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Studies on Certain Heat Induced Changes of Erythrocyte Membrane with Supplementation of Ascorbic Acid and Alteration of Dietary Protein Level

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Abstract: ***The present study was aimed to determine the effect of ascorbic acid on certain changes of erythrocyte membrane of heat exposed rat, fed on altered dietary protein levels. Elevated cholesterol to phospholipid (C/PL) ratios (7.56 ±0.26; p<0.001 & 10.22 ±0.36; p<0.001) in erythrocyte membrane were found both adequately (18%) protein-fed rats and protein-restricted (6%) rats respectively, following chronic exposure to heat. Prior supplementation with ascorbic acid potentiated the effect of chronic heat exposure on cholesterol content (58.47±0.98; p<0.01& 58.38 ±1.35; p<0.001) of erythrocyte membrane of both adequately protein-fed rats and protein-restricted rats respectively, while it tended to restore partially phospholipid content (7.65 ±0.57; p<0.05) of the erythrocyte membrane in adequately protein-fed rats only. The increased activity of AchE was observed, unlike ATPases which showed depressed activity. Significant decrease in iodine number and increase in conjugated diene formation and increased alanine liberation were found in erythrocyte membrane of chronic heat exposed rat. Acute exposure showed the elevated formation of conjugated diene (4.96 ±0.13; p^a<0.01) in rats fed a 6% protein diet and decreased in iodine number (36.39 ±1.09; p^a<0.05) in adequately protein-fed rats only.***

Keywords: Cholesterol; Phospholipid; Dietary protein; Ascorbic acid, Heat stress.

I. INTRODUCTION

Temperature is an important environmental factor which plays a vital role in the reproduction, growth, development and destruction of all living organisms. It is predicted that the trend of increase of temperature in summer will continue globally [1, 2]. Heat wave resulted death of large number livestock in different countries and creates an economic burden in the livestock industry worldwide [3, 4]. Mild elevation of temperature favours the cells to readjust the homeostatic equilibrium [5]. Fever appears to be an evolutionarily conserved response to bacterial or viral infection and may have survival benefits [6]. Again, when an individual is exposed to temperature above the zone of thermal neutrality, heat appears as a stress and changes occur in different physiological systems, such as cardiovascular, respiratory, endocrines, neural etc. [7]. Diagnosing heat-related illness can be difficult because symptoms vary depending on the extent and magnitude of exposure and individual variability to similar heat loads [8]. Environmental stress like heat or cold stress was found to have influence on blood cells. Erythrocytes are known to haemolyse *in vitro* at 48-50^{0}C. Significant haemolysis and increased fragility were not observed at any temperature after incubation for 4 hrs. [9]. The increased osmotic fragility was observed after incubation for 24 hrs at and above 42^{0}C. Thus, it is important to consider not only the temperature but also the duration of heating [9]. Again, red cells are always under high oxygen pressure and extremely susceptible to peroxidation and the conditions that favour peroxidation, are seemingly optimal in red cells [10]. Noble *et al*. reported that the concentrations of total fatty acids and free cholesterol in the plasma of calves are decreased following short-term exposure to heat [11]. Lower concentrations of total fatty acids and free cholesterol are maintained on prolonged heat exposure. Heating of the suspension of pig erythrocyte ghosts induces at least six thermodynamically irreversible transitions. Each of these transitions is believed to be due to a localized structural transition induced by thermal stress [12]. The greatest elevation of cell membrane fluidity occurred during immediately upon the heat treatment and is considered as a rate-limiting step of heat-induced haemolysis [13]. Erythrocytes provide valuable information on the cell maintenance and turnover, oxidative stress parameters, membrane composition and integrity [14]. Bernabucci *et al*. suggested that erythrocytes are an appropriate and sensitive model to study the oxidative status of transtition dairy cows exposed to hot environment [15]. On the other hand, studies demonstrated that heat stress

induces oxidative stress in the body [16, 17, and 18]. Acute heat-stressed broiler chickens had a 2-fold increase in malondialdehyde, a marker of lipid peroxidation, in skeletal muscle [19]. End-product of lipid peroxidation, acts as a heat shock protein (hsp) inducer [20]. Membrane cholesterol contents modify the protective power of flavonoids against oxidative stress in erythrocytes [21].The antioxidant power of flavonoids, in turn depend on the depletion or incorporation of cholesterol in the erythrocyte membrane [22]. Further, it is known that ascorbic acid along with electrolyte supplementation was found to ameliorate the heat stress in buffaloes and plays a key role in the modulation of glutathione oxidase-reductase system [23]. Any influence on this system will accordingly modulate oxidative reaction in erythrocytes. Heat stress deforms the cell constituent molecules such as membrane lipids, proteins or nucleic acids [24]. Thus, the membrane stability may also depend on ascorbic acid status of the membrane. The lipid status of the erythrocyte membrane and its peroxidation may be other contributory factors for its stability. Phospholipids, sphingolipids and cholesterol are also involved in stress-induced second messenger generation [25, 26]. Mild changes in temperature leads to change in lipid raft and resulted in altered fluidity and consequently, heat shock factor synthesis [27]. In addition, dietary protein and lipid level is also known to have profound influence on antioxidant system and membrane composition and stability [28].

Accordingly, the aim of the present investigation is to study the effect *in vivo* of heat stress on biochemical changes of erythrocyte membrane in terms of lipid status, lipid peroxidation, glutathione metabolism and activities of membrane marker enzymes on dietary protein adequacy and inadequacy. It is also intended to note whether ascorbic acid supplementation has any protective effects against chronic and acutely heat-induced membrane changes.

II. MATERIALS AND METHODS

A. Chemicals

Bovine serum albumin (BSA), 5, 5′-dithiobistrinitrobenzoic acid (DTNB), Folin-Ciocalteau reagent, tris, disodium ethylene tetra acetate (EDTA), reduced glutathione (GSH), were purchased from SRL, Mumbai, India. 5-Sulphosalicylic acid dihydrate and 2-thiobarbituric acid (TBA) were purchased from Spectrochem, Mumbai, India. Other chemicals used throughout the investigation were of analytical grade.

B. Animals and diets

Male growing rats of Wistar strain weighing 100-120g were used for the present study. The animals were kept in a well- ventilated room with 12 hrs. day - light cycle. The animals were accustomed with this condition for 7 days with adequate amount of food containing protein (casein)18%, carbohydrate(amylum) 71%, fat(groundnut oil) 7%, salt mixture 4% and adequate amount of vitamins mixture as reported elsewhere [29]. The composition of the salt mixture used was as described by Hawk and Oser [30].Then the animals were divided into four groups of equal average body weight. The animals of half of the groups were continued with diet containing 18% protein while those of the remaining groups were maintained on the diet containing 6% protein, and 83% carbohydrate. The 18% protein was used as it was considered as an adequate (normal) dietary protein level which was used on earlier occasions [29]. The 6% protein was used as an inadequate dietary protein level (protein inadequacy) to study the influence of dietary protein inadequacy. This experiment was approved by the guidelines of Institutional Animal Ethics Committee of department of Physiology, University of Calcutta.

C. Heat Exposure

After maintaining for three weeks on experimental diets, rats of experimental groups were exposed to heat stress. From one week before the onset of heat exposure, body weight, food intake and rectal temperature had been recorded on every alternate day till the termination of the heat-exposure period. Rats were exposed to heat stress in a well maintained climatic chamber.

Ascorbic acid was supplemented to the rats at a dose of 20mg per 100g of body weight intraperitoneally from one week before the beginning of heat exposure to increase the antioxidant reserves of the rats, and supplementation was continued till the day before their sacrifice. Effective thermal stress was determined by varying the duration of heat exposure and keeping the exposure temperature constant and vice versa. In both conditions rectal temperature of each rat was recorded at regular time intervals and also after the termination of heat-exposure in each day to know the pattern and degree of heat stress imposed on the rats. Following these approaches, temperature of 43 ± 1^0C with 2 hrs. duration per day for 15 successive days and 43 ± 1^0C temperature with 3 hrs. duration in one day were considered optimum to produce the effect of chronic and acute heat exposure, respectively. Rats were exposed to heat between 2 and 6 p.m. in each day to avoid the diurnal variation of temperature. To maintain the uniformity in the heat stress induced, no experiment was performed in the months of summer (April to June) and winter (December to February). The entire study was carried out with several sets of experiments involving different groups of rats and keeping all the above conditions identical.

D. Measurement of Rectal Temperature

Rectal temperature of each rat of both the treated and control groups was measured with the help of a clinical thermometer. Proper care was taken to avoid faulty measurement due to varied depth of penetration, defecation and accumulated feces in the rectum at the time of temperature study. In case of treated rats rectal temperature was measured twice a day – before and at the end of heat exposure period.

E. Tissue Collection

At the end of experimental period rats were kept fasting for 18 hrs. and then sacrificed by cervical dislocation. Blood was collected immediately from the hepatic vein with a heparinized syringe and kept in polypropylene vials at 4^0C, taking proper care to prevent any chance of haemolysis. To obtain erythrocytes, heparinized blood was centrifuged (1000×g at 4^0C for 10 mins.). Plasma was collected and stored in deep freeze. The buffy layer was removed completely by aspiration. The erythrocytes were washed three times with 20mM Tris-buffered-saline solution. The washed and packed erythrocytes were used for the preparation of ghost membrane.

F. Preparation of Erythrocyte Membrane

Fresh blood obtained from the rat was used to prepare ghost membrane following the procedure as developed by Marchesi and Pallade [31]. The packed red cells were washed thrice in 130mM NaCl and 20mM Tris-HCl (pH 7.4) mixture and recovered by centrifugation at 2500 r.p.m. for 15mins. Saline-washed red cells were lysed in 5mM Tris-HCl (pH 7.4) buffer containing 1mM EDTA and kept at 4^0C for 15 mins. Thirty nine volumes of haemolysing fluid were added to 1 volume of packed red cell, followed by centrifugation at 25,000□g for 30 mins. at 4^0C. The supernatant was discarded and the membrane settled down was resuspended in the same medium and centrifuged again. The same procedure was repeated for 3 to 4 times until the membrane became milky white. Finally, the membrane was suspended in 50mM Tris-HCl buffer (pH 7.4) containing 1mM EDTA and kept frozen. All operations were carried out in cold.

G. Estimation of Membrane Protein

The protein was estimated by modified Lowry method [32]. The absorbance was read at 500nm and the protein content was calculated using a standard curve prepared with aliquots of BSA-solution having known concentration.

H. Extraction of Membrane Lipid

The lipid was extracted from erythrocyte membrane by the method as described by Rose and Oklander [33]. After centrifugation, 5 millilitres of supernatant was evaporated to dryness with N_2 to get the lipid.

1) *Estimation of Membrane Cholesterol:* To extract the membrane cholesterol 0.1ml of membrane suspension was mixed with 2.9ml of ether-acetone (1:1) mixture and the mixture was warmed at 70^0C for 10 mins. It was centrifuged and 2ml of supernatant was taken for drying with N_2. The cholesterol was estimated according to the method as described by Zlatkis *et al.* [34]. The result was then computed using the standard curve prepared with aliquots of cholesterol solution having known concentration.
2) *Estimation of Membrane Phospholipid:* The lipid was extracted from the membrane with ether-alcohol (1:3) mixture. To 0.1ml of membrane suspension 5.9ml of ether-alcohol mixture was added. It was vortexed and incubated at 37^0C for 12 hrs. Following centrifugation, 5ml of supernatant was taken for drying. The residue was digested with 0.5ml of 10(N) H_2SO_4 and 15µl conc. HNO_3 under a low flame of a burner until it became colourless. To it 2ml of distilled water, 0.25ml of 1% ascorbic acid solution and 0.25ml of 1% ammonium molybdate solution were added and mixed. The absorbance was read at 640nm just after 30 mins. of addition of ammonium molybdate. The total procedure was based on the method as developed by Youngburg and Youngburg [35].
3) *Determination of Iodine Number of Membrane Lipids:* Iodine number was determined by the method as described by Plummer [36]. Extraction of lipid was made from packed red cells following the method as described by Rose and Oklander without any modification [33].
4) *Determination of Conjugated Diene Formation in Membrane Lipid:* The conjugated diene was measured according to the procedure as described by Buege and Aust [37]. One millilitre of membrane suspension was mixed with 5ml of chloroform-methanol (2:1) mixture and the mixture was left for 15 mins. with frequent vortexing. It was then centrifuged at 100×g for 5 mins. to separate the mixture into two phases. The upper layer was removed by suction and 3ml of the lower chloroform layer

was taken and put to dryness in a water bath at 40^0C under a stream of N_2; the residue was dissolved with 1.5ml of cyclohexane and the absorbance was read at 233nm against a cyclohexane blank.

5) *Assay of Acetylcholine Esterase (AchE) (EC 3.1.1.8) Activity of Erythrocyte Membrane:* The activity of AchE was assayed by the method as described by Ellman *et al.* [38], with slight modification as follows: To 280□l of 0.1M phosphate buffer (pH 7.4), 20□l of membrane suspension was added, followed by mixing and warming in incubator at 37^0C for 5 mins. To start the reaction, 0.1ml of 3mM S-butylthiocholine iodide was added to it. After incubating for 30 mins. at 37^0C, the reaction was stopped with addition of 0.1ml of 15% cooled PCA (v/v) and centrifuged to get the supernatant. A definite amount (0.1ml) of supernatant was added to 0.9ml of 0.25mM DTNB prepared in 0.1M phosphate buffer (pH 8.0) and the absorbance was read at 412nm 10 mins after the addition of DTNB against a reagent blank. The absorbance values were compared with a standard curve prepared with GSH solution having known concentration to compute the – SH group liberation by AchE.

6) *Assay of ATPases (EC 3.6.7.3) Activities of Erythrocyte Membrane:* The activities of ATPases were assayed according to the method as developed by Ronner *et al.* [39].During assay of three ATPases (Na^+-K^+, Mg^{2+} and Ca^{2+}-Mg^2-ATPases), the total volume of reaction mixture, amount of HEPES buffer (300mM, pH 7.4) and membrane suspension were kept constant. The mixtures were preincubated for 5 mins. in a water bath at 37^0C and the reaction was started with addition of 0.1ml of 10mM ATP solution, and incubated for 30mins. at 37^0C with constant shaking. The reaction was stopped by the addition of 50% ice-cold TCA solution and kept cooled until the liberated Pi was estimated. Heat-treated membrane suspension (at 80^0C for 5 mins.) was used as control so that ATP blank could be determined. All assays were carried out in duplicate. Ouabain (1%) and EGTA (1%) were used as blockers for Na^+-K^+-ATPase, and Ca^{2+}-Mg^{2+}-ATPase respectively, and 330mM NaCl, 1200mM KCl, 50mM $MgCl_2$ and 1mM□$CaCl_2$ were used for the preparation of incubation medium.

7) *Estimation of Alanine Content in Erythrocytes:* Alanine content of erythrocyte was measured enzymatically following the method as described by Davies and Goldberg [40].One ml of cold 1.6M PCA was added to 3ml of chilled saline-washed cell suspension and it was kept in ice for 10min with frequent vortexing. The mixture was centrifuged at 500□g for 10 mins. to get the sample supernatant. To 1ml sample supernatant 200□l of 2M KOH and 800□l of 0.5M. Tris-HCl buffer (pH 9.0) were added and it was then kept in ice for 2 hrs. during which the perchlorate precipitated the alanine present in each sample supernatant. The mixture containing 500□l of the supernatant containing alanine, 500□l of 0.8M Tris-buffer (pH 9.0) containing 0.04M EDTA, 500□l of 6.6 hydrazine hydrate solution, 100□l of 20mM NAD^+ and 100□l of 20 mg/ml of alanine dehydrogenase (30 units/mg) was incubated at 37^0C for 60mins. Following termination of incubation, the alanine content was determined fluorometrically by the reduction of NAD^+ to NADH catalyzed by alanine dehydrogenase that was measured at excitation wavelength of 340nm and emission wavelength of 450nm. The results were then computed by using a standard curve prepared with alanine solution having varied amounts ranging from 0-50 □moles.

I. Statistical analysis

The data obtained from each experiment described above (N<30) were subjected to statistical analysis. The level of significance of the observed changes between the control and treated groups of animals was calculated according to two-tail student's 't'-test and the probability of chance of occurrence (p) was determined according to the Table (Level of significance for two-tail 't'-test) of Fisher and Yates [41]. The data were expressed as means ± SEM. Differences were considered significant at $p<0.05$.

III. RESULTS

A. Lipid Status of Erythrocyte Membrane

It is revealed from the Table 1A that chronic heat exposure elevated the cholesterol content and decreased the phospholipid content of erythrocyte membrane and, consequently, cholesterol to phospholipid (C/PL) ratio was also raised. This pattern of cholesterol and phospholipid distribution in erythrocyte membrane was found in both adequately (18%) protein-fed rats and protein-restricted (6%) rats following chronic exposure to heat. Prior supplementation with ascorbic acid potentiated the effect of chronic heat exposure on cholesterol content of erythrocyte membrane of both adequately protein-fed rats and protein-restricted rats, while it tended to restore partially phospholipid content of the erythrocyte membrane in adequately protein-fed rats and increased it even after restoration in protein-restricted rats. Ascorbic acid supplementation on the other hand, appeared to have no significant effect on the alternation of cholesterol to phospholipid ratio of erythrocyte membrane of rats following chronic exposure to heat. Acute heat exposure unlike chronic heat exposure was found to have no significant effect on cholesterol content of erythrocyte membrane of rats on an 18% protein diet, while it showed less depressing effect on cholesterol content of erythrocyte membrane (Table 1B). The increase in cholesterol to phospholipid ratio in erythrocyte membrane was also less marked in this group of rats following acute exposure to

heat (Table 1B). Ascorbic acid supplementation to this group of rats raised the cholesterol content of erythrocyte membrane and tended to reverse partially the change in its phospholipid content and consequent to this, cholesterol to phospholipid ratio in the erythrocyte membrane was elevated further (Table 1B). The results presented in Table 1B also reveal that protein-restricted rats (6% dietary protein) following acute exposure to heat did not exhibit any change in the cholesterol content of erythrocyte membrane but its phospholipid content was reduced and, as a result, cholesterol to phospholipid ratio in the erythrocyte membrane appeared to be elevated. Ascorbic acid supplementation to this heat-exposed group of rats increased the cholesterol content of erythrocyte membrane and tended to reverse partially the change in membrane's phospholipid content. It is further found from the Table 1B that the elevation in cholesterol to phospholipid ratio in the erythrocyte membrane of protein-restricted rats following acute heat exposure was further accentuated by ascorbic acid supplementa.

B. Formation of Conjugated Diene in Erythrocyte Membrane

The results presented in Table 2A reveal that the formation of conjugated diene increased in erythrocyte membrane following chronic exposure to heat. Further, the formation of conjugated diene appeared quite higher in protein-restricted rats than in adequately protein-fed rats. Acute heat exposure showed no effect on the formation of conjugated diene in 18% protein-fed rats but it elevated the formation of conjugated diene in rats fed a 6% protein diet (Table 2B).

C. Iodine Number of Erythrocyte Membrane Lipids

Iodine number of membrane lipids decreased significantly in both 18% protein-fed and 6% protein-fed rats following chronic exposure to heat (Table 2A).

Acute heat exposure decreased the iodine number in adequately protein-fed rats only, while in protein-restricted rats no marked change was observed (Table 2B).

D. Liberation of Alanine in Erythrocyte Membrane

The liberation of alanine increased in both 18% protein-fed and 6% protein-fed groups of rats following chronic as well as acute exposure to heat (Table 2A and Table 2B).

E. Marker Enzymes of Erythrocyte Membrane

Acetylcholine Esterase (AchE), Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase and Mg^{2+} ATPase Activities

The results presented in Table 3A reveal that the activity of AchE tended to be increased slightly while the activities of Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase and Mg^{2+} ATPase were depressed significantly in both adequately protein-fed and protein-restricted groups of rats following chronic exposure to heat. Prior supplementation of ascorbic acid was found to have no significant reversing effect on the changes in the activities of these enzymes. Only slight restoring tendency in the activity of AchE was observed (Table 3A). Acute heat exposure was found to produce no marked alteration on the activity of AchE, while the activities of Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase and Mg^{2+}- ATPase were depressed significantly in both adequately protein-fed and protein-restricted groups of rats (Table 3B). Supplementation of ascorbic acid showed no reversing effect on the changes in the activities of these enzymes due to acute heat exposure (Table 3B).

IV. DISCUSSION

The phospholipid and cholesterol form an integral part of the erythrocyte membrane. Again, the fatty acid composition of the cell membrane modulates its structure, fluidity and functions [42, 43]. The pattern of alteration of membrane fluidity is influenced by changes in the level of unsaturation of the phospholipid fatty acyl chains, phosphatidyl ethanolamine ratios, cholesterol to phospholipid ratios or protein to lipid ratios and fatty acyl chain length [44]. The fluidity of the membrane in turn has been shown to modulate cellular functions.

In vivo, the ratio of saturated to unsaturated fatty acids and the amount of cholesterol in the membrane are closely regulated. This may represent a mechanism of maintaining membrane fluidity within certain limits for optimal membrane functions known as "homeoviscous adaptation" [45, 46]. There is evidence to suggest that homeoviscous adaptation occurs *in vivo*, maintaining membrane fluidity within narrow limits and may be involved in the "fine tuning" of membrane fluidity, by regulating membrane cholesterol content, phospholipid class distribution, and/or the activities of the membrane-bound desaturase and elongase enzymes. Cholesterol increases the order of lipids in the membrane, leading to reduced fluidity [47].

The increased cholesterol content (Table 1A) of erythrocyte membrane following chronic heat exposure, as observed in the present study, is a result of homeoviscous adaptation response. The composition of the membrane is also influenced by the quality of the diet. The increased membrane cholesterol with concurrent decrease of membrane phospholipid results in the elevation of C/P ratio.

This in turn maintains the fluidity of the erythrocyte membrane as an acclimatory response to heat stress. From the present studies, it is also evident that the increased C/P ratio (Table 1A) in erythrocyte membrane took place significantly in chronic heat exposure only. In case of acute heat exposure, the C/P ratio (Table 1B) increased insignificantly or remained more or less the same as control, probably due to inadequate development of acclimatory changes. The results further indicate that the C/P ratio in erythrocyte membrane was quite higher in protein-restricted group of animals in general, whether exposed to heat or not. As the acclimatory and adaptational changes depend on the length of heat exposure, the deviation of values of the parameter studied appeared to be greater after chronic heat exposure than acute heat exposure. Interestingly, it is found that supplementation of ascorbic acid to the rats prior to the initiation of heat exposure and during the period of heat exposures (in case of chronic heat treatment) tended to restore the C/P ratio in erythrocyte membrane to control level. But, it did not appear to be effective in case of acute heat exposure. Instead, it tended to elevate further the C/P ratio in erythrocyte membrane. In addition, ascorbic acid supplementation tended to restore the phospholipid content (Table 1A) of erythrocyte membrane in chronically heat exposed rats. Further, the matured erythrocytes are incapable to synthesize membrane lipids but can exchange its free cholesterol and outer layer phospholipids lecithin and sphingomyelin, with plasma lipoprotein [48].

The exact cause of decrease of phospholipid content (Tables 1A and 1B) in erythrocyte membrane following exposure to heat is not clear from the present investigation. The decreased phospholipid content of erythrocyte membrane following exposure to heat as noted in the present investigation, may arise from increased phospholipase activity. However, it requires to be ascertained by studying the activities of phospholipase following exposure to heat.

The reduction of iodine number (Tables 2A and 2B) of erythrocyte membrane lipid in response to chronic heat exposure *in vivo* suggests the reduced degree of unsaturation of membrane lipids and, consequently, lower will be the membrane fluidity. Thus, the reduced iodine number as observed in the present study favours the elimination of chance of heat-induced hyperfluidity of the membrane. This suggests that, following chronic exposure to heat, a sort of adaptive mechanism is developed in the erythrocyte membrane for maintenance of fluidity within normal level. This is also supported by the observations of Johnston and Roots who demonstrated that acclimation to higher temperature results in a greater proportion of saturated fatty acids in the major phospholipid classes [49]. Accordingly, the lesser increase in lipid peroxidation in erythrocyte membrane following chronic exposure than acute exposure to heat may be ascribed to the adaptive mechanism developed during chronic exposure to heat.

The increased values of conjugated diene and alanine (Tables 2A and 2B) as noted in the present studies were the outcome of heat-induced oxidative assult where restriction of protein in the diet aggravates the change.

The increased activity of membrane AchE, unlike ATPases (Tables 3A and 3B) which showed depressed activity, can be ascribed to hyperfluidity of the membrane and the favourable allosteric modulation following chronic exposure to heat as the enzyme is localized on the outerside of the membrane.

V. CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

VI. ACKNOWLEDGEMENT

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Table 1a:; effect of chronic heat-exposure on cholesterol and phospholipid contents of erythrocyte membrane.

Dietary protein levels	Groups of animals	Cholesterol (□g/mg membrane protein)	Phospholipid (□g Pi/mg membrane protein)	Cholesterol/ Phospholipid ratio
18%	Control (6)	48.46 □□1.29	9.53 □□0.50	5.08 □□0.16
	Heat-exposed (8)	54.73 □□0.12 (p^a<0.001)	7.24 □□0.48 (p^a<0.01)	7.56 □□0.26 (p^a<0.001)
	Heat-exposed + Ascorbic acid supplementation (8)	58.47 □□0.98 (p^b<0.01) (p^c<0.001)	7.65 □□0.57 (p^c<0.05)	7.64 □□0.13 (p^c<0.001)
6%	Control (6)	45.74 □□0.96	5.65 □□0.16	8.09 □□0.26
	Heat-exposed (8)	51.32 □□0.42 (p^a<0.001)	5.02 □□0.22 (p^a<0.05)	10.22 □□0.36 (p^a<0.001)
	Heat-exposed + Ascorbic acid supplementation (8)	58.38 □□1.35 (p^b<0.001) (p^c<0.001)	5.89 □□0.08 (p^b<0.01)	9.91 □□0.34 (p^c<0.01)

Values are means □□SEM.
p^a – Compared with control.
p^b – Compared with heat-exposed.
p^c – Compared with control.
Figures in the parentheses of the column of Groups of animals indicate the number of animal

Table 1b.: effect of acute heat-exposure on cholesterol and phospholipid content of erythrocyte membrane.

Dietary protein levels	Groups of animals	Cholesterol (□g/mg membrane protein)	Phospholipid (□g Pi/mg membrane protein)	Cholesterol/ Phospholipid ratio
18%	Control (6)	48.38 □□0.89	9.34 □□0.33	5.18 □□0.14
	Heat-exposed (8)	49.20 □□1.10 (p^a<0.05)	8.26 □□0.19 (p^a<0.05)	5.96 □□0.18 (p^a<0.01)
	Heat-exposed + Ascorbic acid supplementation (8)	59.89 □□0.52 (p^b<0.001) (p^c<0.001)	8.64 □□0.20 (p^b>0.05)	6.82 □□0.22 (p^c<0.001) (p^b<0.01)
6%	Control (6)	45.05 □□0.67	6.05 □□0.41	7.45 □□0.21
	Heat-exposed (7)	45.97 □□0.67	5.28 □□0.21 (p^a>0.05)	8.71 □□0.32 (p^a<0.01)
	Heat-exposed + Ascorbic acid supplementation (8)	55.84 □□1.33 (p^b<0.001) (p^c<0.001)	5.67 □□0.12 (p^b>0.05)	9.84 □□0.29 (p^c<0.001) (p^b<0.05)

Values are means □□SEM.
p^a – Compared with control.
p^b – Compared with heat-exposed.
p^c – Compared with control.
Figures in the parentheses of the column of Groups of animals indicate the number of animals.

Table 2a.: effect of chronic heat-exposure on conjugated diene formation and iodine number of erythrocyte membrane.

Dietary protein levels	Groups of animals	Conjugated diene (nmole/mg membrane protein	Iodine number	Alanine liberated (□mole/ml packed RBC
18%	Control (6)	5.74 □□0.38	40.69 □□1.24	0.47 □□0.01
	Heat-exposed (8)	8.62 □□0.19 (p^a<0.001)	30.20 □□0.29 (p^a<0.001)	0.65 □□0.03 (p^a<0.001)
	Heat-exposed + Ascorbic acid supplementation (6)	Not measured	Not measured	Not measured
6%	Control (6)	3.94 □□0.21	31.87 □□0.24	0.52 □□0.01
	Heat-exposed (8)	6.11 □□0.08 (p^a<0.001)	26.77 □□0.65 (p^a<0.001)	0.70 □□0.01 (p^a<0.001)
	Heat-exposed + Ascorbic acid supplementation (6)	Not measured	Not measured	Not measured

Values are means □□SEM.
p^a – Compared with control.
p^b – Compared with heat-exposed.
p^c – Compared with control.
Figures in the parentheses of the column of Groups of animals indicate the number of animals.

Table 2b.: Effect of acute heat-exposure on conjugated diene formation and iodine number of erythrocyte membrane.

Dietary protein levels	Groups of animals	Conjugated diene (nmole/mg membrane protein	Iodine number	Alanine liberated (□mole/ml packed RBC)
18%	Control (6)	5.74 □□0.38	41.05 □□1.22	0.47 □□0.01
	Heat-exposed (8)	5.47 □□0.16	36.39 □□1.09 (p^a<0.05)	0.54 □□0.005 (p^a<0.001)
	Heat-exposed + Ascorbic acid supplementation (6)	Not measured	Not measured	Not measured
6%	Control (6)	3.94 □□0.21	32.00 □□0.91	0.52 □□0.01
	Heat-exposed (8)	4.96 □□0.13 (p^a<0.01)	30.08 □□0.49	0.59 □□0.02 (p^a<0.05)
	Heat-exposed + Ascorbic acid supplementation (6)	Not measured	Not measured	Not measured

Values are means □□SEM.
p^a – Compared with control.
p^b – Compared with heat-exposed.
p^c – Compared with control.
Figures in the parentheses of the column of Groups of animals indicate the number of animals.

Table 3a.: Effects of chronic heat-exposure on acetylcholine esterase, $(na^+\text{-}k^+)$-atpase, $(ca^{2+}\text{-}mg^{2+})$-atpase and mg^{2+}-atpase activities of erythrocyte membrane.

Dietary protein levels	Groups of animals	AchE (mmole -SH gr./mg protein/hr.)	$Na^+\text{-}K^+$-ATPase (□mole Pi/mg protein/hr.)	$Ca^{2+}\text{-}Mg^{2+}$-ATPase (□mole Pi/mg protein/hr.)	Mg^{2+}-ATPase (□mole Pi/mg protein/hr.)
18%	Control (5)	1.51 □□0.06	3.41 □□0.12	2.46 □□0.39	0.58 □□0.02
	Heat-exposed (6)	1.80 □□0.02 (p^a<0.01)	1.70 □□0.07 (p^a<0.001)	1.06 □□0.07 (p^a<0.01)	0.29 □□0.03 (p^a<0.001)
	Heat-exposed + Ascorbic acid supplementation (6)	1.64 □□0.02 (p^b<0.001)	1.62 □□0.08 (p^c<0.001)	1.20 □□0.04 (p^c<0.01)	0.21 □□0.01 (p^b<0.05) (p^c<0.001)
6%	Control (5)	1.65 □□0.01	3.39 □□0.11	1.88 □□0.38	0.46 □□0.05
	Heat-exposed (6)	1.75 □□0.04 (p^a<0.05)	0.61 □□0.07 (p^a<0.001)	1.03 □□0.09 (p^a>0.05)	0.22 □□0.02 (p^a<0.01)
	Heat-exposed + Ascorbic acid supplementation (6)	1.67 □□0.07	0.64 □□0.08 (p^c<0.001)	1.27 □□0.04 (p^b<0.05)	0.22 □□0.01 (p^c<0.01)

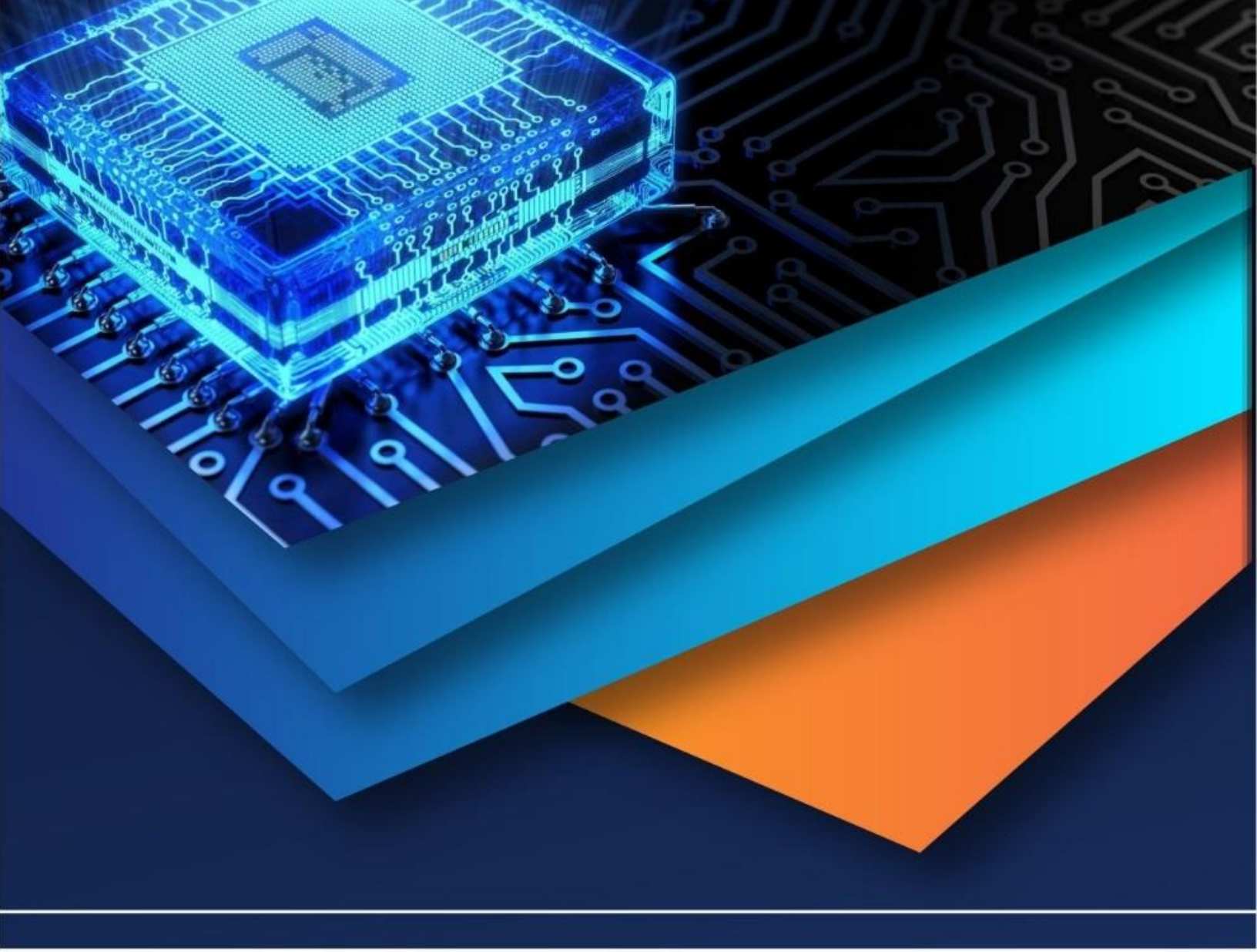
Values are means □□SEM.
p^a – Compared with control.
p^b – Compared with heat-exposed.
p^c – Compared with control.
Figures in the parentheses of the column of Groups of animals indicate the number of animals.

Table 3b.: Effects of acute heat-exposure on acetylcholine esterase, $(na^+\text{-}k^+)$-atpase, $(ca^{2+}\text{-}mg^{2+})$-atpase and mg^{2+}-atpase activities of erythrocyte membrane.

Dietary protein levels	Groups of animals	AchE (mmole -SH gr./mg protein/hr.)	$Na^+\text{-}K^+$-ATPase (□mole Pi/mg protein/hr.)	$Ca^{2+}\text{-}Mg^{2+}$-ATPase (□mole Pi/mg protein/hr.)	Mg^{2+}-ATPase (□mole Pi/mg protein/hr.)
18%	Control (6)	1.51 □□0.06	3.41 □□0.12	2.46 □□0.39	0.58 □□0.02
	Heat-exposed (6)	1.50 □□0.03 (p^a>0.05)	2.32 □□0.10 (p^a<0.001)	1.61 □□0.01 (p^a>0.05)	0.34 □□0.01 (p^a<0.001)
	Heat-exposed + Ascorbic acid supplementation (6)	1.42 □□0.04	1.94 □□0.41 (p^b>0.05) (p^c<0.01)	1.44 □□0.01 (p^c<0.05)	0.35 □□0.04 (p^c<0.001)
6%	Control (6)	1.65 □□0.01	3.39 □□0.11	1.88 □□0.38	0.46 □□0.05
	Heat-exposed (6)	1.69 □□0.02 (p^a>0.05)	1.25 □□0.17 (p^a<0.001)	1.26 □□0.10 (p^b>0.05)	0.23 □□0.02 (p^a<0.01)

	Heat-exposed + Ascorbic acid supplementation (6)	1.64 □□0.01 (p^b>0.05)	1.17 □□0.12 (p^c<0.001)	1.24 □□0.08 (p^c>0.05)	0.25 □□0.03 (p^a<0.01)

Values are means □□SEM.
p^a – Compared with control.
p^b – Compared with heat-exposed.
p^c – Compared with control.
Figures in the parentheses of the column of Groups of animals indicate the number of animals.



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