upregulate antioxidant enzymes (18).

The consumption of a high fructose diet results in an increased hepatic lipogenic turnover and the subsequent manifestation of various metabolic derangements such as dyslipidaemia (hypertriglyceridemia and hypercholesterolemia), hyperglycaemia, and insulin and leptin resistance (19,20). This study sought to determine if the administration of SAC could protect suckling male and female Wistar rats administered 20% fructose solution against the development of metabolic derangements by specifically determining its effects on body mass, blood glucose, triglyceride, and cholesterol concentration, total liver lipid, and NAFLD activity score. The pattern of development and maturation of physiological systems in early life occurs differently in male and females rats, thus we also hypothesised that there would be sexually dimorphic responses to the orally administered treatments.

MATERIALS AND METHODS

Source of SAC

Reagent grade SAC was procured from ChromaDex (Irvine, CA, USA).

Animals, feeding, and housing

Four-day-old male (n=32) and female (n=32) suckling Wistar rat pups from primiparous dams were used in the study. The rat litters were housed in the Central Animal Service animal unit with their respective dams and were allowed *ad libitum* suckling of their mother's milk throughout the experiment. Perspex cages for the housing had stainless steel mesh lids. Bedding made up of clean wood shavings was provided and changed twice per week. Room temperature was maintained at 24±2°C with a 12-h light/dark cycle (lights on from 07:00 h to 19:00 h). The pups were acclimatised for two days prior to the commencement of the experiment. All the experiments conducted in the current study complied with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals). Ethical clearance for the study was granted by the Animal Ethics Screening Committee (AESC) of the University of Witwatersrand. The AESC clearance number was 2015/07/B.

Experimental design

The pups were randomly allocated to and administered one of the following treatment regimens: group I (control), gavaged with 10 mL/kg body mass/d distilled water (DH); group II, gavaged with 10 mL/kg body mass/d 20% fructose solution w/v (FS); group III, gavaged with 150 mg/kg body mass/d of SAC dissolved in distilled water (10 mL/kg body mass); group IV, SAC+FS (Fig. 1). The random allocation was such that there were 8 male and 8 female pups per treatment regimen, and the treatment regimens were administered daily for 15 days from postnatal day (PND) 6 to 20 days.

The pups were weighed daily using an electronic scale (Snowrex Electronic Scale, Clover Scales Pty Ltd., Johannesburg, South Africa) to monitor body mass and to maintain a constant dose of the treatment regimens throughout the treatment period.

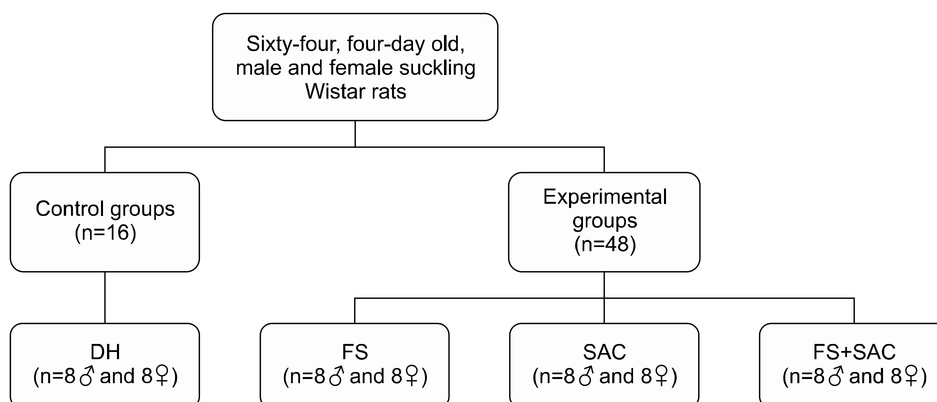

Fig. 1. Experimental design. DH (control), 10 mL/kg body mass per day distilled water; FS, 10 mL/kg body mass per day 20% fructose solution (w/v); SAC, 150 mg/kg body mass per day *S*-allyl cysteine; SAC+FS, 150 mg/kg body mass per day *S*-allyl cysteine+10 mL/kg body mass per day 20% fructose solution (w/v).

Terminal procedures

Determination of terminal body mass and blood parameters: On PND 21, the rat pups' terminal body masses were determined, and blood was obtained from the tail vein via pinprick and used to determine non-fasted blood glucose concentration using a calibrated Contour plus glucometer (Bayer, Isando, South Africa) as per the manufacturer's instructions. The pups were then euthanised by intraperitoneal injection with 100 mg/kg body mass of sodium pentobarbital (Eutha-naze, Bayer). Following euthanasia, blood was collected via cardiac puncture, and a few drops were used to determine blood triglycerides and cholesterol concentration using a calibrated Accutrend triglyceride and cholesterol meter (Roche, Mannheim, Germany) according to the manufacturer's instructions. Thereafter, the plasma was harvested and used to determine the leptin and insulin concentrations.

The liver was divided into two halves. One-half was stored at −20°C for the determination of liver lipid content. The other half of the liver used for histology analysis was preserved in 10% phosphate-buffered formalin and later stained with haematoxylin and eosin. Liver slides were scored using the NAFLD activity score (NAS) criteria, which is a semi-quantitative grading and scoring system, was used to evaluate the progression and severity of NAFLD: steatosis grade 0: <5%, 1: 5% to 33%, 2: 34∼66%, and 3: >66%; foci of lobular inflammation scoring 0: none, 1: <2, 2: 2∼4, and 3: >4; hepatocellular ballooning scoring 0: none, 1: few ballooned cells, and 2: many ballooned cells (21).

Liver lipid content determination: The total liver lipid (ether extract) content was determined by the soxhlet method of extraction as described by the Association of Official Analytical Chemists (AOAC) 2005; method number 920.39 (22).

Determination of plasma insulin and leptin concentration: Plasma insulin and leptin concentrations were determined by the sandwich enzyme-linked immunosorbent assay (ELISA) using a rat leptin kit [rat LEP (leptin) ELISA kit (sensitivity range: 0.03∼8.00 ng/mL), Elabscience®, Wuhan, Hubei, China] and insulin kit [rat INS (insulin) ELISA kit (sensitivity range: 3.125∼200 ng/mL), Elabscience®] according to the manufacturer's instructions. Whole-body insulin sensitivity was calculated using the homoeostasis model assessment of insulin resistance (HOMA-IR) as illustrated in the equation below:

$$\text{HOMA-IR} = \frac{\text{Fasting insulin level (ng/mL)} \times \text{Fasting glucose level (mg/dL)}}{405}$$

(23). To convert mmol/L fasting glucose concentration to mg/dL, mmol/L was multiplied by 18.01.

Statistical analysis

Parametric data are expressed as mean±standard deviation (SD) and non-parametric data are expressed as median and range (min, max). The data was analysed using GraphPad Prism 5 software (Graph-Pad Software Inc., San Diego, CA, USA). Statistical significance was considered at $P \leq 0.05$. A one-way ANOVA was used to analyse body mass, blood parameters, and liver lipid content data followed by multiple-comparisons Bonferroni *post hoc* test. The Kruskal-Wallis test was used to analyse NAS data followed by multiple-comparisons Dunns *post hoc* test.

RESULTS

Fig. 2 shows induction and terminal body masses of male (A) and female (B) rat pups, respectively. While the rat pups (male and female) grew significantly [($P<0.0001$) across treatment regimens] over the experimental period,

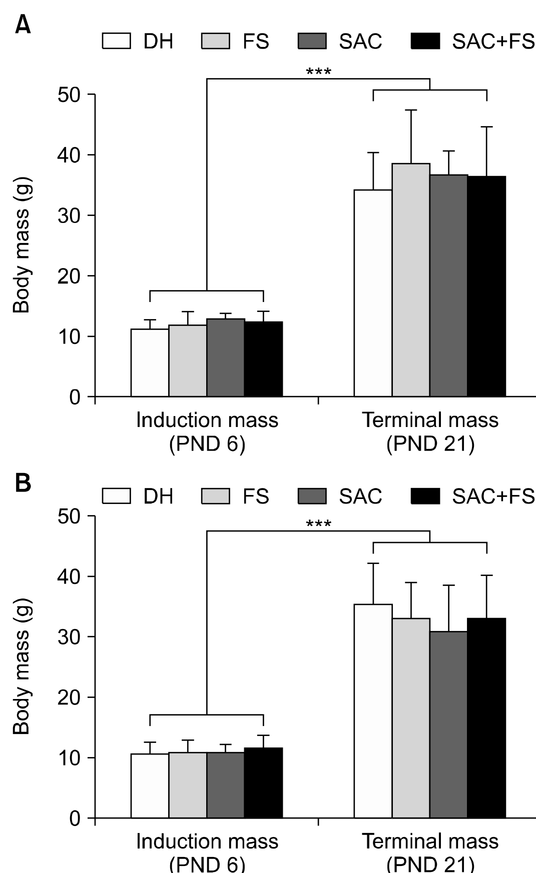

Fig. 2. Terminal body masses of the male (A) and female (B) rat pups. Data presented as mean±SD (n=8). Terminal body masses of male and female rat pups were significantly different from induction body masses (***P<0.0001). DH (control), 10 mL/kg body mass per day distilled water; FS, 10 mL/kg body mass per day 20% fructose solution (w/v); SAC, 150 mg/kg body mass per day *S*-allyl cysteine; SAC+FS, 150 mg/kg body mass per day *S*-allyl cysteine+10 mL/kg body mass per day 20% fructose solution (w/v). PND, postnatal day.

Table 1. Effect of *S*-allyl cysteine on glucose, triglyceride, cholesterol, leptin and insulin concentrations, HOMA-IR index and liver lipid content of non-fasted suckling male (A) and female (B) rat pups orally administered with fructose solution

(A)

Parameter	DH	FS	SAC	SAC+FS	Significance level
Glucose (mmol/L)	6.18±0.51	6.47±0.60	6.65±0.36	6.15±0.40	n.s.
Triglycerides (mmol/L)	3.94±1.57	4.89±1.31	3.82±1.47	4.04±1.21	n.s.
Cholesterol (mmol/L)	5.26±0.78	4.70±0.59	4.45±0.41	4.70±0.68	n.s.
Leptin (ng/mL)	4.28±0.64[ab]	5.21±0.61[a]	4.03±1.04[ab]	3.68±1.13[b]	<0.05
Insulin (ng/mL)	24.78±3.25[a]	35.40±9.87[ab]	46.68±11.13[b]	34.68±7.40[ab]	<0.001
HOMA-IR	7.43±0.92[a]	10.44±3.06[ab]	13.70±2.94[b]	9.47±2.01[a]	<0.01
Liver lipid (% liver mass)	1.66±0.36	1.56±0.10	1.69±0.01	1.57±0.07	n.s.

(B)

Parameter	DH	FS	SAC	SAC+FS	Significance level
Glucose (mmol/L)	6.17±0.43	6.00±0.31	5.92±0.75	6.22±0.59	n.s.
Triglycerides (mmol/L)	4.36±1.34	4.16±0.82	3.63±0.76	4.08±1.18	n.s.
Cholesterol (mmol/L)	5.09±0.68	4.95±0.63	5.27±0.75	4.88±0.80	n.s.
Leptin (ng/mL)	3.87±0.26[b]	3.24±0.10[b]	4.75±0.68[a]	4.67±0.35[a]	<0.05
Insulin (ng/mL)	35.66±10.63[b]	25.50±4.14[a]	44.96±7.40[b]	38.76±6.34[b]	<0.01
HOMA-IR	10.60±0.97[b]	6.80±1.43[a]	11.99±2.03[b]	11.13±1.94[b]	<0.01
Liver lipid (% liver mass)	1.58±0.14[c]	1.36±0.11[b]	1.97±0.04[d]	1.24±0.07[ab]	<0.001

Data presented as mean±SD (n=6~8).
DH (control), 10 mL/kg body mass per day distilled water; FS, 10 mL/kg body mass per day 20% fructose solution (w/v); SAC, 150 mg/kg body mass per day *S*-allyl cysteine; SAC+FS, 150 mg/kg body mass per day *S*-allyl cysteine+10 mL/kg body mass per day 20% fructose solution (w/v); HOMA-IR, homoeostasis model assessment of insulin resistance; n.s., not significant.
Different letters (a-d) are significantly different at $P\leq0.05$ by multiple-comparisons Bonferroni *post hoc* test.

their induction and terminal body masses across treatment regimens were similar.

Table 1 shows non-fasted blood glucose, triglyceride, cholesterol, plasma leptin and insulin concentrations, and HOMA-IR indices of male (A) and female (B) rat pups. In male rat pups, the oral administration of SAC only significantly increased plasma insulin concentration when compared to the control (P=0.0006). Additionally, male rats to which SAC only was orally administered had significantly higher HOMA-IR compared to their counterparts to which the control (DH) and or test treatment regimen SAC+FS were administered (P=0.0009 and P=0.0157, respectively). In female rat pups, the oral administration of FS alone significantly lowered plasma insulin concentration and HOMA-IR (P<0.01). Orally administered SAC prevented the FS-induced decrease in plasma insulin in female rat pups.

Oral administration of FS alone or a combination of SAC+FS significantly decreased the liver lipid content of female rat pups (P<0.001). However, oral administration of SAC only significantly increased liver lipid in female rat pups (P<0.001) (Table 1).

Table 2 shows the male (A) and female (B) rat pups' NAS. The treatment regimens had no effect on the NAS of male and female rat pups. Fig. 3 shows representative liver histology photo sections (hematoxylin and eosin staining, 400× magnification) of male (A) and female (B) rat pups. There were no histological differences in the liver samples of male and female rat pups across treatment regimens.

Table 2. Non-alcoholic fatty liver disease activity scores (NAS) of suckling male (A) and female (B) rat pups following their respective treatment regimens

(A)

Parameter	DH	FS	SAC	SAC+FS
Steatosis score	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)
Ballooning score	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)
Lobular inflammation score	2 (2, 2)	2 (2, 2)	1 (1, 2)	2 (2, 3)
Total NAS	2 (2, 2)	2 (2, 2)	1 (1, 2)	2 (2, 3)

(B)

Parameter	DH	FS	SAC	SAC+FS
Steatosis score	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)
Ballooning score	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)
Lobular inflammation score	1 (1, 3)	1 (1, 2)	2 (2, 2)	2 (2, 2)
Total NAS	1 (1, 3)	1 (1, 2)	2 (2, 2)	2 (2, 2)

Data presented as median and range (min, max) (n=3).
DH (control), 10 mL/kg body mass per day distilled water; FS, 10 mL/kg body mass per day 20% fructose solution (w/v); SAC, 150 mg/kg body mass per day *S*-allyl cysteine; SAC+FS, 150 mg/kg body mass per day *S*-allyl cysteine+10 mL/kg body mass per day 20% fructose solution (w/v).
Total NAS is the sum of values recorded for each category. <2, steatohepatitis; 3~4, uncertain; >5, probable or definite steatohepatitis.

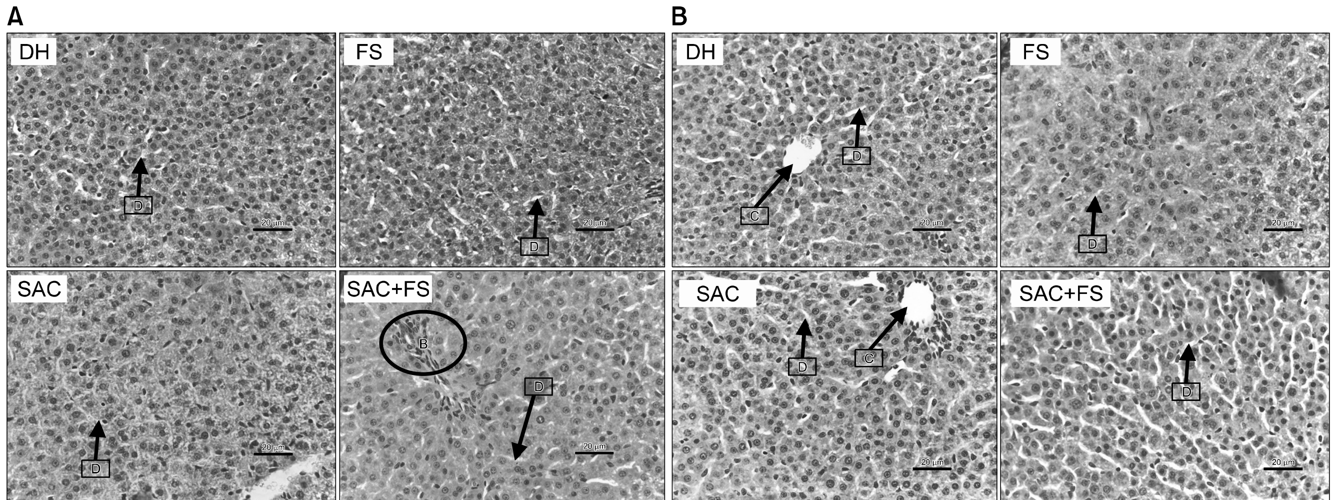

Fig. 3. Liver histology (hematoxylin and eosin staining, 400× magnification) of suckling male (A) and female (B) rats following the treatment regimens. Circle B shows foci of lobular inflammation, arrows C and D point to central veins and sinusoids respectively. No histological or pathological differences were noted in the liver samples from male and female rats across treatment regimens. DH (control), 10 mL/kg body mass per day distilled water; FS, 10 mL/kg body mass per day 20% fructose solution (w/v); SAC, 150 mg/kg body mass per day *S*-allyl cysteine; SAC+FS, 150 mg/kg body mass per day *S*-allyl cysteine+10 mL/kg body mass per day 20% fructose solution (w/v).

DISCUSSION

Fructose feeding during early postnatal life is associated with derangements such as altered growth, obesity, MetS, DM II, and NAFLD immediately in childhood or later in adulthood (8). In the current study, the oral administration of FS in female rat pups resulted in hypoinsulinemia, while the combined oral administration of SAC and FS resulted in normal insulin concentration in female rat pups insulin, which suggests that SAC attenuated the FS-induced hypoinsulinemia. The oral administration of SAC alone resulted in increased plasma insulin concentration in male rat pups suggesting that SAC possesses insulinotropic properties. SAC has been shown to possess antihyperglycaemic, insulinotropic, and antihyperlipidaemic properties in STZ-diabetic rats and high-fat diet NAFLD rat models (14,17,24). The mechanisms by which SAC exerts these beneficial properties have been attributed to its ability to stimulate insulin secretion by pancreatic β-cells, promote regeneration and longevity of pancreatic β-cells, and upregulate lipolytic genes and enzymes in the liver (17,24).

In the current study, none of the treatments regimens had an effect on growth performance (terminal body mass) of the male and female rat pups. This finding suggests that the orally administered FS, SAC, or SAC+FS had no adverse effects on body mass or growth of male and female rat pups. Ghezzi et al. (25) and de Moura et al. (26) contend that younger rats have reduced adiposity and more efficient GLUT4 transporters compared to their older counterparts and are more resistant to diet-induced MetS. In the current study, there were similarities in blood glucose, triglyceride, and cholesterol concentrations of male and female rat pups across treatment regimens. These findings suggest that orally administered treatments (FS, SAC, or SAC+FS) did not result in glucose, triglyceride, and cholesterol related metabolic disturbances in male and female rat pups. In the current study, suckling rat pups were used, thus the absence of derangements in glucose, triglyceride, and cholesterol could have been attributed to resistance to diet-induced MetS due to reduced adiposity and the effectiveness of GLUT4 transporters, despite FS having been orally administered to them.

While insulin, a metabolic regulatory hormone, stimulates glucose uptake, glycogenesis and lipogenesis and inhibits lipolysis in tissues, leptin inhibits satiety (27, 28). In the current study, orally administered FS alone resulted in decreased plasma insulin concentrations and HOMA-IR in female rat pups. These findings suggest that the orally administered FS may have resulted in anti-insulinotropic effects or increased insulin sensitivity in female rat pups. To our knowledge, no study has yet documented decreased plasma insulin in rats following fructose feeding. It is thus our assertion that at this moment the mechanisms by which orally administered FS lowered female rat pups plasma insulin are unknown. However, Teff et al. (28) reported that a high-fructose diet reduced circulating plasma insulin concentrations in normal-weight human adult females by decreasing leptin and dysregulating satiety. In the current study, the plasma leptin concentration of female rat pups to which FS was orally administered was not different compared to female rat pups in the other treatment regimens. Thus suggesting that that the FS did not reduce insulin concentration in the female rats via dysregulation of satiety and other

leptin linked pathways. The current study was conducted during the early postnatal period, which is characterized by progressive maturation of the endocrine system such as the increase in pancreatic β-cells proliferation and secretory function (29). We also propose that the orally administered FS may have reduced the insulin concentrations in female rats by interfering with the proliferation of pancreatic β-cells. Nevertheless, to gain better clarity, the potential mechanisms involved require further interrogation.

The current study reports that orally administered SAC attenuated the FS-induced hypoinsulinemia in female rat pups, which suggests that SAC attenuated the anti-insulinotropic effects of FS in female rat pups. This finding is in agreement with the reported SAC-mediated (as a secretagogue) attenuation of hypoinsulinemia in STZ-diabetic adult rats (13,24). In the current study, the oral administration of SAC only significantly increased plasma insulin concentration and HOMA-IR in male rat pups. Our findings suggest that SAC exerted insulinotropic effects and caused insulin resistance in male rat pups. The mechanisms by which SAC attenuated the insulin-lowering effects of FS in female rat pups and increased plasma insulin concentration and increased HOMA-IR in male rat pups may be attributed to its (SAC's) ability to stimulate insulin secretion by pancreatic β-cells and prevent hepatic insulin inactivation (13,24).

Excessive accumulation of hepatic lipids is associated with hypertriglyceridemia, hypercholesterolemia, and the onset of NAFLD (30). Whereas total liver lipid content provides an indication of the total amount of lipid substrates (triglycerides, total cholesterol, and lipoproteins) in the liver, the NAS evaluates the progression and severity NAFLD (21). In female rat pups, our results show that orally administered SAC increased liver lipid content without affecting liver histology and NAS. These findings suggest that although the orally administered SAC may have altered hepatic lipid accretion, it did not cause NAFLD in the female rat pups. Our findings are in disagreement with those of Saravanan and Ponmurugan (16) who reported that the oral administration of 150 mg/kg SAC to STZ-diabetic male adult rats for 45 days attenuated hepatic hyperlipidaemia (hypercholesterolemia, hypertriglyceridemia, and elevated free fatty acids). However this difference in our findings compared to those by Saravanan and Ponmurugan (16) could be attributed to the differences in age of the rats used (suckling rats in the current study versus adult rats) and the duration of intervention (15 days used by the current study versus 45 days). Koopmans et al. (31) reported that in adult Sprague-Dawley rats, hyperinsulinemia stimulated *de novo* lipogenesis, inhibited glycogenesis, and triggered insulin resistance. We thus hypothesise that the changes in liver lipid content in female rat pups and insulin sensitivity (as indicated by HOMA-IR) in male rat pups to which SAC only was orally administered may be ascribed to the potential insulinotropic effects of SAC as reported by the current study and other previous studies (13,14,24). Although our findings point to the potential health beneficial insulinotropic effects of SAC, it could potentially cause adverse metabolic effects typified by excessive hepatic lipid accretion in suckling female rats and insulin resistance in male rats. Excessive accretion of liver lipids and or insulin resistance increases the risk of developing various metabolic derangements such as MetS and NAFLD.

Currently, we are unable to explain the sexually dimorphic responses to the interventions that were observed in the current study. We speculate that they could be attributed to the dissimilarities in the rate and pattern of early life development of male and female rats (32,33). However, our theory warrants further investigation to fully understand the physiological mechanisms involved in the sexually dimorphic responses of rats. Furthermore, future studies need to investigate the molecular mechanisms, such as gene, protein, and receptor expression that may have been involved in the findings of the current study.

In conclusion, orally administered SAC protected suckling female rat pups against FS-induced hypoinsulinemia and had insulinotropic effects in male rat pups. While SAC displayed potential insulinotropic effects and could potentially be used as an antidiabetic agent, its use should be done with caution as it might potentially result in fatty liver disease and insulin resistance in growing rats.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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