Chapter – 1A

Ultrastructural and Hormonal Changes in the Pineal – Testicular Axis Following Arecoline Administration in Rats¹

INTRODUCTION

Arecoline is the most abundant plant alkaloid of betel nut of *Areca catechu* (Farnworth, 1976). Betel nut is chewed by millions of people living in between the East Coast of Africa and the Western Pacific (Marshall, 1987). Betel quid chewing increases heart rate (Tandon, 1999), water intake (Shen *et al.*, 2001), heightened alertness, basal metabolic rate, capacity to work (Strickland *et al.*, 2003), skin temperature with onset of cardio-acceleratory response, sweating, salivation, palpitation and tremor (Hafeman *et al.*, 2006). Ripe and tender *Areca* nuts reduce antibacterial activity and superoxide anion production of neutrophils (Hung *et al.*, 2000). In rats arecoline has an immunotoxic effect (Selvan *et al.*, 1989), suppresses antibody responses (Selvan and Rao, 1993) and hepatic detoxification system in suckling neonates (Singh *et al.*, 1996a,b), causes cell cycle arrest and DNA fragmentation of mucosal fibroblasts and keratinocytes *in vivo* (Chang *et al.*, 2001a), generates reactive oxygen species resulting in chromosomal aberration and blocks damaged cells from proliferation (Kumpawat *et al.*, 2003), retards the embryonic development of zebra fish in a dose-dependent manner (Chang *et al.*, 2004a), and induces teratogenic effects and abortion (Gracia-Algar *et al.*, 2005). Recently we have also shown immunotoxic and hepatotoxic effects of arecoline in rats (Dasgupta *et al.*, 2006).

Arecoline also affects endocrine functions. Arecoline increases plasma prolactin concentrations which is correlated with the increase of β-endorphine concentration (Risch *et al.*, 1989) and stimulates the hypothalamo-hypophysial-adrenal axis in rats (Calogero *et al.*, 1989). Arecoline induces antidiuretic and natriuretic responses in brain cholinergic neurons (Trimarchi *et al.*, 1991). Betel quid chewing elevates epinephrine
and norepinephrine levels (Chu, 2002) and evokes catecholamines (CA) secretions from the isolated perfused adrenal gland of rat (Lim and Kim, 2006). Arecoline induces abnormal formation of heads in early spermatid stages of mice (Sinha and Rao, 1985). Yang et al. (2004b) have demonstrated Leydig cell stimulation leading to an increased testosterone synthesis in vivo in rats and dose-dependent action of arecoline for increasing testosterone production, employing possibly a non-cAMP-dependent pathway of steroidogenesis, in mouse interstitial cells of Leydig.

Biological activity of testosterone on its target organs (sex accessories) has not been examined under the influence of arecoline. Hence, it was interesting to study the action of arecoline on the pineal-gonadal interrelationship, since the pineal is known to be anti-gonadal in most vertebrates studied (Yilmaz et al., 2000). In the first chapter, these issues have been addressed at ultrastructural, hormonal and other biochemical levels, following arecoline treatment in rats. Moreover the current study is undertaken at both the photophase and scotophase to ascertain whether there is any difference in the action of arecoline between these two phases in rats. This is pertinent especially because the pineal is more active in the scotophase compared to the photophase (Bentley, 1998).

**MATERIALS AND METHODS**

*Experimental design*

Twenty rats were taken and divided into two groups of ten each. Group I served as control and group II rats received arecoline hydrobromide (Methyl 1-methyl 1,2,5,6 tetrahydronicotinate) (Sigma, USA), which was dissolved in physiological saline (0.9% NaCl) and injected intraperitoneally at a dose of 10 mg/kg body weight daily for 10
consecutive days. Each daily dose was equally divided and injected at 11am and 5pm respectively, because of its short half-life (Pradhan et al., '86). The control rats received vehicle (0.9% saline) without arecoline.

**Tissue and blood serum collection**

Experiments were terminated on day 11 of the experiment at 10am. Rats were anesthetized with intraperitoneal injection of sodium barbital and relevant organs were dissected free, weighed by semi-micro analytical balance (METTLER) and frozen at −20°C for studying pineal serotonin, N-acetylserotonin and melatonin, fructose of the coagulating gland and sialic acid of the seminal vesicle. Blood was drawn from the heart, and the serum was separated and stored at −20°C until assayed for indoleamines or hormones (serum serotonin, N-acetylserotonin, melatonin and testosterone).

The experiment was repeated in rats (control, n=10; treated, n=10) to ascertain the effect of arecoline on the pineal-testicular axis at scotophase, because the pineal gland is known to be active at night compared to day (Bentley, '98). So, the second experiment was terminated on day 11 in the scotophase at 12 midnight, and the biochemical parameters were studied. TEM of the pineal, testis or the prostate was not included.
RESULTS

TEM Study

Pineal

(a) Control

The semi-thin sections the pineal parenchyma consisted of the light variety of pinealocytes (equivalent to type 1 pinealocyte), as seen in all the pineal specimens examined. The individual pinealocytes possessed 3-6 cytoplasmic dense granules. In ultrathin sections, the pinealocytes showed an ovoid to elongated euchromatic nucleus with a prominent nucleolus (Fig. 1a) and cytoplasm rich in mitochondria, with long and narrow parallel cisternae of the rough endoplasmic reticulum (RER) (Fig. 1b) and Golgi bodies. Few dense granules, which appeared to be lysosomes (diameter: 0.2-0.5 μm), were present in many pinealocytes, with three to six in number in individual cells (Fig. 1c). In each pinealocyte, two to six electron-dense plate-like synaptic ribbons (SR), oriented parallel to each other, were noted (Fig. 1c). Electron-lucent synaptic-like microvesicles (SLMV) (Fig. 1d) were also attached to the SR. The mean length as well as width of SR were respectively of 0.377 ± 0.02 μm and 32.00 ± 0.87 nm. Synaptic spherules, irregular and nonbar-like in shape were seldom seen. The pinealocyte process terminals, which were located close to the perivascular space, were filled with electron-lucent, small and large SLMV that measured about 30-40 nm and 65-70 nm, respectively (Fig. 1d). The adjacent pinealocytes were connected to each other by desmosomes. The pineal glial supporting cells, astrocytes and oligodendrocytes, contained cytoplasmic processes with distinct nucleus and chromatin materials.
Fig. 1: Transmission electron micrographs showing ultrastructural features of pinealocytes from control (untreated) rats. (a) Ovoid to elongate nuclei (N) with prominent nucleoli of pinealocytes. (b) Part of cytoplasm of a pinealocyte showing elongated cisternae of rough endoplasmic reticulum (arrowheads) and mitochondria (M). (c) Cytoplasm with few dense granules (arrowheads) and nucleus (N). (d) Synaptic ribbons (arrowheads) in cytoplasm. (e) Electron-lucent SLMV (arrowheads) in a bulbous process terminal. Scale bars: 1 μm (a-c) and 0.5 μm (d, e).

(b) Arecoline Treatment

The treated glands contained a relatively higher number of cytoplasmic dense granules examined from 30 pinealocytes. Dead cells with pycnotic nuclei were not observed in any of the pineal glands examined. The pinealocytes showed some apparent signs of degenerative changes, mostly in their cytoplasm. In the majority of the cells, the nucleoli appeared small and inconspicuous. The cytoplasm was found to possess short, dilated cisternae of RER (Fig. 2a), large membrane-bound autophagosome-like bodies containing small vesicles and abnormal mitochondria (Fig. 2b). The latter appeared dark with swollen cristae in some pinealocytes. Golgi bodies were scarcely observed. Numerous cytoplasmic dense granules, seen in light microscope, appeared as lysosomes (8 to10 in number) of various shapes and sizes (diameter: 0.2-0.5 μm) under the
transmission electron microscope (Fig. 2c). Lipid droplets in clusters, with deposition of electron-dense particulate matter around their periphery (Fig. 2d) were seen in many pinealocytes, which corresponded to the dense granules observed under light microscope. Few melanin-like spherical granules with higher electron-density were also observed in some scattered pinealocytes, but their diameter was always larger than that of the lysosomes (about 0.5-0.7 μm). No significant changes in lipid droplets were found.

We had examined 20 profiles of SR present either in the cytoplasm or bulbous end feet of the pinealocytes in each normal and treated specimens. The mean length and width of the SR in the treated group were 0.186 ± 0.03 μm and 44 ± 3.4 nm, respectively. The length of the SR in the treated pinealocytes were smaller but much wider than in the normal untreated pinealocytes (mean length: 0.377 ± 0.02 μm and mean width 32.00 ± 0.87 nm), which were statistically significant (p < 0.01). Additionally the ribbons were partly degenerative (Fig. 2e), swollen (Fig. 2f), bent (Fig. 2g) or irregular (Fig. 2h). Furthermore, in many bulbous process terminals, the SLMV appeared to be less abundant and irregular in outline (Fig. 2f) than those seen in the normal pinealocytes (compare with Fig. 1d), and could not be measured. The mitochondria in the pinealocyte processes were abnormal with swollen cristae (Figs. 2f, h).

No apparent changes were detected at the level of the junctions between the pinealocytes and cytoskeletons, especially the microtubules. This was also the situation with the nerve endings that surrounded the pineal glands. Clear vesicles as well as dense-core vesicles were routinely observed in those nerve endings. Astrocytes and oligodendrocytes that support pinealocytes showed cytoplasmic vacuolations after arecoline treatment.
**Serotonin, N-acetylserotonin and melatonin (control and treated)**

Pineal and serum serotonin(S) levels (a) were increased with decreased N-acetylserotonin (NAS) (b) and melatonin (MEL) (c) concentrations after arecoline treatment as compared to those of the control rats (Table 1, Part A).
Testis (Leydig cell)

(a) Control

In the untreated control rats, the Leydig cells contained a distinct nucleus with moderate number of SER, dense core vesicles (DCV) and a few clear vesicles (CV) (Fig. 3a).

(b) Arecoline Treatment

In the treated rats, the Leydig cells showed hyperactive nucleus with abundance of SER, and clear dense core vesicles (Fig. 3b). No recognizable change in the germ cell types was recorded in the treated recipient.

Ventral prostate

(a) Control

The ventral prostate of the control rat showed moderate number of inconspicuous rough endoplasmic reticulum (RER) (Fig. 3c) and small dense core vesicles in the apical region of the cell (Fig. 3d).

(b) Arecoline Treatment

Following arecoline treatment, the rough endoplasmic reticulum became more conspicuous and abundant (Fig. 3e). Large dense core vesicles having peripheral electron-lucent zone (Fig. 3f) were also noticed after arecoline treatment.
Fig. 4: Transmission electron micrographs of ultrastructural features of Leydig cells from control (untreated) rats showing nucleus (N), with moderate number of smooth endoplasmic reticulum (SER) (arrow), dense core vesicles (DCV) (asterisk) and a few clear vesicles (CV) (arrowhead). (b) Transmission electron micrographs of Leydig cells from treated rats with arecoline showing hyperactive nucleus (N) with abundance of SER (arrow), and clear vesicles (arrowhead) and dense core vesicles (DCV) (*). (c) The untreated epithelial cells of the ventral prostate showing moderate number of rough endoplasmic reticulum (RER) (arrowhead) and (d) many dense core vesicles (arrow). (e) Note the abundance of RER (arrowhead) and (f) dense core vesicles with peripheral electron-lucent zone (arrows) in the treated prostate. Scale bars: 0.5 μm (a, b), 1 μm (c, d, e, f).
Testosterone, fructose and sialic acid (control and treated)

Arecoline treatment significantly increased the concentrations of testosterone, fructose in the coagulating gland, and sialic acid in the seminal vesicle of the rats (Table 1, Part A).

Table 1 Part A: Changes\(^1\) in the concentrations of indoleamines, testosterone, fructose and sialic acid in the photophase of the arecoline recipient rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serotonin (S)</th>
<th>N-acetylserotonin (NAS)</th>
<th>Melatonin (MEL)</th>
<th>Testosterone (ng/ml)</th>
<th>Fructose (μg/g)</th>
<th>Sialic acid (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>(Pineal)</td>
<td>85.62±0.31(^*2)</td>
<td>5.85±0.28(^3)</td>
<td>1.47±0.04(^2)</td>
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<tr>
<td>(Serum)</td>
<td>212.45±5.46(^3)</td>
<td>30.24±1.12(^3)</td>
<td>50.02±0.04(^3)</td>
<td>3.49±0.25</td>
<td>7.21±0.04</td>
<td>3.28±0.04</td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Pineal)</td>
<td>118.27±0.56(^3)</td>
<td>3.25±0.12(^2)</td>
<td>0.68±0.02(^2)</td>
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<tr>
<td>(Serum)</td>
<td>388.02±2.98(^3)</td>
<td>11.21±0.29(^3)</td>
<td>21.22±0.36(^3)</td>
<td>8.92±0.81</td>
<td>22.56±0.04</td>
<td>6.82±0.04</td>
</tr>
</tbody>
</table>

1. Statistically significant (p<0.001) compared to the control group. ANOVA (p<0.01).
2. ng/mg.
3. pg/ml.
*: Mean ± SE.

The results of the second experiment, terminated in the scotophase (24.00 hr), were in the same line with those of the first experiment terminated in photophase, the serotonin being increased with decreased N-acetylserotonin (NAS) and melatonin (MEL), and increased testosterone, fructose and sialic acid concentrations in the treated recipients (Table 1, Part B). The findings were more significant in the scotophase (12 midnight) than the photophase (12 noon).
Table 1 Part B: Changes in the concentrations of indoleamines, testosterone, fructose and sialic acid in the scotophase of the arecoline recipient rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serotonin (S)</th>
<th>N-acetylserotonin (NAS)</th>
<th>Melatonin (MEL)</th>
<th>Testosterone (ng/ml)</th>
<th>Fructose (μg/g)</th>
<th>Sialic acid (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.57±0.04*2</td>
<td>12.52±0.08*2</td>
<td>2.42±6.08</td>
<td>—</td>
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</tr>
<tr>
<td>(Pineal)</td>
<td>212.45±0.08*3</td>
<td>39.28±0.06*3</td>
<td>82.14±0.02*3</td>
<td>1.50±0.08</td>
<td>5.32±0.01</td>
<td>2.62±0.01</td>
</tr>
<tr>
<td>(Serum)</td>
<td>110.28±0.05*2</td>
<td>9.32±0.05*2</td>
<td>1.13±0.09*2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Treated</td>
<td>412.51±0.06*3</td>
<td>19.32±0.02*3</td>
<td>13.21±0.04*3</td>
<td>14.20±0.07</td>
<td>31.21±0.05</td>
<td>10.21±0.12</td>
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<tr>
<td>(Pineal)</td>
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<td>(Serum)</td>
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</table>

1, Statistically significant (p<0.001) compared to the control group. ANOVA (p<0.01).
2, ng/mg,
3, pg/ml,
*: Mean ± SE.

DISCUSSION

In adult rats, only the light pinealocyte is present in contrast to both light and dark pinealocytes known to occur in hamster, rabbit, gerbil and aging rats (Sheridan and Reiter, 1968; Garcia-Maurino and Boya, 1992; Redecker, 1993; Humbert and Pevet, 1995). However, whether the aging factor is responsible for the appearance of the dark pinealocyte cannot be confirmed. Our results indicate that arecoline treatment inhibits pineal activity because it led to remarkable changes at the ultrastructural level of the pinealocyte organelles and synaptic machinery, reported for the first time. Chronic arecoline treatment showed occurrence of numerous, large autophagosomes possessing many small vesicles that might have originated from the cisternae of rough endoplasmic reticulum (RER). Earlier, they were described in the rat and Mongolian gerbil pinealocytes, as acid phosphatase rich cisternae (Krstic, 1985). Their abundance in the arecoline-treated pinealocytes is perhaps due to the physiological demand to dispose off
the disorganized organelles, like mitochondria, affected by the treatment. Similarly, lysosomes were abundant in the treated pinealocytes as compared to that of the normal pinealocytes. In the rat pineal gland, lysosomes are reported to increase in number in the pinealocytes with increasing age (Calvo and Boya, 1984) or when exposed to X-ray irradiation (Logvinov et al., 2004). These findings correspond to our observation of a decrease in melatonin content to be associated with an increase in lysosomal number in the treated pinealocytes. Melatonin is neuroprotective (Parmar et al., 2002), and there is evidence that it can protect the pinealocytes against damage. Neuronal damage in the pineal gland due to stress and other factors (like sleep deprivation) could be alleviated by exogenous melatonin administration (Lan et al., 2001). On the other hand, Redins et al. (2001) noted a decrease in lysosomal number in mice exogenously treated with melatonin. Conversely, the increased number of lysosomes could be indicative of decreased melatonin synthesis which was also observed after arecoline treatment in the present experiment. Moreover, in our study, the damage by arecoline treatment, which caused an increased occurrence of lysosomes, might have resulted from a decrease in the level of melatonin, which was also evident from the present results in rats. This would indicate that melatonin protects the pinealocytes from the adverse effects of cytotoxic agents via production of more lysosomes. The latter may then sequester the unwanted materials in the pineal cytoplasm for digestion.

Arecoline is cytotoxic in nature (Van Wyk et al., 1994; Dasgupta et al., 2006). This alkaloid is reported to cause a decrease in protein synthesis in a dose-dependent manner (Chang et al., 2001b). The observations of changes in the nucleoli size and dilatation of the RER cisternae indicate that the alkaloid has an adverse effect on the cellular protein synthesis machinery. Consequently, the alkaloid showed limited cellular
secretory activity. The rat pinealocyte contains numerous synaptic-like microvesicle (SLMV) reported earlier by Redecker (1993; 1999a). The reduced abundance of SLMV in the pinealocyte process terminals and decrease in melatonin levels in the treated pineal glands were in line with the changes in RER cisternae and paucity of Golgi bodies seen after arecoline treatment. SLMV are known to contain classical neurotransmitters like GABA and glycine (Redecker, 1999 b). So, it is likely that arecoline affects pineal neurotransmission considerably.

In our study, the synaptic changes after treatment of the pineal glands with arecoline were noteworthy. There was significant reduction in the synaptic ribbon (SR) length, and the SR appeared irregular (thick and bent), considerably swollen and often disorganized in the pinealocyte process terminals. Spherical, irregular or nonbar-like shape of the SR was never encountered in normal pinealocyte, as also reported in rat and guinea pigs, (Vollrath, 1981; Luo et al., 1990). SR are plastic and dynamic in nature and subject to change in length and shape in circadian light-dark cycles (Vollrath and Seidel, 1989; Jastrow et al., 1997), under constant illumination (Jastrow et al., 2004) and in seasonal cycles (Martinez-Soriano et al., 2002). The number of SR and synaptic spherules are influenced by serum melatonin and serotonin levels (Vollrath et al., 1985). Also, treatments with select drugs and reagents make them vulnerable to catabolic changes (Peschke et al., 1996). In the rat pineal gland, the SRs are reported to be larger at night than during day time (Jastrow et al., 1997). Since all animals were sacrificed during the same time period (day) of the photopic phase, it is likely that the changes noted in the treated pineal glands were due to the effects of arecoline treatment. Though the mechanism involved in those changes remains elusive, the observations on the reduced length and swollen state of the SR raise the possibility that the SR are vulnerable
to unknown catabolic processes triggered by arecoline treatment. These processes may also attack the SLMV simultaneously, and the limited occurrence of vesicles in the treated pinealocyte process terminals is in favor of this idea. Nevertheless, these findings tend to indicate that arecoline affects SR morphology and consequently the catabolic process of the pinealocytes in rats. Thus, SR may be considered as an index of pinealocyte activity.

Arecoline increased the Na, K-ATP-ase activity in synaptosomal membranes of neurons (Schwarzenfeld et al., 1976; Semenov et al., 1977) and may accelerate the various steps of neurotransmission (vesicular fusion and exocytosis). These subcellular changes in the pinealocytes are known to be related to pineal inactivity (Reiter, 1981). In rat pinealocytes, number of dense core vesicle increases during day when serum melatonin level is low and the situation becomes reverse at night, when the number of dense core vesicles diminishes with an increase in melatonin titre (Karasek et al., 1990). Number of electron dense core vesicles of the pinealocytes varies (increases) with the reproductive status and age of the adult mice (Novaes et al., 1998). Thus, the population of electron dense vesicles, like SR, could also be an index of pinealocyte activity. Moreover, arecoline induces necrosis in the oligodendrocytes of the pinealocyte since nuclear vacuolations were marked after the treatment. Since nuclear vacuolation is an indication of DNA damage (Kumpawat et al., 2003), it is likely that arecoline causes DNA damage and this suggests its genotoxic effect in the pineal gland of rats. Additionally, pineal and serum N-acetylserotonin and melatonin concentrations were also decreased after the treatment in rats. Since serotonin is converted to N-acetylserotonin (NAS) by N-acetyltransferase (NAT) and NAS to melatonin by HIOMT, it is likely that arecoline might have decreased the synthesis of these indoleamines by
inhibition of their respective enzymes in rats. Thus, arecoline decreased both synthesis and release of these indoleamines (NAS and MEL) into circulation, because both the pineal and serum levels of N-acetylserotonin and melatonin levels declined after the treatment. Despite the fall in both synthesis and release of NAS and melatonin, serotonin levels increased after arecoline treatment, which could be due to inhibition of both enzymes, NAT and HIOMT, induced by arecoline. Finocchiaro and others (1990) have also reported a similar increase of pineal metabolic production of hydroxyindole derivatives up to 5-hydroxytryptamine with insignificant stimulation of melatonin biosynthesis induced by pilocarpine which is also a muscarinic cholinergic agonist like arecoline (Pujito et al., 1991). Subsequently, Pujito and others (1991) have reported that pilocarpine stimulates 5-hydroxytryptophan and 5-hydroxytryptamine (serotonin) with inhibition of NAS and melatonin by subsequent inhibition of NAT and HIOMT. Thus, the current finding of the increase of serotonin with decrease of N-acetylserotonin and melatonin after arecoline treatment corroborate with that of the earlier findings of pilocarpine, and indicates that arecoline inhibits the functions of the pinealocytes and the supporting astrocytes and oligodendrocytes, and pineal hormones production in rats.

Cholinergic signalling is known to stimulate phosphoinositide (PI) (Laitinen et al., 1995) which is hydrolyzed into inositol triphosphate (IP₃) and diacylglycerol (DAG) (De Groot, 2001). The latter compounds stimulate PKC, resulting in an enhanced release of serotonin (Finocchiaro and Tenez-Inon, 1991). Simultaneously, IP₃ is known to enhance calmodulin (CAM) secretion through stimulation of calcium release. Since melatonin is calmodulin antagonist (Sotovega et al., 2004), the enhanced secretion of calmodulin induced by IP₃ and PKC might have decreased the melatonin level, which is also evident from the findings of decreased synthesis and release of melatonin in the
arecoline recipient rats. Nevertheless, whether such a mechanism of action of arecoline can explain the present results of increased serotonin and decreased melatonin synthesis, or release, or both, need to be confirmed.

Arecoline treatment also caused stimulation of Leydig cell activity at ultrastructural level by showing hyperactive nucleus with abundance of SER and electron-lucent (clear) secretory vesicles in rats. Moreover, serum testosterone level was increased after the treatment. Yang et al. (2004b) have reported that arecoline stimulates testosterone synthesis in vitro by upregulating the rate limiting enzyme of testosterone through non-cAMP pathway in rats. A similar mechanism of arecoline action in inducing testosterone production cannot be ruled out in our study in rats. Simultaneously, arecoline treatment caused significant stimulation of testosterone target, by showing ultrastructural abundance of RER and large dense core with peripheral electron-lucent secretory vesicles in the prostate. Moreover, fructose and sialic acid concentrations respectively of the coagulating gland and seminal vesicle were increased in the arecoline recipients. Since the latter carbohydrate components are known to be reliable indices of testosterone activity, the present result further confirms the increased testosterone secretion triggered by arecoline in rats. Thus, arecoline stimulates Leydig cell activity both at ultrastructural and testosterone production levels which are confirmed by both testosterone assay by ELISA as well as by biological activity of testosterone on its target organs in rats. Additionally the adrenal cortex, especially the reticularis zone might be involved in contributing androgens induced by arecoline, but arecoline was reported to suppress adrenocortical reticules activity at ultrastructural level in mice (Dasgupta, 2008). Nevertheless, further studies are required to confirm the role of arecoline in the involvement of adrenocortical reticularis. There are evidences that pineal secretory
activity is altered by several hormones like FSH, LH, estrogen, testosterone and prolactin, and that their receptors have also been demonstrated in the pinealocytes of rats. There are also evidences that melatonin receptors are present in rat Leydig cells and prostate (Valenti et al., 1997), and that melatonin is known to suppress testosterone production and testosterone-dependent prostate growth in rats (Gilad et al., 1998). Thus, in the current study, arecoline stimulated Leydig cell activity and testosterone production. Consequently, the prostate activity was presumably stimulated due to inhibition of melatonin synthesis in rats. Since melatonin receptors have been demonstrated in the Leydig cell, and testosterone and other sex hormonal receptors in the pinealocytes, the interdependence of their activities between the pineal and the Leydig cell cannot be ruled out. These findings further confirm the reciprocal relationship between the pineal gland and testis (testosterone) as has been reported in most of the vertebrates studied (Bentley, 1998; Yilmaz et al., 2000; Kus et al., 2000). Arecoline is known to exert its excitatory action by binding the M2-muscarinic receptors on the cell membrane of neurons of the locus coeruleus (Yang et al., 2004a). Atropine, a well known muscarinic receptor inhibitor (Pradhan et al., 1986), has been reported to abolish arecoline action on thyroid-adrenal axis in mice (Dasgupta et al., 2010). Thus, it is likely that arecoline might have exerted its action on the pineal-testicular axis presumably via muscarinic receptor in rats.

**SUMMARY**

Arecoline is an alkaloid of betel nut of *Areca catechu*. Betel nut is chewed by millions of people in the world and it causes oral and hepatic cancers in human.
Arecoline has immunosuppressive, mutagenic and genotoxic effects in laboratory animals and also affects endocrine functions. The objective of this study was to investigate the effects of arecoline on pineal-testicular axis in rats. Since pineal activity is different in the day and at night, the current study is undertaken in both the photophase and scotophase. The findings were evaluated by ultrastructural and hormonal studies of pineal and testicular Leydig cells, with quantitations of fructose and sialic acid of sex accessories. Arecoline treatment caused suppression of pineal activity at ultrastructural level by showing dilatation of the cisternae of the rough endoplasmic reticulum (RER), large autophagosome-like bodies with swollen mitochondrial cristae, numerous lysosomes, degenerated synaptic ribbons and reduced number of synaptic-like microvesicles. Moreover, pineal and serum N-acetylserotonin and melatonin levels were decreased with increased serotonin levels in both the gland and serum. In contrast, testicular Leydig cell activity was stimulated with abundance of smooth endoplasmic reticulum (SER), electron dense core vesicles and vacuolated secretory vesicles, and increased testosterone level in the arecoline recipients. Consequently, the testosterone target, like prostate, was ultrastructurally stimulated with abundance of RER and accumulation of secretory vesicles. Fructose and sialic acid concentrations were also significantly increased in the coagulating gland and seminal vesicle, respectively. These results were more significant in the scotophase than the photophase. The findings suggest that arecoline inhibits pineal activity, but stimulates testicular function (testosterone level) and its target organs presumably via muscarinic cholinergic receptor in rats.
Chapter - 1B

Effect of arecoline on androgen receptors and G1 cell cycle proteins in the prostate gland of male rats
INTRODUCTION

Arecoline has been attributed with adverse effects on the reproductive physiology. It is known to reduce sperm motility (Er et al., 2006), induce abnormality in the shape of sperm heads and stimulate unscheduled DNA synthesis in early spermatids in mice testis (Sinha and Rao, 1985a). Arecoline increases Cox-2 expression in human sperm cells in a dose-dependent fashion (Er et al., 2006). Cox-2 proteins are known to be involved in prostaglandin synthesis, which in turn influences growth of the ventral prostate and seminiferous tubules, sperm motility and contraction of smooth muscles surrounding seminiferous tubules. It has been suggested that arecoline might interact with human sperm membrane and cause a significant decrease in its motility via the Cox-2 pathway (Er et al., 2006). It has been demonstrated that lower doses (20 mg/kg body weight) of arecoline significantly stimulate testosterone production over the basal levels in mice (Yang et al., 2004b). Recent studies have revealed that arecoline increases testosterone secretion from Leydig cells of the rat testis (Saha et al., 2007), by increasing the activity of 17β-hydroxysteroid dehydrogenase and enhancing the expression of StAR (Wang et al., 2008). Increased testosterone, in turn, stimulates an increase in fructose and sialic acid content of the coagulating gland and seminal vesicle, respectively (Saha et al., 2007). A dose-dependent dualistic role of arecoline for testosterone production possibly employs non-cAMP-dependent pathway of steroidogenesis (Yang et al., 2004b). In addition, arecoline can induce cell cycle arrest at G2/M stage (Chang et al., 2001b, 2004b; Lee et al., 2002), which is a consequence of cells with DNA damage.
It is already evident that testosterone influences expression of androgen receptors of the prostate gland (Blok et al., 1992) and induces accumulation of androgen receptor mRNA in the rat ventral prostate (Mora and Mahesh, 1999). Since arecoline has a profound influence on the Leydig cells of the testis, this work attempts to evaluate the effect of arecoline on the circulating testosterone levels in male rats in a dose and time-dependent manner, and its consequential effect on the expression of androgen receptors in the prostate gland of arecoline treated male rats. The study is also an endeavor to unravel the mechanism behind the mutagenic effects of arecoline in the rat prostate gland.

MATERIALS AND METHODS

Experimental Design

Twenty rats were equally divided into 2 groups, A and B. Group A served as control and were injected with normal saline without arecoline. Group B received arecoline hydrobromide injection intraperitoneally at a dose of 10 mg/kg body weight for 10 days, as described before.

Animal Autopsy and Tissue Collection

On day 11, after termination of all the experiments, rats were anaesthetized with sodium barbital. Prostate glands were dissected free washed well with cold PBS and processed for total RNA and protein extraction.
RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction

Prostate tissues was collected from the arecoline treated and untreated rats. 100 mg of tissue samples were frozen quickly in liquid nitrogen and total RNA was isolated using TRI reagent (SIGMA) following manufacturer's instructions. Total RNA was dissolved in DEPC water and quantified by UV spectrophotometry. Reverse transcription reaction was performed at 42°C with 5 μg of RNA in 5X reaction buffer (Fermentas, USA) containing 100-pmol random hexamer primer, 10 mM dNTP mixture (Fermentas, USA), 20 units of RNAse inhibitor (Bioline, USA) and 200 units of RevertAid™ MMuLV Reverse Transcriptase (Fermentas, USA). PCR was initiated using 2.5μg of cDNA in 1X PCR buffer, 10 mM dNTPs and 1 unit of Taq DNA Polymerase (Vivantis, USA). The primers used are shown in Table 2. PCR was carried out for 35 cycles using an annealing temperature of 58°C. Samples were fractioned by 2% agarose gel electrophoresis and quantified using a BioRad Gel Documentation System.

Table 1: Primers used for RT-PCR reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Reference</th>
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<tbody>
<tr>
<td>AR</td>
<td>ACCCTCCCATGGCACATTTT</td>
<td>TTGGTGTGGCACACAGCACAG</td>
<td>Okada et al., 2003</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTACTGGCGCTGCAAGGCT</td>
<td>GCCATGAGGGTCCACCACCCTGT</td>
<td>Okada et al., 2003</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>CTGGCCATGAACTACCTGGA</td>
<td>GTCACACTTTGATCACTCTGG</td>
<td>Kowalski et al., 2002</td>
</tr>
<tr>
<td>CDK4</td>
<td>TGGTGTCCGTCCTATGGGA</td>
<td>GGTAGCTGTAGATTCTGGCT</td>
<td>Corroyer et al., 1996</td>
</tr>
</tbody>
</table>
**Western Blot**

10 mg of prostate tissue samples were lysed with RIPA Buffer (150 mM NaCl, 50 mm Tris, 0.1% Triton X-100 and 0.1 SDS containing protease inhibitors [4-(-2-aminoethyl benzene sulphonyl fluoride), EDTA, leupeptin, aprotinin and bestatin hydrochloride]. Protein concentrations were determined by Bradford assay and equal amount of proteins (30 µg) were fractioned by 10% SDS-polyacrylamide gel electrophoresis. Proteins were electrically transferred to PVDF membranes and blocked for 2 hrs at room temperature with 5% non-fat dry milk. Blots were subsequently incubated with anti-androgen receptor, anti-cyclin D1 and anti-CDK4 antibodies raised in rabbit (Santa Cruz, USA; 1:1000) for 18 hours at 4°C. Immunoreactive proteins were detected after 1 h incubation with horse radish peroxidase-conjugated goat anti-rabbit IgG (1:3000) at 25°C (Chatterji et al., 2004). Corresponding protein bands were visualized by staining the membranes with 0.2% diaminobenzidine in 50 mM Tris (pH 7.4) containing 0.2% H₂O₂. Blots were also incubated with β-tubulin antibody (Santa Cruz, USA) as a loading control.

**RESULTS**

*Overexpression of the androgen receptor in the rat prostate in response to arecoline*

The prostate gland is a major male sex accessory gland, which is under the regulation of neural and hormonal mechanisms (Hernandez et al., 2007). Androgens strongly influence the adult prostate in terms of the maintenance of its morphology and secretory activity (Banerjee et al., 2000). The prostate responds to androgens by widely distributed androgen receptors (AR) in epithelial cells, smooth muscle, and stroma cells
(Prins and Birch, 1993). It has been shown that the androgen-AR complex is translocated to the nucleus to regulate gene transcription (Chatterjee, 2003), and androgens increase both the level and half-life of AR. Since arecoline treatment led to an increase in serum testosterone levels, we attempted to investigate the effect of arecoline on the expression of androgen receptors in the prostate of rats. Figure 1 depicts an increased expression of the androgen receptor in arecoline-treated prostate, as compared to that of untreated ones, both at the mRNA transcript and protein levels, as estimated by RT-PCR and western blotting, respectively. Statistical analysis data has revealed a 1.5 fold increase in AR expression in prostate gland of rats treated with arecoline (p<0.001).

Fig 1: Androgen receptor (AR), Cyclin D1 and CDK 4 expressions analyzed by semi quantitative RT-PCR compared with controls and arecoline treated rats with a statistical significance of p<0.001.
Enhanced cyclin D1 and CDK4 expression in arecoline-treated prostate gland

Studies in rodent and human prostate have shown that androgens and the androgen receptor can regulate the expression of G1 cyclins, CDKs and CDK inhibitors (Chen et al., 1996; Gregory et al., 2001). Increased levels of androgens can stimulate the expression of the cell cycle regulators, especially those that are involved in the G1-S transition, by contributing to the phosphorylation and inactivation of the retinoblastoma gene, whereas castration reduces the expression levels. Concomitantly, we investigated if arecoline, which increased the serum testosterone levels and androgen receptor expression in male rats, would additionally alter the expression of Cyclin D1 and CDK4 in the prostate gland. RT-PCR and western blot data strongly suggests that arecoline increased the expression of both Cyclin D1 and CDK4 expression by 1.5 fold, as compared to the control prostate (Figure 2). Increased CDK4 expression was thus associated with entry into S phase.
DISCUSSION

Arecoline is one of the major ingredients in areca nuts and is responsible for many of the effects of areca nut chewing, including oral carcinogenesis, primarily by deregulating the cell cycle control (Chang et al., 2001a). Although there is no report till date concerning the plasma arecoline concentration in humans, in vitro studies have demonstrated that the arecoline cytotoxicity to gingival keratinocytes was observed at concentrations of 0.8–1.2 mM (Jeng et al., 2003). In addition to causing oral cancer,
arecoline also has various adverse effects on the human reproductive axis. The results reported here show that arecoline upregulates the expression of androgen receptors in the prostate gland, the major target for testosterone, and also increases the expression of cell cycle regulatory proteins associated with cell proliferation, especially cyclin D1 and CDK4. Our results also provide a molecular explanation, at least in part, for the increased tumorigenicity displayed by regular arecoline intake in humans.

In addition to the numerous cytotoxic and genotoxic effects of arecoline, areca nut and betel quid chewing also showed adverse effects on pregnant women who used to chew betel quid. In addition to reduction in weight of the newborns, the adverse pregnancy outcomes included spontaneous abortion, premature delivery, stillbirth, fetal malformation (Yang et al., 2001) and embryo-toxicity (Sinha and Rao, 1985b). Sinha and Rao (1985a) also investigated the influence of arecoline on the morphology of sperm in mice. An increase in the percentage of abnormal sperm was observed following exposure to arecoline, and treatment with arecoline increased DNA synthesis response in the germ cells of mice. A single study by Wang et al. (2008) has reported that arecoline can stimulate testosterone synthesis in rats by acting directly on the Leydig cells. Interestingly, the lowest arecoline-stimulated testosterone concentration was higher than that of the basal testosterone levels observed in the control animals. As reported earlier, the effects of arecoline on the increased testosterone secretion were mostly due to increased steroidogenesis (Wang et al., 2008). Although higher levels of serum testosterone render males more active sexually and less likely to have blood pressure and heart attack, they are often prone to indulge in behavioral imperfections and most often turn out to be extremely aggressive. Sustained high blood levels of testosterone are also
often associated with an increased risk of prostate cancer, and hence detrimental to human health.

The prostate gland is directly dependent on androgens for its development and activity. However, there is no direct evidence implicating increased testosterone levels as a cause for prostate cancer, although it has been shown that higher androgen levels can promote the growth of androgen-dependent prostate tumors. In addition, till date, there is no study regarding the effect of arecoline on the proliferation or secretory activity of the prostate gland. Transmission electron microscope studies revealed cellular proliferation, along with highly dilated rough endoplasmic reticulum cisternae in the secretory epithelial cells of the prostate (Saha et al., 2007). Interestingly, migration of secretory vesicles to the luminal surface and loss of microvilli on the cellular surface are also observed in these cells when compared to the control group. These cellular alterations are related to protein synthesis and release, and are androgen-dependent processes. Therefore, these changes were possibly caused by the increased level of testosterone in response to arecoline treatment. The augmented activity of the gland may thus be attributed to increased serum testosterone levels along with increased parasympathetic stimulations to the prostate gland, both of which are affected by arecoline intake. Testosterone is known to act via the androgen receptors distributed on the prostate gland. Concomitantly, our findings reveal that the androgen receptors of the prostate gland are over-expressed in the arecoline-treated rats as compared to the control rats. Increased expression of androgen receptors in response to arecoline further corroborates the fact that the prostate largely responds to increased serum testosterone, which in turn enhances both the transcription and translation of the androgen receptors.
It is well established that stimulation of androgen receptors by testosterone can trigger rapid activation of the MAPK pathway, and thereby induce a mitogenic response. While the mechanisms supporting the capacity of androgen receptors to induce a mitogenic program may be diverse, it is apparent that ligand-dependent activation of the receptor is important for involvement of the cell cycle machinery in prostate cancer cells (Balk and Knudsen, 2008). Transitions within the mitotic cell cycle are coordinated by the activation of cyclin-dependent kinase (CDK)/cyclin complexes, wherein cyclin binding induces the catalytic activity of the kinase. Mitogenic stimuli typically trigger accumulation of D-type cyclins (cyclins D1, D2, and/or D3), which can bind and activate the early G1 kinases CDK4 or CDK6 (Sherr and Roberts, 2004). Analyses of AR-dependent cell cycle progression in prostate cancer cells have shown that androgen is a critical regulator of the G1-S transition (Balk and Knudsen, 2008) and that androgen stimulates cyclin D1 accumulation and promotes active CDK4/cyclin D1 assembly (Xu et al, 2006). Concomitantly, our results have shown that arecoline upregulated the expression of both cyclin D1 and CDK4 in the prostate gland of male rats, indicating a unique crosstalk mechanism between the arecoline-induced androgen receptor and cell cycle pathways that are essential in coordinating and/or maintaining androgen-dependent cellular proliferation. Arecoline, which has already been shown to cause Cyclin D1 overexpression in squamous cell carcinomas of Taiwanese patients (Kuo et al., 1999), may thus be a putative risk factor for other cancers.

The health implications of mutagenic or carcinogenic chemicals that are consumed by humans are a significant issue. Arecoline has been shown to be a major cause of transforming oral epithelial cells (Yang et al., 2004 c) and generating reactive oxygen species (Nair et al., 2004), leading to oral cancer in several patients. Although
the exact molecular mechanisms are yet to be unraveled, our results reveal that moderate consumption of areca nuts may be temporarily beneficial for male physical or sexual ability, whereas sustained intake seems to be harmful from the point of view of increasing the number of abnormal sperms, eventually leading to infertility. The effects also extend to morphologic and functional transformations of the prostate cells, rendering them over-active and possibly leading to a hyperplastic stage. Arecoline-stimulated androgen receptor over expression, on the other hand, can turn on a totally different set of gene signals to initiate prostate cancer and finally requiring no androgen at all to continue the process.

SUMMARY

Areca nut chewing is the fourth most popular habit in the world, due to its effects as a mild stimulant, causing a feeling of euphoria and slightly heightened alertness. Areca nuts contain several alkaloids and tannins, among which arecoline is the most abundant and is known to have several adverse effects on the human physiology. The objective of this study was to evaluate the effects of arecoline on the prostate gland and determine the putative mechanism of arecoline action in this gland. The findings depicted here show that arecoline treatment leads to a two-fold increase in serum testosterone levels, along with morphological alterations in the ultrastructure and upregulation of androgen receptors of the rat prostate gland. Furthermore, increase in serum testosterone levels and androgen receptors lead to over expression of the G1-S regulatory proteins, cyclin D1 and CDK4. The results implicate arecoline intake as a major risk factor for development of prostate cancer.