Aims and Objectives
Betel nut contains huge amount of arecoline (7.5 mg/g of dry betel nut) which variously affects different endocrine functions. Betel nut is chewed by people irrespective of their problem related to diabetes, hypothyroidism or different stresses. Literature review reveals that millions of people chew betel nut to increase their capacity to work, euphoria arousal response and heightened alertness, in addition, to multiple adverse effects on general physiology. Our knowledge of arecoline action on endocrine functions under such conditions is poor. Therefore, in the current research, the role of arecoline on pineal-testicular activity under normal conditions and conditions of diabetes, hypothyroidism and stress was investigated in rat model.

Precise objectives of the current investigation include the study of:

(i) effect of arecoline on pineal-testicular function in normal rats and its role on the expression of androgen receptors and cell cycle proteins in the prostate gland;
(ii) role of arecoline on pineal-testicular axis in experimentally-induced diabetes;
(iii) effect of arecoline on pineal-testicular activity in experimentally-induced hypothyroid condition; and
(iv) effect of arecoline on pineal-testicular axis under diverse stress (auditory by noise and metabolic stress by food restriction and water deprivation).
Materials and Methods
Animal model

Adult male rats (~100 gm body wt) were collected from the breeding colony and were kept in polythene cages (30 cm x 15 cm x 15 cm) in controlled laboratory conditions (photoperiod, 12L : 12D, and temperature : 25°C) with standard diet (Oser, 1965) and water accessible ad libitum for 5 days for acclimatization and subsequently for the experiments. 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/CPCSEA).

Arecoline administration

Arecoline hydrobromide (Methyl 1-methyl-1,2,5,6 etrahdyronicotinate) (Sigma, U.S.A.), dissolved in normal saline (0.9% NaCl), was injected intraperitoneally at a dose of 10 mg/kg body weight. Each dose (10 mg/100 gm body wt) was divided equally to half (5 mg/100 gm body wt), and each half dose was injected twice daily (11am and 5pm) because of its short half-life (Pradhan et al., 1986).

Animal autopsy, tissue and blood collection

All experimental rats were anaesthetized by sodium barbital injection. Blood was drawn from the heart, and serum was collected and stored at ~20°C until assayed for hormone concentrations. Pineal gland, seminal vesicle and coagulating gland were dissected free and processed for biochemical estimation. Pineal, prostate and testis were also dissected out the processed for Transmission Electron Microscopic study.
Transmission Electron Microscopy

Pineal, prostate and testis were dissected free, cut into small pieces (1x1 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 buffer, the tissue samples were post fixed in 1% osmium tetroxide 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. The measurements of the cytolsamic organelles of the pinealocytes (synaptic ribbons, SR, and synaptic-like microvesicles, SLMV) were done using the software (‘Soft Imaging System’, GmBH, Münster, Germany) equipped with the microscope. In all the specimens, the length as well as the width of the SR (N=20 for each specimen) were measured on the digitized images displayed on the computer screen at a magnification of 28000 X, and the data were expressed as mean ± standard error.

Biochemical Assay

(a) Estimation of pineal and serum indoleamines

Pineal and serum serotonin, N-acetylserotonin and melatonin were assayed by the method of Miller and Maickel (1970). Pineal glands were homogenized in 1 ml of 0.05 N NaOH with a glass homogenizer and the homogenate was added with 6 ml chloroform and centrifuged at 3000 g for 10 min. The organic phase contained 5-methoxy
tryptamine and melatonin, and the aqueous phase contained serotonin, N-acetylserotonin, 5-hydroxyindole acetic acid and 5-methoxyindole acetic acid. 2.5 ml of the organic phase containing melatonin was added with 8.0 ml of n-heptane and 0.3 ml of 5 N HCl and vortexed. Melatonin was transferred to the acid phase. 0.2 ml of the aliquot of the acid phase containing melatonin was added with 0.67 ml of 10 N HCl (containing 15 mg of orthophthalaldehyde (OPT) as fluorescent compound in 100 ml of 10 N HCl) and vortexed. The mixture was heated in a boiling water bath for 10 min, cooled and its fluorescence was measured in a Hitachi spectrofluorometer (Model 650-10M) at 360/470 nm wave length. For serotonin, 0.6 ml of the aliquot in aqueous phase containing serotonin and N-acetyl serotonin was added with 10 ml of ethyl acetate and 0.2 ml of 1.2 N HCl. The ethylacetate phase contained N-acetylserotonin, and the aqueous phase contained serotonin. The serotonin – OPT reaction was carried out in a 0.2 ml aliquot of the aqueous phase and measured as described for melatonin. For N-acetylserotonin, 4 ml of ethylacetate phase was added with 6 ml of n-heptane and 0.3 ml of 1 N HCl and vortexed. N-acetylserotonin was transferred to the acid phase. 0.2 ml aliquot of the acid phase was reacted with OPT as mentioned earlier and was measured as described for melatonin.

(b) Estimation of serum testosterone

Serum testosterone level was assayed by ELISA (Chen et al., 1991) using the pathozyme testosterone kit (Product code OD497) of OMEGA, UK. Serum testosterone level was assayed by ELISA on the principle of competitive binding between testosterone in the test specimen and testosterone-HRP conjugate for a constant amount of rabbit anti-testosterone. Goat anti-rabbit IgG-coated wells were incubated with testosterone standards, controls, arecoline-treated samples, testosterone-HRP conjugate
reagent and rabbit anti-testosterone reagent. During the incubation, a fixed amount of HRP-labeled testosterone competes with the endogenous testosterone in the standard, control and treated serum for a fixed number of binding sites of the specific testosterone antibody. Thus, the amount of testosterone peroxidase conjugate, immunologically bound to the well, progressively decreased as the concentration of testosterone in the experimental samples increased. Unbound testosterone peroxidase conjugate was then removed by washing the wells. A solution of tetramethyl benzidine (TMB) was added, resulting in the development of blue colour. The colour development was stopped with the addition of stop solution (dilute sulphuric acid) and the absorbance was measured by ELISA Reader (Qualigen Plate Reader PR-601, UK) at 450 nm. The intensity of the colour formed was proportional to the amount of enzyme present and was inversely related to the amount of unlabelled testosterone in the sample. A standard curve was obtained by plotting the concentration of the standard versus absorbance. The testosterone concentrations of the control and treated serum were run concurrently with the standards and were calculated from the standard curve.

(c) Estimation of fructose

The concentrations of fructose of the coagulating gland (Roe et al., 1949) and sialic acid of the seminal vesicle (Warren, 1959) were assayed and measured by a spectrophotometer (Perkin-Elmer). For fructose, the coagulating gland was weighed and homogenized in 5 ml distilled water and the homogenate was centrifuged in a cold centrifuge (4°C) at 8000 g for 5 min. 1 ml of the supernatant was added with 1 ml of resorcinol reagent and 7 ml of HCl (30%), and the mixture was heated in a water bath at 80°C for 10 min, and cooled to room temperature. The optical density was measured by a spectrophotometer (Perkin-Elmer) at 520 nm.
(d) Estimation of sialic acid

The seminal vesicle was weighed, and the sialic acid was extracted and oxidized with Na-periodate in concentrated phosphoric acid. The periodate oxidation product was coupled with thiobarbituric acid and the resulting chromophore was extracted into cyclohexanone. The absorption maximum was measured by a spectrophotometer (Perkin-Elmer) at 549 nm for sialic acid. A second absorption maximum at 532 nm due to 2-deoxyribose was also measured for which a correction was made by subtracting the data at 532 nm from the data at 549 nm.

Statistical Analysis

Data were analyzed statistically by one way analysis of variance followed by Tukey's post hoc test and students't-test (Snedecor and Cochran, 1989) to ascertain the degree of significance between experimental groups.