Mechanism of development of residual goitre and associated thyroid disorders

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Abstract

In post salt iodination period, endemic goiter that generally develops for iodine deficiency is found prevalent in many regions, including India, despite adequate iodine intake. Such goiters are termed as residual goiters. Consumption of dietary goitrogens could be the possible regulator in the persistence of residual goitre and associated disorders. Residual goitres exist in Manipur, India for the consumption of bamboo shoots (BS). Therefore, to study the actions of BS on major thyroid hormone synthesizing regulatory factors at cellular and molecular levels on isolated thyrocytes conducted perusing expression of NIS, TPO, Tg and PDS proteins as well as mRNA and protein expression of NIS, TPO, Tg, PDS, MCT8 and PAX8 with and without extra iodine in *in-vitro* studies. Simultaneously, ROS generation in terms of H_2O_2 and antioxidant status, NO, LPO were assayed. Cellular and DNA damage and oxidation, including cell cycle alterations after BS exposure, were also investigated.

Precursors and metabolic end products of cyanogenic glycosides on phytochemical analysis found in BS. It has pleiotropic effects on thyrocytes. It repressed the activities of Na^+-K^+-ATPase, TPO and D2, as well as reduced the expression of NIS, TPO, Tg, PDS proteins and MCT8 and PAX8, at mRNA level. All these effects of BS are mediated through increased ROS levels as H_2O_2 upsurge leading to a perturbed pro-oxidant antioxidant balance in thyrocytes associated with cellular and DNA damage and oxidation. Increased LPO and NO levels upon exposure of BS extract triggered plasma membrane and organelle damage of thyrocytes, which are major sites of NIS, TPO, PDS and MCT8, leading to thyroid disfunction. Cellular and DNA damage/oxidation was observed that leads to disruption of normal cell cycle pattern of thyrocytes which in turn causes changes in apoptotic proteins Bax, Bcl-2 and p53. These changes imply imbalance of cell death and proliferation equilibrium leading to disease conditions as found in goitre upon exposure to BS extract. Extra iodine prevents goitrogenic/anti-thyroid influences of BS on such actions to an extent.

Thyroid diseases among residual goitrous population following consumption of BS evaluated by their iodine nutritional and salt iodization status, serumT_3, T_4 and TSH profiles, TPO- and Tg-Ab antibodies, thyroid ultrasonography, the prevalence of hypo- and hyperthyroidism and cytomorphological features. Goitrous populations are affected by subclinical and overt hypo- and hyperthyroidism with elevated thyroid autoantibodies, hypoechoic thyroid and thyroiditis. This study concludes that residual goitrous individuals are affected by different autoimmune thyroid disorders. Excess thiocyanate from dietary goitrogens of cyanogenic origin (BS) is responsible for the development of residual goitre followed by its progression towards different thyroid autoimmune disorders.

Keywords: Autoimmune diseases, bamboo shoots, goitrogens, iodine, ROS, thiocyanate

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Introduction

The thyroid gland is placed in the lower neck region and is responsible for the synthesis and secretion of thyroid hormones - L-thyroxine (T_4) and L-triiodothyronine (T_3). The structure and functions of this gland are regulated by several factors both through endogenous and exogenous ways. Thyroid hormones have widespread significant physiological activities, including growth and development, metabolism, and other vital physiological functions. Hypo- and/or hyperfunctioning statuses of the gland are associated with several complications as the maintenance of body homeostasis is disturbed and adverse effects on all primary organs[1] Iodine in trace amount is essential for the synthesis of thyroid hormones. The uptake and transport of iodide in the thyroid gland takes place through a basal membrane transporter protein sodium iodide symporter (NIS) and the enzyme that provides necessary energy is Na^+-K^+-ATPase of thyrocyte as well as apical membrane transporter protein pendrin, which, in association with several other proteins thyroglobulin (Tg), and enzyme thyroid peroxidase (TPO) regulate the synthesis followed by secretion of the thyroid hormones.[2] Therefore, the iodine status of the body has an intricate role in sustaining the thyroid gland's normal structural and functional status. To prevent and control iodine deficiency disorders (IDDs) by providing adequate iodine, universal salt iodization has been introduced since 1924; when potassium iodide was added to edible salt by Prof. David Crowie of the University of Michigan. However, after salt iodization, about 300 million people throughout the world were affected by thyroid gland dysfunction, but in the then period, the total goiter prevalence in the general

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population was 15.8%.[3] Environmental factors present in food and water affect the functioning of the thyroid gland by various means. Therefore, to overcome the observed thyroid disorders and decrease the risks, a proper understanding on the interactions of the environmental agents with the thyroid and its dysfunction is found important. In the post-salt iodization period, IDDs found prevalent in several regions of the globe; therefore, a search for other potential causative factors is imperious and is the focus of current research. A number of environmental agents present in food and water called dietary goitrogens is now predicted as exogenous factor that affect the normal functioning of the thyroid gland. Based on epidemiological data supported by experimental evidences it has now been established that exposure to naturally occurring anti-thyroid agents intensifies the goitre endemicity.[4] The foods that contain goitrogens/anti-thyroid substances are responsible for the intensification, persistence, and development of goitre and associated disorders.

Dietary goitrogens have been searched globally and plants of the cruciferous family other than flavonoids rich plants are identified as potent plant- goitrogens for the presence of high quantities of anti-thyroid constituents.[5] Bamboo shoots (BS) belong to the cruciferous family are consumed widely in the south east Asian regions and a common food item in India specially in the north eastern states; in several means as soups, curries, pickles and with meat this plant food is commonly consumed by many tribes and most inhabitants of this area.[6] Bamboo is a universal plant that grow almost all over the world having massive benefits and thus important for rural tribes. The culms of bamboo called bamboo shoots (BS) are quite palatable vegetables having an exclusive taste. Its nutritive values have been investigated and found to contain high fibres, vitamins and alike constituents that has made it popular as a functional food now not only restricted to the eastern part of the ecosphere but has expanded globally. It is now one of the most coveted edibles with plentiful recipes having numerous provisions.[6] Glucosinolates (GLNs), cyanogenic glycosides (CGs) and thiocyanate (SCN) are its key goitrogenic contains that not only disrupt thyroid gland structure and functions but further dissociate to form cyanides triggering severe toxic conditions. It is thus a strong anti-thyroid plant food perhaps acting as a decisive factor accountable for persistence of IDDs in the north eastern part of the country as well as many other regions where it is consumed frequently as an indispensable part of the food.[7] The conceivable mechanism by which the respective stage(s) of thyroid hormone synthesis as iodide trapping and entry, iodination, organification or coupling of iodinated molecules to form T_4 and T_3 are exaggerated under the influence of bamboo shoots and can cause interruption of thyroid hormone biosynthesis and transport still remains unmapped. Effects of usually consumed bamboo shoots - Bambusa Balcooa Roxb as its water extract on thyrocytes at the cellular as well as molecular level to interpret changes in important thyroid hormone synthesizing regulatory factors and to discover a conceivable disruption pathway *in vitro* even with extra iodine is the objective of this study; besides its impact in the development of thyroid diseases among the vulnerable population has been discussed. The upshot of the study may be exploited to comprehend a long-standing public health disorder in the traditional goitre endemic regions where IDDs still prevail in spite of iodine supplementation as well as shed light on the possible mechanism of action of such plant food(s) containing dietary goitrogens with and without iodine supplementation.

MECHANISM OF DEMVELOPMENT OF RESIDUAL GOITRE

Bamboo shoots and its anti-thyroid/goitrogenic constituents

The bamboo shoots that have been used in the study is widely available and consumed by the people of in north east India irrespective of their cast, creed and sex where endemic goitre found prevalent even after effective post salt iodization and was identified based on its features to be Bambusa Balcooa Roxb (BSBR). The amount of anti-thyroid substances viz. cyanogenic glycosides, glucosinolates and thiocyanates in the bamboo shoots extract was measured. The amount of thiocyanate, glucosinolates and cyanogenic glycosides were found to be 99 ± 13 mg/L, 26.5 ± 3.6 mg/L and 523 ± 67 mg/L respectively indicating a total of near to 650 mg of anti-thyroid substances per Kg of bamboo shoots which has been dissolved in 1 litre of ultra-pure water.[8] It indicates that the bamboo shoots as used contain sufficient amounts of anti-thyroid substances of cyanogenic origin in terms of break down products of dietary goitrogens to cause thyroid disruption.

The phytochemical analysis of BSBR extract using liquid chromatography mass spectrometry (LCMS) in high resolution with a quadrupole–time of flight (Q-tof) analyser revealed presence of constituents from 98 families of amines, quinines, terpenoids, protein, reducing sugar, alkaloid, resins, carbohydrate and tannins along with the precursors (tyrosine, coumaric acid) and end products (like p-benzaldehyde, madelonitrile, isothiocyanate) of cyanogenic glycosides (CGs).[8]

A dose response curve of bamboo shoots extract was prepared initially on thyrocytes in culture following MTT assay to determine its inhibition constant (IC_{50}), which was 24 ng/µL. Three doses below the IC_{50} value were selected and the effects of bamboo shoots extract on thyrocytes with these doses 3, 6, and 12 ng/µL, respectively, were used for subsequent studies.

Bamboo shoots on expressions of major thyroid hormone synthesizing regulatory enzymes and elements at cellular level

The expressions of sodium iodide symporter (NIS), thyroid peroxidase (TPO), thyroglobulin (Tg) and pendrin (PDS) of thyrocytes were analysed in presence of thyroid stimulating hormone (TSH) and with and without extra iodine after the

exposure of the bamboo shoots extract to evaluate any alterations at cellular level of those proteins; a graded decline in expression of those proteins were observed, however, there was improvement in expression with extra iodine to a certain extent.[8] A decline in NIS expression indicates less iodine entry in thyrocytes. Thiocyanate (SCN), a constituent of bamboo shoots extracts as well as an end metabolic product of cyanogenic constituents in bamboo shoots extract, inhibits NIS expression.[9] Previous studies where NIS expression decreased reports development of hypothyroidism as well as goitre.[10] Expression of TPO also decreased that regulates several steps in thyroid hormone biosynthesis. A decrease at the translational level of this enzyme indicates disruption in steps of iodination, organification and coupling.[11] Bamboo shoots as mentioned earlier contain thiocyanate, an inhibitor of thyroid iodide transport and organic binding, which along with other cyanogenic end products further inhibits the TPO system. Similar plant foods like cassava and millet are known to have decreased TPO activity *in-vivo*.[12-14] Thyroglobulin (Tg) is a major component required for synthesis of T_3 and T_4 as it is the indispensable substrate required for organification and is the major protein component of the colloid unique to the thyroid follicular lumen.[15] The protein expression of Tg decreased dose dependently on exposure to bamboo shoots extract. Being the thyroid hormone matrix, a decrease in this protein thus indicates a decrease in the bulk of thyroid hormone as a whole, leading to a hypothyroid state. Reduction in action of TPO also prevents the incorporation of iodine into Tg. Individuals with disorders of thyroglobulin synthesis are moderately to severely hypothyroid and cigarette smoke containing SCN also causes decrease in Tg levels.[16] Exogeneous iodide is also known to increase Tg expressions.[17] Pendrin (PDS) which is localized at the apical pole of thyrocytes, in contrast to the basolateral membrane located sodium iodide symporter (NIS); is associated with iodide efflux.[18] The expression of PDS was also decreased dose-dependently when exposed to bamboo shoot extract and such decrease can also be associated with impaired organification. PDS expressions are found to decrease in hypothyroidism as well as thyroid carcinomas associated with impaired iodide organification.[19] A decrease in protein expression of these key regulatory proteins in the thyrocytes is a direct reflection of disruption of thyroid hormone biosynthesis at the cellular level and requires further studies at the transcriptional levels and a look through into the mechanisms that likely causes these disruptions.

Bamboo shoots on expressions of genes on thyroid hormone synthesizing regulatory enzymes and elements at molecular level

Bamboo shoots extract has diminished the expressions of sodium iodide symporter (NIS), thyroid peroxidase (TPO), thyroglobulin (Tg) and pendrin (PDS) of thyrocytes[8] these effects are often mediated on genes both at the transcriptional and translational levels. The determination of mRNA levels of these regulatory elements thus seemed imperative in this context. NIS-mediated iodine uptake initiates the initial step in the biosynthesis of the iodine-containing hormones in vertebrates T_3 and T_4. The study of NIS is of great importance in thyroid pathophysiology.[20]

The mRNA levels of these specific genes were analyzed by real-time quantitative PCR (RT-qPCR) since the quantity of mRNA transcript for a single gene represents how much transcription of that gene has occurred.[20] NIS, TPO, Tg and transcriptional regulator PAX8 expression levels were also studied along with these. MCT8, a thyroid hormone transporter at the basolateral site, was also evaluated at the mRNA level. The expression of NIS decreased significantly ($p< 0.05$). Tg is the major protein of the thyroidal colloid unique to the thyroid follicular lumen and the component required for synthesis of T_3 and T_4 as it is an indispensable substrate for organification and coupling of the iodinated tyrosine molecules.[21] Being the thyroid hormone matrix, the decrease in this protein indicates a reduction in the bulk of thyroid hormone synthesis, leading to a hypothyroid state.[22] Decreases in NIS, TPO and Tg have often been seen together in *in-vitro* models of hypothyroidism[23] and are associated with the development of goitre in the long run as reported with the consumption of bamboo shoots.[7] A decrease in Tg and TPO levels at both transcriptional and translational level[8] therefore indicates an impairment of organification at both these stages.

Pendrin levels decreased significantly ($p <0.05$) only under the highest dose of bamboo shoot extract as used in the study. The role of PDS itself as the iodide transporter at the apical membrane has taken long to be established and few studies have investigated the impacts on the identical.[24] However, bamboo shoots extract modulates PDS levels more significantly at the translational level, this has been indicated from the changes as found in protein and mRNA expression.[8] Nutritional goitrogens are known to reduce PDS as has been reported.[25]

Deficient in monocarboxylate transporter 8 (MCT8) of human and mice have low serum thyroxine (T_4) levels.[26] In our study there was a decrease in deiodinase enzyme activity, signifying the availability of lesser biologically active hormone and in association with a decreased MCT8 level the transport is also depressed. Interruption in MCT8 levels causes abnormal serum thyroid statures.[27] Thiocyanate, a constituent of bamboo shoots extracts and thioamides, is known to interrupt MCT8 and subsidizes low serum thyroid hormone levels.[28] PAX8 expression declined significantly ($p<0.05$) with each increasing doses of bamboo shoot extract.[8] PAX8 comprises a highly preserved 128 amino acid paired domain and displays the expression of Tg, TPO and NIS binding to their promoter regions. The decrease in NIS, TPO, Tg mRNA expression might also be correlated to the associated reduction of PAX8 expression at mRNA level, as it is a regulator implicated in the transcription of these key factors.[29,30]

It may thus be concluded that bamboo shoots extract causes distraction of the thyroid hormone synthesizing

regulatory factors at both the transcriptional and translational levels along with PAX8, a transcriptional regulator of this biosynthetic pathway. Supplementation of iodine bettered the impact of bamboo shoots extract on all these key regulatory elements.[8] Defensibly, iodine being an important component at all the stages of thyroid hormone biosynthesis, when supplemented exogenously enhanced the expressions of the regulatory elements. Bamboo shoots extract thus has an influence on thyroid autoregulation as well, which in company of additional iodine is modified to a degree. Cassava, sweet potatoes and bamboo shoots of Cruciferous origin contain thioglucosides that are metabolized to thiocyanate. These vegetables consequently inhibit iodide transport and the assimilation of iodide into thyroglobulin, leading to an increase in TSH secretion and thyroid cell proliferation. Based on animal experimentation, it has also been observed that these substances can induce thyroid carcinomas.[31] Henceforth, extra iodine can provide more competition to thiocyanate at entry into thyrocytes and the subsequent steps of thyroid hormone synthesis.[8]

Bamboo shoots in the generation of reactive oxygen species in thyrocytes

Bamboo shoots cause interruption in enzyme activities, protein and mRNA expression of major thyroid hormone synthesizing regulatory elements.[8] To find out the possibility how such alterations in the thyrocytes cellular environment might occur is of prime importance for such disruptive changes. Cyanogenic constituents of bamboo shoots generate cyanogenic glycosideds and thioglucosides-mediated reactive oxygen species (ROS).[32] Improper processing of these plant foods brings about severe unpleasant effects on consumption.[6] Reactive oxygen species that generate inherently in the form of hydrogen peroxides (H_2O_2) is obligatory for covalent incorporation of iodide into thyroglobulin molecules through TPO.[33] The mechanism of producing H_2O_2 in thyroid cells is the combination of dual oxidase 2 (DUOX2) and its maturation factor, DUOXA2.[34] TPO gene and protein expression decreased along with NIS expressions, thus the level of ROS in such environment remains an interesting point of study; the concept being that an alteration in the production of ROS through H_2O_2 might be responsible for such inhibitory changes. Besides, sustained production and exposure to H_2O_2 has been postulated to cause increased somatic mutations in the thyroid gland that lead to hypothyroidism, autoimmune diseases and even thyroid carcinomas.[35] Propylthiouracil (PTU) and methimazole (MMI) have been used for decades in the treatment of patients with hyperthyroidism. The two drugs act as reducing agents and inhibit thyroid hormone production. These are thus labelled as TPO inhibitors. These drugs inhibit TPO-H_2O_2 reaction, but not the TPO itself.[36] Therefore, it may be the major mechanism of antithyroid action of methimazole and such other synthetic drugs however, the mechanism of dietary goitrogens is still not clear. With this perception the effect of bamboo shoots extracts on ROS and H_2O_2 generation has been evaluated in this study.

ROS levels are found to increase duration and dose-dependently after exposure to the bamboo shoots extract. Augmented ROS level in thyrocytes for distressed oxidant status has been associated with diminished PAX8 expression and downstream NIS regulation.[29] Augmented ROS levels also act negatively with TPO, reducing its expression at cellular and mRNA levels, resulting in less thyroid hormone synthesis.[37] Increased ROS levels on administration of synthetic antithyroid drugs like methimazole has previously been reported, correspondingly, the association between increased ROS and hypothyroidism/goitre has been recognized. Further, ROS other than H_2O_2, such as $OH^{\cdot}$ and $O^{2\cdot}-$, can be liberated during thyroid hormone synthesis, though not in significant amounts.[35] The produced ROS does not cause adverse effect on the thyrocytes, but at the same time upsurge the level of basal DNA damage in those cells, that in turn can augment the spontaneous mutation rate with functional interruption of thyrocytes as well tumoral development.[38,39] In practice, toxicity due to ROS leakage from thyrocytes during thyroid hormone synthesis is reserved under strict control through intracellular antioxidant defence systems, including catalases, superoxide dismutase, peroxiredoxins, and glutathione peroxidases[40] that have been assessed later in the study. The generation of H_2O_2 is an intrinsic requirement for universal thyroid hormone synthesis or in other words the normal physiology of the thyroid cell necessitates the generation of H_2O_2 by DUOXs and not O^2- as for other nitrogen oxides (NOXs).[41] As observed in this study, increased production likely caused inhibition in TPO activity. In addition, increased production of H_2O_2 leads to increased oxidative damage of the structure and function of TPO.[41] Consequently, the damage of TPO might increase H_2O_2 generation by weakening its inactivation and permitting an amplified generation in response to thyroid stimulating hormone (TSH). This defect reportedly leads to congenital hypothyroidism and goitre, as well as possibly lead to severe thyroid diseases and cancer.[42] ROS facilitates a variety of signal transduction pathways,[43] a recent study indicates that ROS levels comparable to the physiological oxidative load of the cell stands obligatory for preservation of the function and integrity of the thyroid.[40] The oxidative load, however, was found higher than basal conditions in impaired conditions, ensuing in oxidative stress as observed in goitrous thyroids, possibly because of increased H_2O_2 production or impaired intake as iodide is deficient and/or when the function of TPO has been decreased as perceived in this study.[41] All these observations support a role of H_2O_2 during severe occurrence of thyroid disorders and thyroid tumors including microcarcinomas, predominantly under conditions of antioxidant deficiency.[44,45] This is also consistent with a study that exhibited high rate of mutations in the thyroid due to constitutively elevated H_2O_2 levels[46] and

with other studies showing that large quantities of H_2O_2 cause DNA damage in the form of DNA double-strand and single-strand breaks in thyroid follicular cells.[47] In consistent with these, inconsistencies in intracellular redox systems also have association to the molecular mechanisms of goitrogenesis and auto immune diseases. Iodine plays a prime role in the regulation of H_2O_2 generation, by regulating DUOX activity in a dual manner: at low concentrations, it is stimulatory, which favours well-organized iodination of Tg tyrosyl residues;[48] however, when it is present in excess, H_2O_2 formation is momentarily blocked to protect the organism from adverse effects.[49] In accordance, in this study, supply of extra iodine has diminished the H_2O_2 and ROS production. As well as extra supplemented iodine has improved the functions of NIS, TPO and Tg for better functionality than in impaired state and also maintained the basal oxidant state of the thyrocytes by reducing ROS and H_2O_2 levels.[50]

Bamboo shoots on cellular changes including antioxidant status, lipid peroxidation and nitric oxide levels

In addition to elevated H_2O_2 production in thyrocytes after exposure of bamboo shoots extract, GPx, CAT, GSH and SOD activities were diminished dose dependently. GPx and CAT are the major defences against the deleterious effects of ROS in cells. CAT and GPx have the capacity to reduce the effect of H_2O_2 in cultured thyroid cells.[42] Reduced activities of GPx and CAT as found were for high H_2O_2-induced apoptosis of thyroid cells in *in vitro* study.[51] The high oxidative state that develops in the thyrocytes for pro-oxidant- antioxidant imbalance causes damage to these cells. Activity of SOD which is the first line of enzymatic defence against intracellular free radicals[46] was also decreased dose dependently, that subsequently expose the cell membrane and other organelles to oxidative damage. Although under normal conditions, the protective effect of thyroid hormone against oxidative stress can be explained by the function of antioxidants as a defence system in a chronic state of hypothyroidism which is characterized by derailment in the redox potential. Destabilized redox potential in the thyroid environment led to free radical chain reactions and to depression on antioxidant capacity.[46] Therefore, the increase of free radicals is not compensated, as anticipated, by a reduction of antioxidants. An elevated oxidative state in hypothyroid condition has metabolic and biochemical features as found in increased mitochondrial enzyme activity.[52] It is thus plausible that the cells are injured by prolonged oxidative stress that depresses the capability of the organs to synthesize antioxidant molecules or to produce them from extra cellular sources.[53] An elevated LPO level as seen in this study has been previously associated with subclinical hypothyroidism High levels of lipid peroxidation is found in hypothyroidism.[54] Antioxidant enzyme levels are decreased in hypothyroid stage as reported earlier found in present study. An elevated LPO level under the influence of bamboo shoots extract as found might be related with decrease of TPO expression as stated earlier.[55] There are reports that NO donors repressed NIS gene expression by reducing the transcriptional activity of the nuclear factor-KB subunit p65.NO is a recognized inhibitor of I- organification in primary cultures of human and bovine thyroid follicular cells.[56] NO controls Forkhead factor FoxE1 expression that in turn causes inhibition of TPO levels.[57] Therefore, an increase in NO as observed under the influence of bamboo shoots extract is associated with inhibition of NIS and TPO expressions both at protein and mRNA levels. Altered pro-oxidant antioxidant status through increased ROS and H_2O_2 in the thyrocyte environment linked with augmented lipid peroxidation and NO levels under the influence of bamboo shoots extract brings about changes as found in the thyroid hormone synthesizing regulatory factors.[50]

Bamboo shoots on lactate dehydrogenase levels, DNA damage and DNA oxidation

The dose dependent increase in LDH release on exposure of bamboo shoots extract causes the disruption of the plasma membrane and subcellular organelles of thyrocytes; earlier studies reported that the interruption of those organelles in thyrocytes often associated with hypothyroidism.[58] At the basal membrane of thyrocytes, both NIS and Na^+-K^+- ATPase are located, henceforth the disruptions of this membranes are likely to cause interruptions of their function as observed in this study.[8] While TPO is located in the outer apical membrane of thyrocytes and its functions are facilitated in the presence of H_2O_2, this membrane's disruptions affect the TPO-H_2O_2 system, which in turn lead to perturbations of thyroid hormone synthesis through altered TPO function and H_2O_2 production.[41] Pendrin on the other hand is the apical membrane transporter that decreases its expression suggests membrane-oriented alterations in its function. Lipid peroxidation levels also augmented after the exposure of bamboo shoots extract, demonstrating its degradation of membrane lipids and changes in the membrane that affects the membrane bound thyroid hormone synthesizing regulatory factors. A dose dependent increase in DNA damage has also been noted after exposure of bamboo shoots extract.[8] Considerable explanations are existing for thiocyanate, which is recognized to cause DNA damage in considerable concentrations by acting as an intermediate in the damage reactions.[59] In addition, the changed oxidant state with the increase in H_2O_2 is also conducive for DNA damage. Detrimental influences of DNA impairment on NIS and TPO functions are also acknowledged. More than a few selenoproteins act as a defensive barrier for thyrocytes from endogenous H_2O_2,[60] in this scenario, the activity of deiodinase, a seleno-enzyme was also decreased concomitantly. DNA damage that arises can change the configuration of the thyroid hormone synthesizing regulatory factors that lead to the decrease in their expression and self to non-self-recognition as found in autoimmunity.[61] The levels of DNA oxidation were also augmented dose dependently which is

comparable to that of previously observed DNA damage.[62] Increased in ROS and DNA damage goes hand in hand to alter oxidant state of cells that has been observed under the influence of bamboo shoots extract. Supplementation of iodine improves the impairment levels partially, apparently because on supplementation of extra iodine there is improvement of the oxidant status and functionality of the thyroid hormone synthesizing regulatory factors and availability of iodine at each step of the hormone synthesis. The study exposes that the increase in ROS, changed pro-oxidant-antioxidant status, increase in cellular LDH levels and DNA damage and oxidation are all co-related in instigating cellular and molecular changes to the thyroid hormone synthesizing regulatory factors both at the transcriptional and translational levels.[50] All these factors, therefore, in turn cause interference in thyroid hormone biosynthesis.

Bamboo shoots on cell cycle of thyrocytes

The cell cycle is a sequence of well organised events involving cell growth and cell division leading to production of two new daughter cells. Cells on the way to cell division proceed through a sequence of specifically timed and cautiously planned growth, DNA replication, and division phases to yield to clone cells.[63] These phases revealed sum up the two major stages of the cell cycle – Interphase and the actual mitosis. Interphase involves the G1, S and G2 phases with a quiescent G_0 stage. DNA replication is restricted to S phase; G1 is the gap between M phase and S phase, while G2 is the gap between S phase and M phase. These phases hence have varying DNA contents and bears incredible research implication. Mitosis on the other side consists of four-step process (prophase, metaphase, anaphase, telophase) that have consequences in cell division however, normal DNA content.[64] Quantifying DNA with the help of flow cytometer and dyes supports distinguishing stages of the cell cycle and determining the lengths of G1, S, and G2 + M phases. Following a population of cells that have been preselected to be in one specific phase of the cell cycle, DNA content quantities on such a synchronized population of cells discloses how the cells progress through the cycle. The diverse phases of the cell cycle are affected by several issues. This is significant for normal physiology of the cells.[65] Bamboo shoots extract changed the regular pattern of thyrocytes' cell cycle as perceived in our study.[50] The substantial increase in the sub G1 phase cell percentage is indicative of DNA fragmentation and impairment due to apoptosis.[66] G0/G1 phase continued intact from that of control in initial dose and improved to almost control percent after the addition of iodine, demonstrating that cell proliferation had not stopped. In S phase and G2/M phase cell percentage had reduced demonstrating the imbalance between the cell death and proliferation that could be for disruptions in replication or mitosis.[67] The flowcytometry results as noticed are in corroboration with earlier findings and further specified increased DNA fragmentation and disparity in cell death and proliferation of the thyrocytes after the exposure of bamboo shoots extract.[50] Earlier studies on evaluation the cell cycle of thyrocytes is quite scanty thus making it difficult to compare and support the results thus obtained.

Bamboo shoots on certain apoptotic proteins (Bax, Bcl-2 and p53)

Regulation of apoptosis is the crucial link in the pathogenesis of many diseases that includes thyroid disorders, autoimmunity and others.[68] The levels of pro-apoptotic Bax and antiapoptotic Bcl-2 proteins in thyroid cells were evaluated along with p53 level to comprehend the apoptotic regulation induced by bamboo shoots extract on thyrocytes.[50] An equilibrium between cell death and proliferation is decisive for goitre formation that can lead to thyroid autoimmunity in the long run.[69] An imbalance at this point due to DNA damage and disturbed oxidant status might swing the cellular equilibrium towards pathogenesis, including reduced synthesis of thyroid hormone, goitre involution and autoimmune thyroid disorders. In our study, Bax levels were augmented dose dependently, while Bcl-2 levels were diminished markedly and p53 increased subsequently. All these findings are in contour with that of DNA oxidation and damage along with altered redox potential of the cells.[70] The results designate that the thyrocytes might have lost their normal apoptotic control after the exposure of bamboo shoots extract. This loss in apoptotic control results in cellular death and proliferation imbalance as apoptosis.

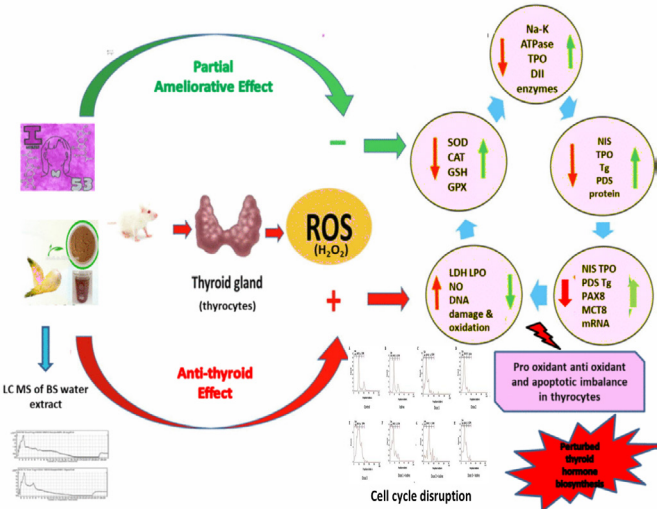
The close associations of proliferations and apoptosis can be bi-directional events and indeed apoptosis is non-automatously induced by proliferating cells.[71] Hyperplasia of thyroid follicular cells due to cellular proliferation is considered as symbol of hypothyroidism and goitre development. The hyperplasia observed in such conditions is likely apoptosis induced hyperplasia, which takes place in such a competitive cellular interaction that the slow-growing cells are eliminated by fast growing cells.[72] In a study, a significant association was found between tissue features of Graves' disease (epithelial hyperplasia, cellular hypertrophy, and colloid content) and the number of apoptotic cells. In a transitional group of cells in the same study there was characterized tissue heterogeneity with hyperactive foci, rich in apoptosis, alternating with regressive areas lacking apoptosis suggesting that even in hyperplastic growth of hypothyroidism/goitre there is apoptotic cells. In this group the participation of apoptosis to the remodelling of thyroid parenchyma in goitrogens, in a tight balance with cell proliferation, has been best defined. Also, the thyroid follicle by assembling apoptotic cells and bodies permitted an uncertain consecutive ordering of apoptosis steps in correlation with Bax-Bcl-2 tissue distribution along with cellular pattern.[73] The observations as noted propose that the commencement of apoptosis resembles to damage to cellular cohesion, a drop in Bcl-2 expression and a delocalization of Bax from a supposed golgi storage location leading to upsurge in its expression.[70] Similar

changes have been observed in thyrocytes on exposure to bamboo shoots extract. It has also been reported that apoptosis is a very rapid and limited process in intact follicles making it a significant delimited process in thyroid diseases especially in goitre involution and auto immunity. Our recent study has concluded that the grade-2 goitrous population of north east India, particularly children and women, have different thyroid diseases including autoimmune thyroid disorders, which exist significantly.[74] This has been attributed to excess thiocyanate ingestion from dietary goitrogens of cyanogenic origin such as in bamboo shoots, which is commonly consumed as has been mentioned repetitively here. It has been observed that this might be responsible for the occurrence of goitre followed by its progression towards different thyroid /auto immune diseases.[74] The results viewed in present study associate strongly with this report, suggesting that an imbalance in apoptotic control of thyrocytes under influence of bamboo shoot extract can lead to thyroid disorders including autoimmunity.

Presence of iodine better the situations by shifting this imbalance towards a more balanced state due to its close connotation with regulatory factors at each step of thyroid hormone synthesis.[50] Addition of iodine to diet has been considered to be the demanding method for prevention of thyroid diseases, however, several epidemiological studies now show that neither is iodine deficiency singularly the cause of these as well as iodine supplementation does not provide complete amelioration of these.[75,76] Multiple studies on how iodine in excess can also disorient this thin line of balance have been reported.[77,78] These observations were also consistent with those obtained from cell cycle analysis where the imbalance in the early cell death and proliferation and their self-interactions has been observed conceivably. Therefore, addition of iodine partially improved the apoptotic balance as observed but could not restore the thyrocytes to their control status.

Conclusion

Bamboo shoots (*Bambusa Balcooa Roxbo*t) water extract showed the presence of precursors and metabolic end products of cyanogenic glycosides on phytochemical analysis. The extract containing goitrogenic/anti-thyroidal biomolecules has pleiotropic effects on the thyrocytes. It repressed the activity of Na^+-K^+-ATPase, TPO and D2, as well as reduced the expression of NIS, TPO, Tg, PDS proteins along with MCT8 and PAX8, at the mRNA level. All these effects of the extract are mediated through increased ROS levels as H_2O_2 upsurge leading to a perturbed pro-oxidant antioxidant balance in the thyrocytes causing associated cellular and DNA damage and oxidation. Increased LPO and NO levels upon exposure of bamboo shoots extract caused plasma membrane and organelle damage of thyrocytes, which are the major sites of NIS, TPO, PDS and MCT8 leading to thyroid dysfunction. Cellular and DNA damage/oxidation was observed that leads to disruption of normal cell cycle pattern



Source: Sarkar D (2021) Cellular and molecular alteration of certatin thyroid hormone synthesizing regulatory elements under the influence of dietary goitrogens in bamboo shoots. Ph.D. Thesis, University of Calcutta.

Figure 1: Mechanism of disruption of thyroid hormone biosynthesis at cellular and molecular level.

of thyrocytes which in turn causes changes in apoptotic proteins Bax, Bcl-2 and p53. These changes imply imbalance of cell death and proliferation equilibrium leading to disease conditions upon exposure to bamboo shoot extract. Extra iodine prevents the goitrogenic/ antithyroidal influences of bamboo shoots on thyroid function to an extent only.

The study provides details to predict mode of action of bamboo shoots and similar natural goitrogens with analogous anti-thyroid compounds. The investigation has been performed in cultured thyroid follicular cells; which adds to the precision of perceptions regarding long standing public health disorders like endemic goitres and associated disorders during post salt iodization in north eastern states and several other goitre endemic regions of the country as well as several other countries in the eastern region of the world where bamboo shoots is consumed as a common food. The role of dietary goitrogens as a factor in mechanism of development at cellular and molecular as well as persistence of residual goitre in the effective post iodization phase has thus been explained on the basis of proper scientific investigation and available information (Figure 1).

DEVELOPMENT OF ASSOCIATED THYROID DISORDERS IN RESIDUAL GOITROUS POPULATION

There are studies in India recalling the existence of mild goitre endemicity in many areas years after salt iodization.[79] The goitres that persist in a population in an area in spite adequate iodine intake even after effective salt iodisation are termed as residual goitre. The aim of this investigation was to commence a comprehensive study analysing functional status by thyroid hormones and TSH profile, serum thyroid antibodies, ultrasonographic features as well as fine needle

aspiration (FNAC) of the thyroid gland in the residual goitrous population of Andro constituency in Manipur of north east India; where salt iodization programme was introduced since 1988 and is still continued. In Andro, bamboo grows abundantly and people often consume its young shoots (raw/fermented) having anti-thyroidal activity. The findings as obtained of goitrous population, the progression of goitrous subjects towards further thyroid diseases in this environmental background has been worked out.

Goitre prevalence

In this study as observed the goitre prevalence in school children was 12.59% (grade 1: 10.78, grade 2: 1.81) while that in women was 16.27% (grade 1: 6.84 and grade 2: 9.43), however, in overall population total goitre rate (TGR) was 13.97% (grade 1: 9.29 and grade 2: 4.68) ensuing palpation method. Consequently, the clinical severity of IDDs has been shifted from severe to mild as public health problem from our previous study.[7] This study further elicited that the prevalence rate was found more in women of reproductive age group than the children. The total number of grade-2 goitrous population in the study region was 187 about 4.68% of total population initially considered for thyroid enlargement; children were 45 (1.81%) and women were 142 (9.43%). The goitrous grade- 2 subjects were examined only in the study to evade misunderstanding on thyroid size of grade-1 goitre that differs from investigator to investigator as it is based on investigators' conventions. Preceding to further investigations on goitrous grade-2 subjects, the state of iodine nutrition of the overall population in the studied areas have been inspected.

Iodine nutritional status

To evaluate the biochemical status of iodine nutrition of the populations, urinary iodine excretion pattern was studied as it is used as a valuable marker for the assessment of biochemical iodine deficiency, because 90% of body's iodine is excreted through urine.[84] In all 160 urine samples collected randomly from the children of 4 (four) different areas and analysed; median urinary iodine (MUI) was 166 ug/L (ranging MUI from 124 to 192 µg/dL in study areas), in overall 22.15% samples it was less than 100 µg/L but more than 50 µg/L and in 6.96% samples it was less than 50 µg/L. The results presented no biochemical iodine deficiency or no inadequacy in iodine intake in the studied population.[85] The people of Andro constituency were thus clinically mild iodine deficient considering the existing goitre prevalence but biochemically there was no iodine deficiency based on iodine excretory pattern.

Salt iodization status

To study the bio-availability of iodine, edible salt samples were collected from house-holds and iodine content was measured and found that all the samples contained iodine more than 30 ppm and no salt samples had iodine less than that of recommended level, or in other words effective salt iodization programme is ubiquitous in the area.[85] Therefore, the endemic goitre that persisted in spite of adequate iodine intake even after effective salt iodization are residual goitre.

Consumption of cyanogenic plant food (bamboo shoots)

Residual goitre was found prevalent in the area in spite of the intake of adequate iodine through iodized salt, therefore to explore the interference of common dietary goitrogen(s) from cyanogenic plant food, that are often consumed by the people in the region, thiocyanate (SCN), the metabolic end product of cyanogenic plant food was measured in urine samples that have been collected for the analysis of iodine. It was noted that the mean thiocyanate level was more than the level as found in non-goitre endemic areas of India 0.728 mg/dL against 0.640 mg/dL; thiocyanate that originates mainly from bamboo shoots has role in goitrogenesis in the region.[7] In the studied population the urinary excretion of iodine and thiocyanate are positively correlated. Recent *in-vitro* studies on thyrocytes reported from our laboratory as mentioned in the preceding section showed that antithyroid /goitrogenic biomolecules of bamboo shoots extract suppressed the activities of major thyroid hormone synthesizing enzymes and regulatory factors as well as suppressed the expression of sodium iodide symporter (NIS), thyroid peroxidase (TPO), PAX8, pendrin, thyroglobulin, MCT8 at mRNA level. All these effects of bamboo shoots extract are translated through the generation of increased ROS that disturb the pro-oxidant and antioxidant equilibrium in thyroid follicular cells causing cellular and DNA damage.[8,50] Free radicals induced damage of thyroid tissue is often associated with thyroid autoimmunity.[86] All these findings directed us to recommend that thiocyanate (SCN-), the metabolic end product of bamboo shoots may develop autoimmune thyroiditis.

Ultrasonography (USG) of thyroid in goitrous population

To evaluate thyroid diseases, ultrasonography (USG) of thyroid is considered an important tool in adults because diminished echogenecity in USG is evidenced to be responsible for autoimmune thyroiditis in adult; however, in children the sensitivity of echogenecity in the diagnosis of autoimmune thyroiditis is less.[87] In this investigation USG conducted on school children (45) and women (140) who had grade-2 residual goitre and found that sixty percent of school children and ninety-five percent of women having grade-2 residual goitre were hypoechoic indicating their vulnerability for autoimmune thyroiditis. Serum thyroid hormone profiles and TSH levels, TPO and TG antibodies were analysed in all the grade- 2 residual goitrous children and women to comprehend their thyroid functional status. Based on results, the thyroid functional status of the studied subjects was grouped into subclinical- and overt hypothyroid and hyperthyroid. It is relevant to indicate here that there is no indication of autoimmunity in those who are affected by endemic goitre.[88]

Prevalence of hypo- and hyperthyroidism in the goitrous population

The present study was focused only on the goitrous population. However, ultrasonographic findings are important in determining the prognosis of mild subclinical hypothyroidism.[89] Our study showed that goitrous grade-2 individuals were hypoechoic. These findings further reveal that there is the chance for the occurrence of more overt hypothyroidism in goitrous grade 2 subjects. In consistence with the above study, the risk of development of thyroid autoimmunity and mild hypothyroidism was found in young people after voluntary iodine prophylaxis though there was decrease in goitre prevalence and thyroid autonomy in younger subjects and frequency of non-autoimmune hyperthyroidism, hypothyroidism in older subjects.[90] The present findings showed that in the study region endemic goitre found present though the total goitre rate (TGR) had reduced from the earlier study and the residual goitrous subjects were affected further by thyroiditis, subclinical and overt hypothyroidism though they were not exposed to excess iodine; they were not biochemically iodine deficient as their iodine intake was adequate.

The definition of subclinical hyperthyroidism (SHyper) is based exclusively on laboratory findings, not on clinical criteria.[91] The prevalence of endogenous SHyper varies considerably, between 0.6% and 16%,[92-94] depending on diagnostic criteria and the age and sex of the population studied. In the study area, in overall the prevalence of SHyper was 4.86%, in women it was 6.25% and in children it was 2.22%. Patients with SHyper may have stable thyroid dysfunction which progress to overt hyperthyroidism over time. The degree of TSH suppression is the best parameter for predicting the progression from subclinical to overt hyperthyroidism.[95,96] In the present study, TSH level of about 11% goitrous subjects was low and about 17% had high, but they were not exposed to iodine deficiency as effective salt iodization program was in operation. Both higher and lower TSH levels predict an increased risk of subsequent thyroid dysfunction.[97] Therefore, as observed in this study, those with altered TSH levels (low or high) were affected by subclinical or overt hypo- and hyperthyroidism.

TPO-Ab or Tg-Ab antibodies in goitrous population

In the overall goitrous grade-2 population 16.21% were subclinically hypothyroid and 2.16% were overt hypothyroid while in overall 17.29% (children 2.22 and women 22.14) had TSH level more than normal or TSH level was deranged. Anti-TPO antibodies are related to levels of stimulating thyroid hormone (TSH) and both alone or in combination have been used to predict development of hypo/hyperthyroidism. It has been reported that altered levels of anti-thyroid antibodies and TSH in euthyroid subjects is associated with the development of hypothyroidism in future.[98,99] In the study, the 41.62% population (children 8.88 and women 52.14%) have both anti TPO and anti-Tg antibodies positive.

Both more than adequate and excessive levels of iodine intake may increase the incidence and prevalence of autoimmune thyroiditis in humans. Autoimmune thyroiditis appeared to be the main cause of overt hypothyroidism. In addition, among subjects who had high levels of TPO-Ab or Tg-Ab at base line, the rate of progression to hypothyroidism correlated directly with iodine intake. Therefore, both more than adequate levels and excessive levels of iodine intake may drive thyroid function from a state of potential autoimmune impairment to overt hypothyroidism.[97] In the study, the median urinary iodine levels were adequate; however, the mean urinary thiocyanate level was higher in the population of non-goitrous areas in India. Iodine in excess has the potentiality for the development of thyroid autoantibodies [100], however no information is available on excess thiocyanate induced thyroid autoimmunity/generation of thyroid antibodies. In a recent study it has been reported from our laboratory, thiocyanate that arises from bamboo shoots cause cellular damage of thyrocytes producing free radicals *in-vitro* studies[8] enhancing the possibility of thyroiditis and generation of thyroid antibodies. Thiocyanate have the same molecular size and ionic charge that of iodine and thus has the possibility to bind with thyroglobulin and thyroid peroxidase that could change their stereochemical configuration leading to change their immunoreactivity and generation of thyroid autoantibodies.

Cytomorphological studies (FNAC) of thyroid in goitrous population

Thyroid functional status of goitrous (grade-2) subjects was examined based on thyroid hormones and TSH profiles, TPO-Ab and Tg-Ab as well as USG. To understand the histological/cytological features of goitrous thyroid further FNAC test was conducted in all the goitrous subjects following the method of Orell and Sterret (2014).[101]

Thyroid status of grade-2 goitrous subjects was interpreted in terms of cytomorphological studies (FNAC) with respective thyroid diseases (Table 1). The prevalence of colloid goitre was highest, followed by lymphocytic thyroiditis, Hashimoto's thyroiditis, subacute thyroiditis and other types viz. autoimmune thyroiditis, papillary carcinoma, medullary carcinoma, simple diffused hyperplasia, adenomoid nodular goitre in equal proportion. The FNAC results revealed that the goitrous populations are affected by thyroiditis of different types. Thyroiditis is caused by an attack on the thyroid, causing inflammation and damage to the thyroid cells. Antibodies that attack the thyroid cause most types of thyroiditis. As such, thyroiditis is often an autoimmune disease. In the study, overall, 41.62% goitrous grade-2 population (children 8.88 and women 52.14%) have both anti-TPO and anti-Tg antibodies positive.

Table 1: Occurrence of thyroid status following FNAC of residual goitrous children (45) and women (140) in Andro Constituency, Manipur.[74]

Thyroid status	*Children [45]*	*Women [140]*	*Total [185]*
Colloid goitre	22 (48.88%)	54 (38.57%)	76 (41.08%)
Lymphocytic thyroiditis	23 (51.11%)	47 (33.57%)	70 (37.83%)
Hashimoto s thyroiditis	–	15 (10.71%)	15 (8.10%)
Autoimmune thyroiditis	–	8 (5.71%)	8 (4.32%)
Papillary carcinoma	–	3 (2.14%)	3 (1.62%)
Medullary carcinoma	–	3 (2.14%)	3 (1.62%)
Simple diffused hyperplasia	–	3 (2.14%)	3 (1.62%)
Adenomoid nodular goiter	–	3 (2.14%)	3 (1.62%)
Sub-acute thyroiditis	–	4 (2.85%)	4 (2.16%)

(Values in parentheses are percentages)

Conclusion

This study reveals that in spite of adequate iodine intake endemic goitre persists. Further investigation on grade-2 goitrous population showed subclinical and overt hypo- and hyperthyroidism with elevated thyroid auto antibodies, hypoechoic thyroid and thyroiditis found prevalent. In general, there is no evidence of autoimmunity in those affected by endemic goitre.[88] This study concludes that goitrous individuals particularly children and women were affected by different thyroid diseases including autoimmune thyroid disorders. Excess thiocyanate from dietary goitrogens of cyanogenic origin (bamboo shoots) might be responsible for the occurrence of goitre followed by its progression towards different thyroid /autoimmune diseases.

Concluding Remarks

In this study the goitrogenic/antithyroid constituents of bamboo shoots that develops residual goitres exists during post salt iodization period have been analysed phytochemically. The cellular and molecular mechanism by which bamboo shoots extracts disrupt thyroid hormone synthesis in cultured thyroid follicular cells in presence and absence of iodine in the medium in *in vitro* have been evaluated. In consistence with these, further investigation continued in population who are affected by goitre despite their adequate iodine intake but for frequently consuming bamboo shoots. Goitrous population showed subclinical and overt hypo- and hyperthyroidism with elevated thyroid auto antibodies, hypoechoic thyroid and thyroiditis. In general, there is no evidence of autoimmunity in those affected by endemic goitre. This study concludes that goitrous individuals particularly children and women were affected by different thyroid diseases including autoimmune thyroid disorders. Excess thiocyanate from dietary goitrogens of cyanogenic origin (bamboo shoots) might be responsible for the occurrence of residual goitre followed by its progression towards different thyroid /auto immune diseases.

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Conflict of interest

There is no conflict of interest in the study.

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The author is the Founder Fellow of The Physiological Society of India.

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