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RESEARCH ARTICLE

Mechanism of delayed puberty in rats whose mothers consumed *Hibiscus sabdariffa* during lactation

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Abstract

Context: Extract of the calyx of *Hibiscus sabdariffa* Linn. (HS) (Malvaceae) has been reported to decrease fluid and food intake in lactating rats through a mechanism not yet fully understood. It has also been reported that rat pups undernourished during lactation have delayed puberty onset, suggesting a link between nutrition and onset of puberty. There is paucity of data addressing the effect of maternal consumption of HS during lactation on the onset of puberty in the female offspring.

Objective: The present study was designed to investigate whether consumption of HS during lactation will affect the onset of puberty and to examine the possible mechanism underlying this.

Materials and methods: Lactating Sprague-Dawley rats were randomly grouped into three on postnatal day one. One group had tap water (control); another had 0.6 g aqueous HS extract/100 mL, while the third had 1.8 g aqueous HS extract/100 mL as their drinking solution throughout lactation. Maternal fluid consumption, food consumption, weight gain, plasma Na^+ and corticosterone concentrations were determined. Offspring weights were recorded at 0, 21, 28, 35, and 42 days. Ages at onset of puberty and body weights were also recorded.

Results: A decreased maternal fluid and food intake and an increased maternal plasma Na^+ and corticosterone concentration were observed in HS dams. The HS treated female offspring showed delayed onset of puberty.

Discussion and Conclusion: The accelerated growth and delayed puberty in the HS offspring may be through increased corticosterone and decreased leptin delivery through breast milk.

Keywords: *Developmental programming; onset of puberty; postnatal weight gain; fluid and food intake; plasma sodium; plasma corticosterone; Hibiscus sabdariffa*

Introduction

Hibiscus sabdariffa Linn. (HS) (Malvaceae) is an annual, erect, herbaceous sub-shrub that is cultivated in the tropical and subtropical regions of the world for its stem, fiber, calyx, leaves and seeds. Extracts of HS are widely used in folk medicine for the treatment of a variety of ailments such as hypertension, hepatic disorders and febrile conditions (Daffalah & al-Mustafa, 1996). The effectiveness of HS in the treatment of these ailments has been attributable to the various constituents of HS like flavonoids, anthocyanins, organic acids, vitamins A and C, zinc, copper, manganese, magnesium, potassium, sodium and iron (Daffalah & al-Mustafa, 1996; Appel, 2003; Adigun et al., 2006). The flavonoids have been reported to inhibit the enzyme, 11βHSD2 (11-β hydroxysteroid dehydrogenase type-2), which inactivates glucocorticoids (Zhang & Wang, 1997; Guo & Reidenberg, 1998; Wang et al., 2002).

The anthocyanins of HS have been reported to have a protective effect against *tert*butylhydroperoxide-induced hepatic toxicity in rats (Wang et al., 2000),

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were able to quench the free radicals of 1, 1-diphenyl-2-picrylhydrazyl, reduced the cytotoxicity induced by *tert*-butylhydroperoxide in rat primary hepatocytes and attenuated hepatotoxicity in rats (Wang et al., 2000). Also, administration of the anthocyanins has been reported to significantly reduce the activities of the serum enzymes indicative of liver damage, ameliorated histological lesions, reduced oxidative liver damage and was effective in significantly mitigating the pathotoxicity induced by paracetamol in rats (Ali et al., 2003). It has also been reported that anthocyanins protect against DNA damage induced by *tert*-butylhydroperoxide in rat smooth muscle and hepatoma cells (Lazze et al., 2003). Hou et al. (2005) and Chang et al. (2005) also reported that anthocyanins induced a dose- and time-dependent apoptosis in human cancer cells especially human leukemia cells (HL-60) as characterized by cell morphology, DNA fragmentation, activation of caspase-3, -8, and -9, and inactivation of poly(ADP)ribose polymerase (PARP), thus enhancing the understanding for anticancer function of *Hibiscus* anthocyanins.

A sweetened aqueous extract of the dry calyx of HS is generally called *zobo* in Nigeria and is commonly produced, sold and consumed without caution by both males and females, young and old. It is consumed as a substitute for carbonated drinks and fruit juices, not necessarily for medicinal reasons. Anecdotal reports by those women who consume this extract during lactation suggest that they do so because of the folkloric belief that it makes them "feel lighter". Extract of HS has also been reported to decrease fluid and food intake in both pregnant, non-pregnant and lactating rats through a mechanism not yet fully understood (Orisakwe et al., 2003; 2004; Ojokoh, 2006; Iyare & Adegoke, 2008a, 2008b).

HS has been reported to be effective at decreasing the levels of total plasma lipids, cholesterol, and triglycerides, suggesting the possibility that HS may function as an anti-obesity agent (Kim et al., 2003; Olatunji et al., 2005; Hirunpanich et al., 2006). This anti-obesity effect of HS has been confirmed by Alarcon-Aguilar et al. (2007) when they observed significantly reduced body weight gain in monosodium glutamate-induced obese mice. It is not known, however, whether maternal consumption of HS during lactation will affect offspring postnatal weight gain.

The period of lactation (the first 21 days of life) when the mother can pass along contaminants to her offspring represents a critical period of reproductive development for the rat pups as the male and female reproductive systems undergo substantial development during this period (Pelletier, 2001; Rodriguez et al., 2002; Cyr et al., 2002). Kennedy and Mitra (1963) have earlier reported that rat pups undernourished during lactation have delayed puberty onset. This suggests a link between nutrition and the onset of puberty. It follows that when aqueous extract of HS is administered to lactating rats, it may lead to decreased food consumption in these rats and a consequent poor nutrition of the suckling pups with the attendant developmental sequelae (Barker et al., 1993; Gluckman & Hanson, 2004; Armitage et al., 2005a; 2005b). It is not known whether maternal consumption of aqueous HS during lactation, which may cause decreased food consumption, will affect postnatal growth and onset of puberty in the female offspring. The present study was therefore designed to investigate this and the possible mechanism underlying it.

Materials and methods

Plant material

Matured dry dark-red calyces of HS were purchased from a local market in Lagos, Nigeria and authenticated by T.I. Adeleke of the Department of Pharmacognosy, University of Lagos, Nigeria where a voucher specimen number PCG H455 was deposited. The extraction procedure used was as described previously by Adegunloye and coworkers (1996), but with slight modifications. Dry calyx of HS (30 g) was brewed in 400 mL of just boiled tap water for 45 min. The resulting decoction was filtered using a filtration sieve and the filtrate was evaporated to dryness giving a dark red powder (yield 48.87%). This dark red powder was stored in a refrigerator until needed for the preparation of the respective drinking solutions.

The dark red powder (0.6 g and 1.8 g) was then weighed and dissolved in 100 mL of tap water and given to the respective groups of rats as their drinking solutions.

Experimental animals

Inbred pregnant female Sprague-Dawley rats (n = 36), aged between 10-12 weeks and weighing 125 ± 5.5 g (mean ± SEM), were used for this study. The rats were housed individually in cages under standard environmental conditions. From day 1 of pregnancy untill delivery, animals had *ad libitum* access to food and water. On the day of delivery, the dams and their pups were divided randomly into three groups of 12 dams each. The first group (Control) was given tap water to drink throughout lactation while the second and third groups were given 0.6 and 1.8 g extract/100 mL, respectively, to drink throughout lactation. All groups received normal rat chow *ad libitum*. Fluid and food intake and dam weights were measured daily throughout lactation. Each dam in each group was allowed nine pups to nurse throughout the lactation period so as to eliminate the effects of undernutrition and overnutrition of some of

the pups. After 21 days, the pups were weaned to tap water. After weaning, the female pups were kept in groups of three per cage. Pups' weights were recorded at birth, weaning and weekly thereafter untill onset of puberty. Pubertal development starts soon after weaning, so from postnatal day 30 onwards, the young female rats were inspected daily for vaginal opening since onset of puberty is defined as the age (in days) at which vagina opening occurs (Engelbregt et al., 2000).

This study was approved by the ethics and experimentation review board of the College of Medicine, University of Nigeria, Enugu Campus, Nigeria.

Plasma Na^+ and corticosterone determination

On postnatal day 18, six rats in each group were anesthetized by chloroform inhalation and the thoracic cavity was quickly opened and the heart exposed. Blood samples were withdrawn by cardiac puncture using a 19 G hypodermic needle. The needle was removed and the blood ejected into a heparin-containing specimen bottle. The blood was immediately spun at 3000 rpm for 10 min in a centrifuge. The plasma was then gently withdrawn using a Pasteur pipette and stored at –20°C until ready for use.

The sodium ion content of the plasma was assessed by flame photometry while the corticosterone concentration was assayed by enzyme immunoassay (EIA).

Statistical analysis

Results are expressed as mean ± standard error of mean (M ± SEM). For data comparison, the one way analysis of variance (ANOVA) was used, followed by a *post hoc* Student's Newman-Keuls test. P <0.05 was taken as statistically significant.

Results

Results of the present study show a significant reduction (P <0.05) in fluid and food intake by the dams that consumed HS compared with the control group throughout lactation (Table 1). The fluid and food intake did not appear to be dose dependent.

The weights in all groups of dams at all postpartum periods of measurement were significantly greater than the respective pregravid weights (P <0.05; Table 2). There was no significant difference for any group of rats between weight measurements at postpartum days 7, 14 and 21 compared with postpartum day 0 within each group. There was also no significant difference between the postpartum weights of the control rats and the HS rats at the periods measured. However, the high dose HS (1.8 g/100 mL) was significantly lower (P <0.05) than that of the low dose (0.6 g/100 mL) HS rats.

Table 1. Effect of consumption of *Hibiscus sabdariffa* during lactation on maternal fluid and food intake.

	Mean fluid (mL/day) and food (g/day) intake					
	1st week of lactation		2nd week of lactation		3rd week of Lactation	
Groups	Fluid intake	Food intake	Fluid intake	Food intake	Fluid intake	Food intake
Control	27.7 ± 0.8	19.3 ± 0.6	33.2 ± 1.3	24.5 ± 0.9	32.5 ± 1.4	23.8 ± 0.8
0.6 g/100 mL	16.2 ± 1.4*	16.2 ± 0.7*	20.3 ± 0.8*	20.3 ± 0.7*	20.8 ± 1.1*	20.0 ± 0.9*
1.8 g/100 mL	16.5 ± 0.7*	15.2 ± 0.8*	16.8 ± 0.5*†	15.3 ± 0.6*†	20.2 ± 0.9*	18.2 ± 1.0*

Control group, tap water; no HS. *N=6* each. Values are expressed as mean ± SEM; *, P < 0.05 versus control; †, P < 0.05 versus control and 0.6 g/100 mL.

Table 2. Effect of consumption of aqueous HS on maternal weight changes during lactation.

	Maternal weight changes (g)				
Groups	Pregravid wt	PPD 0	PPD 7	PPD 14	PPD 21
Control	125.0 ± 4.5	180.0 ± 12.7*	189.8 ± 6.9*	194.0 ± 9.5*	183.7 ± 8.3*
0.6 g/100 mL	128.8 ± 8.6	177.5 ± 7.8*	185.5 ± 2.5†*	200.5 ± 5.5†*	202.0 ± 3.5†*
1.8 g/100 mL	122.5 ± 2.3	167.5 ± 7.8*	172.9 ± 5.7*	183.8 ± 7.8*	185.1 ± 7.1*

N=6/group; Wt, weight; PPD, postpartum day; *, P < 0.05 versus pregravid weight; †, P < 0.05 versus 1.8 g/100 mL group.

Table 3. Effect of maternal consumption of HS during lactation on absolute postnatal weight of the offspring.

	Mean absolute postnatal weight (g)				
Groups	PND 0	PND 21	PND 28	PND 35	PND 42
Control	5.61 ± 0.14	22.78 ± 0.65	32.78 ± 0.77	48.33 ± 0.93	55.83 ± 1.44
0.6 g/100 mL	5.72 ± 0.09	26.89 ± 1.80*	41.83 ± 2.46*	76.67 ± 1.44†	94.58 ± 5.80†
1.8 g/100 mL	5.67 ± 0.14	28.61 ± 1.31*	44.44 ± 1.34*	62.22 ± 0.98*	82.5 ± 0.63*

PND, Postnatal day; *N* = 9/group; *, P < 0.05 versus Control; †, P < 0.05 versus control and 1.8 g/100 mL.

Table 4. Effect of maternal consumption of HS during lactation on absolute postnatal weight gain of the offspring.

	Postnatal weight gain (g)			
Groups	PND 21	PND 28	PND 35	PND 42
Control	17.17 ± 0.70	27.17 ± 0.81	42.72 ± 0.86	50.22 ± 1.42
0.6 g/100 mL	21.17 ± 1.81*	36.11 ± 2.44*	70.94 ± 1.45†	88.86 ± 5.79†
1.8 g/100 mL	22.94 ± 1.31*	38.78 ± 1.28*	56.56 ± 1.04*	76.83 ± 0.60*

PND, Postnatal day; *N* = 9/group; *, P < 0.05 versus control; †, P < 0.05 versus control and 1.8 g/100 mL.

Results show a significant increase (P <0.05) in the absolute weight of the offspring of the HS dams at all periods of measurements (that is, postnatal days 21, 28, 35, and 42) compared with the offspring of the control dams (Table 3). There was no difference between the weights of the offspring of both HS groups at the periods measured except at postnatal days 35 and 42, when the weight of the offspring of the low dose (0.6 g/100 mL) HS group was larger (P < 0.05) than the weight of the offspring of the high dose (1.8 g/100 mL) HS group.

The weight gain by the offspring of dams in both of the HS groups was significantly greater (P <0.05) than the weight gain by the offspring of the dams of the control group (Table 4). There was no difference in weight gain between the offspring of dams in both HS groups throughout the periods of measurement except at postnatal days 35 and 42, when the weight gain of the offspring of the dams in the low dose (0.6 g/100 mL) HS group was greater (P <0.05) than the weight gain by the offspring of the dams in the high dose (1.8 g/100 mL) HS group.

The rate of weight gain per day in the offspring of the HS dams was significantly greater (P <0.05) than the rate of weight gain in the control offspring at all periods of measurement except at PND 35 when the rate of weight gain by the offspring of the high dose (1.8 g/100 mL) HS dams was similar to that of the control offspring but lower (P <0.05) than that of the low dose (0.6 g/100 mL) HS group (Table 5).

The plasma Na^+ concentrations in the HS dams were significantly higher (P <0.05) than that of the control dams. There was no difference between the two HS groups (Table 6).

The plasma concentration of corticosterone was significantly greater (P <0.05) in the high dose (1.8 g/100 mL) HS dams compared with both the control and the low dose (0.6 g/100 mL) HS dams whereas that of the low dose HS dams was not significantly different from that of the control dams (Table 6).

There was a significant delay (P < 0.05) in the onset of puberty and an elevated body weight in the HS groups compared with the control group (Table 7). There was no difference between the two HS groups, suggesting that the effect is not dose dependent.

Table 5. Effect of maternal consumption of HS during lactation on rate of weight gain of the offspring.

	Mean rate of weight gain (g/day)			
Groups	PND 21	PND 28	PND 35	PND 42
Control	0.82 ± 0.03	1.43 ± 0.06	2.22 ± 0.23	1.07 ± 0.26
0.6 g/100 mL	1.01 ± 0.09*	2.13 ± 0.27*	4.98 ± 0.42†	2.56 ± 0.88*
1.8 g/100 mL	1.09 ± 0.06*	2.26 ± 0.18*	2.54 ± 0.19	2.90 ± 0.18*

PND, Postnatal day; *N* = 9/group; *, P < 0.05 versus control; †, P < 0.05 versus control and 1.8 g/100 mL.

Table 6. Effect of consumption of HS during lactation on maternal plasma Na^+ and corticosterone concentration.

Doses of HS consumed	Na^+ (mmol/L)	Corticosterone (ng/mL)
Control	138.17 ± 1.01	13.5 ± 0.5
0.6 g/100 mL	145.5 ± 1.06*	14.83 ± 0.6
1.8 g/100 mL	149.33 ± 1.76*	16.83 ± 0.48†

N = 6/group; *, P < 0.05 versus control; †, P < 0.05 versus control and 0.6 g/100 mL.

Table 7. Effect of maternal consumption of HS during lactation on offspring age and body weight at onset of puberty.

	0.6 g/100 mL	1.8 g/100 mL	Control
Age (days)	49.67 ± 1.55*	48.78 ± 1.70*	43.11 ± 1.84
Weight (g)	116.11 ± 5.70*	100.28 ± 4.92*	58.89 ± 1.96

N = 9 each; Values are expressed as mean ± SEM; PPD, postpartum day; *, P < 0.05 compared with control.

Discussion

During lactation, the action of 11βHSD2 at inactivating glucocorticoids in the mammary gland decreases by over 75% (Quirk et al., 1990). Since glucocorticoids induce the expression of milk proteins such as casein and lactalbumin (Ono & Oka, 1980), the postpartum decrease in 11βHSD2 activity, and hence increased active glucocorticoids, is a prerequisite for the endocrine induction of lactation (Quirk et al., 1990). The report by several workers that flavonoids inhibit the activity of 11βHSD2 (Zhang & Wang, 1997; Guo & Reidenberg, 1998; Wang et al., 2002) suggests that consumption of aqueous extract of HS, reported to be rich in flavonoids (Dafallah & Al-Mustafa, 1996; Appel, 2003; Adigun et al., 2006), during lactation, further increases the concentration of active glucocorticoids. This may explain the increased corticosterone level as observed in the present study. This may have contributed to the process of lactation and thus enhanced nutrient delivery to the suckling neonates.

The decreased food consumption in the two groups of HS dams in the present study may have been due to hypernatremia-induced dehydration-anorexia (Ross & Desai, 2005). The hypernatremia observed in the present study in the HS dams may have been due to a

variety of reasons. The water deprivation following the decreased fluid intake by the HS rats may have caused a state of hypernatremia in these rats (Ross & Desai, 2005) as observed in the present study. Also, aqueous extract of HS has been shown to be rich in Na^+ (Adigun et al., 2006) suggesting that consumption of aqueous extract of HS increases the body's Na^+ load. Mojiminiyi and coworkers (2000), in their investigation of the diuretic action of aqueous extract of HS, observed that rats that consumed this extract had elevated plasma Na^+ concentration. They concluded that this raised plasma Na^+ concentration was due to the diuretic action of this extract.

According to several workers (Zhang & Wang, 1997; Guo & Reidenberg, 1998; Wang et al., 2002), flavonoids inhibit the action of 11βHSD2. This enzyme, localized to mineralocorticoid target cells in the kidney, colon and parotid glands as well as to the pancreas and placenta (Mercer & Krozowski, 1992; Brown et al., 1993; Albiston et al., 1994) was identified as the enzymatic "gatekeeper" that catalyses the conversion of the active glucocorticoid into the inactive form, thus excluding active glucocorticoids from the non-specific mineralocorticoid receptors which display little inherent specificity for their normal ligand, aldosterone (Krozowski & Funder, 1983; Arriza et al., 1987). Thus, consumption of aqueous extract of HS which has been shown to be rich in flavonoids (Dafallah & Al-Mustafa, 1996; Appel, 2003; Adigun et al., 2006) leads to the inhibition of the action of this enzyme, thereby allowing glucocorticoids (which normally circulate at concentrations higher than aldosterone) to gain access to the mineralocorticoid receptors. This causes hyperstimulation of these receptors resulting in excessive Na^+ reabsorption and retention (Funder et al., 1988). This mechanism may also have contributed to the elevation of plasma Na^+ concentration observed in the rats that consumed aqueous extract of HS in this study.

The decreased food consumption in the HS dams in the present study may have led to decreased leptin levels in these dams since it has been reported that circulating leptin level falls with malnutrition (Maffei et al., 1995; Frederich et al., 1995). In rats, breast milk leptin, absorbed by the stomach of the suckling neonates (Casabiell et al., 1997; Oliver et al., 2002; Sanchez et al., 2005) and transported to the circulation (Casabiell et al., 1997), is the main source of neonatal leptin during the first half of lactation (Miralles et al., 2006). Oral administration of leptin, at doses close to the physiological concentration in breast milk, has been shown to reduce food intake in suckling neonatal rats (Sanchez et al., 2005) suggesting that leptin supplied by the mother through breast milk could regulate short-term feeding in the suckling neonate during the first half of lactation (Sanchez et al., 2005). Since breast milk leptin correlates positively with maternal leptin at anytime during lactation (Houseknecht et al., 1997; Casabiell et al., 1997; Uysal et al., 2002; Miralles et al., 2006), the amount of leptin in the suckling neonates in the first half of lactation, therefore, depends on maternal nutrition.

It can therefore be speculated that, compared with the control, the decreased food consumption induced by aqueous HS consumption during lactation in the HS dams may have led to decreased leptin transport to the suckling neonates. This may have led to increased consumption of milk by the suckling pups of the HS dams. This, coupled with the possible corticosterone-enhanced lactation in the HS dams and the growth promoting constituents (flavonoids, vitamins A and C and iron) (Ceesay et al., 1997; Hilakivi-Klarke et al., 1998; Christian et al., 2003; Jain et al., 2008; Wu et al., 2008) in the aqueous HS extract, may have been responsible for the accelerated rate of weight gain (and hence the increased weight gain) in the offspring of the HS dams during the period of lactation.

The first half of lactation is a very critical period for the development of the rat pups. During this period, there is a postnatal leptin surge that is responsible (and required) for the correct development and later functioning of hypothalamic neurocircuitry that controls energy homeostasis and reproductive functions which are immature during this period (Myers et al., 2005). It has been speculated that alteration of the amplitude or timing of this surge; as might be expected in the face of maternal malnutrition, since maternal leptin is the main source of neonatal leptin during this period; could alter the development of these hypothalamic circuits so as to predispose to weight gain or increased adiposity and reproductive abnormalities later in life, especially when these offspring are exposed to normal nutrition or overnutrition (Myers et al., 2005). Since the weaning of the offspring of the HS dams to ad libitum food and water may have represented a deviation from the nutritional level, in terms of the maternal leptin reaching them through the breast milk, to which they were exposed during lactation, the increased rate of weight gain (and thus increased body weight) in these offspring after weaning may be a reflection of the inability of leptin to appropriately regulate energy homeostasis since the pathway through which it normally does this may have been poorly developed. These offspring, therefore, overeat, increase body weight and thus show a propensity towards obesity later in life.

Vagina opening is considered a good marker of the onset of puberty in the female rat (Engelbregt et al., 2002). In the present study, there was a delayed onset of puberty in the offspring of the HS dams through mechanisms that may not be unconnected with the ability of HS extract to decrease fluid and food intake (and consequently creating osmotic and nutrient stresses) since malnutrition during the lactation period has been shown to delay the onset of puberty (Engelbregt et al., 2001). Osmotic and

nutrient stresses are known to result in elevated glucocorticoid level. Elevated plasma glucocorticoid level, as observed in the present study, has been shown to cause a permanent resetting of endocrine systems, such as the somatotrophic and hypothalamo-pituitary adrenal axes (Edwards et al., 1993; Seckl, 1998; Phillips et al., 1998; Barker, 2000; Lesage et al., 2001; Fowden & Forhead, 2004) and a delay in the onset of puberty in female offspring (Smith & Waddell, 2000; Wellberg et al., 2001). It is also possible that the delayed puberty observed in the female offspring of the HS dams may also be due to the poor development of the hypothalamic neurocircuitry that controls energy homeostasis and reproductive functions which normally matures during lactation.

From the result of the present study, it can be hypothesized that since the offspring of the HS dams were at no time during their development given aqueous HS to drink directly, their accelerated postnatal growth and delayed puberty may have been induced during lactation possibly through increased corticosterone and decreased leptin delivery to them through breast milk.

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Declaration of interest

The authors report no conflicts of interest.

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