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Delayed Puberty Onset in Rats that Consumed Aqueous Extract of *Hibiscus sabdariffa* During the Juvenile-Pubertal Period

[1]E.E. Iyare and [2]U.I. Nwagha
[1]Department of Physiology,
[2]Department of Physiology/Obstetrics and Gynaecology,
College of Medicine, University of Nigeria, Enugu Campus, Enugu State, Nigeria

Abstract: The report that intervention during the juvenile-pubertal period in rats modifies the phenotype induced by prenatal nutrition suggests some degree of plasticity in the juvenile-pubertal period. It is not known whether consumption of aqueous extract of *Hibiscus sabdariffa* (HS) during the juvenile-pubertal period will affect the growth and onset of puberty in rats. The present study was therefore designed to investigate this. Weaned 21 days old rats, whose mothers were given food and water *ad libitum* during pregnancy and lactation, were divided randomly into three groups of nine rats each. Control group had tap water only while the extract groups had 0.6 g extract/100 mL and 1.8 g extract/100 mL as their drinking solution throughout the juvenile-pubertal period [postnatal day (PND) 21-puberty onset]. From PND 30 onwards, the rats were inspected daily for vaginal opening, which was used as the index for puberty onset. Rats in the HS groups (0.6 and 1.8 g/100 mL) drank less fluid (solution of HS extract and water) and consumed less food compared with the control group at all periods of measurement. The weight of 0.6 g/100 mL was not different from the control whereas 1.8 g/100 mL was lower at PND 28, similar at PND 35 and higher at PND 42 compared with the control. Puberty onset in the HS groups was significantly delayed compared with the control. It is concluded that consumption of aqueous extract of HS during the juvenile-pubertal period decreased fluid and food consumption, increased weight gain and delayed puberty onset in rats.

Key words: Puberty onset, *Hibiscus sabdariffa*, juvenile-pubertal period, food intake, fluid intake

INTRODUCTION

Nutritional perturbations during the developmental period (*in utero* and lactational) have been widely reported to be associated with increased risk of metabolic and reproductive diseases later in life (Armitage *et al*., 2005; Gluckman and Hanson, 2004; Engelbregt *et al*., 2001). These perturbations alter the developmental trajectory of the developing offspring by inducing in them metabolic adaptations that are necessary to ensure optimum survival when these offspring are faced with such nutritional perturbations in postnatal life (Gluckman and Hanson, 2004). According to Gluckman and Hanson (2004), a mismatch between the nutritional planes to which these offspring are metabolically adapted and that to which they are born or reared underlie the early origins of metabolic diseases.

In humans and animals, the nature of the developmental adaptation differs with respect to the developmental stage of the offspring at the time of the exposure (Erhuma *et al*., 2007; Roseboom *et al*., 2006). It has been reported that metabolic adaptations induced by nutritional perturbations during pregnancy can be reversed by intervention during lactation. For example, Vickers *et al*. (2005) have shown that leptin administration to neonatal rats reversed the metabolic adaptation induced by maternal undernutrition during pregnancy.

Extracts of *Hibiscus sabdariffa* (HS; Family: Malvaceae) are widely used in folk medicine for the treatment of a variety of ailments (Daffalah and Al-Mustafa, 1996). The effectiveness of HS in the treatment of these ailments have been attributable to the various constituents of HS like flavonoids, anthocyanins, organic acids, Na^+, vitamins A and C and Fe (Adigun *et al*., 2006; Appel, 2003; Daffalah and Al-Mustafa, 1996). It has previously been reported that consumption of this extract by pregnant and lactating rats decreased their fluid and food intake and led to delayed puberty onset in their female offspring (Iyare and Adegoke, 2008a, b).

Corresponding Author: Eghosa E. Iyare, Department of Physiology, College of Medicine, University of Nigeria, Enugu Campus, Enugu, Nigeria Tel: +234 8052820041, +234 42 309472

Burdge *et al.* (2009) have reported that intervention during the juvenile-pubertal period in rats modifies the phenotype induced by prenatal nutrition. This suggests some degree of plasticity in the juvenile-pubertal period. It is not known whether phenotypes induced (or programmed) at weaning without exposure to nutritional perturbations during pregnancy and lactation, by way of maternal consumption of HS as shown previously (Iyare and Adegoke, 2008a, b), will be affected by consumption of HS during the juvenile-pubertal period. The present study was therefore designed to investigate whether consumption of HS during the juvenile-pubertal period will affect the growth and onset of puberty in rats.

MATERIALS AND METHODS

Study was conducted in January-June, 2009.

Animals and treatment: Eighteen in-bred virgin female Sprague-Dawley (SD) rats age between 10-12 weeks and weighing 125±5.5 g (Mean±SEM; range 110-155 g) with two consecutive regular 4 day estrous cycles were used for this study. They were housed individually in cages under standard environmental conditions. The estrous cycles were monitored and males of proven fertility (male rats that have been previously used successfully to mate consecutively with female rats to produce live and healthy offsprings) were introduced into the cages of the female rats that were expected to get into the estrus phase within 12 h to allow for mating. Day 1 of pregnancy was taken as the day spermatozoa were seen in the vaginal smear of the rats (Iyare and Adegoke, 2008c). From day 1 of pregnancy through postnatal day 20 (PND 20), animals were given food and water *ad libitum*. On PND 21 the young rats were weaned and divided randomly into three groups of nine rats each. Control group had tap water only while the extract groups had 0.6 g extract/100 mL and 1.8 g extract/100 mL as their drinking solution throughout the juvenile-pubertal period (PND 21-puberty onset). All groups had normal rat chow and their respective drinking solutions (that is water, for control rats and HS extract solutions, for the HS rats) *ad libitum*. From PND 30 onwards, the rats were inspected daily for vaginal opening, which was used as the index for puberty onset (Engelbregt *et al.*, 2001). The age at vaginal opening (onset of puberty) was recorded and body weight was measured.

All procedures used in this study conformed with the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals (American Physiological Society, 2002) and were approved by the Departmental Committee on the Use and Care of Animals.

Extraction procedure: Mature dry dark-red calyces of HS were purchased in a local market in Enugu, Nigeria. It was authenticated by a resource staff at the Department of Botany, University of Nigeria, Nsukka, Nigeria. The extraction procedure used in our laboratory was as described previously (Iyare and Iyare, 2006a, b). Briefly, 30 g of the dry petals of HS was brewed in 400 mL of boiled tap water for 45 min. The resulting decoction was filtered.

The concentrations in the extract groups (0.6 and 1.8 g/100 mL) were then derived as follows: 10 mL of filtrate was added to 48 mL of tap water to make approximately 0.6 g/100 mL tap water while 10 mL was added to 9 mL of tap water to make approximately 1.8 g/100 mL tap water.

Aqueous extract of HS was used in this study because we wanted to simulate what was obtainable in our environment. Nigerians consume a beverage (*zobo* drink) made from an aqueous extract of HS.

The first dose (0.6 g/100 mL) was chosen because according to Mojiminiyi *et al.* (2000), it gave an equivalent concentration with the commercially available local beverage (*zobo* drink) that is commonly produced and consumed in Nigeria. The second dose (1.8 g/100 mL) was chosen in order to see if there will be any degree of dose dependent effect.

Statistical analysis: For data comparison between the three groups, the one way Analysis of Variance (ANOVA) was used followed by a post-hoc Students Newman Keuls test. $p<0.05$ was taken as statistically significant.

RESULTS

Fluid and food intake: Rats in the HS groups (0.6 and 1.8 g/100 mL) drank less fluid compared with the control group at PND 28 ($p<0.05$ and $p<0.005$, respectively) and 35 ($p<0.05$ for both) (Table 1). At PND 42, the fluid intake in the HS groups was not significantly different from that of the control ($p>0.05$ for both HS groups). There was no difference in fluid intake between the two HS groups at PND 28, 35 and 42 ($p>0.05$ at each period of measurement).

The food intakes by the rats in the HS groups (0.6 and 1.8 g/100 mL) were significantly lower than that of the control dams at all periods of measurement ($p<0.05$ and $p<0.01$, respectively at PND 28, $p<0.05$ and $p<0.01$, respectively at PND 35, $p<0.05$ for both at PND 42)

Table 1: Effect of consumption of aqueous extract of *Hibiscus sabdariffa* during the juvenile-pubertal period on fluid and food intake

	Fluid intake (mL day^{-1})			Food intake (g day^{-1})		
Groups	PND 28	PND 35	PND 42	PND 28	PND 35	PND 42
Control	7.67±0.60	11.22±0.66	13.17±0.81	6.50±0.26	9.89±0.42	11.22±0.36
0.6 g/100 mL	6.06±0.73*	9.56±0.58*	11.44±0.85	5.44±0.47*	8.44±0.50*	9.67±0.5*
1.8 g/100 mL	5.33±0.65*	9.33±0.64*	13.06±0.90	3.94±0.40*^{P}	6.94±0.99*	9.83±0.64*

N: 9 each. Values are expressed as M±SEM, *p<0.05 compared with control, ^{P}p<0.05 compared with 0.6 g/100 mL

Table 2: Effect of consumption of aqueous extract of *Hibiscus sabdariffa* during the juvenile-pubertal period on absolute body weight

	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	5.69±0.17	24.72±1.06	32.22±0.65	50.56±2.94	57.22±2.65
1.8 g/100 mL	5.83±0.12	24.17±0.63	26.67±1.02*^{P}	46.67±1.02	65.28±1.11*

^{P}HS: *Hibiscus sabdariffa*, N: 9 each. Values are expressed as M±SEM, *p<0.05 compared with control, ^{P}p<0.05 compared with 0.6 g/100 mL

Table 3: Effect of consumption of aqueous extract of *Hibiscus sabdariffa* during the juvenile-pubertal period on some body parameters at onset of puberty

	Groups		
Body parameters	Control	0.6 g/100 mL	1.8 g/100 mL
Age (days)	43.11±1.84	60.78±2.05*^{P}	54.22±1.41*
Weight (g)	58.89±1.96	93.06±2.49*^{P}	77.22±1.69*

HS: *Hibiscus sabdariffa*, N: 9 each. Values are expressed as M±SEM, *p<0.05 compared with control, ^{P}p<0.05 compared with 1.8 g/100 mL

(Table 1). There was no difference in food intake between the two HS groups except at PND 28 when the food intake in the high dose HS group (1.8 g/100 mL) was significantly lower than that of the low dose HS group (0.6 g/100 mL) (p<0.05).

Postnatal weight: There was no significant difference in the weights of the rats at PND 0 (birth) and PND 21 (weaning) (Table 2). This was not surprising because these rats were still being nursed by their mothers that were given *ad libitum* food and water. There was no difference in postnatal weights between the low dose HS group (0.6 g/100 mL) and the control rats at PND 28, 35 and 42. The postnatal weight of the rats in the high dose HS group (1.8 g/100 mL) at PND 28 was significantly lower than that of the rats in both the control and the low dose HS groups (0.6 g/100 mL) (p<0.05), whereas at PND 35, it was not significantly different from both (p>0.05). At PND 42, the weight of the rats in the high dose HS group (1.8 g/100 mL) was significantly higher than those of the control and low dose HS groups (0.6 g/100 mL) (p<0.05).

Age and body weight at vagina opening: The age at vagina opening in rats in the low dose HS group (0.6 g/100 mL) was significantly higher than those of the high dose HS (1.8 g/100 mL) (p<0.05) and control groups (p<0.0005) (Table 3). The age at vagina opening in rats in the high dose HS group (1.8 g/100 mL) was also significantly higher than that of the control rats (p<0.05).

The body weight at vagina opening in rats in the low dose HS group (0.6 g/100 mL) was also significantly higher than those of the high dose HS (1.8 g/100 mL) (p<0.01) and control groups (p<0.0005) (Table 3). The age at vagina opening in rats in the high dose HS group (1.8 g/100 mL) was also significantly higher than that of the control rats (p<0.01).

DISCUSSION

Since food consumption in the rat is dependent on the hydration of the gut (Schoorlemmer and Evered, 2002) the decreased consumption of the HS fluid may have caused a decreased hydration of the gut which may have resulted in the decreased food consumption. Also, since HS extract has been reported to be rich in Na^{+} (Adigun *et al*., 2006) and consumption of hypertonic fluid is known to reduce food consumption (Steiger *et al*., 2000; Kraly *et al*., 1998), the decreased food consumption observed in the present study following HS consumption, may also have been due to increased hypertonicity of the fluid reaching the gut by way of HS consumption. It is also possible that dehydration-anorexia may also have contributed to the decreased food consumption observed in the rats that consumed the HS fluid. This is so because rats that consumed aqueous HS extract have been shown to have hypernatremia (Iyare and Adegoke, 2008a, b) which has been reported to cause dehydration-anorexia (Ross and Desai, 2005).

The decreased fluid and food consumption observed in the rats that drank aqueous HS fluid in the present study was expected to result in decreased weight gain in these rats. This, however, was not the case, suggesting that the HS fluid consumed by these rats may have contributed to the growth (or increased weight gain) of these rats. This is so because aqueous HS extract has been shown to contain substances like flavonoids, iron, vitamins A and C (Adigun *et al*., 2006; Appel, 2003; Daffalah and Al-Mustafa, 1996; Morton, 1987; Clydesdale,

1979; Duke and Francis, 1973; Fuleki and Francis, 1968) which have been shown to increase body weight gain (Jain *et al*., 2008; Wu *et al*., 2008; Christian *et al*., 2003; Hilakivi-Klarke *et al*., 1998; Ceesay *et al*., 1997).

Like several other workers (Leonhardt *et al*., 2002; Engelbregt *et al*., 2000, 2001, 2002), we defined puberty onset as the age in days at which vagina opening occurred because it is the first visible sign that pubertal development is taking place even though Gruaz *et al*. (1998) suggested that vaginal opening may only be a sign of the increase in estrogen secretion in the rat and not necessarily a reflection of the state of reproductive capacity.

In the present study, there was a significantly delayed puberty onset in the rats that consumed aqueous HS extract. Several factors may have contributed to this delayed puberty onset. For example, it is well established that rats that consume aqueous extract of HS are nutritionally and osmotically stressed and thus have elevated corticosteroid concentration (Iyare and Adegoke, 2008a, b). Since glucocorticoid has been reported by Tohei and Kogo (1999) to inhibit ovarian function and the differentiation of granulosa cells by follicle stimulating hormone in immature female rats and Leonhardt *et al*. (2002) postulated that increased circulating glucocorticoid level in female offspring could mediate the impaired development of Graafian follicles induced by maternal malnutrition, the observed delay in vagina opening (used as the index of puberty onset) in the rats that consumed aqueous HS extract in the present study may have been due to the elevated glucocorticoid level in these rats.

Leptin has been postulated to be the metabolic signal that triggers puberty when the energy reserve is adequate to support pregnancy (Chehab *et al*., 1997; Campfield *et al*., 1996; Bronson and Manning, 1991) by interacting with the Gonadotropin Releasing Hormone (GnRH) neuron (Lee, 1995) and causing changes in the frequency and magnitude of GnRH pulses which has been shown to herald the onset of puberty (Terasawa, 1995). Decreased food consumption is known to cause a fall in plasma leptin level (Maffei *et al*., 1995; Frederich *et al*., 1995). It is possible therefore that the decreased food consumption observed in the rats that consumed aqueous HS extract in the present study may have decreased plasma leptin level and consequently the delayed puberty onset.

It is well established that estrogens inhibit the secretion of GnRH, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) (Ganong, 2005) thus affecting the growth and functions of the ovarian follicles. Phytoestrogens are a group of biologically active plant substances with a chemical structure that is similar to that of estradiol, an endogenous estrogen. This structural similarity accounts for the ability of these compounds to bind to estrogen receptors and exert various estrogenic and antiestrogenic effects (Usui, 2006). Extract of HS has been reported to be rich in phytoestrogens (Adigun *et al*., 2006; Appel, 2003; Daffalah and Al-Mustafa, 1996). This suggests that in rats that consumed aqueous HS extract, the phytoestrogens may have inhibited the GnRH, FSH and/or LH which consequently resulted in a decrease in the outflow of estrogens from these follicles. This may have resulted in the delay in the attainment of the threshold estrogen concentration required for the cornification of the cells of the vagina which causes vagina opening (Engelbregt *et al*., 2002).

In conclusion, the results of the present study may suggest that consumption of aqueous extract of HS during the juvenile-pubertal period may: (1) decrease food consumption through dehydration-anorexia possibly by stimulating gut receptors or Central Nervous System receptors, (2) increase weight gain directly or indirectly through the actions of the growth promoting constituents in the extract, (3) delay puberty onset through the effect of the phytoestrogens in the aqueous HS extract at inhibiting the GnRH, FSH and/or LH. Further work still needs to be carried out to establish the nature of the constituents of aqueous HS extract mediating these effects.

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