Morphological and functional alterations of female reproduction after regular exposure of bamboo shoots of North East India

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ABSTRACT

Objective: To evaluate the effect of daily consumption of bamboo shoots (BS) on the morphological features and functional status of the female reproductive system in adult with respect to thyroid. Methods: Adult female rats were divided into control and experimental groups of six each. Control group was given normal diet while experimental group was fed BS by 1/3rd replacement of 180 g of their food i.e. 60 g of BS containing 35 g of goitrogens of cyanogenic origin such that each rat likely consumed 6 mg/100 g of body weight per day for a period of 45 d. Morphological features like changes in body weight and organ weight were noted. Key steroidogenic enzyme levels viz $\Delta^3\beta$ hydroxysteroid dehydrogenase (HSD) and $\beta$ HSD along with serum estradiol, estriol and progesterone levels were measured. Estrous cyclicity of the animals monitored regularly followed by histological analysis of thyroid, ovary and uterus at the end of experimentation. Results: Increase in body weight, thyroid gland weight, thyroid stimulating hormone, decrease in serum triiodothyronine and thyroxine, a decrease in ovarian as well as uterine weight and the activity of steroidogenic enzymes $\Delta^3\beta$ HSD and $\beta$ HSD along with diminished serum estradiol, estriol and progesterone levels were noted; while histological plates showed prominent degenerative changes in both the ovary and uterus. Estrous cyclicity of the treated animals were irregular and almost stopped at diestrous stage of the cycle in the latter stage of the treatment as compared to control. Conclusions: Overall results indicates that BS rich in cyanogenic constituents induces biochemical hypothyroidism in the experimental animals that acts in corroboration to cause morphological and functional alteration of reproductive organs indicating its likely impact in fertility on continued use.

1. Introduction

The female reproductive system is designed to carry out many functions, the most important of which is preservation of fertility[1]. The female reproductive system is concerned with maintenance of morphological female characters and ovulation; maintenance of menstrual cyclicity, implantation, pregnancy, birth, lactation and menopause are important processes of the functionality of this system[2]. Several endogenous[3-6] and exogenous factors[7,8] regulate the normal functioning of the female reproductive system like follicle stimulating hormone[3], luteinizing hormone, thyroid hormone[6], food consumed[9], stress and environmental pollutants, etc. Bamboo-shoots (BS) are one such consumed crucifers
aids their labelling as nutraceuticals or natural medicines that are creating ripples among health advocates and scientists alike. Recent studies have also revealed that BS has a number of health perks: increasing appetite and digestion, reducing obesity and risks of cardiovascular disease\cite{16} as well as anti-cancer constituents\cite{17-20}.

In opposition to all the nutraceutical advantages of BS, it is an acknowledged natural goitrogen with anti-thyroid constituents like cyanogenic glycosides\cite{21}, glucosinolates and thiocyanates\cite{22} and has been reported to cause endemic goiter in Manipur, North East, and India on regular consumption even in presence of adequate iodine\cite{23}. Hypothalamic-pituitary-thyroid axis and hypothalamic-pituitary-ovarian axis function in close conjunction and as thyroid hormone receptors exist in the ovarian tissue; several instances of hypothyroidism causing reproductive disturbances are available including menstrual cycles characterized mainly by polymenorrhea, especially anovulatory cycles\cite{24} and an increase in fetal wastage in mature women\cite{25}. Changes in sex hormone-binding globulin, prolactin, gonadotropin-releasing hormone, and sex steroid serum levels are also associated with dysfunction of the thyroid gland\cite{26}. Abnormalities in thyroid hormone levels can thus greatly influence fertility in both sexes. BS has also been reported to induce infertility in male rats\cite{27} and is used widely in third world country tribes to ameliorate several reproductive problems\cite{28}.

The effect of sub-chronic exposure of BS in female reproduction is yet to be clearly elucidated experimentally and no conclusive experimental or clinical data is available about its effects on female reproduction on regular ingestion. Its indigenous uses by various communities further fuel the cause of potentiating the exact role that BS plays over the female reproductive system. Moreover as mentioned BS has anti-thyroid properties, indicating that it is likely to have an impact on the female reproductive system through suppression of secretion of thyroid hormones. On attaining clear information regarding its effect on the female reproductive system, inklings to how on consumption of natural goitrogens like BS induces reproductive problems are available including menstrual cycles characterized mainly by polymenorrhea, especially anovulatory cycles and an increase in fetal wastage in mature women. Changes in sex hormone-binding globulin, prolactin, gonadotropin-releasing hormone, and sex steroid serum levels are also associated with dysfunction of the thyroid gland. Abnormalities in thyroid hormone levels can thus greatly influence fertility in both sexes. BS has also been reported to induce infertility in male rats and is used widely in third world country tribes to ameliorate several reproductive problems.

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2. Materials and methods

2.1. Animals

For investigation, 12 adult female rats (6 for experimental and 6 for control studies) weighing (90±10) g were used. The animals were maintained as per national guidelines and protocols as approved by the Institutional Ethics Committee of the Department of Physiology, University of Calcutta. They were housed in two cages having six each; in air conditioned rooms maintaining 12 h light/12 h dark cycles. The rats were allowed free access to drink water and were fed on standardized normal laboratory diet (20% protein) which consisted of 70% wheat, 20% Bengal gram, 5% fish meal powder, 4% dry yeast powder, 0.75% refined til oil and 0.25% shark liver oil with required amount of potassium iodide\cite{29}. Control rats were fed a normal laboratory diet and experimental group was fed BS as mentioned below for 45 d.

2.2. Preparation of bamboo shoot containing food\cite{22}

The experimental group of rats was fed BS by replacing 1/3rd portion of normal diet. Sixty gram of BS were weighed, equal amount of water was added to it and boiled for 15 min. It was then mixed with 180 g of normal food as mentioned. Thus, 60 g of BS containing near to 35 mg/100 g of goitrogens was given to six rats, such that ideally, 6 rats received a daily dose of 6 mg/100 g of body weight antithyroid substances.

2.3. Food consumption pattern\cite{30}

Food consumption of each rat per day was recorded daily during the period of treatment. Calculation of food consumption for each rat (g/rat/day) was calculated as:

\[
\text{Food given(g)} - \text{Food wasted(g)} = \text{Food consumption(g)}
\]

2.4. Measurement of urinary iodine and thiocyanate

Urine samples were collected by placing animals in metabolic cages. The urine samples were ashed after digestion, and iodide was estimated by its catalytic action on the reduction of ceric ion Ce\(^{4+}\) to cerous ion Ce\(^{3+}\), maintaining internal quality control by the method of Karmakar et al.\cite{31}.

Thiocyanate concentrations in urine samples were measured using the method of Aldridge\cite{32} as modified by Michajlovskij et al.\cite{33}.

2.5. Study of estrous cycle of female rats\cite{34}

Normal saline (0.9%) was taken in a blunt tipped dropper and was pushed into vagina of each rat of both the experimental as well as the control groups. A smear was obtained by spreading on a slide. The smear was dried and then stained using hematoxylin-eosin. The slide was then observed under a microscope to identify the stage of estrous cycle. The entire protocol was performed for 45 consecutive days and the data was recorded.

2.6. Sacrifice of the animal

The animals were sacrificed on the consecutive day of the last treatment following IEC protocol. Blood was collected from the hepatic portal vein of each animal and stored separately. Before sacrifice the body weight of each rat was taken. The ovaries and uterus from each animal was collected and stored separately.

2.7. Histological study

On the day of sacrifice, thyroid, ovary and uterus of both experimental and control groups of animals were removed, weighed and fixed in Bouin’s solution. The tissues were embedded in paraffin and sectioned on a microtome. The sections were stained in hematoxylin-eosin and observed under a light microscope (Model –CH20i Olympus; serial number 8A06177) at 400 × and 100 × as required. The photomicrograph of the sections were using

\(×\)
the Nikon Cool P1 500 Digital Camera.

2.8. Assay of 3 β hydroxysteroid dehydrogenase (HSD) and 17 β HSD activity

The ovarian tissue was homogenized with a fluid containing 20% spectroscopic graded alcohol, 5 mM potassium phosphate and 1 mM EDTA at a tissue concentration of 100 mg/mL. Homogenizing mixture and centrifuged at 1 000 r/min for 30 min in an ultracentrifuge at a constant temperature of 4 °C. The supernatant was then used for assay. The activity was determined by the optical measurement of the rate of reduction of NAD. The reaction system contained in a final volume of 3 mL: 100 µM NAD, 30 µg of substrate of 3 β HSD or 17 β HSD as the case is each in 0.02 Ml of purified doxin and a suitable quantity of enzyme (200-500 µL) to initiate the reaction. The final pH of the system was 9.1. The reaction was carried out in silica cuvette of 1 cm light path, in a spectrophotometer (Shimadzu, UV-mini 1240) at 340 nM absorbance. The activities were measured at 15 s intervals against a blank containing all components except steroids, linear initial velocities were determined graphically. One unit of enzyme activity is the amount causing change in absorbance of 0.001/min when enzymes serve as substrates. Quantitative assay of tissue protein was done by Lowry method[37].

2.9. Enzymes linked immunosorbsent assay of serum estradiol (E2) level, estriol (E3) level and progesterone level

This assay employs the competitive inhibition enzyme immunoassay technique. A polyclonal antibody for goat anti-rabbit IgG had been pre-coated onto a microplate. Horseradish peroxidase (HRP) labelled human E2, unlabelled human E2 (standards or samples) and a polyclonal antibody for E2 was added. Then the competitive inhibition reaction was launched between HRP labelled E2 and unlabelled human E2 with the antibody. Next, a TMB substrate solution was added to each well and a change in colour was exhibited. More of amount of human E2 present in the samples, lesser the HRP labelled human E2 could bind to the antibody. The substrate solution was added to the wells respectively. A colour developed in reversal to the amount of human E2 bound in the initial step. The colour development was stopped and the intensity of the colour was measured at 450 nm as per manufacturer’s protocol via kits obtained from bio vision life sciences. The E2 level is also measured by the above procedures.

Progesterone was assayed using ELISA kits obtained from Equipar Diagnostics, Italy as per manufacturer’s method.

2.10. Measurement of serum thyroxine (T4) and triiodothyronine (T3) and thyroid stimulating hormone (TSH)

The serum obtained from the experimental and control group of animals were used to measure T4 and T3 by Enzyme Linked Immunoabsorbent assay (ELISA) kits obtained from RFCL Limited, India as per manufacturer’s instructions.

Thyroid stimulating hormone level was measured using Cusabio Biotech Limited, Rat TSH kit (Lot no.C0710270665) as per manufacturer’s protocol, following principle of ELISA.

2.11. Statistical analysis

Results were expressed as mean±SD. Significance of difference between the control and treated groups were estimated using student’s two tail t-test. The level of significance was determined on the basis of P values and the level of significance was tested at 0.05.

3. Results

3.1. Body weight

There was a significant increase in body weight of BS fed animals in comparison to control animals (Table 1).

3.2. Organ weight

There was a significant increase in thyroid weight, however there was decrease in ovarian weight and uterine weight of BS fed animals when compared with control animals (Table 1).

Table 1

| Group | Initial BW (g) | Gain of BW (%) | Final BW (g) | Weight of thyroid (mg/100 g BW) | Weight of ovary (mg/100 g BW) | Weight of uterus (mg/100 g BW) |
|-------|----------------|----------------|-------------|-------------------------------|-------------------------------|-------------------------------|
| Control | 92.25±3.97 | 22.62 | 113.12±1.12 | 84.3±12.26 | 75.23±7.12 | 156.0±7.52 |
| BS fed | 96.4±3.02 | 40.41 | 135.42±1.23 | 126.8±3.68 | 62.34±0.95 | 138.2±0.91 |

This significant difference was found in food consumption rate in control ($\mu$g/d/L) and BS fed animals ($\mu$g/d/L), with only a slight decrease being noted in the BS fed group.

3.3. Food consumption pattern

No significant difference was found in food consumption rate in control ($\mu$g/d/L) and BS fed animals ($\mu$g/d/L), with only a slight decrease being noted in the BS fed group.

3.4. Serum TSH and thyroid hormone levels

A significant decrease in both serum T3 and T4 levels of BS fed animals was found on assessment with the control animals (Table 2).

Table 2

| Group | TSH (µU/mL) | T4 (µg/dL) | T3 (ng/mL) | Urinary iodine levels (µg/100 mL) | Urinary thiocyanate levels (µg/mL) |
|-------|-------------|------------|------------|----------------------------------|----------------------------------|
| Control | 0.384±0.023 | 6.72±0.71  | 0.85±0.05  | 35.26±1.63                      | 0.50±0.04                       |
| BS fed | 0.532±0.087 | 4.67±0.54  | 0.69±0.01  | 42.87±0.97                      | 1.43±0.12                       |

Values are expressed as mean±SD. A two tailed ‘t’ test was performed and the significant differences were found between control and treated groups ($P<0.05$).
3.5. Urinary iodine and thiocyanate

A significant increase in the levels of urinary iodine and thiocyanate was found in the BS fed group in comparison to the control group. (Table 2).

3.6. HSDs

There was a significant decrease in ovarian $\Delta^5 3\beta$ HSD activity and ovarian $17\beta$ HSD activity of BS fed animals when evaluated against the control animals (Table 3).

Table 3

| Group      | $3\beta$ HSD (OD/min/mg of protein) | $17\beta$ HSD (OD/min/mg of protein) | Estradiol (ng/mL) | Estriol (ng/mL) | Progesterone (ng/mL) |
|------------|------------------------------------|--------------------------------------|-------------------|-----------------|----------------------|
| Control    | 0.100±0.010                        | 0.116±0.050                          | 78.15±1.47        | 0.13±0.02       | 43.00±1.52           |
| BS fed     | 0.061±0.003*                       | 0.111±0.020*                         | 71.52±1.38        | 0.08±0.01*      | 31.00±1.83*          |

Values are expressed as mean±SD. A two tailed 't' test was performed and significant differences were found between control and treated groups ($P<0.05$).

3.7. Serum estrogen and progesterone levels

A significant decrease in serum estradiol ($E_2$), estriol ($E_3$) levels and progesterone levels (Table 3) of BS fed animals was found on assessment with the control animals.

3.8. Estrous cycle

The study of the vaginal smear for a period of 45 d shows that the animals of control group had regular (5±1) d of estrous cycle while in the BS fed group constant diestrous phase was observed almost on/from day 40 (Figure 1).

The uterine microphotograph showed that the BS fed animals had an increased perimetrium and endometrium, diminished uterine glands, an atrophied lumen and decreased stratum vasculare distinctly different from that of control animals (Figure 3).

3.9. Histology of thyroid, ovary and uterus

The photomicrograph of the ovary revealed that in contrast to the control animals, the BS fed animals had degenerated thecal cells, disintegrated granulosa cells, followed by diminished cumulous oophorous and an immature oocyte (Figure 2).

The photomicrograph of the thyroid of BS fed animals showed features similar of a hypothyroid state including irregularly-shaped follicles with decreased colloid content and hypertrophied and hyperplastic nature of follicular epithelial cells in contrast to evenly filled regular shaped follicles of the control animals (Figure 4).

4. Discussion

Commonly known for their industrial uses, a rare known fact of bamboos is the use of its young shoots as a food that can be eaten fresh, fermented, or canned. BS are now being utilized worldwide as a potential health food and as a nutraceutical due to its rich nutrient content. It contains carbohydrates (5.70%), proteins (3.90%),
minerals (1.10%) while several other essential components such as vitamins, amino acids, sterols, polyphenols and steroids can be extracted from it to a lesser extent[40]. BS however contains an approximate 600 mg/kg wet weight of cyanogenic glycosides of the taxiphyllin group, glucosinolates and thiocyanates[22]. All these are anti-thyroid components of naturally occurring goitrogenic plants and are capable of disrupting the bodies’ ability to utilize iodine, blocking thyroid hormone synthesis and release[22]. Thus, being an established anti-thyroidal agent it is expected to bring about changes by perturbing balance of the hypothalamo-pituitary-thyroid axis and hypothalamo-pituitary-ovarian axis or by any of its several other components which in their way can impinge on the female reproductive system that are liable to occur on BS consumption.

No significant changes was observed in the food consumption pattern of BS fed animals in conjunction to control animals indicating that there was no disparity in the amount of food intakes of both groups and that sufficient amount of goitrogens were consumed by BS fed animals.

Urinary iodine and thiocyanate levels both increased significantly in BS fed group in contrast to control animals. The cyanogenic glycosides and glucosinolates present in BS are readily converted to thiocyanates which appear in the blood and urine[22]. The free thiocyanate present in BS also adds to these levels. On the other hand thiocyanate is a well-established inhibitor of iodide in the thyroid gland, causing its excess efflux and appearance in urine[41]. Thus, elevated levels of iodine and thiocyanate in the urine are also markers of sufficient goitrogen consumption by the experimental group of animals. Current findings are consistent with previous studies which have reported similar increase in urinary iodine and thiocyanate levels on consumption of BS[22].

There was a significant increase in body weight and decrease of T₃, T₄ levels and increase in TSH levels of BS fed animals in comparison to the control group. This effect could likely be due to the anti-thyroidal properties of BS which can induce hypothyroidism as patients with an underactive thyroid tend to have a very low basal metabolic rate; one of the most noticeable symptoms of hypothyroidism is weight gain and difficulty in losing extra weight[42]. Increase in TSH levels, thyroid weight as well as hyperplastic and hypertrophic nature of thyroid morphology on BS consumption are strong indicators of hypothyroidism induction[43]. A significant decrease in ovarian weight was also observed in comparison with the control group. A study on effect of rape seed (another natural goitrogen containing cyanogenic glycosides) on pig’s female reproduction also showed that there is decrease in ovarian weight following its consumption[44]. On treatment with synthetic anti-thyroid drug like thiourea also decreases ovarian weight in Chinese hamsters[45].

The histological comparison of the control ovary with that of the BS fed ovary showed changes in the granulosa cells lining of the ovary, which lack discrete cellular arrangement. T₃ receptors have been identified in the granulosa cells of ovary. It could be assumed that this impaired folliculogenesis is due to insufficient thyroid hormone production that hampers the differentiation and not the propagation of granulosa cells as suggested by Dijkstra et al.[46]. Certain parts of the ovarian lining in BS fed ovary were disrupted and thecal cells were degenerated. This could be due to decrease in estrogens level which are the primary steroid hormones in this region. Vice-versa, the estrogens concentration could have decreased due to the changes here. Absence of cumulous oophorous and decreased membrane granulosa were also the prominent features of the BS fed ovary in comparison to the control suggesting that there was less maturation of the oocyte for which the deficiency of oophorous found responsible.

There was a significant decrease in uterine weight in comparison with that of control. In a similar study cassava, a cruciferous vegetable containing cyanogenic glycosides was fed to rabbits and vaginal and left oviductal weight was reported to have decreased which are similar to weight reduction as observed in the present study[47]. It is also known that uterine regression is associated with a hypothyroid state and is also conspicuous in thyroidectomized female[42]. Available results suggest that thyroid hormones have a direct effect on the uterus which regulates the responsiveness of the organ to estradiol[48]. Thus, BS via its induction of a hypothyroid state impinges on the uterine weight and function.

The lumen of the BS fed uterus showed an atrophied appearance while the cellular mass in the endometrium and perimetrium were increased in respect of the control uterus. Uterus in hypothyroid sheep has been showed to have endometrial hyperplasia and smooth muscle hypertrophy[49]; a similar condition is probable to have occurred here. Stratum vasculare and uterine glands were declined in BS fed uterus in comparison to the control uterus. On treatment with PTU a drug which induces hypothyroidism and also on thyroidectomy in rats poorly developed uterus was seen[42]. Inuwa et al.[50] reported that absolute epithelial cell volume as well the height of the luminal epithelium in hypothyroid rats decreased but endometrium thickness was significantly increased. Thus it might be concluded that thyroid hormones might be importantly concerned in the maintenance of the normal structure of uterine epithelial cells. In the present study BS presumably affected the thyroid hormones and almost similar results have been documented.

Δ₃ 3 β HSD activity in BS fed animals was significantly reduced than that of control animals. Flaxseed having similar constituents as that of BS reduced Δ₃ 3 β HSD activity in males[51]. Glucosinolates present in Christmas Bush (Chromolaena odorata) plant similar to BS have also been reported to decrease LH, FSH, and testosterone as well as Δ₃ 3 β HSD activity in males[52]. Thus, it may be assumed that such alterations have also been mediated in females. In accordance to Δ₃ 3 β HSD, 17 β HSD activity also decreased significantly in experimental animals than in control animals. 17 β HSD expression is controlled by gonadotrophins, androgens and estrogens themselves[53]. Constituents of BS like glucosinolates are known to decrease gonadotrophins and androgens[52] although these were not assayed in the present investigation but estrogen and progesterone levels were observed to decline. Consequently it may be presumed that 17 β HSD being under multihormonal control can
be regulated by any of its modulators which themselves could be
affected by BS constituents.

Both the serum estrogens and progesterone levels were decreased
significantly in BS fed animals in comparison with control animals.
A possible mechanism could be via phytosterols and dietary fibres
which are present in BS. Phytoestrogens are the known effectors which
decrease cholesterol and sex steroids in plasma[54,55]. Decrease in
Δ⁴ 3β HSD and 17β HSD levels is also likely to have resulted in
decreased conversion of precursors of progesterone and estrogens to
the hormones themselves.

In this study, experimental animals were fed BS for 45 d. The
estrus cycle was regular till (18±2) d, then became irregular, finally
cessing from day 40 at diestrous phase in the BS fed animals.
Similar finding was reported by United States Government in
relevance to public health[56]. It was stated there that on consumption
of dietary cyanogens (also present in BS) there is prolongation of
diestrous phase with reduction in estrous phase. This finding is also
correlated with the fact that estrogens levels declined in the treated
animals. Adult female rats, made hypothyroid, also reportedly
correlated with the fact that estrogens levels declined in the treated
animals. Adult female rats, made hypothyroid, also reportedly
have irregular estrous cycles and ovarian atrophy[57]. PTU treated
hypothyroid animals also have been shown to have irregular cycles
leading towards diestrous[58]. Thus changes in the estrus cycle
observed in the study are consistent with those seen in hypothryroid
conditions and substantiates the role of BS as a potent goitrogen.

The overall results reveal that BS consumption caused an increase
in body weight and thyroid weight, decrease in ovarian weight along
with a decrease in uterine weight followed by inhibition of Δ⁴ 3β
HSD and 17α HSD activity and decrease of serum estriol, estradiol
and progesterone levels. The estrus cycle was predominantly
irregular and ceased at diestrous towards the end of the study.
Therefore, BS either through its direct action or via its action as an
anti-thyroid agent brings about some significant changes in female
reproduction putting to question the regular, ubiquitous consumption
of it as a health food. Degenerative changes were seen in both the
uterus and ovary indicating that it has a disrupting role which could
possibly lead to infertility in women. However, it is difficult to come
to exact conclusive statements about its accurate regulating effect
without measuring other essential parameters such as FSH, LH,
aromatase, etc. Further studies along with in-vitro studies can shed
more light into the role of BS consumption on female reproduction.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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