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ORIGINAL ARTICLE

Arecoline plays dual role on adrenal function and glucose-glycogen homeostasis under thermal stress in mice

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ABSTRACT

Arecoline has biomedical importance, but it has untoward side effects on endocrine functions. The aim is to investigate its role on adrenal activity under thermal stress by ultrastructural and hormonal parameters in mice. Cold (4 °C) or heat (37 °C) stress, or arecoline (10 mg/kg body wt), each for 7 days in cold or heat stress stimulated adrenocortical activity ultrastructurally with an elevation of corticosterone level. Adrenomedullary activity was suppressed in cold stress with depletion of catecholamine levels. In heat stress, adrenomedullary activity was stimulated ultrastructurally with an elevation of catecholamine levels. Arecoline treatment alone, or in cold or heat stress suppressed adrenomedullary activity, judged by ultrastructural and hormonal parameters. Arecoline treatment caused hypoglycemia with an elevation of glycogen level, but cold or heat stress, or arecoline treatment in thermal stress caused hyperglycemia, with a fall in glycogen profile. Thus, arecoline in thermal stress plays a dual role on adrenal function and glucose-glycogen homeostasis in mice.

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KEYWORDS
Arecoline; adrenal; carbohydrate; thermal stress; mice

Introduction

Adrenal is known to be involved in thermal stress in animals (Chesterjones and Phillips 1988). Acute heat stress in rats causes a significant increase in plasma adrenocorticotropic hormone (ACTH), arginine vasopressin (AVP), and serum aldosterone and corticosterone levels (Harikai *et al*. 2003, Koko *et al*. 2004). Chronic heat stress increases adrenal cortisol level (Venditti *et al*. 2006). Heat stress during exercise increases sympathetic neural activity (as reflected in the rise of NE level) without stimulating the release of epinephrine (E) from the adrenal gland (Rowell *et al*. 1987). In contrast, patients suffering from heat stroke had higher levels of norepinephrine (NE) and epinephrine (E) than in the control (Al-Hadramy and Ali 1989). Heat stress increases NE and E levels significantly (Gisolfi *et al*. 1991). Long-term repeated heat exposure significantly enhances excretion rates of cortisol, NE and E compared with the control in humans (Melmed *et al*. 2011). Average heart rate, blood lactate and glucose, plasma epinephrine and norepinephrine as well as serum cortisol concentrations are higher in the hot trial running test compared to those recorded at moderate condition. Muscle glycogen utilisation may be elevated by heat stress (Morris *et al*. 2005).

Cold stress, acute or chronic, increases plasma levels of ACTH (Hauger *et al*. 1990), adrenal cortisol (Venditti *et al*. 2006) and plasma corticoids (Dronjak *et al*. 2004, Goundasheva *et al*. 1994) by activating the hypothalamic-pituitary-adrenal (HPA) axis transiently (Fukuhara *et al*. 1996a). Cold stress produces much larger proportionate increment in norepinephrine (NE) level than epinephrine and dopamine (Hata *et al*. 1991). Long-term isolation of rats exposed to 2 h of cold stress leads to a significant elevation of plasma ACTH, corticosterone, epinephrine (E) and norepinephrine (NE) levels compared with the control (Dronjak *et al*. 2004). Chronic local cold stimulation to the soles of paws of inbred Wistar-Kyoto rats (proposed model of stress vulnerability) also increases plasma ACTH, corticosterone (Pacak and Palkovit 2001), epinephrine and norepinephrine (Laverty and Taylor 1968) levels. Both intermittent cold stress (cold exposure interrupted by hourly-interval daily at room temperature) as well as continuous cold exposure fail to alter plasma epinephrine level, but markedly increases NE level which is significantly greater in intermittent than continuous cold exposure (Fukuhara *et al*. 1996a). Adrenal tyrosine hydroxylase (TH) activity is significantly increased in rats exposed to intermittent or continuous cold. TH activity is also increased only in continuous cold exposure in brown rat (Fukuhara *et al*. 1996a, 1996b).

Arecoline is a plant alkaloid, present abundantly in the betel nut of *Areca catechu* (Rooban *et al*. 2005) which is chewed raw or with betel quid by millions of people for stress reduction and heightened alertness. Arecoline is used for the treatment of presenile dementia with Alzheimer's disease (Soncrant *et al*. 1993) and schizophrenia (Sullivan *et al*. 2000). It has a wide spectrum of untoward effects, such as oral carcinoma, immunosuppression, antioxidant depression and hepatotoxicity (Dasgupta *et al*. 2006) in mice. Arecoline

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has multiple actions on endocrine functions, while it stimulates adrenal and testicular functions, suppresses pineal and thyroid activities in rats and mice (Saha *et al*. 2007, Dasgupta *et al*. 2010a, 2010b). There are also reports that arecoline aggravates hypothyroidism in metabolic stress (Dasgupta *et al*. 2017) and ameliorates hypothyroid condition in cold stress. Recently Saha *et al*. (2017) have reported that, arecoline cannot exert its action on pineal-testicular function in noise in rats. Thus, it is pertinent to examine the role of arecoline on adrenocortical and adrenomedullary functions including their target on glucose-glycogen homeostasis in cold and heat stress in mice.

Materials and methods

Animal model

Adult male albino Swiss mice (~25 gm body weight, 90 days old) were collected from the standard breeding colony, Calcutta and were kept in polythene cage (30 cm ×15 cm ×15 cm) in controlled laboratory conditions (12L: 12 D at 25 °C) with standard diet (Oser 1965) and water accessible *ad libidum* throughout the experiments. Mice were kept in the laboratory for 10 days for acclimatisation prior to experiments. Animals were handled for 10 days before starting the experiments to acclimatise with the stress generated during experimental manipulation.

Arecoline administration

Arecoline hydrobromide (Methyl 1-methyl 1,2,5,6 tetrahydronicotinate) (Sigma, USA), dissolved in physiological saline (0.9% NaCl), was injected intraperitoneally at a dose of (10 mg/kg body wt, i.e. 1 mg/100 gm body weight). Each dose (1 mg/100 gm body wt) was divided equally to half (0.5 mg/100 gm body wt) and each half dose was injected twice daily at 09 h and 18 h because of its short half-life (Pradhan *et al*. 1986).

Experimental protocol

Fifty-six Swiss albino mice (*Mus musculus Albinus*) were divided into 7 groups with 8 specimens each. Group I served as untreated control 1; group II is second control which received vehicle only (normal physiological saline, 0.9%); group III mice were treated with arecoline (10 mg/kg body wt daily) for 7 consecutive days. Group IV mice were exposed to cold stress (in the cold room at 4 °C for 2 h twice, each at 9 h and 18 h daily) for 7 consecutive days. Group V specimens were exposed to cold stress (as in group IV) and simultaneously treated with arecoline in the same dose and duration as in group III. Group VI mice were exposed to heat stress (37 °C for 60 min, twice daily, each at 9 h and 18 h) for 7 days and group VII mice were exposed to heat stress (as in group VI) and simultaneously treated with the same dose and duration of arecoline (as in group III).

Animal autopsy, tissue and blood collection

Animal experiments were carried out following the "Principles of Laboratory Animal Care" (NIH publication No. 85–23, revised in '85) and Indian Laws of Animal Protection (Registration No. 885/ac/05/PCSEA). All the experimental mice were anesthetized by intramuscular injection of sodium barbital (0.1 mg/100 gm body wt). Blood was drawn from heart and serum was collected and stored at –20 °C until assayed. Adrenal glands were dissected out, and right adrenals were weighed by Mettler balance and processed for measurements of epinephrine and norepinephrine levels. Right adrenals were saved for TEM study. A small piece of liver was weighed by Mettler balance and processed for liver glycogen quantitation.

Transmission electron microscopy (TEM)

Left adrenal glands were cut into small pieces (1 × 1 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 and the sections were contrasted with uranyl acetate and alkaline lead citrate, and viewed under a Morgagni 268 D transmission electron microscope (Fei Company, The Netherlands) at an operating voltage of 80 KV. TEM study of adrenal cortex in heat stress was not included.

Serum corticosterone

Serum corticosterone concentrations were measured by the fluorometric method of Glick *et al*. (1964). Briefly, 1 ml of serum was used for estimation of corticosterone; 3 ml of isooctane was added to the sample, vortexed, centrifuged and the top aqueous layer was discarded. Chloroform was added to the remaining solution, vortexed and the upper layer was discarded. 0.1 N NaOH was now added to this solution, vortexed and the upper layer was discarded. In freshly prepared 2 ml of an acid-alcohol mixture (1.75 ml ethyl alcohol was added to 3.25 ml conc. H_2SO_4 to obtain 5 ml of acid-alcohol mixture); 5 ml of purified sample was added and vortexed to form a fluorescence compound. The fluorescence compound was read at 462/518 nm after 45 min in a Hitachi fluorescence spectrophotometer (Model 650-10M) with excitation and emission slit at 6 nm and sensitivity at 0.1 μg level.

Adrenal catecholamines

Catecholamines (norepinephrine and epinephrine) concentrations were measured from the adrenal gland, instead of serum (Ray and Maiti 2001), because plasma norepinephrine is also secreted from extra-adrenomedullary source such as

sympathetic nerve terminals that richly innervate the CNS, hypothalamus, spleen and heart (Melmed *et al*. 2011). Pineal gland also produces both norepinephrine and epinephrine in turtles (Mahata and Mahata 1992). Chromaffin cells are also localized in different areas of the body besides adrenal medulla, and are increased in pheochromocytoma at least in humans (Melmed *et al*. 2011). Norepinephrine and epinephrine were extracted with acidified *n*-butanol and purified with activated alumina that separated catecholamines from other amines (Cox and Perhach 1973, Fukuhara *et al*. 1996a). Purified samples were oxidized with NaI-I_2 solution at pH 7.0 for norepinephrine and at pH 4.0 for epinephrine resulting in the formation of adrenochromes. Sorenson's M/15 phosphate buffer (pH 7.0) and McIlvaine's citrate-phosphate buffer (pH 4.0) were used for norepinephrine and epinephrine respectively (Laverty and Taylor 1968). Oxidation was stopped using sodium sulfite as an antioxidant to form fluorescent product. The oxidized product was then exposed to strong alkali (NaOH and Na_2-EDTA) for tautomerisation 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 stabilises the lutins and increases the fluorescence. Noradrenalin fluorescence was read at 380/480 nm 30 min after adding acetic acid and adrenalin fluorescence was recorded at 410/500 nm immediately after adding acetic acid; both were measured by Hitachi Fluorescence Spectrophotometer (Model 650-10M) with excitation slit at 10 nm, emission slit at 2 nm, and sensitivity at 0.1 μg level.

Liver glycogen

Liver glycogen levels were measured by the method of Hassid and Abraham (Hassid and Abraham 1957). A small piece of liver was immersed in 30% KOH solution in a centrifuge tube and boiled in water bath for 20 to 30 min. Then 0.5 ml of saturated sodium sulfate was added and glycogen was precipitated by the addition of 1.1–1.2 ml of 95% ethanol and vortexed. The tubes were heated to boil, cooled and centrifuged at 3000 rpm for 10 min. The mother liquor was decanted and precipitated, and glycogen was redissolved in 2 ml of distilled water, precipitated again with 2.5 ml of 95% ethanol, centrifuged and the supernatant was discarded. The precipitate was cooled, diluted in water in a volumetric flask and vortexed. Glycogen solution was further diluted with water in a separate volumetric flask to yield glycogen concentration of ∼3–30 r/ml. Five ml of the aliquot, equivalent to 15–150 r for glucose, was taken in a separate tube. The other tube contained 5 ml of water and served as blank. The tube containing 5 ml of glucose (10 r of hexose) served as standard. All the tubes were cooled and added with 10 ml each of 0.2% anthrone reagent (1.2 gm anthrone in 100 ml of 95% sulfuric acid). All the tubes were then 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 [as referred in the original method of D.L. Morris (1948), Science, 107, 254].

Blood glucose

Blood (serum) glucose levels were measured by the glucose oxidase-peroxidase (GOD-POD) enzymatic method (Trinder 1969) 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 colored quinone-amine compound. The OD 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.

Statistical analysis

Data were analysed statistically by one-way analysis of variance (ANOVA) followed by *post hoc* "*t*" test (Snedecor and Cochran 1989). Data were presented as mean ± SEM and p values $<.01$ was *c*onsidered statistically significant.

Results

Adrenal cortex (TEM study)

Control

Ultrastructural study of the adrenal cortex of control mice was described earlier (Dasgupta *et al*. 2010b). The epithelial cells of the fascicular layer showed an abundance of typical round mitochondria and numerous clustered cytoplasmic vacuoles depleted of their contents (lipid?) (Figure 1(A)).

Treated: arecoline, cold stress and arecoline in cold stress

Arecoline treatment showed abundance of enlarged mitochondria with dilated cristae and scanty vacuoles in the fascicular cells of the adrenal cortex (Figure 1(B)), unlike in the control (Figure 1(A)). Cold stress showed heterochromatic nucleus with numerous enlarged mitochondria containing conspicuous cristae (Figure 1(C)). Arecoline treatment in cold stress showed abundance of enlarged mitochondria with extensively dilated cristae, appearance of long smooth endoplasmic reticulum (SER) and several cytoplasmic vacuoles (V) in the fascicular cells of the adrenal cortex (Figure 1(D)).

Adrenal medulla

Control

Ultrastructural studies of the adrenal chromaffin cells in mice were described earlier by Dasgupta *et al*. (2010b). A large vesicular oval euchromatic nucleus surrounded by numerous chromaffin granules was observed (Figure 2(A)).

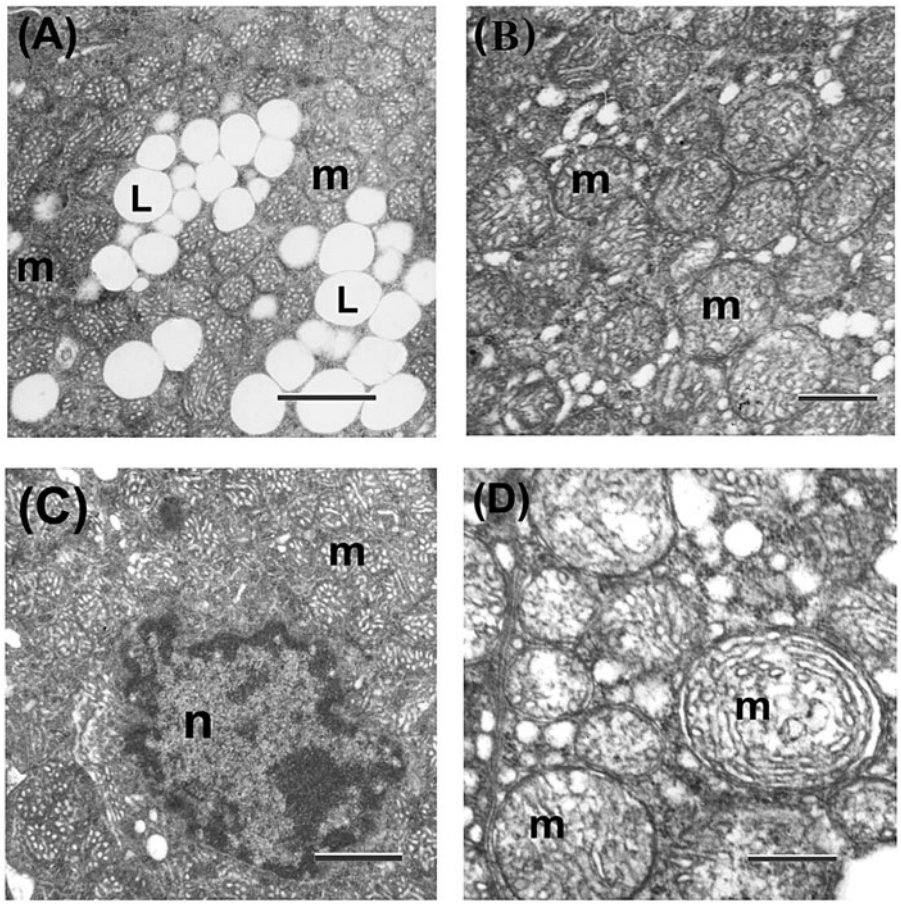

Figure 1. Transmission electron micrographs of the adrenal gland of mice. (A) Zona fasciculata of the adrenal cortex of control mice showing numerous lipid (L) droplets represented by vacuoles, with abundance of typical round mitochondria (m). (B) Arecoline treatment showing mitochondria (m) with electron-dense matrix of the swollen cristae and lack of lipids in the fascicular cells. (C) Adrenal cortex of cold-stressed mice showing hyperchromatic nucleus (n) and numerous mitochondria (m) with well-developed cristae and negligible lipid droplets (L). (D) Arecoline treatment in cold stress showing enlarged mitochondria (m) with conspicuously dialated cristae, appearance of long smooth endoplasmic reticulum and several cytoplasmic vacuoles (V), in the fascicular cells. [Scale bars: (A): 1 μm, (B): 0.5 μm, (C): 1 μm, (D): 0.5 μm].

Treated: arecoline, cold stress and arecoline in cold stress

Ultrastructural study arecoline treatment showed the reduced size of the round hyperchromatic nucleus with nucleolus located in the center of the nucleus, loaded with cytoplasmic vacuoles surrounded by depleted chromaffin granules (Figure 2(B)), compared to control mice (Figure 2(A)). In cold stress, chromaffin granules (E? /NE?) were abundant with scanty vacuoles in the cytoplasm (Figure 2(C)). Arecoline treatment in cold stress showed scanty chromaffin granules, each surrounded by the huge size of vacuoles (Figure 2(D)).

Biochemical study

Corticosterone, epinephrine and norepinephrine

Serum corticosterone (Figure 3(a)) levels were significantly increased after arecoline treatment, cold-stress or arecoline treatment in cold-stress. Corticosterone level was maximally increased in cold stress as compared to arecoline or arecoline in cold stress. Adrenal epinephrine (Figure 3(b)) and norepinephrine (Figure 3(c)) levels were significantly declined after arecoline treatment, but epinephrine level remained unaltered with a significant decrease of norepinephrine level in cold stress. Arecoline treatment in cold stress significantly decreased both epinephrine and norepinephrine levels.

Blood glucose and liver glycogen

Blood glucose profile (Figure 4(b)) was declined after arecoline treatment, but increased in cold stress or after arecoline treatment in cold stress compared to control. Blood glucose level was maximally increased after cold stress as compared to arecoline treatment in cold stress. Liver glycogen level (Figure 4(b)) was significantly increased in arecoline recipients, but significantly decreased in both cold and arecoline treatment in cold stress.

Heat stress TEM study

Electron microscopic study of the adrenal cortex was not included.

Adrenal medulla

Control

Ultrastructural findings of the control adrenal medulla were described earlier in cold stress experiment (Figure 2(A)).

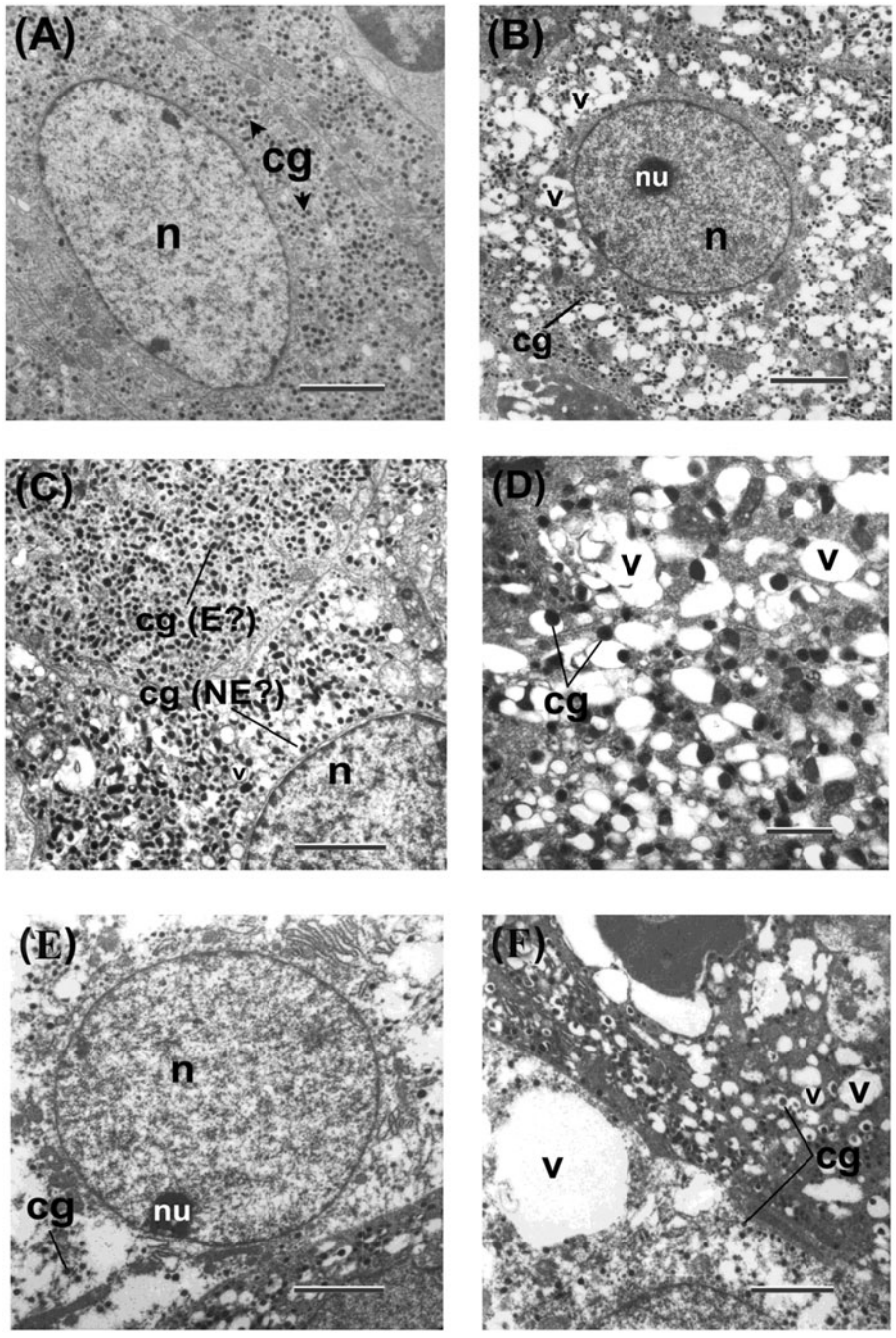

Figure 2. (A) Electron micrograph of a chromaffin cell of the adrenal medulla of untreated mice showing a large vesicular euchromatic nucleus (n) and numerous chromaffin granules (cg) (arrowheads). (B) Chromaffin cells of the arecoline-treated adrenal medulla showing reduced size of the round hyperchromatic nucleus (n) loaded with cytoplasmic vacuoles (V) surrounded by depleted chromaffin granules (CG). (C) Adrenal medulla of cold-stressed mice showing abundance of numerous chromaffin granules (cg E? /NE?). (D) Arecoline treatment of cold-stressed mice showing drastic depletion of chromaffin granules (cg), represented by large vacuoles (V) in the cytoplasm. (E) Adrenal medulla of heat-stressed mice showing large hyperchromatic nucleus (n) with the large nucleolus (nu) near the nuclear membrane (nm, arrow), conspicuous rough endoplasmic reticulum (RER arrow) and drastic depletion of chromaffin granules (CG). (F) Adrenal medulla of heat-stressed mice treated with arecoline showing disorganized electron dense cytoplasmic mass (cm), abundance of vacuoles (V) of abnormal size and scanty chromaffin granules (cg). [Scale bars: (A), (B) and (C) 2 μm, (D) 1 μm, (E) and (F) 0.5 μm].

Treated: arecoline, heat stress and arecoline in heat stress

Ultrastructural study arecoline treatment showed ultrastructural degeneration of chromaffin cells with reduced size of the hyperchromatic nucleus, with nucleolus located in the center of the nucleus, and clustered and depleted chromatin granules (CG) surrounding the cytoplasmic vacuoles (Figure 2(B)) compared to control (Figure 2(A)). Heat stress caused ultrastructural stimulation of the chromaffin cells by showing enlarged euchromatic nucleus with the conspicuous nucleolus migrated towards the nuclear membrane. Drastic depletion of chromaffin granules (CG) and hyper vacuolation of cell cytoplasm were seen (Figure 2(E)). Arecoline treatment in heat-stress also showed ultrastructural degeneration of chromaffin cells with abundance of unusually large vacuoles, compact disorganised electron-dense cytoplasmic mass and scanty chromaffin granules (CG) (Figure 2(F)).

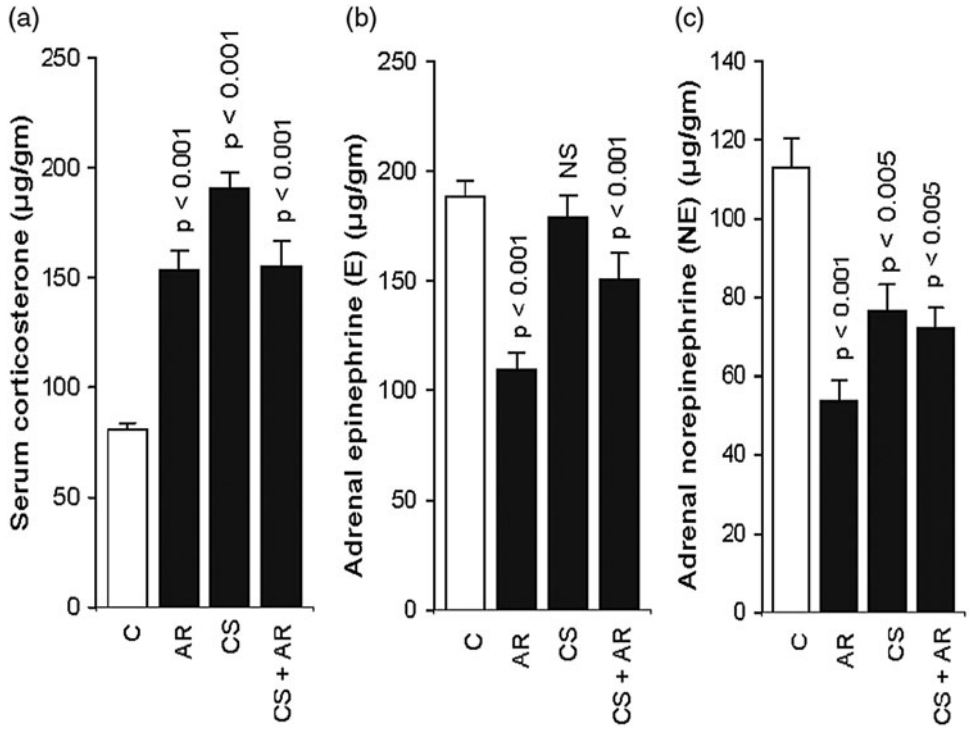

Figure 3. Histograms showing changes in adrenal hormone levels, [corticosterone (a), epinephrine (b) and norepinephrine (c)], following arecoline (AR), cold stress (CS) and arecoline + cold stress (CS + AR) compared to control mice. (NS: Not Significant; for other legends see Figure 2).

Biochemical study

Corticosterone, epinephrine and norepinephrine

Serum corticosterone levels (Figure 5(a)) were significantly increased after arecoline treatment, heat stress or arecoline treatment in heat stress, but more significantly in heat stress than in arecoline or arecoline in heat stress treatments. Adrenal epinephrine (Figure 5(b)) and norepinephrine (Figure 5(c)) levels were significantly decreased in arecoline recipients, increased in heat stress and again decreased after arecoline treatment in heat stress.

Blood glucose and liver glycogen

Blood glucose level (Figure 6(a)) was significantly decreased after arecoline treatment, but significantly increased in heat stress or after arecoline treatment in heat stress, with maximum increment noticed in heat stress. Liver glycogen level (Figure 6(b)) was significantly increased after arecoline treatment, but significantly decreased both in heat stress or after arecoline treatment in heat stress. All the findings are summarized in Figure 7.

Discussion

Adrenal, especially the cortex, responds promptly to stress and plays a vital role in counteracting stress of diverse character in all the vertebrate animals examined (Bentley 1998). In the present study, mice when exposed to cold or heat stress continuously for 7 days caused ultrastructural stimulation of the adrenocortical cells, which has been evident from drastic depletion of lipid droplets, and abundance of smooth endoplasmic reticulum (SER) and mitochondria with dilated cristae in the fascicular cells. Lipids containing cholesterol and cholesterol esters, are used as substrates and are mobilised towards mitochondria for synthesis of steroid hormones under condition of stress (Silva *et al*. 2004). Consequently, corticosterone level was elevated in heat stress, as has been reported earlier in heat or cold stress in rats (Koko *et al*. 2004). ACTH is known to cause protrusion of the outer membrane of mitochondria (often penetrating the lipid droplets) in cold stress, probably for the transport of cholesterol from the lipid droplet to the inner mitochondrial membrane "desmolase complex" for facilitating side-chain cleavage of cholesterol to produce pregnenolone (Merry 1975) which in turn probably produced corticosterone in cold/heat stressed mice. Arecoline treatment alone, or in cold or heat stress, also showed adrenocortical stimulation with the similar ultrastructural and hormonal changes as observed in cold or heat stress. Heat stress is known to stimulate glucocorticoid release into circulation (Silva *et al*. 2004) which is accelerated in humid condition (Harikai *et al*. 2003). Adrenocortical activity was maximally stimulated both in cold and heat stress alone as compared to that of arecoline treatment alone or arecoline treatment in cold or heat stress, suggesting that cold or heat stress is more potent than arecoline. Further, cold stress is probably more potent than heat stress, because adrenocortical stimulation was more intense in cold stress than in heat stress. As adrenocortical responses to cold/heat stress is greater than arecoline alone or arecoline in thermal stress, it is likely that arecoline cannot cause further stress beyond that of cold or heat stress alone in mice. Chronic cold exposure increases ACTH (Hauger *et al*. 1990) and adrenal cortisol production (Won and Lin 1995). Acute cold exposure increases corticotropin-releasing hormone (CRH) production by increasing CRHmRNA in rat hypothalamus

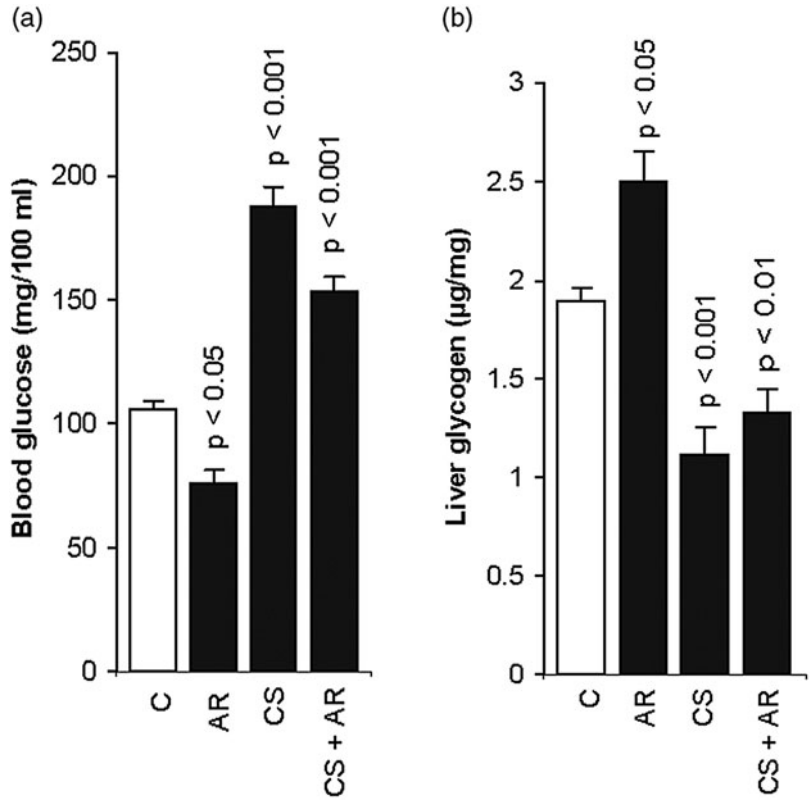

Figure 4. Histograms showing changes in blood glucose (a) and liver glycogen (b) levels following arecoline, cold stress and arecoline + cold stress experiments compared to control mice. (For legends see Figure 2).

(Zoeller *et al*. 1990). In addition to CRH, vasopressin (Pacak and Palkovit 2001) is reported to stimulate ACTH release from the anterior pituitary. In our study, hypothalamic CRH, pituitary ACTH and vasopressin might have been involved in inducing adrenocortical hyperactivity in thermal stress or after arecoline treatment in thermal stress in mice.

Adrenomedullary response to thermal stress (cold/heat) is different from that of adrenocortical response, because adrenomedullary activity was suppressed in cold stress but stimulated in hear stress, unlike stimulation of adrenocortical activity both in cold and heat stress in mice. Adrenomedullary activity was suppressed by showing huge ultrastructural accumulation of fine chromaffin granules, containing probably epinephrine (?) followed by depletion of adrenal norepinephrine level. Presence of abundance of chromaffin granules (epinephrine?) could be due to enhanced synthesis and/or impaired rate of release of epinephrine into circulation which was reflected in the unchanged adrenal epinephrine level. In contrast, depletion of norepinephrine level in cold stress could be due to its decreased rate of synthesis and/or enhanced rate of its release into circulation, because chromaffin granules, containing probably norepinephrine (?), were depleted from the chromaffin cells resulting in the formation of cytoplasmic vacuoles in the chromaffin cells. Our findings are consistent with those of earlier report of depletion of only norepinephrine (NE) containing chromaffin granules in cold stress in hamsters (AL-Lami and Farman 1975). Intermittent and continuous cold exposures activate the sympathoneural system apparently without causing significant change in adrenomedullary epinephrine release (Fukuhara *et al*. 1996b). Hypothalamic catecholamines are also depleted in cold stress (Palkovit *et al*. 1995). The latter stress produces much larger disproportionate decrease of norepinephrine than epinephrine (Pacak and Palkovit 2001). Nevertheless, the current findings suggest that the stimulus generated by cold stress, does not evoke a generalised stress response, but selective activation of peripheral sympathetic system (Palkovit *et al*. 1995) that leads to the rapid release of only norepinephrine rather than its synthesis in mice. Arecoline alone or in cold stress also caused ultrastructural depletion of chromaffin granules represented by an abundance of cytoplasmic vacuoles. Since both epinephrine and norepinephrine concentrations were also declined after arecoline treatment unlike only norepinephrine in cold stress, it is likely that arecoline might be more potent stressful agent than cold stress in mice. In contrast to the inhibited response of adrenomedullary activity to cold stress, heat stress stimulated adrenomedullary activity by showing enlarged nuclear size, peripheral localisation of nucleolus towards the nuclear membrane and abundance of rough endoplasmic reticulum (RER) with drastic depletion of chromaffin granules, containing probably epinephrine (?) and norepinephrine (?). Increased nuclear size of the chromaffin cells could be due to its hyperactivity. This finding corroborated by the significant elevation of adrenal epinephrine and norepinephrine profiles in heat stress. In contrast to heat stress, arecoline treatment in heat stress, suppressed adrenomedullary activity with ultrastructural degeneration of

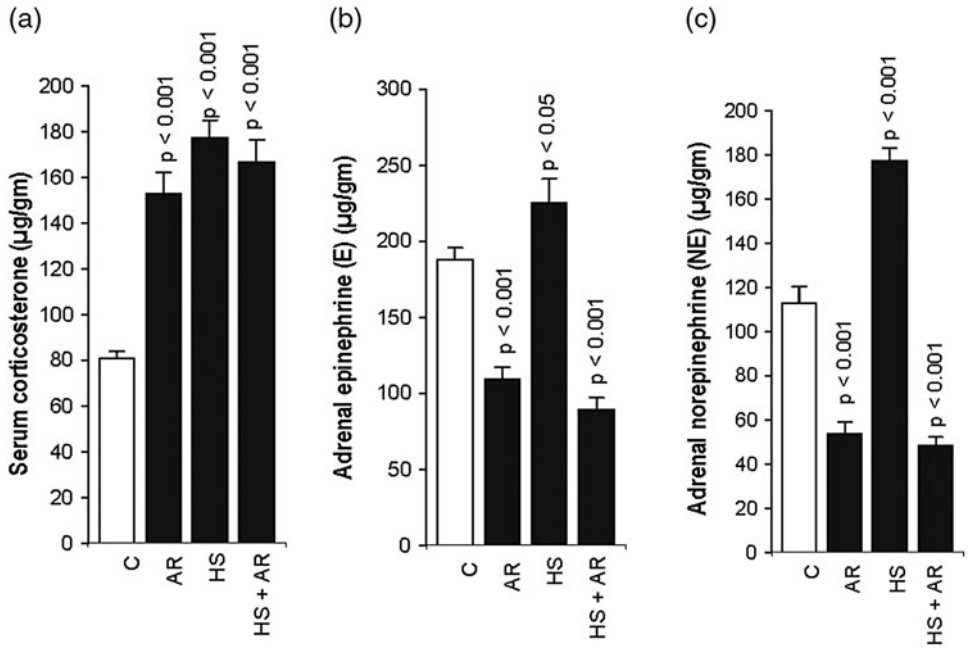

Figure 5. Histograms showing changes in adrenal hormone levels, [corticosterone (a), epinephrine (b) and norepinephrine (c)], following arecoline (AR), heat stress (HS) and arecoline + heat stress (AR + HS) compared to control mice. (For legends see Figure 2).

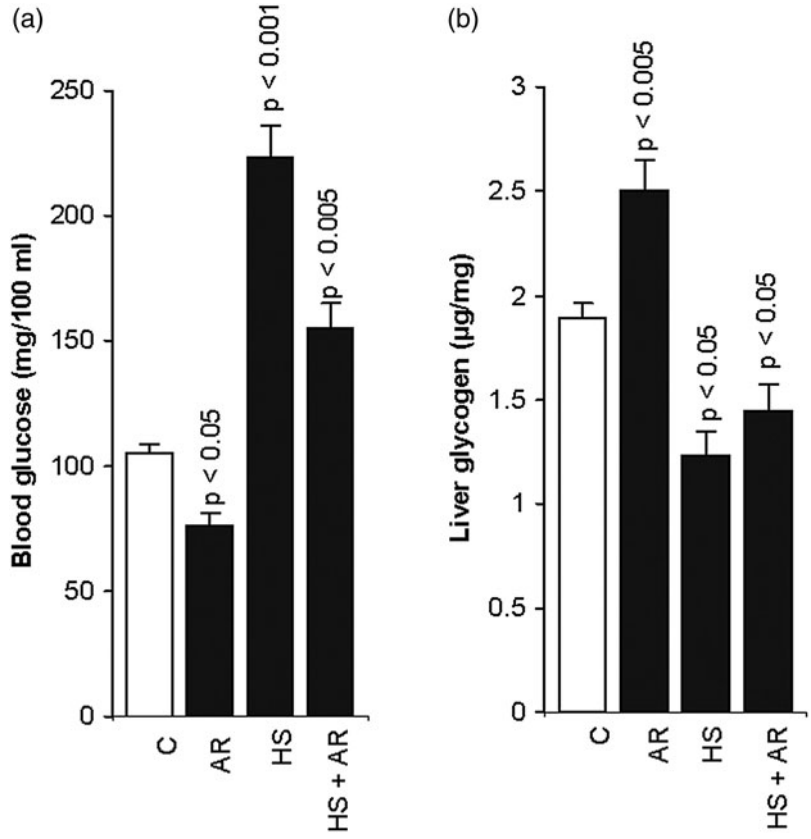

Figure 6. Histograms showing changes in blood glucose (a) and liver glycogen (b) levels following arecoline (AR), heat stress (HS) and arecoline + heat stress (AR + HS) compared to control mice. (For legends see Figure 1).

chromaffin cells, scanty chromaffin granules followed by depletion of both E and NE levels. There are evidences that adrenomedullary enzymes are involved in the biosynthesis of epinephrine and norepinephrine (Axelrod 1975). Tyrosine hydroxylase (TH) is known to be the initial and major rate-limiting enzyme of catecholamines (CAM) biosynthesis which is increased during enhanced CAM biosynthesis in chronic or repeated stress (De Groot and Jameson 2001, Larsen *et al*. 2003). A recent report confirms that long-term stress leads to an increase of tyrosine hydroxylase (TH)

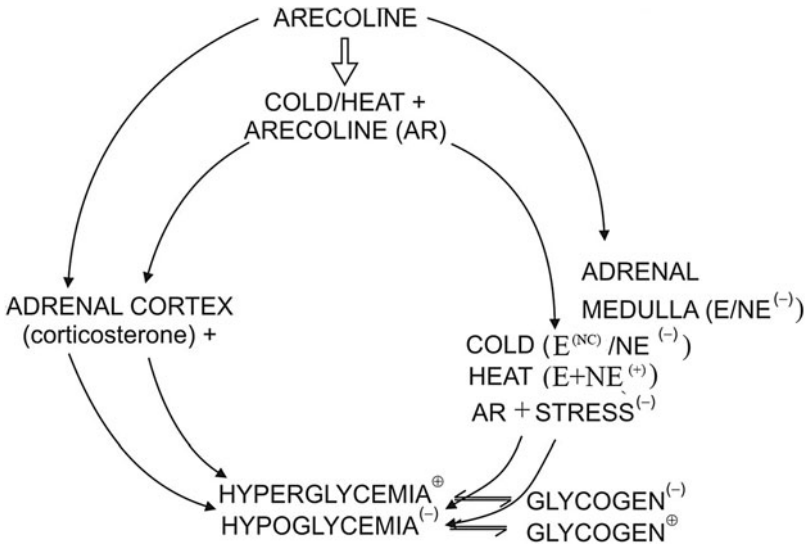

Figure 7. Summarized results of arecoline, thermal stress and arecoline in thermal stress on adrenal cortex, adrenal medulla, glucose and glycogen profiles in mice.

activity in the adrenal medulla (Xu *et al*. 2007). Furthermore, phenyl-N-methyl transferase (PNMT) is required for biosynthesis of catecholamines (Axelrod 1975). As corticosterone is known to stimulate CAM production, its involvement in enhancing the rate of catecholamine biosynthesis via stimulation of TH and PNMT cannot be ruled out in heat stress in mice. In addition to corticosterone, pineal serotonin also might have been involved in the induction of CAM synthesis in heat stress in albino mice. Whether pineal plays any protective role and/or is involved in counteracting thermal stress in rats remains unknown, because pineal is reported to be stimulated in noise stress in chicks (Ray *et al*. 2018). In contrast, decreased production of CAM in cold stress or after arecoline treatment alone or arecoline treatment in cold/heat stress could be due to decreased TH, PNMT or pineal serotonin in mice. As arecoline is highly toxic (Pradhan *et al*. 1986, Dasgupta *et al*. 2006) its influence on adrenomedullary dysfunction in cold/heat stress cannot be ruled out in mice.

Role of adrenal hormones in thermal stress or after arecoline treatment alone or in thermal stress on carbohydrate metabolism cannot be ruled out, because adrenal hormones are hyperglycemic known in vertebrates (Norris and Carr 2013). In our study, we have shown that thermal stress (cold/heat) induced hyperglycemia that resulted in the decline of liver glycogen in mice. Arecoline administration caused hypoglycemia followed by a rise in liver glycogen level, which could be due to its over utilisation to counteract stress, and/or due to enhanced rate of glycogenesis via glycogen synthase (Norris and Carr 2013). Our results of cold and heat stress are in agreement with those of earlier report of hyperglycemia in cold (Smythe *et al*. 1989) or heat (Jentjens *et al*. 2002) stress. Corticosterone, epinephrine and norepinephrine are strong hyperglycemic hormones (Larsen *et al*. 2003, Das 2007, Norris and Carr 2013). In the present study, corticosterone levels were increased both in cold and heat stress suggesting their involvement in the induction of hyperglycemia in stressed mice. Epinephrine (E) and norepinephrine NE) levels were elevated only in heat stress, suggesting their active participation in the induction of hyperglycemia in heat stress in mice. Additionally, E and NE are reported to have a protective role in stress. Our study further reveals that liver glycogen level was reciprocally altered with those of blood glucose profile during thermal stress, because liver glycogen level was declined during hyperglycemia in thermal stress, but was elevated during hypoglycemia observed after arecoline treatment. Alteration in liver glycogen profile is intimately associated with glycemia (Das 2007). Blood glucose is known to be converted to glycogen by glycogen synthase (Norris and Carr 2013). Thus, in our study, declined glycogen level could be due to increased glycogenolysis that resulted in hyperglycemia in thermal stress in mice, but increased liver glycogen level might have been occurred due to its enhanced synthesis by stimulation of glycogen synthase activity that resulted in hypoglycemia in thermal stress. Ryzhavskii *et al*. (1981) also reported low liver glycogen level in cold stress. Oxidation rate of ingested carbohydrate is impaired in heat compared to a cool environment (Jentjens *et al*. 2002). Thus, alteration of oxidation rate of carbohydrates could be responsible for alteration in the interrelationship of glucose-glycogen profiles in stress or after arecoline treatment in mice. Arecoline alone caused hypoglycemia despite the elevation of adrenal hormones. Arecoline, cold or heat are well-known stressful agents which are known to alter insulin and glucagon levels (Bentley 1998). Insulin and glucagon respectively have hypoglycemic and hyperglycemic actions on carbohydrate metabolism (Das 2007). Their probable involvement in the alteration of glucose-glycogen homeostasis in cold or thermal stress or after arecoline treatment alone or in cold or thermal stress, cannot be ruled out in mice. Generally adrenal and pancreatic hormones are involved in carbohydrate metabolism (Norris and Carr 2013). Since arecoline causes adrenocortical disturbance especially in thermal stress, betel nut chewing (vis-à-vis arecoline) should be avoided especially in those who have Cushing's syndrome.

Conclusion

It is concluded that (a) thermal stress has a dual role on adrenal function, because both cold and heat stress stimulates adrenal cortex, but cold stress inhibits adrenomedullary activity in mice. (b) Arecoline alone or in thermal stress stimulates adrenocortical activity, but inhibits adrenomedullary function. (c) Both the thermal stress alone or in arecoline treatment cause disturbance in glucose-glycogen homeostasis in mice.

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