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ARECOLINE CANNOT ALTER PINEAL-TESTICULAR RESPONSES TO METABOLIC STRESS IN WISTAR RATS

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Abstract

Context. Betel nut is consumed by millions of people for stress reduction and increased capacity to work. One of its components is arecoline which is useful for Alzheimer and schizophrenia; it also influences endocrine and gonadal functions.

Objective. Objective is to examine whether arecoline can influence pineal-testicular function in metabolic stress.

Design. Rats were deprived of food or water or treated them with arecoline, each separately for 5 days.

Subjects. Pineal and testis with sex accessories were studied.

Methods. Ultrastructural (pineal, testis, Leydig cells and prostate), hormonal (melatonin and testosterone) and other parameters (fructose and sialic acid) were examined. Pineal indoleamines were quantitated by fluorometric method; testosterone by ELISA, and carbohydrate fractions by spectrophotometric methods.

Results. Inanition/ water deprivation caused pineal stimulation ultrastructurally (with enlarged synaptic ribbons) and elevation of melatonin level, but reproductive dysfunction by ultrastructural degeneration of Leydig cells and prostate with fall of testosterone, fructose and sialic acid concentrations. Arecoline treatment showed reversed changes to those of metabolic stress, but arecoline treatment in metabolic stress showed same results as in metabolic stress.

Conclusion. The findings suggest that arecoline cannot alter the action of metabolic stress on pineal-testicular activity in rats.

Key words: Pineal, testis, arecoline, metabolic stress, rat.

INTRODUCTION

Many people regularly consume a substantial amount of Areca nut for psychophysiological gain in routine daily work. The alkaloid, arecoline, is also known to have therapeutic value for the treatment of Schizophrenia and presenile Alzheimer diseases (1), but it also has some adverse side effects, like increased risk of oral mucosal cancer, hepatotoxicity, immunosuppression and depression of antioxidant production (2). In addition arecoline causes endocrine disorder including male gonadal functions in rats and mice (3-5). Arecoline has been reported to suppress pineal activity but stimulates testis functions (5) including prostate growth by hyperplasia (6). It induces hypothyroidism (3), but it stimulates adrenocortical function (4) in mice. A protective effect of arecoline has also been reported to occur in experimentally induced male diabetic rat (7).

Food deprivation reduces LH dependent testosterone release in bulls, pulsatile LH and testosterone secretions in adult male rhesus monkey (Macaca mulatta) and serum FSH level in rats (8). Decrease of testis, seminal vesicle and ventral scent gland size in Mongolian gerbils and body mass in Syrian hamsters under short photoperiod have been reported to occur following water deprivation. Parents having long-term consequence on reproductive capabilities and survival of offsprings (9) and retardation of genital reflexes (10) also occur under water deprivation stress in rats. Chronic swimming results in a significant reduction in testis, epididymis, prostate and seminal vesicle stromal indices leading to reproductive dysfunction in male albino Wistar rats (11). There is loss of fertility with increased volume of prostate mucosa in male rats subjected to psychological and physiological chronic stress, or chronic heat stress, both *in vivo* and *in vitro* (12-14). Since arecoline has diverse side effects on endocrine functions including gonads, it is pertinent to examine whether or not arecoline can alter pineal-testicular activity in metabolic stress in rats.

MATERIALS

Animal model

Sexually mature male Wistar rats (100 days

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old, ~100 g body wt) were collected from the breeding colony of the animal house of Department of Zoology, Calcutta University, and were housed in polythene cage (40 cm x 20 cm x 15 cm) in controlled laboratory conditions (temperature 25˚C, photoperiod 12 L:12D) with standard diet (5). Food and water were available ad libitum 5 days for acclimatization with the laboratory conditions.

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 daily for 5 days. Each dose was divided equally to half, and each half dose was injected twice daily at 09 h and 18h because of its short half-life (2).

Inanition

Animals were deprived of daily ration of food consecutively for 5 days, but water was accessible ad libitum throughout the experiment.

Water deprivation

Animals were deprived of daily water intake consecutively for 5 days, but fed with standard diet ad libitum throughout the experiment.

Experimental Design

Thirty six rats were divided equally in six groups. Group A rats served as control which received normal saline without arecoline. Group B received arecoline at a dose of 10 mg/kg body weight daily for 5 days. Group C rats were not fed (inanition) for 5 days, and group D rats were unfed and treated with arecoline as in group B for 5 days. Group E rats were deprived of water for 5 days. Group F rats were deprived of water and treated with arecoline as in group B for 5 days.

METHODS

Transmission Electron Microscopy

Pineal, testis and prostate glands were dissected out, cut into small pieces (~1 mm^3) 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, tissue samples were post-fixed in 1% osmium tetroxide for 2 h at 4˚C. Tissues were then dehydrated through ascending grades of ethanol, infiltrated and embedded in araldite CY 212. 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. For all the specimens, digitized images of cellular organelles (n=20 for each specimen) were recorded on the displayed computer screen at a magnification of 28000 X.

Biochemical assays

All the experiments were terminated on Day 6 of the experiments. Rats were anesthetized by intramuscular injection of sodium barbital (1 mg/kg body weight). Blood was drawn from the heart; serum was collected and stored at –20˚C until assayed for testosterone, melatonin, N-acetyl serotonin and serotonin. Coagulating gland and seminal vesicles were dissected out, weighed on a semi-micro analytical balance (Mettler, Switzerland) and stored at -20˚C for sialic acid and fructose assays. Pineal gland was also dissected out to estimate the indoleamines level.

Estimation of pineal and serum indoleamines

Pineal and serum serotonin, N-acetyl serotonin and melatonin levels were assayed by the fluorometric method as reported earlier by us (5). Pineal glands were homogenized in 1 mL of 0.05 N NaOH with a glass homogenizer and the homogenate was added with 6mL 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-acetyl serotonin, 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 by a Hitachi spectrofluorometer (Model 650-10M) at 360/470 nm wavelength. 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 ethyl acetate phase contained N-acetyl serotonin, and the aqueous phase contained serotonin. The serotonin – OPT reaction was carried out in 0.2 mL aliquot of the aqueous phase and measured as described for melatonin. For N-acetyl serotonin, 4 mL

of ethyl acetate phase was added with 6 mL of n-heptane and 0.3 mL of 1 N HCl and vortexed. N-acetyl serotonin was transferred to the acid phase. 0.2 mL aliquot of the acid phase was reacted with OPT as described earlier and was measured as followed for melatonin.

Estimation of serum testosterone

Serum testosterone level was assayed by ELISA reported by Saha *et al.* (5) 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, control and treated samples, testosterone-HRP conjugate reagent and rabbit anti-testosterone reagent. During 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 Plate Reader (Qualigen, 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. Testosterone concentrations of the control and treated serum were run concurrently with the standards and were calculated from the standard curve.

Estimation of fructose

Fructose concentration of the coagulating gland was assayed as reported by Saha *et al.* (5). Briefly, the coagulating gland was weighed and homogenized in 5 mL distilled water. The homogenate was centrifuged at 8000xg for 5 min at 4˚C. 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 80˚C water bath for 10 min. The reaction mixture was cooled to room temperature and optical density was measured by a spectrophotometer (Perkin-Elmer) at 520 nm.

Estimation of sialic acid

Sialic acid content of the seminal vesicle was assayed from the homogenate of the control and treated samples (arecoline and arecoline treatment in metabolic stress) as reported earlier by us (5). The extracts were oxidized with sodium periodate in concentrated phosphoric acid. The periodate oxidation product was coupled with thiobarbituric acid and the resulting chromophore was extracted using cyclohexanone. The absorption maximum for sialic acid was measured at 549 nm. A second absorption maximum was also measured at 532 nm, due to the presence of 2-deoxyribose. The correction was made by subtracting the data at 532 nm from the data at 549 nm. Actual quantity of sialic acid was calculated.

Coefficients of inter-assay and intra-assay variations of hormones were recorded at 4.8% (maximum) and at 5.2% (maximum) levels respectively. Recovery of standards (indoleamines, testosterone, fructose and sialic acid) was recorded at the level of 94%.

Statistical Analysis

All the data were expressed as mean ± S.E.M. Data were analyzed statistically by one way analysis of variance (ANOVA) followed by Tukey's and Student's t-test (15) to determine the degree of significance between the control and experimental groups.

RESULTS

Inanition

Pineal (TEM study)

Control

Pinealocyte of control rat showed elongated euchromatic nucleus with a prominent nucleolus. Mitochondria and rough endoplasmic reticulum(RER) were abundant with several electron dense plate-like synaptic ribbons (SR), oriented parallel to each other(mean length: 0.377 ± 0.02 μm) and width (36.21 ± 0.91 nm) (Fig. 1A).

Inanition

The pinealocyte showed enlarged nucleus with double layer of nuclear membrane (NM), nuclear lobe (NL) and two prominent electron dense nucleoli (NU), mitochondria (M) with cristae and synaptic-like microvesicles (SLMV) were seen (Fig. 1B). RER with cisternae, Golgi body and long SR were seen in the pinealocyte cytoplasm (Fig. 1C). The mean length (0.521 ± 0.04 μm) and mean width (55 ± 0.12 nm) of synaptic ribbons were increased significantly ($p <$

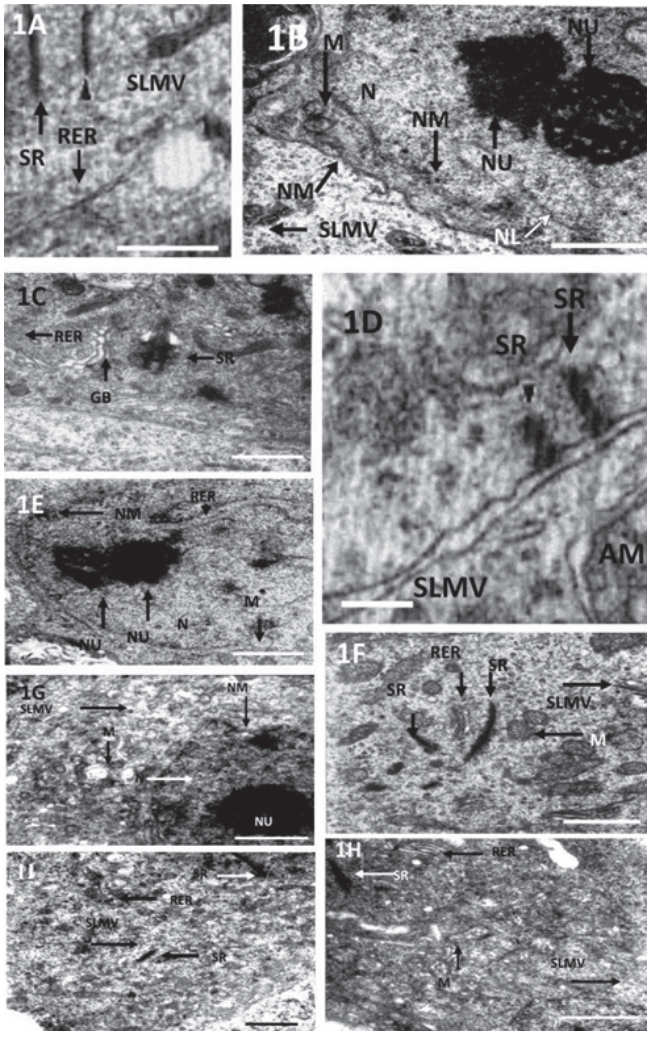

Figure 1. Inanition stress: Transmission electron micrographs (TEM) of the pinealocytes of rat. (A) Control: The pinealocyte showing electron lucent synaptic-like microvesicles (SLMV), rough endoplasmic reticulum (RER) and electron dense synaptic ribbons (SR). (B) Inanition: The pinealocyte showing enlarged euchromatic nucleus (N) with nuclear lobes (NL), conspicuous double nuclear-membrane (NM) and two large electron dense nucleoli (Nu), with mitochondria (M) and synaptic-like microvesicles. (C) Inanition: Rough endoplasmic reticulum (RER), Golgi body (GB) and enlarged SR are also seen in the pinealocyte. (D) Arecoline treatment showing reduced and crumpled SR, small SLMV and extremely dilated cristae of the mitochondria (M). (E) Inanition + arecoline treatment: The pinealocyte showing enlarged euchromatic nucleus (N) with distinct nuclear membrane (NM) and two electron dense nucleoli (Nu) and conspicuous RER. (F) Several long synaptic ribbons (SR) of various size, abundance of RER with conspicuous cisternae, mitochondria (M) and SLMV are also seen in the hyperactive pinealocyte after inanition and arecoline treatment. (G) Water-deprivation showing large euchromatic nucleus (N), conspicuous nuclear membrane (NM), large electron dense nucleolus (Nu), abundance of mitochondria (M) and SLMV in the hyperactive pinealocyte, and (H) abundance of RER with conspicuous cisternae, mitochondria (M) with well developed cristae and SLMV. (I) Water deprivation + arecoline: showing elongated SR, abundance of RER with conspicuous cisternae and SLMV in the pinealocyte cytoplasm. Scale bars: 1.5 μm (A, D), 1 μm (B-I).

0.001) compared with control rats.

Arecoline treatment

The pinealocyte cytoplasm showed abnormal mitochondria (AM) with unusually dilated cristae, degenerated SR with their decreased length and increased width (mean length = 0.198 ± 0.02 μm and mean width = 46 ± 3.9 nm) ($p < 0.01$), and small SLMV as compared with control (Fig. 1D).

Arecoline treatment in inanition

Pinealocyte cytoplasm showed enlarged euchromatic nucleus with distinct nuclear membrane (NM) and two electron dense nucleoli (NU) (Fig. 1E). Mitochondria (M) with well-developed cristae and RER with conspicuous cisternae were abundant. Several long synaptic ribbons (SR) of various size with tapering ends were seen (Figs. 1E & F). The mean length (0.751 ± 0.02 μm) and width (59 ± 0.12 nm) of SR were significantly increased ($p < 0.01$) compared with control.

Water deprivation

Pinealocytes showed enlarged nucleus with conspicuous nuclear membrane and large electron dense nucleolus. Abundance of mitochondria with well-developed cristae and RER with conspicuous cisternae were noticed. Large SRs with numerous SLMV were also observed. The mean length (0.498 ± 0.04 μm) and width (50.00 ± 0.12 nm) of synaptic ribbons were significantly increased ($p < 0.01$) compared with control rats (Figs. 1G, H).

Arecoline treatment in water deprivation

Pinealocytes showed large nucleus, RER with conspicuous cisternae and mitochondria with well-developed cristae. RER and mitochondria were more abundant than in water-deprivation. SLMV were frequently seen. The synaptic ribbons were elongated with a mean length of 0.590 ± 0.04 μm and a mean width of 56.00 ± 0.16 nm and were significantly increased ($p < 0.01$) compared with water deprivation (Fig. 1G, H). These manifestations were more intense after arecoline treatment in water deprivation.

Serotonin, NAS and melatonin (control, inanition and water deprivation)

Pineal and serum serotonin (Figs 2 a, b) and melatonin (Figs 2 e, f) levels were significantly increased with decreased level of N-acetyl serotonin (NAS) (Figs 2 c, d) after inanition stress compared with control. Arecoline treatment increased serotonin level but decreased both NAS and melatonin concentrations compared with control. Arecoline treatment during

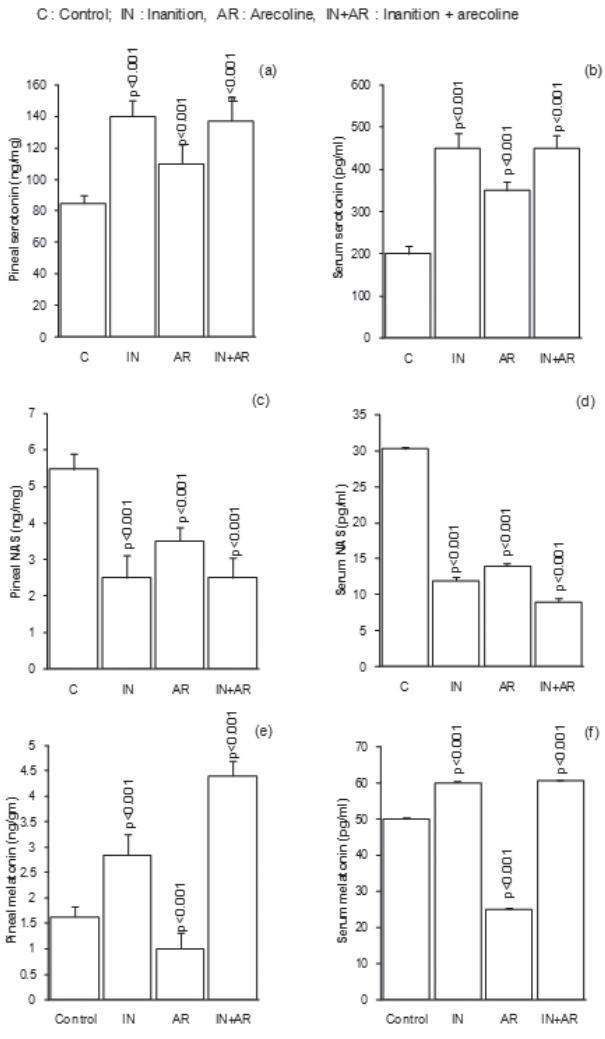

Figure 2. Changes in pineal and serum levels of serotonin (a, b), N-acetyl serotonin (c, d) and melatonin (e, f) following inanition (IN), arecoline (AR) and arecoline + inanition (IN+AR), water deprivation and arecoline + water deprivation compared to control (c) in rats. Bar diagrams represent mean + SE values. Level of significance P-values (control vs treated samples) are shown on top of each bar.

inanition showed same results as in inanition stress, without significant difference between inanition and arecoline treatment during inanition (Fig. 2). Results of pineal and serum indoleamines level in water deprivation were the same as in inanition. Pineal and serum NAS and melatonin levels were declined with elevation of serotonin after arecoline treatment or arecoline treatment in water deprivation stress were the same as observed after arecoline treatment in inanition.

Testis (Leydig cells) (TEM study)

Control

Leydig cells have ovoid nucleus with smooth endoplasmic reticulum (SER), dense core vesicles (DCV) and a few clear vesicles (CV) (Fig. 3A).

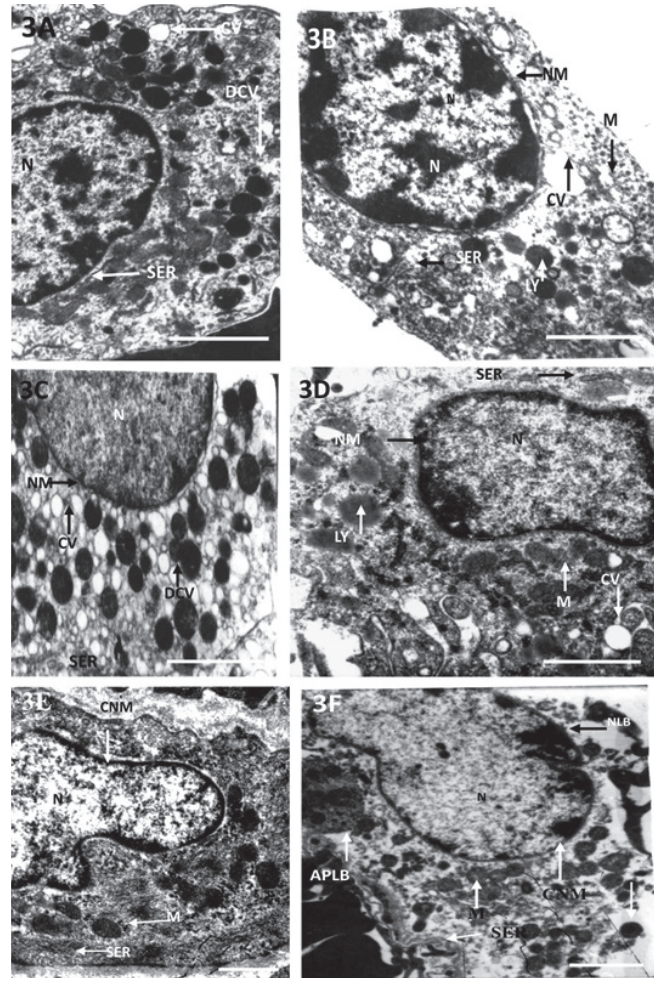

Figure 3. Inanition: TEM of the Leydig cell of rat. (A) Control : showing normal euchromatic nucleus (N) with smooth endoplasmic reticulum (SER and few dense core vesicles (DCV). (B) Inanition: degenerated Leydig cell with dissociated nuclear membrane (NM), extremely hyperchromatic nucleus (N), degenerated mitochondria (M) and SER, lysosomes (Ly) and large clear vesicles (CV). (C) Arecoline treatment showing enlarged euchromatic nucleus with distinct nuclear membrane and numerous DCV, CV and SER. (D) Inanition + arecoline treatment showing degenerated Leydig cell with crumpled and pycnotic nucleus (N), thick nuclear membrane (NM), degenerated SER and mitochondria (M), large lysosome (LY) body, scanty dense core vesicles (DCV) and large clear vesicles (CV) (compare with Fig. 3C). (E) Water deprivation: showing crumpled pycnotic nucleus (N) with crumpled nuclear membrane (CNM), scanty degenerated mitochondria (M) and SER. (F) Water deprivation + arecoline: Leydig cell showing degenerated pycnotic nucleus (N) with crumpled nuclear membrane (CNM) and fragmented nucleolus-like body (NLB), autophagosome like body (APLB), lysosome (Ly), and degenerated mitochondria (M) and smooth endoplasmic reticulum (SER) in the cytoplasm. Scale bars: 1 µm (A-G).

Inanition

Leydig cells were degenerated and showed hyperchromatic pycnotic nucleus with dissociated nuclear membrane and disorganized mitochondria, SER, DCV and CV. Large clear vesicles with lysosomes were seen (Fig. 3B).

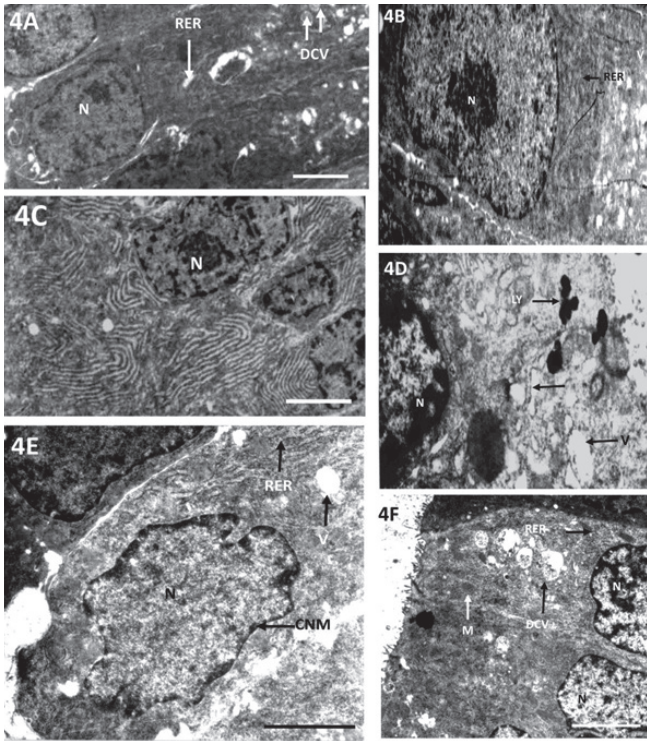

Figure 4. Inanition: TEM of epithelial cell of the ventral prostate gland of rat. (A) Control : tall columnar cells with basal round euchromatic nucleus (N), RER and scanty DCV. (B) Inanition: hyperchromatic pycnotic nucleus (N) with degenerated RER and numerous cytoplasmic vacuoles (V) (arrow). (C) Arecoline treatment: very tall columnar cells with abundance of RER oriented in the form of circular bundle occupying major area of the cell cytoplasm. (D) Inanition + arecoline: hyperchromatic and pycnotic nucleus (N), degenerated RER (arrow), numerous electron dense lysosomes (Ly) and cytoplasmic vacuoles (V) (arrow) in the cell cytoplasm (compare with Fig. 4C). (E) Water deprivation: pycnotic nucleus (N) with crumpled nuclear membrane (CNM) and disorganized RER. Vacuoles (V) are rarely seen. (F) Water deprivation + arecoline: degenerated manifestations with pycnotic nucleus (N), degenerated RER, mitochondria (M) and DCV. Scale bars: 1 µm (A-F).

Arecoline treatment

Leydig cells showed hyperactive nucleus along with abundance of SER, DCV and CV compared with control rats (Fig. 3C).

Arecoline treatment in inanition

Leydig cells were degenerated and showed crumpled pycnotic nucleus with thick nuclear membrane (NM). Subcellular organelles looked compact with disorganized SER, mitochondria and dense core vesicles. Lysosomes were also noticed in the cytoplasm (Fig. 3D).

Water deprivation

Leydig cells showed pycnotic nucleus with crumpled nuclear membrane (NCM). The round/ oval shape of the nucleus was lost. Cytoplasm became compact and showed disorganized SER and mitochondria. Dense core and clear vesicles were scanty (Fig. 3E).

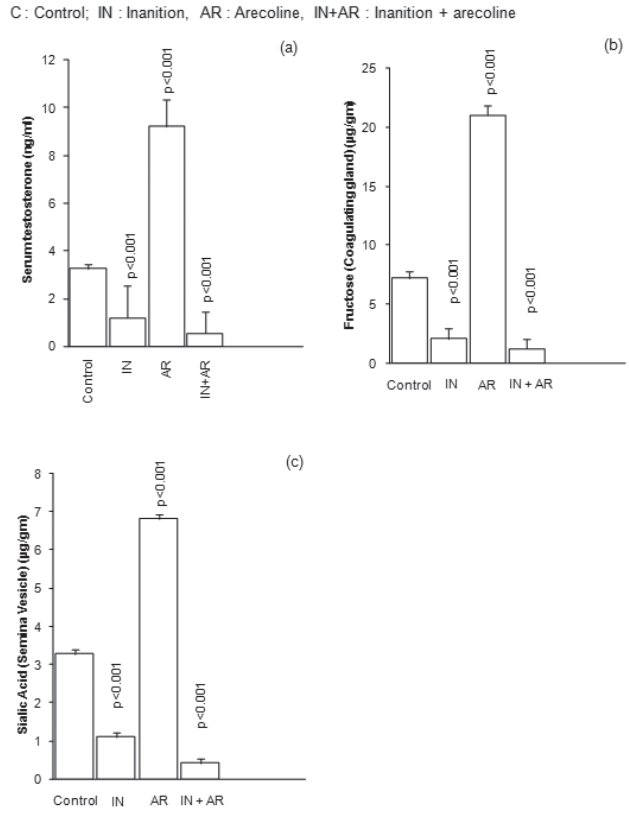

Figure 5. Changes in serum testosterone level (a), and fructose content of the coagulating gland (b) and sialic acid content of the seminal vesicle (c) following inanition (IN), arecoline (AR) and arecoline in inanition (IN+AR), (d) water deprivation and (e) water deprivation + arecoline treatment in rats.

Arecoline treatment in water deprivation

Cell boundaries were not distinguishable. Nucleus with crumpled nuclear membrane (CNM) showed fragmented electron dense nucleolus-like body (NLB). Autophagosome-like bodies (APLB), degenerated mitochondria and smooth endoplasmic reticulum were also observed (Fig. 3F).

Ventral Prostate (TEM study)

Control

It is composed of very tall columnar cells oriented perpendicular to the basement membrane, contained basal ovoid euchromatic nucleus and showed moderate number of inconspicuous RER (Fig. 4A).

Inanition

Perpendicular pattern of cell orientation was lost. Nucleus showed electron dense pycnotic nucleolus (N). Other organelles were scanty (Fig. 4B).

Arecoline treatment

Very tall columnar cells appeared with regular pattern of orientation. Euchromatic nucleus and abundance of RER were seen in the cytoplasm (Fig. 4C).

Arecoline treatment in inanition

Cell boundaries were not distinguishable. Hyperchromatic and pycnotic nucleus (N) with thick nuclear membrane, disorganized RER and mitochondria (M), abundance of lysosomes (LY) and clear vesicles were observed (Fig. 4D).

Ventral prostate

Water deprivation

Cells had pycnotic nucleus with crumpled nuclear membrane (CNM). Rough endoplasmic reticulum looked disorganized. Mitochondria, dense core vesicles and clear vesicles were scanty in the disorganized cytoplasm (Fig. 4E).

Arecoline treatment

Subcellular changes of prostate epithelium were the same as reported earlier by Saha *et al.* 2007 (5).

Arecoline treatment in water deprivation

Epithelial cell cytoplasm looked compact and organelles were not clearly distinguishable. SER and mitochondria looked disorganized when visible. Dense core vesicles were vacuolated with cell debris stored within the vesicles (Fig. 4E).

Testosterone, fructose and sialic acid

Serum testosterone concentration was significantly decreased in inanition or water deprivation, increased in arecoline treatment and declined after arecoline treatment in inanition or water deprivation stress (Fig. 5a). Fructose content of the coagulating gland (Fig. 5b) and sialic acid level of the seminal vesicle (Fig. 5c) were significantly declined in inanition or water deprivation stress, increased in arecoline treatment, and declined further after arecoline treatment in inanition or water deprivation stress (Fig. 5).

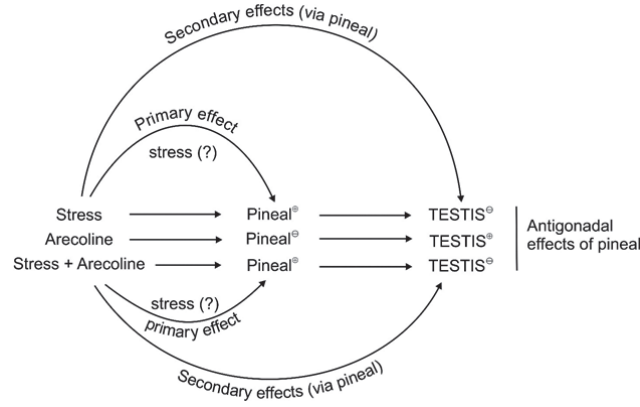

Figure 6. Summary of the results of pineal and testes interactions after arecoline treatment in metabolic stress in rats.

DISCUSSION

Betel nut chewers chew 3-4 or more unripe nuts daily for life and consequently consume a huge quantity of toxic arecoline, contained in the betel nut (7.5 mg/gm nut) (16). Earlier we have shown that arecoline suppresses pineal activity, but stimulates testis and testosterone dependent sex accessory functions in rats (5). Current experiments showed that inanition and water deprivation, both significantly caused stimulation of the pineal gland, confirmed from the findings of abundance of synaptic-like microvesicles (SLMV), and increased length and width of synaptic ribbons, because (SLMV) are known to appear abundantly in active pinealocytes (17), and length of synaptic ribbons (SR) are increased in hyperactive pinealocytes (18). Movement of rough endoplasmic reticulum (RER) with dilated cisternae towards the perinuclear area of the pinealocytes is related to the secretory activity or selective aggregation of raw materials required probably for biosynthesis of pineal indoleamines (19). The latter finding is correlated with the increased production of serotonin, N-acetyl serotonin (NAS) and melatonin levels recorded both in the pineal gland and blood serum, except depletion of NAS that may be related to the rate of conversion of serotonin to NAS and/or NAS to melatonin, influenced by the respective enzymes (NAT and HOMT). Since indoleamine levels were increased in both the pineal gland and blood, it is likely that metabolic stress induced probably both the synthesis and release of these indoleamines into circulation. Arecoline treatment alone caused pineal suppression, as evident from ultrastructural changes leading to degeneration of pinealocytes followed by depletion of NAS and melatonin levels, and thus confirms our earlier findings of pineal dysfunction in rats (5). In contrast, arecoline treatment under conditions of inanition and water deprivation caused stimulation of pineal activity both at ultrastructural and pineal hormonal levels, because SLMV and RER were abundant with enlarged size of SR marked in the hyperactive pinealocytes and elevation of pineal hormonal levels in stress recipients. It is known that N-acetyl transferase (NAT) and hydroxyindole-o-methyl transferase (HIOMT) are required respectively for conversion of serotonin to NAS and NAS to melatonin (19, 20). There are evidences that NAT (21) and HIOMT activities are enhanced in stress. Thus, elevation of serotonin and melatonin levels in metabolic stress or after arecoline treatment in metabolic stress could be due to enhanced activities of NAT and HIOMT in rats. Conversely, fall of NAS and melatonin levels after arecoline treatment in rats without stress could be due to inhibition of NAT

and HIOMT activities of the pinealocytes.

Earlier we have demonstrated that arecoline induced hepatotoxicity and depletion of the concentrations of antioxidant enzymes, i.e., glutathione, S-transferase (GST), superoxide dismutase (SOD), catalase and non-enzymatic antioxidant glutathione (2). Moreover, thiol depletion as well as attack of oxygen free radicals could be responsible mainly for arecoline intoxication (2). Lack of antioxidant enzymes, glutathione, thiol and ROS induced by arecoline treatment resulted in pineal dysfunction in rats. Arecoline treatment in metabolic stress showed pineal stimulation, probably by counteracting arecoline intoxication by involving stress hormones (adrenal) (?) that impaired antioxidant production in metabolic stress in rats.

Metabolic stress has significant effect on testes, and testicular hormone-dependent male sex accessories functions, because inanition and water-deprivation caused Leydig cell dysfunction at ultrastructural level, judged from reduced Leydig cell nuclear size with scanty cytoplasm, SER and mitochondria observed after inanition or water deprivation stress in rats. Furthermore, serum testosterone level was significantly declined after exposure to metabolic stress in rats. Additionally, testosterone-dependent sex accessories functions were suppressed, because cell size was drastically reduced with scanty RER and mitochondria, and presence of abundance of lysozymes in the prostate epithelium. It has been reported that mitochondria are swollen with few cristae seen in the Leydig cells during chronic stress. Furthermore, fructose contents of the coagulating gland and sialic acid level of the seminal vesicle were significantly declined in rats exposed to both inanition and food deprivation stress in rats. We have shown that arecoline stimulates Leydig cell function with elevation of testosterone level and sex accessories function ultrastructurally with the rise of fructose and sialic acid concentrations (5). Saha *et al.* (6) have further shown that arecoline stimulates prostate growth by increasing expression of testosterone receptors which augmented cell proliferation confirmed by an increase in the expression of Ki67 protein and overexpression of cyclin D1 and CDK4 cell cycle regulatory proteins in rats. Arecoline treatment in metabolic stress failed to counteract the inhibitory effect of stress on testes and sex accessories functions in rats. Gonadotropins, especially LH, is known to regulate Leydig cell activity and testosterone production (20). FSH and LH levels are increased in arecoline recipient rats (6). Thus, testes and sex accessories dysfunctions following arecoline treatment in metabolic stress could be due to low FSH and LH productions.

Pineal is known to act as antigonadal in most of the animals studied (5, 22-24) which is also confirmed from the present findings, because pineal activity was stimulated with testicular dysfunction following metabolic stress, after arecoline treatment in metabolic stress or after arecoline treatment in rats. Melatonin receptors are reported to be present in the Leydig cells(25) and in the prostate epithelium (26). Therefore, increased production of melatonin in metabolic stress might be responsible for decreased production of testosterone which resulted in the inhibition of prostate growth in rats. Adrenocortical and adrenomedullary hormonal levels are increased following arecoline treatment in mice (4). Corticosterone receptors are present in the Leydig cells of the rat testis (27). Glucocorticoid or cortiscosterone is antigonadal and reduces testosterone level in stress (28). Thus, in addition to melatonin, increased levels of glucocorticoids hormones might be responsible for testis and testosterone-dependent male sex accessories dysfunctions in rats exposed to metabolic stress. Calorie restriction causes oxidative damage (29). Thus, it is likely that metabolic stress probably induced oxidative damage which in turn might have caused testicular damage via antigonadal actions of pineal, adrenal cortex and adrenal medullary hormones in rats. Also in our experiment, arecoline might have exerted its action primarily on the pineal which in turn acted secondarily on the testis, eventually resulting in gonadal suppression via stimulation of pineal melatonin production in rats.

Melatonin is known to have protective effect against stress (2), but it was not observed in our current experiment, because pineal hyperactivity could not prevent testicular dysfunction in metabolic stress in rats.

In conclusion, (1) metabolic stress by inanition or water-deprivation, both can stimulate pineal function but cause testicular dysfunction in rats. (2) Though arecoline inhibits pineal activity and stimulates testis and sex accessories functions in normal rats, but it cannot prevent or counteract the effects of metabolic stress on pineal-testis activity in male rats.

Conflict of interest

The authors declared that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ethical approval

Animal experiments were conducted following the 'Principles of Laboratory Animal Care' (NIH Publication No. 85-23 revised in 1985) as well as Indian Laws of Animal Protection (Registration No. 885/ac/05/PCSEA).

Institutional approval

University of Calcutta has no reservation to publish the article.

Informed consent

We have no reservation to publish the article.

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