DUAL ACTION OF ARECOLINE ON ADRENAL FUNCTION AND GLUCOSE-GLYCOGEN HOMEOSTASIS IN METABOLIC STRESS IN MICE

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Abstract

Background. People chew betel nut (*Areca catechu*) for physical work and stress reduction, but it contains arecoline, which has both therapeutic value and untoward effects on endocrine and gonadal functions.

Objective. Aim of the present study is to investigate its role on adrenal with its target in metabolic stress in mice.

Materials and methods. Mice were deprived of water / food, each for 5 days / treated with arecoline (10 mg / kg body wt daily for 5 days) / arecoline after water or food deprivation, for 5 days each.

Results. Water or food-deprivation caused adrenocortical hyperactivity, evident from abundance of enlarged mitochondria and smooth endoplasmic reticulum (SER) with elevation of corticosterone level (C: 68.31 ± 2.30 , WD: 159.31 ± 4.10 / FD: 194.12 ± 3.40 $\mu\text{g}/\text{mL}$). Arecoline treatment alone or in water deprivation (C: 68.31 ± 2.30 , AR: 144.50 ± 4.33 , AR+WD: 194.42 ± 3.35 $\mu\text{g}/\text{mL}$) / food deprivation (AR + FD: 180.89 ± 4.51 $\mu\text{g}/\text{mL}$) stress also stimulated adrenocortical activity as recorded in metabolic stress. In contrast, adrenomedullary activity was not altered following water/ food deprivation. Arecoline treatment alone or in metabolic stress suppressed adrenomedullary activity by showing depletion of chromaffin granules (E/NE?), epinephrine (E) and norepinephrine (NE) concentrations. Both the stress decreased blood glucose and liver glycogen levels. Arecoline treatment decreased blood glucose level, with a rise in liver glycogen level, but elevated blood glucose level in water deprivation unlike in starvation.

Conclusion. Arecoline alone or in metabolic stress involves adrenal and probably other endocrine glands (pancreas, posterior pituitary and rennin-angiotensin system) to maintain homeostasis in metabolic stress in mice.

Key words: Arecoline, adrenal, blood glucose, glycogen, metabolic stress, mice.

INTRODUCTION

Daily water restriction decreases body weight (1), and increases relative adrenal weight including

weight of the adrenal capsule by hyperplasia (2), plasma rennin angiotensin II (AII) (3) and arginin-vasopressin (AVP) levels (1, 4) in adult rats. AVP and AII are the main hormones which stimulate growth of the zona glomerulosa of the adrenal cortex and aldosterone secretion (5). Extracellular and intracellular fluid volumes are decreased following water deprivation that alters hormonal secretion and consequently regulates fluid and electrolyte balance (6). During water deprivation plasma ACTH (7), corticosterone (8) and epinephrine (2) concentrations are elevated, but hypothalamic corticotropin releasing hormone (CRH) mRNA level is decreased without any change in anterior pituitary POMC (Pro-opiomelanocortin) mRNA level in rats (1).

De Boer *et al.* (9) have reported that plasma norepinephrine and glucose levels are declined in food deprivation compared to the fed rats. A marked reduction occurs in cortisol responses to prolonged fasting in humans (10) and sheep (11), or to both food and water deprivations in rats (1). In contrast, plasma corticosterone level is increased (8), but CRH mRNA level is decreased without any change in ACTH and catecholamine levels (12). Basal plasma vasopressin level remains normal in food-deprived rats (1).

Millions of people, especially in developing countries chew betel nut of *Areca catechu* for increased capacity of work, euphoria and stress reduction (13). In addition, arecoline has therapeutic value for the treatment of Alzheimer with presenile dementia (memory enhancer) and schizophrenia (19). Betel nut contains abundance of arecoline which is highly toxic (13). It has several untoward side effects such as, immunosuppression, hepatotoxicity and antioxidant suppression in mice (14). Arecoline also affects endocrine and gonadal functions such as, hypothyroidism, adrenal (15) and testicular stimulations (16) with protective function in experimentally induced

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diabetic mice (18).

We have reported that arecoline aggravates hypothyroidism in metabolic stress in mice (19). Arecoline is known to act as stress (20). The aim of the current work is to investigate whether arecoline plays any different role on adrenocortical and adrenomedullary functions under metabolic stress from that of its action in normal rats. Further, as glucose is the chief source of energy intimately connected with metabolic stress, it is pertinent to study glucose-glycogen homeostasis during stress. Adrenocortical and adrenomedullary functions were evaluated ultrastructurally and by quantitation of hormones. Glucose and glycogen levels were also quantitated in metabolic stress.

MATERIALS AND METHODS

Animal model

Forty two adult male mice (~ 25 gm body weight, 90 days old) were collected from the breeding colony and were kept in polythene cage (30 cm x 15 cm x 15 cm) (6 mice per cage) in controlled laboratory conditions (12L:12D, and 25°C temperature) with standard diet (16) and water accessible ad libitum for 5 days for acclimatization. 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). Animals were handled for 5 days to reduce handling stress during experimental manipulation.

Experimental protocol

Forty two mice were divided into 7 groups with 6 each. Group I served as untreated control which received food and water ad libitum for 5 days. Group 2 served as saline treated control which received food and water ad libitum and were injected peritoneally with normal saline (0.9%) (0.1 mL daily) for 5 days. Group 3 mice were provided with water and food accessible ad libitum and treated with intraperitoneal injection of arecoline (Arecoline hydrobromide, methyl 1-methyl 1,2,5,6 tetrahydronicotinate) (Sigma, U.S.A.) at a dose of 20 mg/kg body wt daily for 5 days (16). Each dose (i.e., 2 mg/100 gm body wt) was divided equally to half (i.e., 1 mg/100 gm body wt) and each half dose was injected twice daily (at 09 h and 19 h) due to its short half-life (20). Group 4 mice were deprived of water but with food for 5 days. Group 5 mice were deprived of water but with food and injected with arecoline in the same dose as in group 3 for 5 days. Group 6 mice were

deprived of food but with water accessible ad libitum for 5 days. Group 7 mice were deprived of food but with water accessible and injected with arecoline (in the same dose as in group 3) for 5 days. All the experiments were repeated twice as independent replicates. All the data of each parameter were pooled, averaged and expressed as Mean+SE.

Animal autopsy, tissue and blood collection

All experimental mice were autopsied on day 6 by anaesthetising with sodium barbital injection (0.1 mg/100 gm body wt). Blood was drawn from heart, collected in chilled tubes, and aliquots of serum were collected and stored at -20°C until assayed. A small piece of liver was dissected out and weighed on a semimicro-analytical balance (Mettler, Switzerland) and saved for glycogen estimation. Left adrenal glands of each specimen were dissected out and processed for transmission electron microscopic (TEM) study.

Adrenal glands (left) were quickly dissected out, 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 the buffer, tissue samples were post-fixed in 1% osmium tetroxide in the same buffer for 2 h at 4°C. Tissues were dehydrated through ascending grades of ethanol, infiltrated and embedded in araldite CY 212. Thick sections (1 µm) were cut, stained with toluidine blue and observed under a light microscope. Thin sections (60-80 nm) were cut by ultratome (Reichert Ultracut E), mounted on copper grids, and 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.

Spectrofluorometric assay of corticosterone and catecholamines of the adrenal gland

Serum corticosterone

Serum corticosterone concentrations were studied by the fluorometric method of Glick *et al.* (21). 1 ml of serum was taken and 3 mL of isooctane were added to the sample, vortexed, centrifuged and top aqueous layer was discarded. Chloroform was added to the remaining solution, vortexed and the upper layer was discarded. To this solution was added 0.1 N NaOH, vortexed and the upper layer was discarded. In freshly prepared 2 mL of acid-alcohol mixture (1.75 mL ethyl alcohol was added to 3.25 mL conc. H₂SO₄ to obtain 5 mL of acid-alcohol mixture); 5 mL of purified sample was added and vortexed to form a fluorescence

compound. The latter was read at 462/518 nm after 45 minutes in a Hitachi fluorescence spectrophotometer (Model 650-10M) with excitation and emission slit at 6 nm and sensitivity at 0.1 μ g.

Adrenal catecholamines

Catecholamines (norepinephrine and epinephrine) concentrations were measured from the adrenal gland, instead of serum (22), because plasma norepinephrine is also secreted from extra-adrenomedullary sources, such as sympathetic nerve terminals that richly innervate the CNS, hypothalamus, spleen and heart (23). The pineal gland also produces both norepinephrine and epinephrine in turtles (24). The chromaffin cells are also localized in different areas of the body besides adrenal medulla, and are increased in pheochromocytoma at least in humans (23).

Norepinephrine and epinephrine were extracted with acidified n-butanol and purified with activated alumina that separated catecholamines from other amines (25). Purified samples were oxidized with NaI-I² solution at pH 7.0 for norepinephrine and at pH 4.0 for epinephrine, essential for complete oxidation, using Sorenson's M/15 phosphate buffer (pH 7.0) for norepinephrine and McIlvaine's citrate-phosphate buffer (pH 4.0) for epinephrine, resulting in the formation of adrenochromes (25). Oxidation was stopped using sodium sulfite as an antioxidant to form the fluorescent product. The oxidized product was then exposed to strong alkali (NaOH and Na₂-EDTA) for tautomerization of the adrenochrome to the corresponding lutins. To achieve peak fluorescence, sulfite and alkali were added together and left for 5 min for norepinephrine and 1 min for epinephrine. Oxidation of lutins was prevented by adding glacial acetic acid, which stabilizes the lutins and increases the fluorescence. Noradrenolutin fluorescence was read at 380/480 nm, 30 min after adding acetic acid and adrenolutin fluorescence was recorded at 410/500 nm immediately after adding acetic acid; and both were measured by Hitachi fluorescence spectrophotometer (Model 650-10 M) with excitation slit at 10 nm, emission slit at 2 nm, and sensitivity at 1 μ g.

Blood glucose

Serum glucose levels were measured by the glucose oxidase-peroxidase (GOD-POD) enzymatic method of Trinder (26) using the AUTOSPAN Kit (Span Diagnostic Ltd. Surat, India). Glucose was oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. In a subsequent peroxidase

catalyzed reaction, oxygen liberated was accepted by the chromogen system to give a red coloured quinoneamine compound. The red colour so developed was measured by a Spectrophotometer (Smart spec 3000, BIORAD, Australia) at 505 nm (490-550 nm) against a reagent blank and was directly proportional to the glucose concentration.

Liver glycogen

Liver glycogen levels were measured by the method of Hassid and Abraham as adopted by Dasgupta *et al.* (25). Liver tissue was taken in 30% KOH solution in a centrifuge tube and dissolved by heating the tube in a boiling water bath for 20 to 30 mins. 0.5 mL of saturated sodium sulfate was added and glycogen was precipitated by addition of 1.1 to 1.2 mL of 95% ethanol and vortexed. Tubes were heated to boil, cooled and centrifuged at 3000 r.p.m. for 10 min. The mother liquor was decanted and precipitated; glycogen was redissolved in 2 mL of distilled water, precipitated with 2.5 mL of 95% ethanol, centrifuged and the supernatant was discarded. The precipitate was cooled, diluted with water in a volumetric flask and vortexed. Glycogen solution was further diluted with water in a second volumetric flask to yield glycogen concentration of approximately 3 to 30 r/mL.

A 5 mL of the aliquot, equivalent to 15 to 150 r of glucose, was taken in a new tube. The other tube contained 5 ml of water served as blank. A tube containing 5 ml of glucose (containing 100 r of hexose) served as standard. All the tubes were cooled and added with 10 mL each of 0.2% anthrone reagent (1.2g anthrone in 100 mL of 95% sulfuric acid), vortexed and heated in the boiling water bath for 10 min. All the samples were cooled and O.D. was recorded at 620 nm by a spectrophotometer (PERKIN, Elmer). The amount of glucose was converted to glycogen by dividing with the factor 1.11.

Statistical analysis

Data were analyzed statistically by ANOVA followed by post hoc "t" test (27). Data were presented as mean+SEM and P-value <0.01 was considered statistically significant.

RESULTS

Gross observation: Mice, exposed to water deprivations were found to consume less quantities of food than the control during the later part (3-4 day) of the experiment. Since the results of ultrastructural, hormonal,

blood glucose and liver glycogen parameters were not significantly different between the two control groups (Groups I and II), the data of both the control groups were pooled, averaged and presented as a single control.

Water deprivation

Adrenal gland

Adrenal cortex

TEM study

Control

Adrenal cortex of the control mice showed abundance of small round mitochondria (m) with tubulo-vesicular cristae and clustered of cytoplasmic vacuoles, depleted of their lipid droplets (L) (Fig. 1A). The reticularis cells were seen in anastomosing cords with ovoid to spherical deeply stained nuclei.

Arecoline treatment

Arecoline treatment showed ultrastructural stimulation of the fascicular cells with enlarged nuclei and mitochondria (m) with swollen cristae. Smooth endoplasmic reticulum with dilated cisternae (SER),

Figure 1. Transmission electron micrographs of the adrenal cortex of mice. (A): control mice showing numerous small round mitochondria (m) with clustered of cytoplasmic vacuoles depleted of lipid contents (L) in the fascicular cells. (B) Arecoline treatment showing abundance of large round mitochondria (m) with dilated cristae and scanty lipid droplet in the zona fascicular cells. (C): adrenal cortex of water-deprived mice showing numerous mitochondria (m) with conspicuous cristae. (D): arecoline treatment in water-deprived mice showing abundance of enlarged mitochondria (m) with extremely dilated cristae and abundance of cytoplasmic vacuoles (v), depleted of their contents (lipids ?), seen in the fascicular cells. [Scale bars: (A): 1 μ m, (B): 0.5 μ m, (C): 1 μ m, (D): 0.5 μ m].

Golgi bodies and scanty vacuoles were also seen in the cell cytoplasm (Fig. 1B).

Water deprivation and arecoline treatment in water deprivation

Water deprivation showed abundance of round mitochondria (m) with conspicuous cristae in the fascicular cells (Fig. 1C). Arecoline treatment in water-deprived mice showed extremely enlarged mitochondria with dilated cristae and abundance of vacuoles, depleted of their contents (lipids ?), in the fascicular cells (Fig. 1D).

Adrenal medulla

TEM study

Control

Chromaffin cells of the control mice showed large, round euchromatic nucleus (n) and numerous round chromaffin granules in the cytoplasm. Several mitochondria (m) with inconspicuous cristae, rough endoplasmic reticulum (rER) (Fig. 2A) and epinephrine (E?) and norepinephrine containing granules (NE?) (Fig. 2B) were present.

Arecoline treatment

Arecoline treatment showed reduced nuclear size (n) with central nucleolus (nu) (Fig. 2C) and smaller chromaffin granules – epinephrine (?) and norepinephrine (?) intermingled with abundance of vacuoles in the cytoplasm (Figs. 2C, D). Chromaffin granule number was significantly decreased after arecoline treatment (38.02 ± 1.8) compared with the control (80.95 ± 4.32) ($P < 0.001$). Vacuole number was significantly increased in arecoline treatment (56.21 ± 1.33) compared with the control (3.10 ± 0.5) ($P < 0.001$).

Water deprivation and arecoline treatment in water deprivation

Water deprivation showed crumpled nucleus with huge aggregation of chromaffin granules, electron dense epinephrine (E?) containing granules and electron dense core surrounded by electron lucent peripheral halo of the norepinephrine (NE?) containing granules, and numerous vacuoles (Fig. 3A) compared to control (Fig. 2A). Arecoline treatment in water-deprived mice showed further aggregation of chromaffin granules (E/NE?) with abundance of large empty vacuoles (V) located in the compact electron dense scanty cytoplasm (Fig. 3B). Extremely dilated and degranulated cisternae of the rough endoplasmic reticulum (rER) and dissoluted mitochondria (m) were noticed in the cell cytoplasm (Fig. 3C). Chromaffin granule or vacuole number could not be studied due to the degenerated changes in the subcellular organelles of the adrenomedullary cells.

Figure 2. Transmission electron micrographs of the chromaffin cell of the control mice showing (A): large vesicular nucleus (n), rough endoplasmic reticulum (rER), several mitochondria (m) and numerous large round electron-dense chromaffin granules in the cytoplasm and (B): The same at higher magnification showing chromaffin granules, one with electron dense core of epinephrine (E ?) and the other with electron dense core surrounded by wide electron lucent peripheral halo of the norepinephrine (NE ?) granules. C-D: Chromaffin cells of the arecoline-treated adrenal medulla. (C): A chromaffin cell showing reduced hyperchromatic nucleus (n) with a nucleolus (nu), abundance of cytoplasmic vacuoles (v) and numerous chromaffin granules (cg) (low magnification) and (D): the same at higher magnification showing abundance of vacuoles, depleted of their contents (asterisk), and drastic depletion of chromaffin granules (NE/E ?). [Scale bars: (A): 1 μ m, (B): 0.5 μ m, (C): 2 μ m, (D): 0.5 μ m].

Arecoline, water deprivation and arecoline treatment in water deprivation

Corticosterone, epinephrine and norepinephrine

Serum corticosterone levels (Fig. 4a) were significantly increased after arecoline treatment (P-value <0.001), moderately increased following water-deprivation (P-value <0.001) and maximally increased after arecoline-treatment in water-deprived mice (P-value <0.001). Adrenal epinephrine (Fig. 4b) and norepinephrine (Fig. 4c) levels were declined after arecoline treatment (P-value <0.001) and remained unaltered in water deprivation. Epinephrine level was increased (P-value <0.001), without significant change in norepinephrine level (NS), after arecoline treatment in water-deprived mice.

Figure 3. (A): Chromaffin cell of the adrenal medulla of the water-deprived mice. Crumpled nucleus (n) with numerous very small chromaffin (E/NE?) granules are seen. (B): A chromaffin cell of the water-deprived mice treated with arecoline showing extremely aggregated very small chromaffin granules (electron dense core containing E ? and electron dense core with peripheral halo containing NE ?) with abundance of very large empty vacuoles (v). (C): Chromaffin cell showing abundance of unusually dilated and degenerated cisternae of rER (arrowheads) and dissolute mitochondria (m) with disorganized cristae. [Scale Bar: 1 μ m].

Arecoline, water deprivation and arecoline in water deprivation

Blood glucose and liver glycogen

Blood glucose levels (Fig. 4d) were significantly decreased following arecoline treatment (P < 0.05) and water deprivation (P-value <0.001), but significantly increased after arecoline treatment in water-deprived mice (P-value <0.01). Liver glycogen levels (Fig. 4e) were increased after treatment of arecoline (P < 0.05), but significantly decreased following water-deprivation or arecoline treatment in water-deprived mice (P-value: <0.05) without showing much difference between water deprivation and arecoline treatment in water deprivation.

Food deprivation

Adrenal gland

TEM study

Adrenal cortex

Control

Ultrastructure of the adrenocortical cells of the control and arecoline-treated mice was described earlier (Fig. 1A).

Figure 4. Histograms showing changes in (a) corticosterone, (b) epinephrine and (c) norepinephrine, (d) blood glucose and (e) liver glycogen levels following arecoline, water deprivation and arecoline in water deprivation experiments, compared to control, in mice. (NS: Not Significant; for other legends see Fig. 3) (ANOVA: $p < 0.01$).

Figure 5. Electron micrographs of the fascicular cells of the adrenal cortex of mice. For control see Fig. 1A and for arecoline treatment see Fig. 1B. (A): Food-deprived mice showing abundance of large mitochondria (m) with dilated cristae and abundance of vacuoles (v) depleted of lipids (?). (B): Arecoline treatment in food-deprived mice showing abundance of very large mitochondria (m) with extremely dilated cristae and abundance of cytoplasmic vacuoles. [Scale bars: 0.5 µm].

Arecoline, food deprivation and arecoline treatment in food deprivation

Arecoline treatment showed ultrastructural stimulation of the fascicular and reticularis cells (Fig. 1B). In food deprived mice, the adrenal cortex showed enlarged nucleus (n) and abundance of large round mitochondria with extremely dilated cristae in the zona fascicular cells and numerous cytoplasmic vacuoles (V) (Fig. 5A) compared to the control (Fig. 1A). Food-deprived mice treated with arecoline showed very large mitochondria (m) with further dilatation of the mitochondrial cristae and numerous cytoplasmic vacuoles (Fig. 5B).

Adrenal medulla

Control

Adreno-medullary chromaffin cells of the control mice (Figs. 2A-B) were described earlier.

Figure 6. Electron micrographs of a chromaffin cell of the adrenal medulla of mice. For control see Fig. 2A and for arecoline treatment see Fig. 2B. (A): Chromaffin cell of the adrenal medulla of food-deprived mice showing abundance of dissolved mitochondria (m) with indistinct cristae, many empty vacuoles and numerous electron dense chromaffin granules (arrows). (B): Arecoline treatment in food deprived mice showing dissolved mitochondria (m), with extremely indistinct cristae, abundance of vacuoles (v) of various size and drastic depletion of chromaffin granules (arrows). [Scale bars: 1 µm].

Arecoline, food deprivation and arecoline treatment in food deprivation

Arecoline treatment showed hyperchromatic round nucleus with central nucleolus and depletion of chromaffin granules (E/NE?) represented by vacuoles as described earlier (Figs. 2C-D).

Food deprivation caused significant increase of chromaffin granules (250.55 ± 8.99) compared with control (80.95 ± 4.32) ($P < 0.001$). Vacuole number was also increased in food deprivation (control: 3.10 ± 0.05 , treated: 90.25 ± 3.55) ($P < 0.01$). Arecoline treatment in food-deprived mice showed depletions of chromaffin granules (control: 80.95 ± 4.32 , treated: 50.55 ± 2.11) ($P < 0.01$), but with abundance of vacuoles (v) (75.22 ± 4.25) compared with the control (3.10 ± 0.05) ($P < 0.001$). Mitochondria (m) with dissolved cristae were seen (Fig. 6B).

Figure 7. Histograms showing changes in (a) corticosterone, (b) epinephrine and (c) norepinephrine levels, following arecoline, food deprivation and arecoline + food deprivation experiments compared to control. (ANOVA: $p < 0.01$).

Corticosterone, epinephrine and norepinephrine

Serum corticosterone level (Fig. 7a) was significantly increased after arecoline treatment (P-value: <0.001), and further increased in food deprivation (P-value: <0.001) or after arecoline treatment in food-deprived mice (P-value : <0.001). Adrenal epinephrine (Fig. 7b) and norepinephrine (Fig. 7c) levels were decreased after arecoline treatment (P-value : <0.001), remained unaltered after food deprivation (NS) and decreased after arecoline treatment in food-deprived mice (P-value : <0.001).

Blood glucose and liver glycogen

Blood glucose level (Fig. 8a) was significantly declined following arecoline treatment (P-value: <0.05) and further declined after food deprivation (P-value: <0.01) or arecoline treatment in food-deprived mice (P-value: <0.05). Liver glycogen level (Fig. 8b) was significantly increased after arecoline treatment (P-value: <0.05), decreased after food deprivation (P-value: <0.05) and remained unaltered after arecoline treatment in food-deprived mice (NS).

DISCUSSION

Betel nut, containing arecoline, is widely used by millions of people for increased capacity to work and euphoria, but it has several untoward side effects such as immunosuppression, antioxidant depression and hypothyroidism in mice (14, 15). In contrast arecoline stimulates adrenal and testis functions in mice and rats (16, 17, 25). As arecoline is known to be a potent stressful agent its role on adrenal activity and carbohydrate metabolism in

Figure 8. Histograms showing changes in (a) blood glucose and (b) liver glycogen levels following arecoline, food deprivation and arecoline in food-deprivation experiments compared to the control, mice. (ANOVA: $p < 0.01$).

metabolic stress was investigated. The findings revealed that water deprivation or food deprivation stress caused adrenocortical stimulation, evaluated from ultrastructural stimulation of the fascicular and reticular cells, evident from abundance of enlarged mitochondria with dilated cristae, smooth endoplasmic reticulum (sER), Golgi body and cytoplasmic vacuoles (depleted of their contents, lipids?). Simultaneously, serum corticosterone levels were elevated significantly in metabolic stress. These manifestations were further increased after arecoline treatment in metabolic stress, as compared with that of metabolic stress or arecoline treatment alone in mice. Our findings corroborate with the earlier reports of elevation of serum corticosterone level following starvation in rat pups and morning (unfed) cortisol level in children (28). Adrenocortical cells are known to have tubulo-lamellar or tubulo-vesicular internal membranes in the mitochondria, with smooth endoplasmic reticulum, liposomes and lipid-storing droplets constituting the substrates (29, 30). Morphology of the mitochondria is related to enzyme activity and steroid-biosynthetic capacity of the adrenal (31). Fascicular/reticularis cells with prominent mitochondrial inner vesicular membranes suggest an increase of steroidogenesis as observed after ACTH or CRH administration (31). Since 3β hydroxysteroid dehydrogenase enzyme is involved in corticoidogenesis (34), its involvement in enhanced production of corticoids induced by metabolic stress cannot be ruled out. In addition, higher titre of corticoids in metabolic stress or after arecoline treatment could be due to its enhanced release into circulation in mice.

It has been reported that water restriction activates hypothalamic-pituitary-adrenal activity and

results in increased Fos expression in vasopressin (VP) magnocellular and parvocellular neurons, but not the CRH parvocellular neurons in the paraventricular nucleus of the hypothalamus (35). Anterior pituitary pro-opiomelanocortin (POMC) mRNA, which is a precursor of ACTH, MSH, lipotropin and β -endorphin (<http://en.wikipedia.org/wiki/POMC>), is also significantly increased after food deprivation (2). Increased pituitary POMC mRNA might have stimulated glucocorticoid production *via* stimulation of ACTH in metabolic stress in mice. Leptin, a potent anorexigenic hormone of the anterior pituitary (AP), may signal nutritional changes and facilitates rapid responses by the neuroendocrine system to nutritional cues. Pituitary leptin mRNA in male rats is reduced after fasting, accompanied by an increase in corticotropes (36). A similar mechanism might have operated in inducing adrenocortical stimulation in metabolic stress or after arecoline treatment in metabolic stress in mice. Nevertheless, since adrenocortical responses to metabolic stress were higher after arecoline treatment alone or arecoline treatment in metabolic stress as compared with the metabolic stress alone, it is likely that arecoline may have synergistic stimulatory effect on adrenocortical activity in metabolic stress in mice.

Arecoline treatment caused depletion of both epinephrine (E) and norepinephrine (NE) granules from the chromaffin cells observed under TEM, which could be due to the discharge of E and NE hormones into circulation, resulting in the loss of these granules (hormones) from the adrenomedullary chromaffin cells. The present finding corroborates with the decreased levels of epinephrine and norepinephrine from the adrenal medulla of the arecoline recipient mice. Water deprivation (dehydration) or food deprivation stress for 5 days caused ultrastructural degeneration of chromaffin cells with reduced nuclear size, accumulation and condensation of cytoplasmic granules and vacuoles without any change in epinephrine and norepinephrine levels in mice as has been reported earlier by Kiss *et al.* (1). Results of medullary hormones after arecoline treatment in metabolic stress are different, because arecoline treatment in water deprivation increased only epinephrine level, whereas both epinephrine and norepinephrine levels were declined after arecoline treatment in food deprivation stress; the latter findings corroborate with the ultrastructural degeneration of chromaffin cells observed in both types of stress. Rise of epinephrine level in the adrenal gland could be due to its retarded synthesis and release into circulation in water deprivation, in contrast to inhibition of both

synthesis and/or release of medullary hormones into circulation resulting in the fall of E and NE hormones following arecoline treatment in food deprivation stress. Further work is necessary to confirm our findings of adrenomedullary hormones following arecoline treatment in metabolic stress. Since tyrosine hydroxylase and phenyl-N-methyl transferase (PNMT) are required for synthesis of norepinephrine and epinephrine, their involvement in the alteration of NE and E levels in metabolic stress or after arecoline treatment in metabolic stress cannot be ignored in mice. Nevertheless, arecoline inhibits adrenomedullary activity unlike stimulation of adrenocortical activity in metabolic stress in mice. Further, arecoline may have synergistic inhibitory effect on adrenomedullary function in metabolic stress as the changes were more significant after arecoline treatment in metabolic stress than the metabolic stress alone in mice.

Arecoline treatment caused hypoglycemia followed by a rise in liver glycogen level in mice. Epinephrine, glucagon and corticosterone are known to cause hyperglycemia while insulin induces hypoglycemia (34). Thus, decreased levels of epinephrine and/or glucagon (?), and/or lack of food might be responsible for induction of hypoglycemia induced by arecoline treatment in mice. Involvement of corticosterone in causing hypoglycemia is unlikely, because hypoglycemia occurred in spite of increased corticosterone levels in mice. Thus, food deprivation itself seems to be primarily responsible for causing hypoglycemia in mice. Epinephrine (E) might also contribute its influence to induce hypoglycemia, because E level was declined in arecoline recipients. Higher demand for overutilization of blood glucose is required to counteract stress and that might also be responsible for causing hypoglycemia in metabolic stress in mice. In contrast to hypoglycemia, liver glycogen level was elevated after arecoline treatment that could be due to its enhanced glycogenesis resulting in hypoglycemia in the arecoline recipient mice. Water or food deprivation stress caused decrease of both blood glucose as well as liver glycogen levels as reported earlier (25). Involvement of epinephrine (and/or norepinephrine?) does not arise because their levels remained unchanged in both the metabolic stress. Neither adrenocortical involvement is indicated, because hypoglycemia occurred despite elevation of corticosterone level in metabolic stress. Hyperglycemia followed by low liver glycogen in water deprivation, or hypoglycemia without any change in glycogen level in food deprivation after arecoline treatment could be due to more epinephrine production in the former and

less epinephrine and norepinephrine (?) productions in the latter indicating involvement of catecholamines following arecoline treatment in metabolic stress in mice.

It is known that blood glucose level is lowered by food deprivation (10). In our study, food deprivation also caused reduction of blood glucose level to a great extent compared with arecoline treatment alone. Arecoline treatment in unfed mice did not cause further loss of glucose. Liver glycogen reserve also became low during food deprivation. Glucocorticoid and catecholamines are known to stimulate hepatic glycogenolysis that results in hyperglycemia (33). In our study, corticosterone release was greatly increased in fasting which in turn might have produced more glucose from liver resulting in loss of liver glycogen, but actually glucose level was decreased instead of increase, and that could presumably be due to overutilization of glucose in food deprivation stress to restore glucose homeostasis. Since the results of arecoline treatment in fasted mice were same as observed after fasting, it is likely that the same mechanism might have operated to explain hypoglycemia in mice.

In conclusion, arecoline plays a dual role on the adrenal gland (1), because it stimulates adrenocortical activity, but inhibits adrenomedullary function in mice (2). Water or food deprivation stress causes adrenocortical hyperactivity, without disturbing adrenal medulla (3). Arecoline exerts its synergistic action on both adrenocortical and medullary functions in metabolic stress (4). Arecoline alone or in metabolic stress disturbs blood glucose-glycogen homeostasis in mice (5). Thus, betel nut chewing during metabolic stress induced by food or water deprivation should be avoided.

Conflict of interest

The authors declare that they have no conflict of interest.

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