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Ultrastructural and hormonal modulations of the thyroid gland following arecoline treatment in albino mice

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ABSTRACT

Arecoline is a plant alkaloid of betel nut *Areca catechu*. Arecoline has immunosuppressive, hepatotoxic, mutagenic and teratogenic effects, and disturbs some endocrine organs in rats. The objective is to investigate the untoward effects of arecoline on the thyroid gland in mice. Intraperitoneal injection of arecoline (10 mg/kg body weight only once) increased the serum T_3 and T_4 levels and decreased the serum TSH 20, 40 or 60 min after the treatment, with maximum effect at 40 min. Chronic arecoline treatment (10 mg/kg body weight daily for 15 days) caused light microscopic and ultrastructural degenerations of thyrofollicular cells with depletion of T_3 and T_4 levels followed by the elevation of the TSH level. Atropine (arecoline antagonist) injection prevented the changes (hyperactivity) induced by acute (40 min) arecline treatment. Arecoline initially stimulates thyroid activity, and eventually inhibits the activity; atropine prevents thyroid dysfunction induced by arecoline. Arecoline action is mediated probably via muscarinic cholinergic receptor—hypothalamic–pituitary–thyroid axis in mice.

1. Introduction

Arecoline is a major plant alkaloid of betel nut *Areca catechu* (Bhonsle et al., 1992). Millions of people of South East Asia, Indonesia and East Africa chew betel nut to increase the capacity to work (Pradhan et al., 1986; Marshall, 1987). It has multiple actions in humans and rats. It causes oral and throat cancer if taken 6 betel nuts a day for long (Sullivan, 2000), genotoxicity (Sharan, 1996), hepatotoxicity, disturbance in antioxidant production (Singh et al., 2000), antibacterial activity (Huang et al., 2002), c-jun protooncogene expression in human oral mucosal fibroblasts (Ho et al., 2000), mutagenicity (Kumpawat et al., 2003), and teratogenesis leading to abortion (Garcia-Algar et al., 2005). Arecoline arrests splenic lymphocytic cycle resulting in the immunosuppression and induces hepatoxicity with depression of antioxidants in mice (Dasgupta et al., 2006). Arecoline has some therapeutic value for the treatment of patients with Alzheimer cum presenile dementia (memory enhancing effect) (Mondadore et al., 1994) and schizophrenia (Sullivan, 2000).

Arecoline also causes endocrine and brain dysfunctions. It increases plasma concentration of beta-endorphin immunoreactivity, which is correlated with the increase in plasma prolaction concentration in humans (Risch et al., 1982). It stimulates adrenocortical and adrenomedullary activities in rats (Calogero et al., 1989; Lim and Kim, 2006) and testosterone production (Saha et al., 2007). But in mice arecoline has been reported to cause ultrastructural degeneration of adrenal cortex (unpublished observation). Pineal activity is inhibited by arecoline in rats (Saha et al., 2007). The importance of the current work is to ascertain the untoward effects of arecoline on other endocrine organs, if any, that may be relevant to those who consume betel nut almost daily throughout life. There is no information concerning the action of arecoline on the thyroid gland of vertebrate animals. Neither its mode of action on endocrine organs is known. In the current article, these problems are resolved by investigating the light microscopic, ultrastructural and hormonal changes of thyroid gland in the mouse model. The mode of arecoline action was investigated by using muscarinic receptor inhibitor, because arecoline is known to mediate its action via muscarinic cholinergic receptor (Calogero et al., 1989).

2. Materials and methods

2.1. Animal model

Adult male mice (90 days, ~25 g body weight) were collected from the breeding colony and were kept in polythene cage (30 cm × 15 cm × 15 cm) in controlled laboratory conditions (photoperiod, 12L: 12D, and temperature: 25 °C) with standard diet (Oser, 1965) and water accessible *ad libidum* 5 days for acclimatization and subsequently for experiments.

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2.2. Arecoline administration

Arecoline hydrobromide (Methyl 1-methyl 1,2,5,6-tetrahydronicotinate) (Sigma, U.S.A.), dissolved in physiological saline (0.9% NaCl), was injected intraperitoneally at a dose of 10 mg/kg body weight. Each dose (1 mg/100 g body weight) was divided equally to half (0.5 mg/100 g body weight), and each half dose was injected twice daily at 9 AM and 6 PM because of its short half-life (Pradhan et al., 1986).

2.3. Experimental protocol

Experiments, I, II and III each, were carried out in triplicate, and the data were pooled, averaged and presented as mean of each experiments.

2.3.1. Acute treatment

The objective was to examine the dose and time-dependent actions of arecoline. Thirty-six mice were divided in six groups of six each. Groups I, II and III served as controls which received vehicle (saline) only. Arecoline was injected to the remainder of the groups (Groups IV, V and VI) of animals only once. Experiments were terminated 20, 40 and 60 min, respectively, after the treatments. Each experiment was repeated thrice. Control mice were handled once as handled for the treated mice during arecoline and atropine injections in order to avoid stress due to handling both in the control and treated mice.

2.3.2. Chronic treatment

The objective was to ascertain whether arecoline action in chronic treatment was different from that of the acute treatment. Twelve mice were equally divided in two groups: Group I served as control which received vehicle (saline) only and Group II mice were injected with arecoline in the same dose (10 mg/kg body daily) for 15 days.

2.3.3. Acute atropine + arecoline treatments

The objective was to investigate the mode of action of arecoline by inhibiting its action with an arecoline antagonist, atropine, which is also a muscarinic cholinergic receptor inhibitor, because arecoline is known to act via muscarinic cholinergic receptor (Calogero et al., 1989).

For thyroid study, arecoline was treated for 40 min, because the effect of arecoline was maximum at 40 min, recorded from the experiment I. Eighteen mice were divided in 3 groups (I, II and III) of 6 each. Group I served as control; group II animals were treated with arecoline (10 mg/kg body weight) for 40 min. Group III mice were treated with atropine sulphate at a dose of 0.5 mg/kg body weight for 30 min and subsequently with arecoline (10 mg/kg body weight) for 40 min (total: 70 min).

2.4. Animal autopsy, tissue and blood collection

Animal experiments were carried out following the 'Principles of Laboratory Animal Care' (NIH publication No. 85-23, revised in 1985) and Indian Laws of Animal protection (Registration No. 885/ac/05/PCSEA). All experimental mice were anaesthetised by sodium barbital injection. For acute experiment (I), blood was drawn from heart, and serum was collected and stored at −20 °C until assayed for hormones. Thyroid gland was studied by TEM only in chronic experiment (II). Thyroid glands were dissected free and processed for TEM study. Other parameters (serum hormones) were same as in experiment I. Parameters of experiment III were also same as in experiment I.

2.5. Light microscopy

Thyroid gland was fixed in Bouins Fluid for 24 h and processed for routine microtomy. 5 μm thick paraffin sections were stained by haematoxylin and eosin and were examined under light microscope.

2.6. Transmission electron microscopy

Thyroid glands were dissected free, cut into small pieces (1 mm × 1 mm) and fixed by immersion in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 6–8 h at 4 °C. After washing in the buffer, the tissue samples were post-fixed in 1% osmium tetroxide in the same buffer for 2 h at 4 °C. Tissues were dehydrated through ascending grades of ethanol, infiltrated and embedded in araldite CY 212. Thick sections (1 μm) were cut, stained with toluidine blue and observed under a light microscope. Thin sections (60–80 nm) were contrasted with uranyl acetate and alkaline lead citrate, and viewed under a Morgagni 268D transmission electron microscope (Fei Company, The Netherlands) at an operating voltage of 80 KV.

2.7. Hormone estimations

Serum TSH (Soos and Siddle, 1982), T_3 (Walker, 1977) and T_4 (Schurrs and Van Weeman, 1977) levels were quantitated by ELISA.

TSH, total triiodothyronine (T_3) and total thyroxine (T_4) were quantitated by EIA kit (PATHOZYME-TSH, T_3 and T_4) of OMEGA, UK (Product No. OD387).

Specific anti-TSH antibodies were coated onto microtitration wells. Test sera were applied. Then Goat anti-TSH labelled with horseradish Peroxidase (Conjugate) was added. After incubation, the wells were washed with wash buffer to remove the unbound labelled antibodies. On addition of the substrate, tetramethyl benzidine (TMB), a colour developed only in those wells in which the enzyme conjugate was present, indicating the presence of TSH. The enzyme reaction was stopped by the addition of dilute sulphuric acid and the absorbance was measured at 450 nm.

T_3 and T_4 specific Goat anti-Mouse IgG antibodies were coated onto microtitration wells. Test sera were applied along with antibody reagent coating T_3 and T_4 to bind to the wells. T_3 and T_4 horseradish Peroxidase enzyme conjugates were added which competed with the released serum T_3 and T_4 for available binding sites on the solid phase. After incubation, the wells were washed with wash buffer to remove any unbound T_3/T_4 or T_3/T_4 enzyme conjugate. On addition of the substrate tetramethyl benzidine (TMB), a colour was developed only in those wells in which the enzyme was present, indicating a lack of serum $T_3//T_4$. The reaction was stopped by the addition of dilute sulphuric acid and the absorbance was measured at 450 nm.

2.8. Statistical analysis

Data were analyzed statistically by one way analysis of variance (ANOVA) followed by post-hoc "*t*" test (Snedecor, 1971). Data were presented as mean ± S.E.M. and *P*-value <0.01 was considered statistically significant.

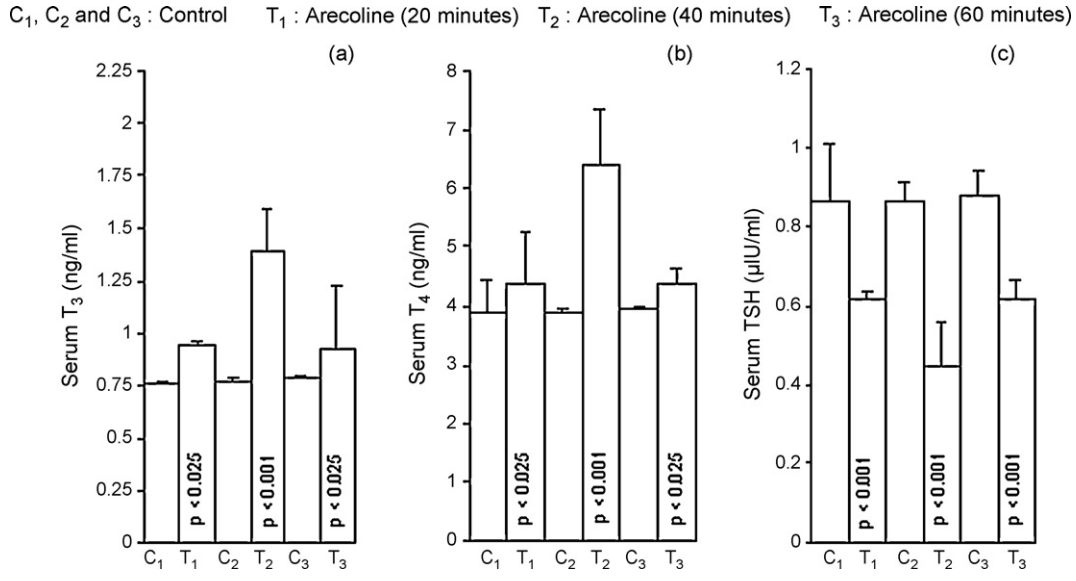

Fig. 1. Arecoline treatment showing elevations of serum T_3 (a) and T_4 (b) levels with depletion of TSH (c) 20, 40 and 60 min with a maximum effect at 40 min after the treatments in mice. (ANOVA, $p < 0.001$ considered significant.)

3. Results

3.1. Acute treatment

3.1.1. Thyroid and pituitary TSH hormones

3.1.1.1. Serum T_3, T_4 and TSH. Arecoline treatment increased serum T_3 (Fig. 1a) and T_4 (Fig. 1b) levels with decreased TSH level (Fig. 1c) after 20, 40 or 60 min of the treatment, but most significantly at 40 min interval as compared to other time intervals. T_4/T_3 ratio was not significantly altered after the acute treatment of arecoline for 20 min (control: 4.87, arecoline: 4.73), 40 min (control: 4.86, arecoline: 4.67) and 60 min (control 5.00, arecoline: 4.84).

3.2. Chronic treatment

3.2.1. Thyroid gland

3.2.1.1. Light microscopy. Follicles are regularly oriented in the thyroid gland of control mice. Each follicle is lined by a single layer of large cuboidal epithelial cells with mostly round and conspicuous nucleus, regularly located towards the basement membrane. Follicular lumen contain(s) homogenous colloid material (Fig. 2A).

Chronic arecoline treatment decreased follicle size as evident from increased absolute number of intact follicles per microscopic focus in the treated (15 ± 1.10) compared to the control (intact follicle number 10.12 ± 1.21) mice. Healthy follicles were not seen. Arecoline also caused reduced epithelial cell size with haphazardly located inconspicuous nuclei. Colloid content was also decreased (Fig. 2B).

3.2.1.2. TEM study. In control mice, thyro-follicular cell possessed round-to-spherical euchromatic nuclei (approx. diameter: 5 μm) with prominent one or two nucleoli (Fig. 3A). Mitochondria, Golgi bodies and rough endoplasmic reticulum (RER) were abundant in the follicular cell cytoplasm (Fig. 3B). The rough-surfaced endoplasmic reticulum was extensive (Fig. 3B) and located towards the basal lamina of the follicular epithelium. Many electron dense secretory granules were located in the apical part (Fig. 3A) or centre (Fig. 3B) of the cell. Microvilli from the apical region of the cell were projected into the lumen (Fig. 3A) that contained electron dense colloidal substance (Fig. 3A). Parafollicular C cells were located on

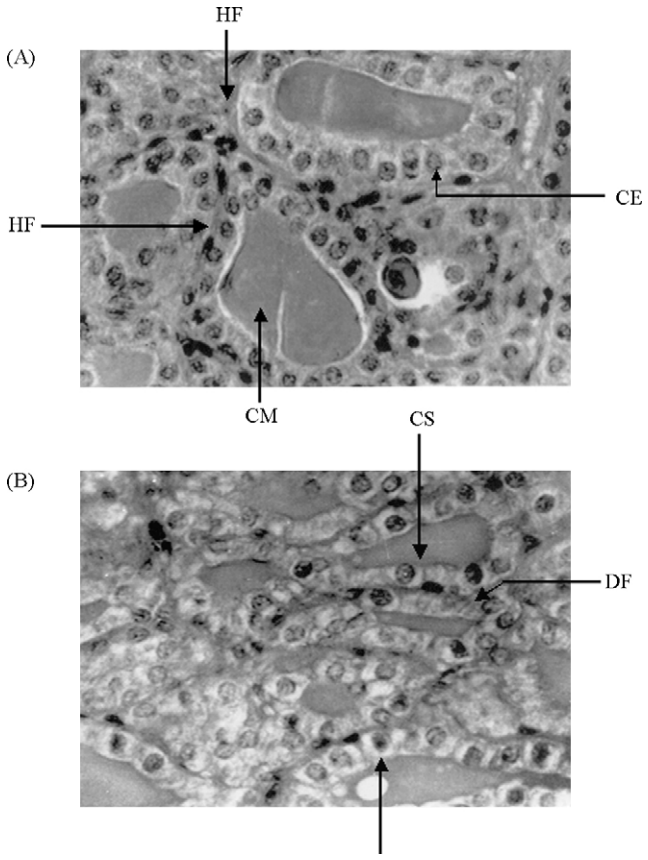

Fig. 2. (A) Light microscopic photograph of the thyroid gland of control mice showing numerous large healthy follicles (hf) per microscopic focus. Each large follicle is lined by a single layer of large cuboidal epithelial cells (ce) with regular orientation of prominent round nucleus located towards the basement membrane. Colloidal materials (cm) are seen in the follicular lumen (400×). (B) Arecoline treatment showing increased number of disorganized follicles (DF) per microscopic focus. The follicles are irregular in shape (ic) with reduced cell size (cs) and haphazardly located inconspicuous nuclei (n) (400×).

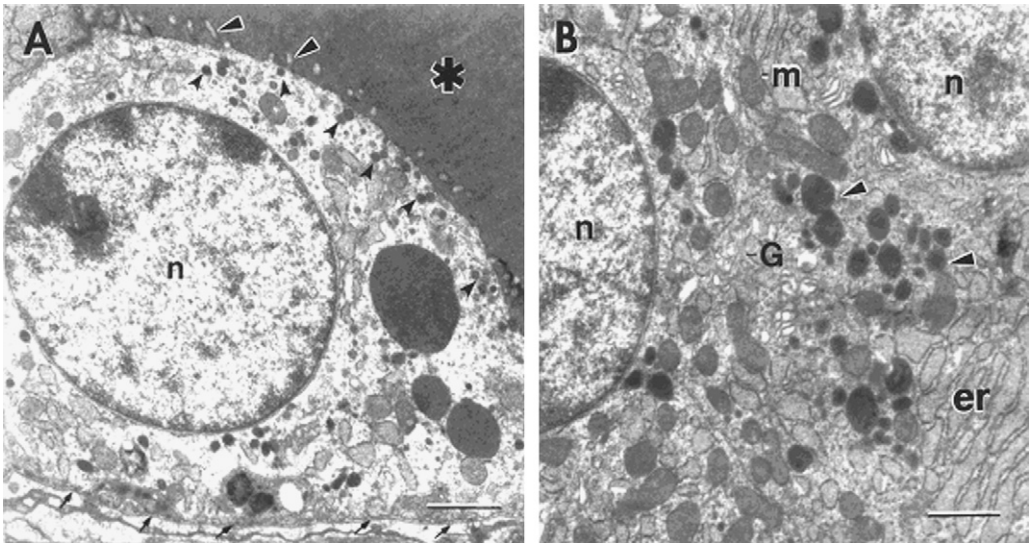

Fig. 3. Electron micrographs of the untreated thyroid follicular cells of mice. (A) The secretory granules are located mainly in the apical cytoplasm (black arrowheads). Arrows indicate the basal lamina of the cell. The lumen is relatively electron dense (asterisk) with microvilli (black and white arrowheads) projected from the cell. n, nucleus. (B) Showing various organelles: Golgi bodies (G), mitochondria (m) and rough-endoplasmic reticulum (er). The secretory granules (arrowheads) are located in the centre of the cytoplasm. [Scale bars: 1 μm].

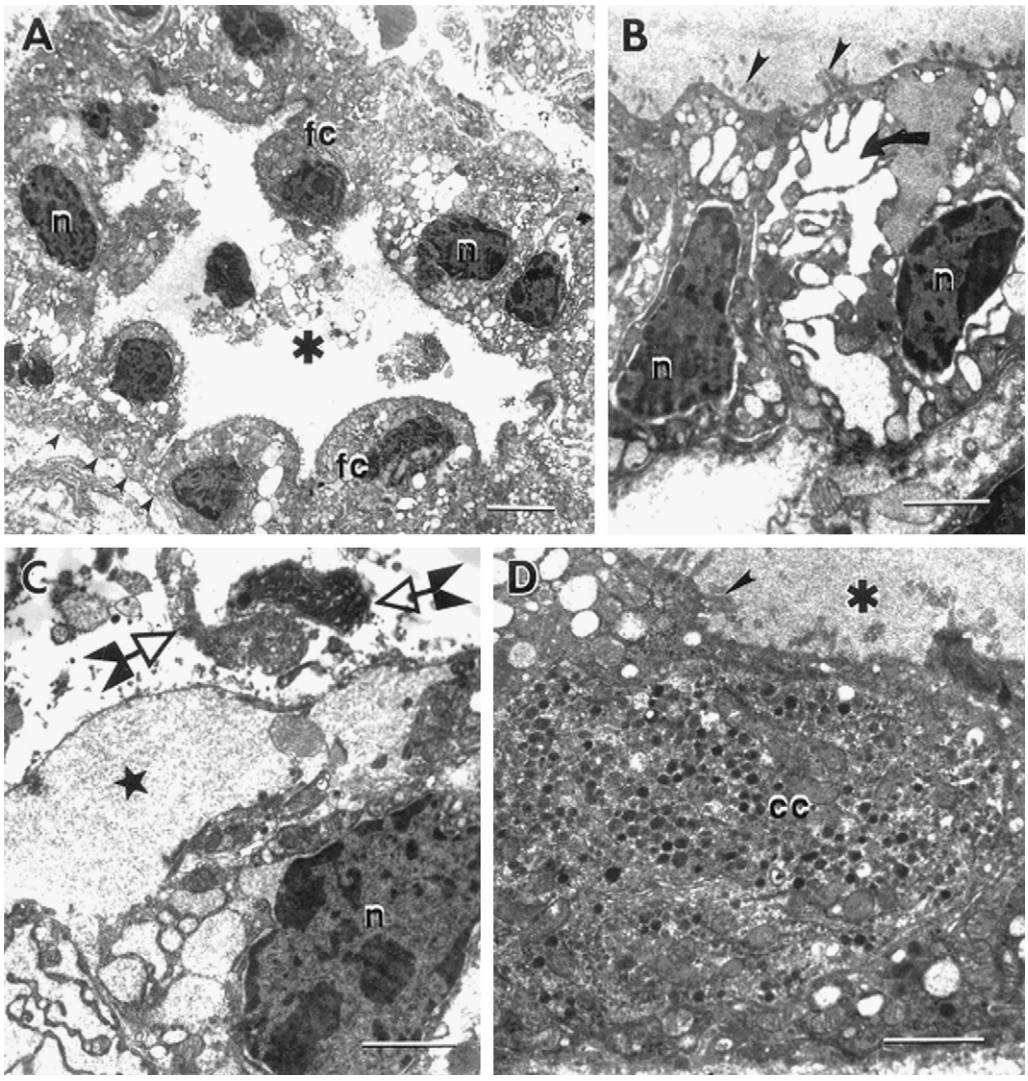

Fig. 4. Electron micrographs of the treated thyroid follicle (A) and follicular cells (B–D). (A) Follicular cells (fc) appear vacuolated with shrunken nuclei (n). The lumen of the follicle (asterisk) is electron-lucent and contains cellular debris. (B) Degenerated follicular cells containing hyperchromatic nuclei (n) and dilated cisternae of the rough endoplasmic reticulum (arrow). Arrowheads indicate microvilli of the follicular cells. (C) A degenerated follicular cell with empty apical cytoplasm (asterisk) and hyperchromatic nucleus (n). Note that the lumen contains cellular debris (arrows). (D) A C-cell (CC) located near the lumen of the follicle (asterisk). Arrowheads indicate microvilli of the follicular cell. [Scale bars: 3 μm (A), 1 μm (B–D)].

the basal lamina of the follicular epithelium and possessed numerous small, secretory granules of uniform size.

After arecoline treatment the follicles became smaller and highly variable in size (Fig. 4A). In many follicles, the basal lamina of the follicular epithelium was not intact (Fig. 4A). The follicular cells had many vacuoles. Their nuclei were shrunken and irregular, and showed condensed chromatin materials (Fig. 4B). There was evidence of loss of cellular content in the disorganized follicular cells (Fig. 4C). The cytoplasm of these cells possessed extensively dilated cisternae of the rough-surfaced endoplasmic reticulum (Fig. 4B and C) with few non-secretory granules marked in the degenerated follicular epithelium (Fig. 4A–C). The cellular debris of the follicles was desquamated into the luminal colloid (Fig. 4C). The parafollicular C cells were unusually located towards the lumen (Fig. 4D).

3.2.1.3. Serum T_3, T_4 and TSH. Serum T_3 (Fig. 5a) and T_4 (Fig. 5b) levels were declined with the elevation of TSH (Fig. 5c) level after chronic arecoline treatment in mice. The T_4/T_3 ratio was also reduced after chronic arecoline treatment (control: 5.00, arecoline: 4.11, percent change: −17.8%).

3.3. Acute co-treatments of atropine and arecoline

3.3.1. Thyroid and pituitary (TSH) hormones

3.3.1.1. Serum T_3, T_4 and TSH. Thyroid hormones and TSH levels of the control mice are presented in Fig. 5a–c. Arecoline treatment increased T_3 and T_4 levels, and decreased TSH level in mice (Fig. 5a–c). But atropine pretreatment showed reversed changes in T_3, T_4 and TSH levels to those of arecoline alone recipient mice (Fig. 5a–c). But the ratio of T_4/T_3 was not significantly altered after the acute treatment of arecoline alone or co-treatment of arecoline and atropine in mice (control: 4.87, Arecoline: 4.81 or arecoline and atropine: 4.72).

4. Discussion

Arecoline certainly has untoward effects on the thyroid gland of mice. This plant alkaloid has a dual action, because it initially stimulates thyroid function, but fails to maintain it subsequently. The initial stimulation was evident from the rise of serum T_3 and T_4 levels accompanied by a fall in TSH levels after the acute treatment of arecoline in mice. Arecoline initially stimulates the thyroid

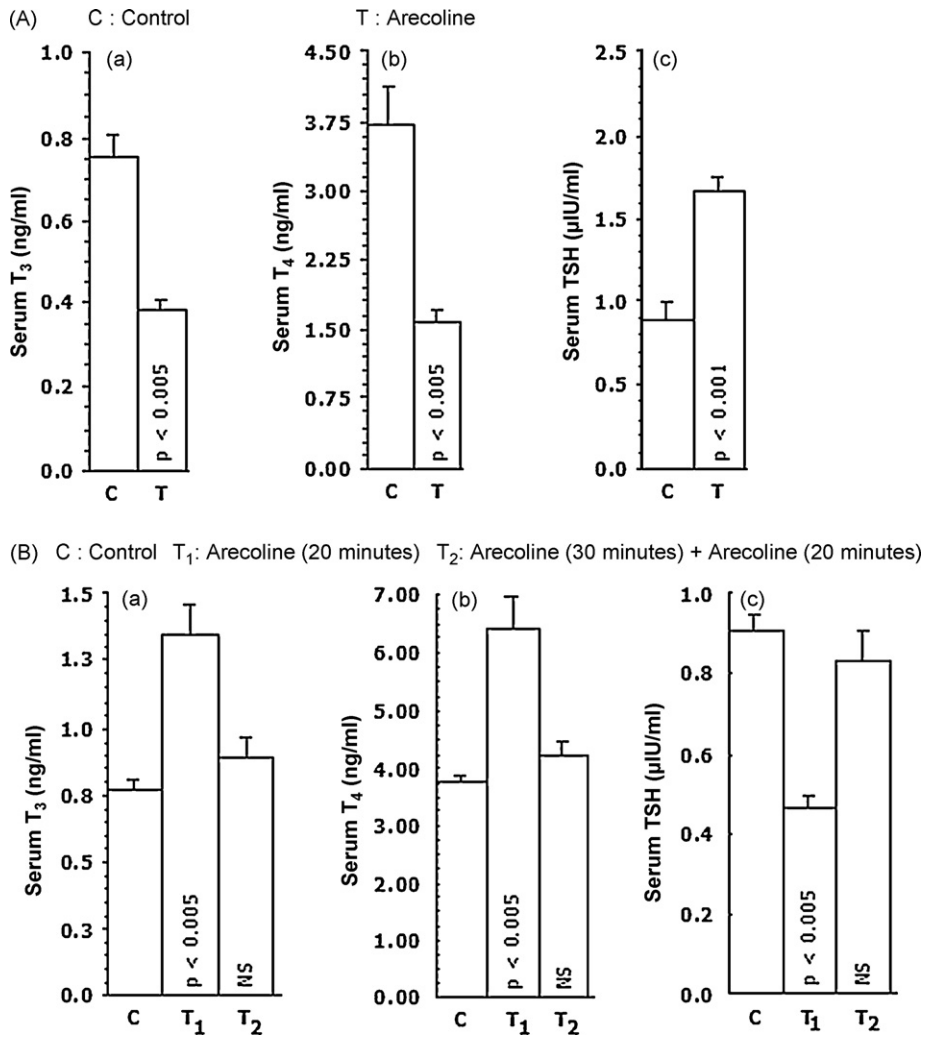

Fig. 5. (A) Chronic treatment of arecoline showing depletions of serum T_3 (a) and T_4 (b) levels with the elevation of serum TSH (c) level in mice (ANOVA, $p < 0.001$ considered significant). (B) Arecoline alone in acute treatment showing elevations of T_3 (a) and T_4 (b) followed by depletion of TSH levels) (c) in mice, but atropine pretreatment prevented the changes in T_3, T_4 and TSH levels (ANOVA, $p < 0.005$ considered significant).

gland, but subsequently inhibits its function which was evident from the light microscopic, ultrastructural and hormonal studies of the thyroid gland of mice when treated continuously for 15 days (chronic treatment). Under light microscope thyro-follicular number and size were reduced per microscopic focus with irregular orientation of the nuclei located in the reduced size of the epithelial cells. Ultrastructurally, follicular degeneration was evident from the presence of disorganized follicular cells with shrunken hyperchromatic nuclei and extensive dilatation of the cisternae of the rough endoplasmic reticulum (RER). The secretory granules and secretory vesicles, and colloid materials became scanty. Abundance of desquamated cell debris was seen in the thyro-follicular lumen after chronic arecoline treatment. Arecoline is cytotoxic (Van Wyk et al., 1994) and known to decrease protein synthesis in a dose-dependent manner (Chang et al., 2001). Our findings of the changes of nuclear size and RER cisternae also support the adverse toxic effects of arecoline on protein synthesis machinery of the thyro-follicular cells and its secretory activity in the chronic treatment of mice. The parafollicular C-cells showed sign of degeneration, because they were located away from the basement membrane towards the thyro-follicular lumen. Light microscopic and ultrastructural degenerations of thyro-follicular cells lead to a significant decline in serum T_3 and T_4 production after the chronic treatment of arecoline in mice.

A single betel nut contains 7.5 mg of arecoline (Farnworth, 1976; Rooban et al., 2005). Average weight of an unripe single betel nut is 1 g. A single betel nut chewer generally consumes 2–3 nuts daily (i.e. 7.5 mg × 1 g × 2–3 nuts = 15–22.5 mg). This is an estimated average amount of arecoline consumed daily by a single betel nut chewer. The actual amount of arecoline consumed daily by a single human subject needs to be ascertained. In Alzheimer's patient, arecoline is treated at a dose of 9.5–61 mg/day for enhanced verbal memory (Asthana et al., 1995). In our experiment the dose of arecoline used (1 mg/100 g body weight) was much less in mice than the daily dose of arecoline consumed by betel chewers or even compared to the dose used for the treatment of Alzheimer's disease in humans. Additionally, arecoline at a dose of less than 1 mg/100 g body weight was ineffective in mice, so the dose of arecoline used in our current study cannot be considered as unphysiologic.

Arecoline infusion in acute high dose (5 mg i.v. over 30 min/after 6 h) caused elevations of plasma ACTH, cortisol and β-endorphin levels, but without any change in these compounds in chronic treatment for 2 weeks followed by 1 week in cognitive responders with Alzheimer's disease (AD). Acute high dose of arecoline activates the hypothalamic–pituitary–adrenal axis (HPA) that likely represent a 'stress response', but cognition-enhancing low dose of arecoline do not produce a glucocorticoid response (Asthana et al., 1995). In our study, arecoline, both in acute and chronic

administrations, caused elevations of corticosterone, epinephrine and norepinephrine concentrations indicating 'stress response' in mice (DasGupta, 2008). Thyroid activity including T_3 and T_4 levels is also suppressed under diverse stress in vertebrates (Kudo et al., 1987; St. Aubin and Geraci, 1988; Scollon et al., 2004; Ray et al., 2008). Corticosterone inhibits thyroid activity presumably by suppressing TSH and/or thyroid hormone production as reported earlier in other vertebrates (Ray et al., 2006). Thus alteration of ACTH and/or adrenal hormones might be responsible for up- and down-regulations of thyroid activity in arecoline recipient mice. Dual actions of arecoline on thyroid activity probably depend on the amplitude of the toxic effect of this compound, because arecoline in acute treatment (20–40 min) is less hepatotoxic than the chronic treatment (15 days) (Dasgupta et al., 2006). Thus arecoline in acute treatment (40 min) initially stimulated thyroid, because toxic effect of arecoline in such treatment (i.e. in low dose: 1 mg/mice and in very short time: 46 min) was probably not strong enough to cause thyroid suppression which was observed only in chronic treatment (i.e. in high dose at 10 mg/mice in 10 days) due to its high toxicity in mice.

We examined the mechanism of action of arecoline by injecting atropine, which is a well known arecoline antagonist and a muscarinic cholinergic receptor blocker (Calogero et al., 1989). Atropine pretreatment in the arecoline recipients prevented thyroid suppression including the fall of T_3 and T_4 and rise of TSH induced by arecoline alone. Thus, atropine can prevent arecoline action on thyroid activity, or in TSH level by blocking arecoline action via muscarinic cholinergic receptors in mice.

Arecoline acts via muscarinic cholinergic receptor which is found in all effector cells (Guyton and Hall, 2001) including cell membranes of neurons of the *Locus coeruleus* (Yang et al., 2000) and Fischer rat thyroid cells (FRT) (Jiménez et al., 2001). The latter authors have also reported that muscarinic acetylcholine receptor subtype M_3 is present in FRT cells (Calogero et al., 1989; Asthana et al., 1995). Like arecoline, carbamylcholine (Cchol), carbachol (cch) and cytidine-5′-diphosphate (CTD) choline act via stimulations of muscarinic acetylcholine receptors (mAchR) (Jiménez et al., 2002). Muscarinic receptors are involved in the increase of TSH level, because atropine blocked cytidine-5′-diphosphate (CDP) choline-induced TSH stimulation (Cauvan and Savci, 2004). Muscarinic receptors are known to activate cell regulation via cyclic AMP cascade and causes phospholipase C (PLC) to release two second messengers from phosphotidylinositol 4,5-biphosphate such as diaceyl glycerol (DAG) and inositol 1,4,5-triphosphase (IP_3), itself activating Ca^{2+} release from intracellular stores (Lurent et al., 1991). In our study arecoline in acute treatment stimulated thyroid activity, but caused thyroid dysfunction (hypothyroidism) in chronic treatment probably by involving phospholipase C, IP_3 and Ca^{2+} cascade in mice. Thyroid hormone secretion is regulated by hypothalamo–hypophysial system (Larsen et al., 2003). Thus arecoline might have altered thyroid activity via TRH-TSH axis in mice. Nevertheless, it is cautioned that betel nut chewing should be avoided in order to save from hypothyroidism.

In essence (1) arecoline promptly stimulated thyroid activity, but chronic treatment of arecoline caused thyroid dysfunction (hypothyroidism) probably due to its strong toxic effects resulting in the fall of T_3 and T_4 with increased TSH levels in mice. Arecoline stimulates thyroid activity in mice within 40 min of the treatment, but not subsequently in 60 min, indicating short half-life of arecoline in mice thyroid. (2) But arecoline in chronic treatment causes hypothyroidism in mice. (3) Arecoline has dual and reversible actions on thyroid activity, because it stimulates initially and inhibits subsequently in mice. (4) Arecoline mediates its action on thyroid function probably via muscarinic cholinergic receptors in mice. (5) Betel nut chewing causes hypothyroidism and hence it should be avoided.

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