CHAPTER - 1

Ultrastructural and hormonal modulations of thyroid and adrenal glands with alterations of glycemia and liver glycogen following arecoline treatment in albino mice
Introduction

Arecoline is a major plant alkaloid of betel nut or 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, 1986; Marshall, 1987). Areca quid extract or arecoline has multiple actions on cell immunity of lymphoid and adrenal organs (Selvan et al. 1980; Selvan and Rao, 1993), and causes salivation, tremor, genial euphoria, palpitation, bronchial asthma, stimulations of electrolyte excretion, basal metabolic rate and heart rate (Trimarchi et al. 1991; Taylor et al., 1992; Chu, 2002; Strickland et al., 2003; Hafeman et al. 2005). It causes oral and throat cancer if taken 6 betel nuts a day for long (Sullivan, 2000). Arecoline has several other effects such as 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 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 has been reported to disturb endocrine and brain functions. It increases plasma concentration of beta-endorphin immunoreactivity, which is correlated with the increase in plasma prolaction concentrations in human (Risch et al., 1982). Calogero et al. (1989) have reported that arecoline stimulates plasma ACTH and corticosterone release into circulation by stimulating hypothalamo-hypophysial axis in rats. Arecoline causes a reduction in the level of norepinephrine of the cortex and subcortex of mice (Molinengo et al. 1988). Plasma epinephrine level is also increased after arecoline treatment in normal human subject (Polinsky et al., 1991). Betel nut chewing increases plasma concentrations of norepinephrine and epinephrine in human (Chu, 2001). Recently Lim and Kim (2006) have reported that arecoline in low dose does not inhibit catecholamine release from the perfused rat adrenal, but it does so in high dose. 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. The current information concerning the action of arecoline on the thyroid gland, adrenal cortex and adrenal medulla and carbohydrate metabolism is still inadequate. Moreover, the mode of action of arecoline is not known. In the current article, these problems are resolved by investigating the ultrastructural and hormonal changes of thyroid and adrenal glands, glycemia and liver glycogen in the mouse model. The mode of arecoline action was investigated by using muscarinic receptor inhibitor, since arecoline action is known to be mediated by muscarinic cholinergic receptor (Calogero et al., 1989).

Materials and Methods

Animal model

Adult male mice (90 days, ~25 gm body wt) were maintained as described in Materials and Methods (pg. 18).

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 gm body wt) was divided equally to half (0.5 mg/100 gm body wt), and each half dose was injected twice daily at 9 AM and 6 PM because of its short half-life (Pradhan et al., 1986).

Experimental protocol

(Each experiment, A, B or C, was carried out in triplicate, and the data represented the mean of all the experiments).
A. Acute treatment

The objective was to examine the time-dependent actions of arecoline.

Thirty six mice were divided in six groups of six each. Arecoline was injected to the animals of the groups IV, V and VI once only. Groups I, II & III served as control which received vehicle (saline) only. Animals were autopsied respectively at 20, 40 and 60 minutes after the treatments. This experiment was repeated thrice. Transmission electron microscopy (TEM) of the thyroid and adrenal glands were not carried out anticipating that the changes within 60 minutes of the treatments may not be easily recognizable at ultrastructural level.

B. 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 wt) daily for 15 days.

C. 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 a muscarinic receptor inhibitor because arecoline is known to act via muscarinic receptor (Calogero et al., 1989). Half life of atropine is 2 hours in serum.
(a) For thyroid study, mice were treated with arecoline for 40 minutes, because the effect of arecoline was maximum at 40 minutes, recorded from experiment A. Eighteen mice were divided in 3 groups (I, II & III) of 6 each. Group I served as control; group II animals were treated with arecoline (10 mg/kg body wt) for 40 minutes. Group III mice were treated with atropine sulphate at a dose of 0.5 mg/kg body weight for 30 minutes and subsequently with arecoline (10 mg/kg body wt) for 40 minutes (total: 70 minutes).

(b) For adrenal, arecoline was treated for 20 minutes, because the effect of arecoline was maximum at 20 minutes of the treatment, recorded from experiment A. This experiment was conducted with a new set of 18 mice, divided in three groups [(I: control, II: Arecoline for 20 minutes and III: Atropine for 30 minutes + arecoline for 20 minutes (total: 50 minutes)]. The remainders were same as for experiment number C(a).

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 (A), blood was drawn from the heart, and serum was collected and stored at –20°C until assayed for hormones and blood glucose. Liver was dissected free and stored at –20°C for glycogen assay. For chronic experiment (B), thyroid
and adrenal glands were dissected free and processed for TEM study. Other parameters (liver glycogen, serum hormones and glucose levels) were same in all the experiments A, B and C. TEM studies were not carried for experiments A and C.

**Transmission Electron Microscopy**

Thyroid and adrenal glands were processed for transmission electron microscopy by the procedure as described in Materials and Methods (pg. 19).

**Hormonal estimations**

Serum TSH (Soos and Siddle, 1982), T₃ (Waker, 1977) and T₄ (Schurrs and van Weeman, 1977) levels were quantitated by ELISA. TSH, T₃ and T₄ were quantitated by EIA kit (PATHOZYME-TSH, T₃ and T₄) of OMÉGA, UK. Serum corticosterone concentrations were studied by the method of Glick et al. (1964). Catecholamine (norepinephrine and epinephrine) concentrations were measured (Cox and Perháč, 1973) from the adrenal gland, instead of serum, for reasons as described in Materials and Methods (pg. 21). Blood glucose and liver glycogen levels were estimated by the methods of Trinder (1969) and Hassid and Abraham (1957) respectively. (For details, see Materials and Methods, pg. 20-23.)

**Statistical analysis**

Data were analyzed statistically by two way analysis of variance (ANOVA) followed by student’s t test (Snedecor, 1971). The data were presented as mean ± SEM and P-value <0.01 was considered statistically significant.

**Results**

**A. Acute treatment**

(a) Thyroid and pituitary TSH hormones

**Serum T₃, T₄ and TSH**

In normal adult male mice, serum T₃ level (Fig. 1a) is extremely lower than T₄ (Fig.
1b). Arecoline treatment increased serum T₃ (Fig. 1a) and T₄ (Fig. 1b) levels with decreased TSH level (Fig. 1c) at 20, 40 or 60 minutes of the treatment, but most significantly at 40 minutes interval as compared to other intervals.

![Graph of serum T₃, T₄, and TSH levels](image)

**Fig. 1:** Swiss albino mice were intraperitoneally injected with 10 mg/kg-body weight arecoline for 20, 40, 60 mins. Serum (a) T₃, (b) T₄ and (c) TSH levels were determined by EIA. Values are mean ± SEM of three independent experiments performed six times. (ANOVA for T₃, T₄ and TSH is p<0.01.)

(b) Adrenal cortex

**Serum corticosterone**

Serum corticosterone level of control mice is presented in Fig. 2a. Arecoline treatment increased serum corticosterone levels (Fig. 2a) at 20 and 40 minutes without any change at 60 minutes of the treatment, but most significantly at 20 minutes as compared to 40 minutes after the treatment.

![Graph of serum corticosterone levels](image)

**Fig. 2a:**

Note alterations of serum corticosterone (a), at 20 and 40 minutes, without any change at 60 minutes of arecoline treatment. (ANOVA: p < 0.01).
(c) Adrenal medulla

**Adrenal epinephrine and norepinephrine**

In the control mice, adrenal epinephrine level (Fig. 2c) is relatively higher than norepinephrine (Fig. 2b). Both norepinephrine (Fig. 2b) and epinephrine (Fig. 2c) levels were increased at 20 minutes without any significant change at 40 or 60 minutes of the treatment.

(d) **Blood glucose and liver glycogen**

Arecoline significantly increased the blood glucose level at 20 and 40 minutes, but most significantly at 20 minutes as compared to 40 minutes, without any change at 60 minutes of the treatment (Fig. 2d). Liver glycogen level was significantly decreased at all intervals of the treatment (20, 40 and 60 minutes), but most significantly at 20 minutes interval as compared to other intervals. The changes became gradually less significant from 20 minutes through 60 minutes of the treatment (Fig. 2e).
B. Chronic treatment

(a) Thyroid gland

Transmission Electron Microscopy (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 the basal lamina of the follicular epithelium and possessed numerous small, secretory granules of uniform size (Fig. 3C).

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 with narrow cytoplasm between them. Their nuclei were shrunken and irregular, and showed condensed chromatin materials (Fig. 4B). There was evidence of loss of cellular content and the secretory granules could not be detected in the disorganized follicular cells (Fig. 4C). The cytoplasm of these cells possessed extensively dilated cisternae of the rER (Fig. 4B, 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 and their granules were disorganized (Fig 4D) and occasionally aggregated.
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; cf. Fig. 4) 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 (rER). The secretory granules (arrowheads) are located in the centre of the cytoplasm.

(C): A parafollicular cell (cc) on the basal lamina (indicated by arrow) of the follicular epithelium containing numerous cytoplasmic granules (cyg).

[Scale bars for all: 1 µm]
Fig. 4: Electron micrographs of the arecoline 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 (appears empty) 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: (A): 5 µm, (B):1 µm, (C):1 µm, (D):1 µm].
Serum $T_3$, $T_4$ and TSH

Serum $T_3$ (Fig. 5a) and $T_4$ (Fig. 5b) levels were declined with elevation of TSH (Fig. 5c) level after chronic arecoline treatment in mice.

(b) Adrenal cortex

TEM study

In the adrenal cortex of the untreated mice, the cells of zona fasciculata (Fig. 6A) showed large mitochondria with moderate quantity of electron-dense matrix and tubular cristae (Fig. 6A) which are tubulo-lamellar in glomerulosa cells and tubulo-vesicular in fasciculate/reticularis cell type. The cells of the zona reticularis were arranged in regular anastomosing cords. Their nuclei were ovoid to spherical in shape and deeply stained, and the cytoplasm possessed elongated mitochondria and large lipofuscin granules (Fig. 6B).

In the arecoline treated animals, the cells of the zona fasciculata (Fig. 6C, D) showed signs of degeneration, because mitochondria were noted with highly electron-dense matrix and disorganization of their cristae (Fig. 6C). Also, there was a paucity of organelles in those cells, notably smooth-surfaced endoplasmic reticulum and Golgi bodies (Fig. 6D). The cells of the zona reticularis showed an apparent dissolution of their plasma membrane boundary, and their arrangement into cords was not evident (Fig. 6E). The nuclei were somewhat irregular and the cytoplasm contained numerous small lipid droplets (Fig. 6F).
Fig. 6: (A): Electron micrograph of mitochondria (m) in a cell of the zona fasciculata of the adrenal cortex of the untreated mice. (B): Electron micrograph of a cell of the zona reticularis (untreated), showing deeply stained oval nucleus (n), elongated mitochondria (m) and large lipofuscin granules (l). (C): showing electron-dense mitochondria (between arrows) in situ in the zona fasciculata of the treated animal. (D): Disorganized mitochondria with electron-dense matrix and swollen cristae in the treated zona fasciculata cell. (E) (lower magnification): the treated reticularis showing loss of individual cell boundaries with small irregular nuclei (n). (F) (higher magnification): showing the other area of the treated reticularis with numerous lipid droplets (lipid has been extracted during tissue processing) and deeply stained drastically reduced irregular nuclei (n).

Scale bars: (A): 0.5 um. (B): 1 um. (C): 1 um. (D): 0.5 um. (E): 2 um. (F): 5 um.
(c) Adrenal medulla

**TEM study**

The adrenal medulla of the control mice showed a large euchromatic nucleus and numerous small secretory vesicles in the cytoplasm of the chromaffin cells (Fig. 7A). Small mitochondria and well-developed rER were also present in those cells. Two types of chromaffin cells were distinguished: one possessing many small, homogenous vesicles (mean diameter: $0.21 \pm 0.07$) supposedly contain epinephrine granules (Fig. 7A) and the other type with large dense-core vesicles (mean diameter: $0.251 \pm 0.04$) supposedly containing norepinephrine granules (Fig. 7B).

![Fig. 7A-B](image)

**Fig. 7A-B**: Electron micrograph of (A) a chromaffin cell of the untreated mice showing a large vesicular euchromatic nucleus (n) and numerous mitochondria and epinephrine containing homogeneous granules (arrowhead). (B): at higher magnification, the cytoplasm showed abundance of dense core heterogeneous vesicles (arrows) and supposedly containing norepinephrine. [Scale bars for A: 0.5 μm, B: 0.5 μm]

In the treated mice, ultrastructural studies revealed that both types of chromaffin cells were affected. The cytoplasm of norepinephrine-containing cells appeared highly vacuolated, probably due to the discharge of secretion from the vesicles (Fig. 7C, D). Similarly, there was a depletion of the content from the epinephrine containing vesicles (Fig. 7E).
Using Wilcoxon paired t-test, there is a significant decrease in the number of both NE granules ($t = 9.875$, $p < 0.001$) and E granules ($t = 83.707$, $p < 0.001$) between control and treated glands (Fig. 7F).
Fig. 8a-c: Changes in serum corticosterone (a), adrenal norepinephrine (b) and epinephrine (c) levels after chronic arecoline treatment in mice (ANOVA for all: p < 0.01).

C: Control  T: Arecoline

(a)  (b)  (c)

Serum corticosterone level was increased after arecoline treatment in mice (Fig. 8a). Both norepinephrine (Fig. 8b) and epinephrine (Fig. 8c) levels of adrenal were decreased significantly after chronic treatment with arecoline.

(d) Blood glucose and liver glycogen

Blood glucose (Fig. 8d) and liver glycogen levels (Fig. 8e) of the control mice are presented in Fig. 8d and e). Blood glucose level was significantly decreased (Fig 8d) with increased liver glycogen level (Fig. 8e) after the chronic treatment of arecoline in mice.

C: Control  T: Arecoline

(d)  (e)

Blood glucose (mg/100)  Liver glycogen (mg/gm)

Fig. 8d-e: Changes in blood glucose (d) and liver glycogen level (e) after chronic arecoline treatment in mice (ANOVA for both p < 0.01).
C. Combined atropine and arecoline treatments

(a) Thyroid and pituitary (TSH) hormones

*Serum T₃, T₄ and TSH*

Arecoline treatment increased T₃ and T₄ levels and decreased TSH level in the serum of mice when compared to control. (Fig. 9a, b and c). But atropine pretreatment prevented these hormonal changes induced by arecoline alone (Fig. 9a, b and c).

![Fig. 9: Arecoline treatment alone (acute) showing elevation of T₃ (a) and T₄ (b) with depletion of TSH levels (c) in mice, but atropine pretreatment failed to show any perceptible change in the thyroid or TSH hormone levels compared to those of the mice receiving arecoline alone. (ANOVA for all: p < 0.01).](image)

(b) Adrenal cortex

*Serum corticosterone*

Arecoline treatment increased serum corticosterone level but atropine pretreatment prevented corticosterone rise induced by arecoline (Fig. 10a).

![Fig. 10a: Arecoline alone in acute treatment showing an increased serum corticosterone. But atropine pretreatment failed to show any perceptible change in adrenal hormones, compared to arecoline treatment alone in mice. (ANOVA for all: p < 0.01).](image)
(c) Adrenal medulla

Adrenal norepinephrine and epinephrine

Adrenal norepinephrine and epinephrine levels were increased after arecoline treatment, but atropine pretreatment prevented hormonal elevations induced by arecoline (Fig. 10b and c).

![Fig. 10b-c: Arecoline alone in acute treatment showing elevation of adrenal norepinephrine (b) and epinephrine (c) levels. But atropine pretreatment failed to show any perceptible change in adrenal hormones compared to arecoline treatment alone in mice (ANOVA for both: p < 0.01).](image)

C: Control
T<sub>1</sub>: Arecoline (20 min)
T<sub>2</sub>: Atropine (30 min) + Arecoline (20 min)

(d) Blood glucose and liver glycogen

Arecoline treatment for 20 minutes increased blood glucose level and decreased liver glycogen level in mice, but atropine pretreatment showed reversed changes in blood glucose and liver glycogen levels to those of arecoline treatment alone in mice (Fig. 10d and 10e).

![Fig. 10d-e: Arecoline alone in acute treatment showing hyperglycemia (d) and decreased liver glycogen (e) levels in mice. But atropine pretreatment failed to show any perceptible change in glycemia or liver glycogen level compared to arecoline treatment alone in mice. (ANOVA for all: p < 0.01).](image)

C: Control
T<sub>1</sub>: Arecoline (20 min)
T<sub>2</sub>: Atropine (30 min) + Arecoline (20 min)
Discussion

Arecoline has a dual action, since it initially stimulates thyroid and adrenal functions, but fails to maintain it subsequently. The initial stimulation is evident from the rise of serum T3 and T4 levels accompanied by a fall in TSH levels as well as from the elevations of serum corticosterone and adrenal norepinephrine and epinephrine levels after acute treatment of arecoline in mice. Although both the glands were stimulated but the degree of their responses to arecoline treatment was different, because the thyroid gland showed stimulations from 20 minutes through 60 minutes with a peak at 40 minutes, but the adrenal activity was peak at 20 minutes and declined at 40 minutes and remained unchanged at 60 minutes after the treatment.

Although arecoline initially stimulates the thyroid-adrenal axis, but subsequently inhibits the axis which is evident from the ultrastructural and hormonal studies of these endocrine glands of mice when treated continuously for 15 days (chronic treatment). In the thyroid gland, follicular degeneration was evident from the presence of disorganized follicular cells with shrunken hyperchromatic nuclei, extensive dilatation of the cisternae of the rough endoplasmic reticulum (RER), negligible secretory granules and drastic reduction of colloid materials with desquamated cell debris observed after 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). The findings on the changes in nuclear size and rER cisternae indicate that the alkaloid has an adverse effects on protein synthesis machinery and consequently showed decreased secretory activity. The parafollicular C-cells showed sign of degeneration, because they were located away from the basement membrane towards the thyro-follicular lumen. Additionally, serum T3 and T4 levels were declined after chronic treatment of arecoline. Ultrastructural degeneration followed by a fall in T3 and T4 levels certainly indicates that arecoline in prolonged treatment inhibits thyroid activity in mice. The increment of TSH in arecoline treated rats can be considered a feedback response caused by reduction of thyroid hormone (Sakai et al., 2000).

Chronic arecoline treatment also inhibits adrenocortical activity in mice, because it showed degenerative changes at ultrastructural level by showing disorganized, swollen
mitochondrial cristae, paucity of smooth endoplasmic reticulum (sER) and Golgi bodies in
the zona fasciculata. The reticularis showed dissolution of plasma membrane boundary and
disorganized cords of cells with irregular shaped nuclei and huge accumulation of lipid
droplets. Whereas the corticosterone level was increased in the blood serum, as observed in
the present study, but it was decreased in the adrenal gland (unpublished data, control: 9.10
\(\mu g/gm\), treated: 6.20 \(\mu g/gm\), \(p < 0.005\)). Since corticosterone level was not increased in the
adrenal gland, but in the blood serum, it appears that corticosterone synthesis might be
inhibited but its release might be stimulated at least in the chronic treatment in mice. As both
the mitochondria and smooth endoplasmic reticulum (sER) are involved in corticoidogenesis
(De Groot et al., 2001; Larsen et al., 2003), the damage of these cytoplasmic organelle in the
arecoline recipient indicate that arecoline also affects corticoid syntheses, especially
corticosterone, including sex hormone, presumably by the suppression of steroid synthesizing
enzymes present in the mitochondria and sER. Aldosterone was presumably not affected,
because arecoline did not show degenerative changes in the glomerularis layer of the adrenal
cortex. Thus, the ultrastructural findings also coroborate with the findings of corticosterone
estimation which was decreased in the adrenal gland of the arecoline recipients. In contrast,
arecoline in acute treatment increased corticosterone concentration in both the adrenal gland
(unpublished data, control: 10.30 \(\mu g/gm\), treated: 18.20 \(\mu g/gm\), \(p < 0.01\)) and blood serum in
the current study in mice (vide supra). Increased concentrations of corticosterone in both the
adrenal gland and serum in acute treatment indicate that arecoline can stimulate corticosterone
synthesis and release at least initially, but fails to stimulate the synthesis eventually because in
chronic treatment it decreased the synthesis of corticosterone. . This was evident from data on
the ultrastructure and corticosterone concentration of the adrenal gland of the recipients mice
(vide supra). Calogero et al. (1989) have reported enhanced corticosterone release after acute
arecoline treatment in rats. Corticosterone release is also known to be enhanced during stress
(Ray and Maiti, 2001). Since arecoline acts as stress (Wilson et al., 1998; Calogero et al.,
1989), it might have stimulated the release mechanism of corticosterone in the current study.
Although earlier authors (Selven et al., 1980; Calogero et al., 1989) reported adrenocortical
hypertrophy with increased serum corticosterone concentration after arecoline treatment in
mice, but this observation cannot confirm the enhanced synthesis of corticosterone without studying the corticosterone concentration with its rate-limiting enzyme, 11β-hydroxylase, in the adrenal gland (cortex) of mice.

Ultrastructural study of the adrenal medulla showed depletions of epinephrine and norepinephrine containing granules, resulting in the formation of vacuoles in the chromaffin cell cytoplasm of mice. Such subcellular changes are known to be related to the discharge of epinephrine and norepinephrine hormones into circulation, resulting in the loss of catecholamine hormones from the chromaffin cells of the adrenal medulla (Ghosh, 1977). Moreover, estimations of adrenal norepinephrine and epinephrine also showed depletions of their concentrations after chronic arecoline treatment in mice, and thus supports the ultrastructural observations of depletions of both epinephrine and norepinephrine vesicles from the chromaffin cells of the arecoline recipient mice. Arecoline presumably stimulated at least the release of the adrenomedullary hormones into circulation, because depletions of both norepinephrine and epinephrine vesicles from the chromaffin cells at ultrastructural level and their decreased concentrations from the adrenal gland were observed after chronic arecoline treatment in mice. Arecoline presumably could not stimulate the synthesis of adrenomedullary norepinephrine (NE) and epinephrine (E), because accumulations of NE or E vesicles at ultrastructural level or increased levels of these hormones in the adrenal gland were not observed after chronic treatment of arecoline. Decreased hormonal level could be due to the decreased dopamine β-hydroxylase (DBH) activity, responsible for the conversion of L-dopamine to norepinephrine, and/or decreased phenylethanolamine-N-methyltransferase (PNMT) activity, necessary for the conversion of norepinephrine to epinephrine (Axelrod, 1975). PNMT activity is known to be stimulated by exogenous ACTH in rat (Pohorecky and Wurtman, 1968) and corticosterone in birds and mammals (Zachariasen and Newcomer, 1975; Bentley, 1998). In the current study, adrenocortical activity was suppressed, as evident from the findings of adrenal ultrastructures and corticosterone concentration (vide supra) after chronic treatment of arecoline in mice. Thus, the decreased adrenal corticosterone level might be responsible for the inhibition in the synthesis of norepinephrine and epinephrine by
inhibition of DBH and/or PNMT activity in chronic arecoline treated mice. Nevertheless, all these evidences indicate that arecoline inhibits adrenomedullary activity, at least at the level of their synthesis in mice. Catecholamine secretion is known to be enhanced by Ca^{2+} influx through L-type Ca^{2+} channels in culture bovine chromaffin cells (Tallarida and Murray, 1987) and reduced by blocking the Ca^{2+} influx in the perfused rat adrenal chromaffin cells after arecoline treatment (Lim and Kim, 2006). In the current study, alteration of catecholamine secretion (stimulation in acute treatment and inhibition in chronic treatment) induced by arecoline might have resulted from the alteration of Ca^{2+} influx in the chromaffin cells of mice. Stress in general primarily affects both adrenal cortex and adrenal medulla in most of the vertebrates studied (Axelrod and Reisine, 1984; Bentley, 1998; Ray and Maiti, 2001). Stress also affects thyroid gland as evident from the current study. Thus arecoline might have acted as stress, like other stress agents (Calogero et al., 1989), which eventually affected thyroid and adrenal activities in mice. Arecoline also inhibits pinealocyte function and melatonin production in rats (Saha et al., 2006). Since melatonin has neuroprotective effects (Parmar et al., 2004), arecoline might have caused thyro-follicular and adrenal cell damage due to the decreased melatonin production in mice.

Apart from the actions of arecoline on thyroid and adrenal glands, it has some action on glycemia in mice, because arecoline initially induced hyperglycemia from 20 minutes to 40 minutes, but failed to maintain it subsequently after 60 minutes of the treatment, and eventually caused hypoglycemia when the treatment was continued for long (15 days) in chronic treatment in mice. Whereas liver glycogen level was reversely altered, being depleted at 20, 40 or 60 minutes of the treatment, but was elevated when the treatment was continued for 15 days in mice. Thyroid hormone accelerates the rate of absorption of monosaccharides from the GI tract after digestion and induces hyperglycemia by diminishing the glycogen store from the liver through glycogenolysis, both resulting in the hyperglycemia (Turner and Bagnara, 1976). It is well known that blood glucose production occurs directly from the food source after digestion and the excess of blood glucose is stored in the liver as glycogen. Subsequently glycogen is converted back to blood glucose by glycogenolysis (Ganong, 2001;
Nelson et al., 2005). Thyroid (T<sub>3</sub>, T<sub>4</sub>), adrenocortical (corticosterone) and adrenomedullary (norepinephrine and epinephrine) hormones are potent hyperglycemic agents in vertebrates (Bentley, 1998; Ray and Maiti, 2001). Since concentration of these hormones were increased initially in acute treatment of arecoline, and decreased subsequently in chronic treatment, their influence in inducing hyperglycemia initially and hypoglycemia subsequently cannot be ignored in mice in the present study.

In order to examine the mechanism of action of arecoline, atropine, a well known arecoline antagonist and a muscarinic cholinergic receptor blocker (Calogero et al., 1989), was used in the present study. Atropine pretreatment in the arecoline recipient mice failed to show any perceptible change in the thyroid-adrenal axis or in carbohydrate metabolism as compared to that of the arecoline treatment alone. This was because atropine pretreatment in arecoline injected mice did not cause any perceptible alteration in serum T<sub>3</sub> and T<sub>4</sub>, TSH, serum corticosterone or adrenal norepinephrine and epinephrine levels, or in hypoglycemia and glycogen level which were all significantly altered after arecoline treatment alone in mice. Thus, atropine prevents arecoline action on thyroid- adrenal axis, or on TSH level and consequently on carbohydrate metabolism (glycemia-glycogen interaction) by blocking arecoline action via muscarinic cholinergic receptor in mice. Calogero et al. (1989) have reported that arecoline is a muscarinic cholinergic agonist which acts via muscarinic receptors. Yang and others (2000) have reported that arecoline exerts its excitatory action by binding M<sub>2</sub>-muscarinic receptors on the cell membranes of neurons of the locus coeruleus. These authors also suggest that arecoline acts by stimulating hypothalamo (CRF) – hypophysial (ACTH) – adrenocortical (corticosterone) axis in rats. Thus, in the current study, arecoline might have exerted its action via muscarinic cholinergic receptors and hypothalamo-hypophysial axis on the thyroid- adrenal activity, and consequently on carbohydrate metabolism in mice.

In essence (1) arecoline treatment affects both thyroid and adrenal glands promptly, but the adrenal gland is more sensitive to arecoline than the thyroid in mice, because adrenal activity reaches peak at 20 minutes, but thyroid did so at 40 minutes after arecoline treatment.
(2) Arecoline has short half-life, because it can exert its action up to 60 minutes, but not beyond this time period. (3) Arecoline action on the thyroid and adrenal functions can be extended, if treated chronically. (4) Arecoline has dual and reversible actions on the thyroid-adrenal axis, because it stimulates the axis initially but inhibits subsequently. (5) Arecoline also has dual and reversible actions on the glycemia-glycogen interaction in mice.

Summary

Arecoline, an alkaloid of betel nut disturbs some endocrine functions. The objective of the current work is to investigate the untoward effects of arecoline on endocrine organs such as, thyroid and adrenal and their consequence on carbohydrate metabolism in mice. Intraperitoneal injection of arecoline in acute treatment increased serum T3 and T4 levels with decreased serum TSH at 20, 40 or 60 minutes, increased serum corticosterone at 20 and 40 minutes, and adrenal norepinephrine and epinephrine levels only at 20 minutes after the treatment. Hyperglycemia was noted at 20 and 40 minutes interval, with depletions of liver glycogen at 20, 40 and 60 minutes of the treatment. Chronic arecoline treatment caused ultrastructural degeneration of thyro-follicular cells with depletions of T3 and T4 levels followed by elevation of the TSH level. The treatment also caused ultrastructural degenerations of fasciculata and reticularis zones of the adrenal cortex and medulla with the elevation of serum corticosterone, and depletions of adrenal norepinephrine and epinephrine levels. Hyperglycemia with decreased glycogen in acute treatment followed by hypoglycemia with increased glycogen in chronic treatment were observed. To confirm the action of arecoline, arecoline antagonist, atropine, was injected. Atropine pretreatment prevented the changes of thyroid-adrenal activities or glycemia and glycogen induced by arecoline. The findings indicate that arecoline initially stimulates the thyroid and adrenal activities with hyperglycemia and depletion of liver glycogen, but inhibits this axis (except corticosterone) with hypoglycemia and elevation of liver glycogen eventually, and atropine prevents arecoline action. Arecoline action is mediated presumably via muscarinic cholinergic receptor—hypothalamo-hypophysial axis and consequently on carbohydrate metabolism in mice.