Neurochemistry International 74 (2014) 46–52

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Intermittent cold-induced hippocampal oxidative stress is associated with changes in the plasma lipid composition and is modifiable by vitamins C and E in old rats

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ARTICLE INFO

Article history:
Received 2 January 2014
Received in revised form 30 April 2014
Accepted 5 May 2014
Available online 13 May 2014

Keywords:
Hippocampus
Intermittent-cold exposure
Lipid peroxidation
Lipid profile
Vitamin C
Vitamin E

ABSTRACT

This study primarily investigated the effects of intermittent cold exposure (ICE) on oxidative stress (OS) in the hippocampus(HC) and plasma lipid profile of old male rats. Secondly, it evaluated structural changes in the hippocampus region of the rat's brain. Thirdly, it attempted an evaluation of the effectiveness of the combined supplement of vitamins C and E in alleviating cold stress in terms of these biochemical parameters. Thirty male rats aged 24 months were divided into groups of five each: control (CON), cold-exposed at 10 °C (C10), cold-exposed at 5 °C (C5), supplemented control (CON+S), and supplemented cold-exposed at either 5 °C (C5+S) or 10 °C (C10+S). The rats were on a daily supplement of vitamin C and vitamin E. Cold exposure lasted 2 h/day for 4 weeks. Rats showed increased levels of hydrogen peroxide (H_2O_2), and thiobarbituric acid reactive substances (TBARS) in the HC at 10 °C with further increase at 5 °C. Cold also induced neuronal loss in the hippocampus with concomitant elevations in total cholesterol (TCH), triglycerides (TG) and low-density lipoproteins (LDL-C) levels, and a depletion in high-density lipoprotein (HDL-C). A notable feature was the hyperglycaemic effects of ICE and depleted levels of vitamins C and E in the hippocampus and plasma while supplementation increased their levels. More importantly, a positive correlation was observed between plasmatic LDL-C, TCH and TG and hippocampal TBARS and H_2O_2 levels. Further, intensity of cold emerged as a significant factor impacting the responses to vitamin C and E supplementation. These results suggest that cold-induced changes in the plasma lipid profile correlate with OS in the hippocampus, and that vitamin C and E together are effective in protecting from metabolic and possible cognitive consequences in the old under cold exposures.

1. Introduction

Brain tissue is vulnerable to oxidative damage since it consumes high levels of oxygen and to lipid peroxidation which is readily peroxidized (Özmen et al., 2007). It is known that, oxidative stress (OS) resulting from exposure to extreme cold can alter neuronal function and this alteration has been associated with neurochemical changes that take place in the different regions of the brain. Incidentally, cold-induced thermoregulation is also associated with increased lipid metabolism and is age-dependent (McDonald et al., 2004). Low temperature exposures lead to an increased metabolic rate (Selman et al., 2000), elevated reactive oxygen species (ROS) (Venditti et al., 2004) and OS-induced tissue damage (Topp et al., 2000). It is possible that cold-stimulated high metabolic rates can alter blood lipid profiles and accelerate the development and progression of OS-related occupational hazards such as reduced cognitive performance in terms of memory, vigilance and concentration (Pilcher et al., 2002; Palinkas, 2001). In an earlier study, we demonstrated the hypolipidemic effects of vitamin E supplements with age in rats (Asha Devi et al., 2003a). Incidentally, LPO and cholesterol metabolism have been related to a number of OS-related diseases in the older brain.

Animal studies have shown that the structure and function of hippocampus (HC), a brain region that is important for cognition is adversely affected by conditions such as obesity and hyperlipidemia (Morisson et al., 2010; Farr et al., 2008). Further, the mechanisms that are responsible for hyperlipidemia impacting the HC may be related to situations that cause increased LPO and OS under situations such as intermittent cold exposures (ICE).

Abbreviations: ICE, intermittent cold exposure; HC, hippocampus; HDL-C, high density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LPO, lipid peroxidation; OS, oxidative stress; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; TCH, total cholesterol; TG, triglycerides.

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http://dx.doi.org/10.1016/j.neuint.2014.05.001
0197-0186/

Vitamin E (α-tocopherol) is the most important antioxidant that is distributed in the lipid phase of the cells and can react directly with the free radicals, including hydrogen peroxide (H_2O_2) and superoxide (O_2^-) produced by oxidative stress, and can interrupt lipid peroxidation (LPO) by scavenging the peroxidation intermediates (Asha Devi and Ravi, 2004). Studies indicate that vitamin E has a critical role in maintaining metabolic integrity of neurons and has been shown to reduce degeneration of hippocampal neurons following oxidative stress (Crouzin et al., 2010). α-Tocopherol along with vitamin C (ascorbic acid), a water-soluble vitamin is likely to exert a synergistic action since vitamin E is reduced to a tocopheroxyl radical and subsequently reduced back to α-tocopherol by ascorbic acid. The synergistic effect of the two vitamins is seen in tissues of rats that are subjected to oxidative stress situations such as cold exposure (Asha Devi et al., 2012) and diabetes with age (Naziroğlu et al., 2011). Animals experience neuronal injury in the HC as a consequence of exposure to intermittent cold stress since structural changes are noticed in the HC with regard to stress (Sapolsky, 2000). However, the effectiveness of a combined supplement of vitamin C and E on the hippocampal responses and its correlation with plasma lipid profile under cold exposures has not been evaluated in the old subjects.

In the present study we evaluated whether there is a correlation between the plasma lipid changes and hippocampal oxidative stress parameters in the old rats that were intermittently exposed to cold temperatures. Since exposure to cold is known to raise the metabolic rate the degree of response might vary at the two temperatures. Further, a difference of 5 °C could be more stressful with the progression of age due to weakened thermoregulatory capacity. In this study, TBARS, a LPO product and hydrogen peroxide (H_2O_2) levels indicative of cellular metabolism were checked for ICE-induced OS in the HC along with the probable neuronal loss under ICE. Further, the study examined whether a combined supplement of vitamin C and E would have a protective effect on oxidative stress in the hippocampus and plasma lipid profile as well.

2. Materials and methods

All fine chemicals and α-tocopherol were obtained from Sigma–Aldrich (St. Louis, MO). Ascorbic acid was procured from Spectrochem (Mumbai). All other chemicals were of analytical grade and solvents were of spectral grade.

2.1. Animal maintenance and experimental design

All animal procedures were approved by the Institutional Animal Ethics Committee (IAEC), Bangalore University, Bangalore.

Thirty young (1-month-old) male albino *Wistar* rats of equal body mass were procured from the Central Animal Facility, IISc, Bangalore, and maintained until they were 24 months of age. They were housed at 24 ± 2 °C, relative humidity of 70 ± 1%, and had free access to food (Amruth Feeds, India) and water. After acclimation for 4 weeks in our animal facility, they were assigned to six groups of five animals each: animals at housing room temperature (RT, 25 °C, CON), animals exposed to 10 °C (C10), animals exposed to 5 °C (C5), animals supplemented (CON+S) and exposed to 10 °C (C10+S) and 5 °C (C5+S). The supplement was a daily oral gavage of 400 mg vitamin C and 50 IU of vitamin E/kg body weight using a microsyringe. The purpose of including the CON+S sub-group in all of our studies was to determine whether combined supplementation has an antioxidant effect and to emphasize their influence, if any, under cold-induced oxidative injury. Food consumption and body weights were measured on a daily and weekly basis respectively.

2.2. Cold exposure schedule

During the light phase of the L:D cycle, rats of the ICE stress groups were transported in their home cages, with food, water and bedding into a temperature-controlled chamber and exposed to either 5 °C or 10 °C for 2 h before being returned to their housing facility. The procedure was repeated every day for 4 consecutive weeks. Rats in the control condition remained in the housing facility during this period. Temperatures and periods of cold exposure were selected as per the literature (Şahin and Gümüşlü, 2004) and based on our earlier studies (Manjula et al., 2013) on whole body exposure and in vitro studies on noxious cold exposures of sensory neurons from rat dorsal root ganglion (Naziroğlu and Özgül, 2012).

2.3. Tissue preparation

Immediately following the last exposure, animals were mildly etherized and the brain was quickly removed from the calvarium by opening the lateral sides of the skull. The brain was dissected on ice-cold surface and the hippocampus was isolated approximately between bregma −1.20 mm and −5.28 mm using the anterior commissure as the reference point (Glowenski and Iversen, 1966). A 5% homogenate was prepared in ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA using an ice-chilled Potter homogenizer with a teflon pestle. 100 μl of diluted homogenate was used for TBARS assay.

2.4. Biochemical estimations

2.4.1. Blood glucose

Glucose was measured by the method of Nelson (1944). In brief, blood was deproteinised by adding a mixture of barium hydroxide and zinc sulphate and was then filtered. The filtrate was treated with alkaline copper sulphate solution and boiled for 20 min. This was followed by the addition of arsenomolybdate and diluted to an appropriate volume and the color developed was measured at 540 nm in a spectrophotometer (ELICO, SL 159, India).

2.4.2. Plasma

2 ml of blood was drawn into EDTA-coated tubes and centrifuged at 600×*g* for 10 min at 4 °C. The separated plasma was used for measuring the lipid profile.

2.4.2.1. Measurement of plasma lipid profile. Total cholesterol (TCH), high density lipoprotein (HDL-C), triglycerides (TG), and low density lipoproteins (LDL-C) were measured using cogent kits from Span Diagnostics Ltd., India. LDL-C was calculated using Friedewald's method (1972).

2.4.3. Hippocampus

2.4.3.1. Vitamin E and C. Vitamin E in the HC was measured by the method of Desai (1984) and as described in detail earlier (Asha Devi et al., 2003b). In brief, HC was homogenised in isotonic KCl and the extraction of vitamin E from HC was performed on TLC plates by the modified method of Kayden and Traber (1993). Standard α-tocopherol was spotted and the plates were run using 2:1 benzene-ethyl acetate as solvent system. Reference plates were sprayed with 0.001% rhodamine 6G in purified methanol to identify α-tocopherol. The silica gel corresponding to the reference plates was eluted in purified ethanol and centrifuged at 600×*g* for 5 min and 0.2% bathophenanthroline reagent, 0.001% ferric chloride and orthophosphoric acid were added to the extract. The absorbency of samples and the standard were obtained at 536 nm. Tissue and plasma vitamin E were expressed in terms of μg/g tissue and μg/ml plasma.

Vitamin C was determined by the method of Omaye et al. (1979) by the oxidation of ascorbic acid to dehydroascorbic acid which reacts with 2,4,-dinitrophenyl hydrazine to form 2,4-dinitrophenyl hydrazone. Absorption was read at 520 nm and vitamin C was expressed as μg/g tissue and μg/ml plasma.

2.4.3.2. Thiobarbituric acid reactive substances (TBARS). The generation of TBARS of which malondialdehyde (MDA) is a product of LPO was measured spectrophotometrically (ELICO, SL 159, India) by the method of Ohkawa et al. (1979). TBARS was expressed in terms of nmol MDA/mg protein, using a standard curve prepared from measurements with a standard solution, 1,1,3,3-tetramethoxypropane.

2.4.3.3. Hydrogen peroxide assay. The procedure used was a modified version of Bruce and Winge (1984) as explained elsewhere (Jolitha et al., 2009). The rate of H_2O_2 released by the mitochondria was measured fluorimetrically (Genway, Model 6299, UK), following the method of Hyslop and Skar (1984) by the oxidation of p-hydroxyphenylacetate, coupled to the enzymatic reduction of H_2O_2 by horseradish peroxidase. H_2O_2 release rate was expressed in terms of nmol/mg protein/min.

2.4.3.4. Protein measurement. Protein concentration in the HC was estimated by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

2.5. Histological examination

Isolated hippocampus was fixed in a neutral 10% formalin solution. After hydration procedures, the tissue specimens were embedded in paraffin wax and 5 μm thickness sections were obtained and stained with hematoxylin and eosin. CA1, CA3 and dentate gyrus (DG) sites in the hippocampus were identified with the aid of stereotaxic atlas (Paxinos and Watson, 2004). Measurements were obtained from 3 images per rat. The number of pyknotic neurons in the sections was counted using light microscopy (Olympus IX, Japan) under a 40-fold magnification objective. Three sections were taken from each animal and the average number of pyknotic neurons in a fixed field was counted. The acidophilic neurons were identified by very intense cytoplasmic eosinophilia along with nuclear membrane disruption and were estimated on a 0–3 grading scale wherein 0 = none, <10% = 0.5 (slight), 26–45% = 1.0 (mild), 46–54% = 2 (moderate), 55–75% = 2.5 (moderate-to-severe) and >75% = 3 (severe), as previously reported by Fujikawa et al. (2000). Histological evaluations were performed in a blind manner.

2.6. Statistical analyses

The mean and standard error of the mean (SEM) values were calculated for the five animals in each subgroup. The data were subjected to two-way analysis of variance (ANOVA) and Bonferroni post hoc test to compare intergroup significance by using GraphPad prism software package. Pearson's correlation coefficient was used to determine the relationship between plasma lipid profile and the extent of changes in the variables in the hippocampus. Probability values $p < 0.05$ were considered significant.

3. Results

Food intake and body mass patterns of rats when exposed to ICE are shown in the Supplementary table (Table S1). Cold-exposed rats gained weight compared to controls. Increases in feeding efficiency were evident ($F = 303.8$, $p < 0.0001$) in the cold-exposed and supplemented rats.

3.1. Blood glucose

The Supplementary figure (Fig. S) shows the glucose levels in the cold stressed animals at 5 °C and 10 °C. Glucose increased significantly ($F = 5.12$, $p < 0.005$) in the cold-exposed, the extent of increase being higher at 5 °C than at 10 °C. However, supplementation resulted in the glucose levels returning to the control level.

3.2. Plasma lipid profile

The mean levels of plasma TCH, TG and LDL-C were significantly ($F = 5.147$, $p < 0.005$) increased in the cold-exposed compared to the controls and the extent of increase was higher at 5 °C than at 10 °C. Supplementation, however, resulted in decreases ($p < 0.005$) compared to unsupplemented levels. Unlike the above parameters, HDL-C reduced significantly ($p < 0.005$) in rats exposed at 10 °C and 5 °C as well as in relation to their controls. Supplementation increased HDL-C ($p < 0.005$) and the extent was higher at 5 °C than at 10 °C (Table 1).

The changes in the ratios of lipid profile are shown in Table 2. The magnitude of decrease in the LDL-C/HDL-C, TC/HDL-C and TG/HDL-C ratios in the supplemented cold-exposed samples was greater at 5 °C than at 10 °C ($F = 58.79$, $p < 0.0001$).

3.3. Hippocampus

3.3.1. Vitamins C and E

The mean values of tissue vitamin C and E in the cold exposed are shown in Table 3. Vitamins C ($F = 54.6$, $p < 0.0001$) and E ($F = 79.03$, $p < 0.0001$) decreased significantly at 10 °C with a further decrease at 5 °C with regard to their controls. Supplementation, however, increased the levels of the two vitamins.

The Supplementary table (Table S2) represents the plasma vitamin C and E levels in the cold stressed and control rats. Cold exposure resulted in significantly ($p < 0.0001$) decreased levels of plasma vitamins, and the decrease was directly proportional to the degree of severity of cold exposure. On the contrary, supplementation with vitamin E and C to the cold-exposed samples resulted in significantly increased levels of antioxidants.

Table 1
Influence of vitamin C and vitamin E supplementation on the lipid profile following cold exposure in old rats.

Plasma	Groups					
	CON	CON+S	C10	C10+S	C5	C5+S
TCH (mg/dl)	158.4 ± 0.9^{a}	153.5 ± 3.9^{a}	182 ± 4.0^{b}	155 ± 1.4^{a}	198.1 ± 4.6^{c}	154 ± 1.0^{a}
HDL-C (mg/dl)	27.9 ± 0.2^{a}	29.8 ± 0.7^{a}	19.6 ± 0.6^{b}	27.9 ± 0.7^{a}	18.3 ± 1.1^{b}	27.6 ± 0.4^{a}
TG (mg/dl)	106 ± 1.5^{a}	90.6 ± 0.9^{b}	125 ± 1.6^{c}	97.4 ± 3.2^{ab}	130 ± 2.0^{c}	107 ± 2.1^{ab}
LDL-C (mg/dl)	109.3 ± 0.9^{a}	107 ± 4.5^{a}	137 ± 4.2^{b}	108 ± 2.1^{a}	153 ± 5.8^{b}	104 ± 0.7^{a}

Each value represents mean ± SEM ($n = 5$). CON, control; C5, ICE at 5 °C; C10, ICE at 10 °C; S, supplement, vitamins C and E; TCH, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high density-lipoprotein cholesterol. Significance between group means was analyzed by two-way ANOVA followed by Bonferroni's multiple comparison's test. Values between groups are represented as superscripts in lower case and considered significant at $p < 0.05$. Means with a common superscript are not significantly different ($p > 0.05$).

Table 2
Influence of vitamin C and vitamin E supplementation on the mutual ratios of plasma lipid parameters following cold exposure in old rats.

Ratio	Groups					
	CON	CON+S	C10	C10+S	C5	C5+S
TCH/HDL-C	5.67 ± 0.05[a]	5.16 ± 0.10[a]	9.32 ± 0.16[c]	5.56 ± 0.15[a]	10.9 ± 0.87[b]	5.57 ± 0.03[a]
TG/HDL-C	3.79 ± 0.05[a]	3.04 ± 0.02[b]	6.42 ± 0.11[d]	3.48 ± 0.02[a]	7.16 ± 0.34[c]	3.90 ± 0.07[a]
LDL-C/HDL-C	3.91 ± 0.04[a]	3.55 ± 0.10[a]	7.04 ± 0.14[c]	3.86 ± 0.15[a]	8.52 ± 0.81[b]	3.79 ± 0.25[a]
LDL-C/TG	1.08 ± 0.02[a]	1.16 ± 0.02[a]	1.30 ± 0.02[b]	1.10 ± 0.05[a]	1.39 ± 0.01[b]	0.97 ± 0.00[a]

Each value represents mean ± SEM ($n = 5$). CON, control; C5, ICE at 5 °C; C10, ICE at 10 °C; S, supplement, vitamins C and E. Significance between group means was analyzed by two-way ANOVA followed by Bonferroni's multiple comparison's test. Values between groups are represented as superscripts in lower case and considered significant at $p < 0.05$. Means with a common superscript are not significantly different ($p > 0.05$).

Table 3
Influence of vitamin C and vitamin E supplementation on the concentration of vitamins in the hippocampus following cold exposure.

Vitamin	Groups					
	CON	CON+S	C10	C10+S	C5	C5+S
Vit. E (μg/g)	18.96 ± 1.2[a]	38.42 ± 2.4[b]	16.55 ± 1.5[a]	30.85 ± 1.9[b]	13.80 ± 1.0[a]	31.77 ± 2.7[b]
Vit. C (μg/g)	234.0 ± 10.0[a]	451.6 ± 18.1[b]	181.1 ± 12[ac]	299.0 ± 7.4[ad]	142.6 ± 13.0[c]	274.8 ± 12[ad]

Each value represents mean ± SEM ($n = 5$). CON, control; C5, cold exposure at 5 °C; C10, cold exposure at 10 °C; S, supplement, vitamin C and E. Significance between group means was tested by two-way ANOVA followed by Bonferroni's multiple comparison test. Values between groups are represented as superscripts in lower case and considered significant at $p < 0.05$. Means with a common superscript are not significantly different ($p > 0.05$).

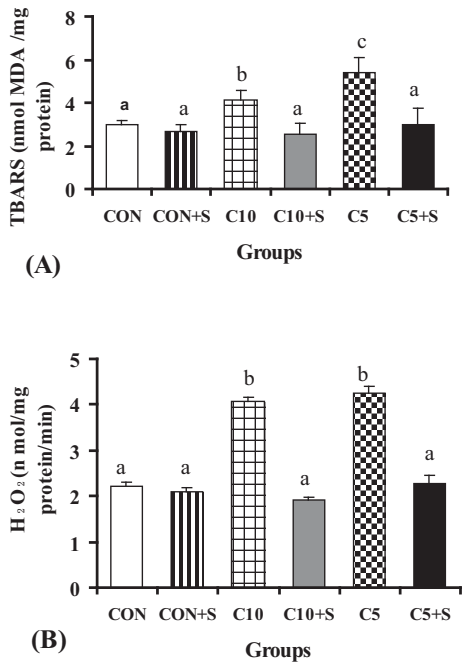

Fig. 1. Effects of intermittent cold exposure on the TBARS (A) and H_2O_2 (B) levels in the hippocampus of old rats. Each value represents mean ± SEM ($n = 5$). CON, control; C5, ICE at 5 °C; C10, ICE at 10 °C; S, supplement, vitamins C and E. Significance between group means was analyzed by two-way ANOVA followed by Bonferroni's multiple comparison's test. Means with a different letter are different from the others ($p < 0.05$) and with a common letter are not significantly different ($p > 0.05$).

3.3.2. Lipid peroxidation level (TBARS)

The levels of TBARS are shown in Fig. 1A. There was significant increase ($F = 58.79$, $p < 0.0001$) compared to the controls in the levels of TBARS in the hippocampus of cold-exposed rats while the supplementation reduced these levels. However, the magnitude of these changes was more at 5 °C than at 10 °C.

3.3.3. Hydrogen peroxide level (H_2O_2)

The changes in the H_2O_2 generation under cold exposures are shown in Fig. 1B. H_2O_2 levels were higher in the cold-exposed than in controls. H_2O_2 was significantly elevated above the control level ($F = 102.7$, $p < 0.0001$). Supplementation induced a modulatory effect by significantly reducing the H_2O_2 values compared to the unsupplemented.

Table 4
Pearson correlation coefficient assessed between parameters measured in all experimental groups in hippocampus of aging rats.

	H_2O_2	TBARS	TC	HDL-C	TG	LDL-C
G	0.23	0.32	0.24	−0.30	0.34	0.34
H_2O_2		0.95**	0.97**	−0.98**	0.94**	0.97**
TBARS			0.99**	−0.95**	0.93**	0.98**
TC				−0.97**	0.92**	0.99**
HDL-C					−0.96**	−0.97**
TG						0.91*

Data are presented as 'r' values. ($n = 5$).
* Correlation is significant at 0.05 level (2-tailed).
** Correlation is significant at 0.01 level (2-tailed).G, group; H_2O_2, hydrogen peroxide; TBARS, thiobarbituric acid reactive substances; TCH, total cholesterol; TG, triglycerides; LDL-C low-density lipoprotein cholesterol; HDL-C, high density-lipoprotein cholesterol.

3.3.3.1. Correlation between measured plasmatic lipid parameters.

TCH showed a moderate negative correlation with HDL (−0.57, $p < 0.01$) and a positive correlation with TG (+0.82, $p < 0.01$) and LDL (+0.97, $p < 0.01$). TG showed a positive correlation with LDL (+0.82, $p < 0.01$) (Table 4).

3.3.3.2. Plasmatic lipid profile versus lipid peroxidation in the hippocampus.

A positive correlation was observed with TBARS in the HC and plasma TCH ($r = +0.88$; $p < 0.01$), LDL-C (+0.83, $p < 0.01$), and TG (+0.84; $p < 0.01$) with a moderate negative correlation with HDL-C (−0.52, $p < 0.01$) (Table 4).

3.3.3.3. Histology.

Examples of H & -E staining in the hippocampus of controls and cold-exposed rats are shown in Fig. 2. Compared to controls, cold exposure at 10 °C increased the number of damaged neurons (Fig. 2B) and was more in the dentate gyrus compared to the CA1 and CA3 regions (Table 5). Supplementation was not effective in reducing the damaged neurons in the cold-exposed rats. Compared to 10 °C, rats at 5 °C showed more of damaged neurons. Supplementation, however, significantly reduced ($p < 0.05$) the

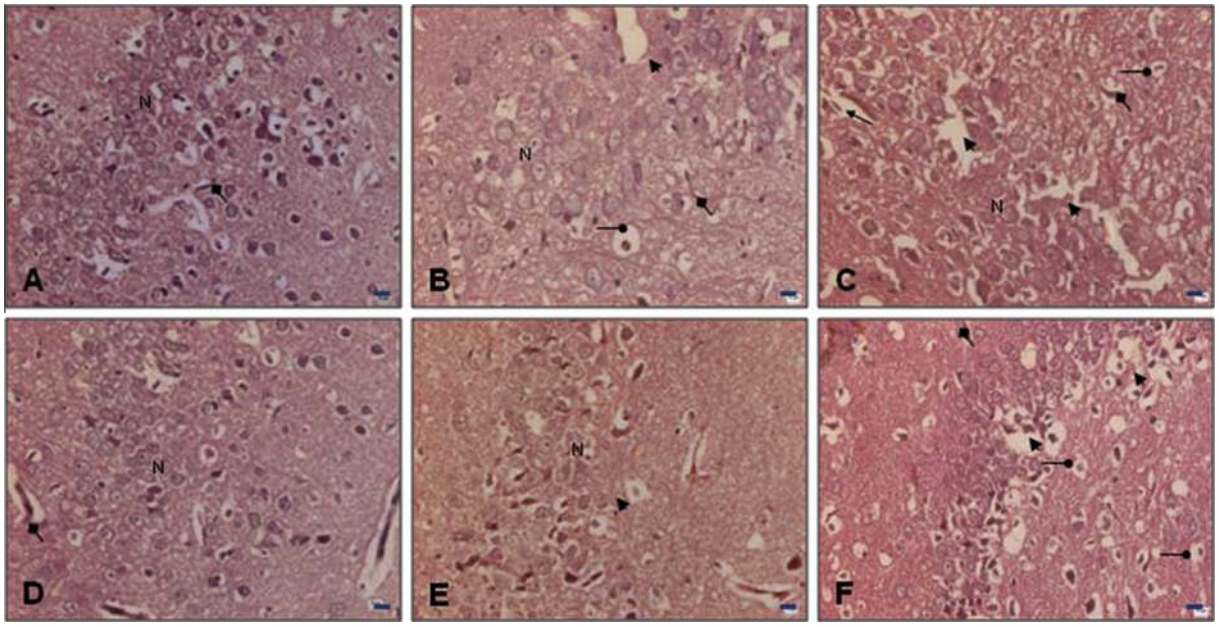

Fig. 2. Photomicrographs of hematoxylin and eosin staining (20X) of the hippocampus of old rat. (A) control; (B) ICE at 10 °C; (C) ICE at 5 °C; (D) supplemented controls; (E) supplemented and cold-exposed at 10 °C; (F) supplemented and cold-exposed at 5 °C. Supplement, vitamins C and E. N indicates neurons; Diamond-head arrow indicates blood vessels; arrow indicates perivascular space; arrow head indicates interstitial spaces; oval-head arrows show dark neurons. Scale = 10 μm.

Table 5
Morphological examination for damaged neurons in the hippocampus of experimental groups of old rats.

Group	DG		CA1		CA3	
	%	G	%	G	%	G
CON	40.7 ± 0.9^{a}	1.5	36.2 ± 2.7^{a}	1.5	29.2 ± 1.6^{a}	1.5
CON+S	32.1 ± 0.7^{b}	1.5	29.7 ± 1.6^{b}	1.5	22.3 ± 1.6^{b}	1.0
C10	53.5 ± 1.5^{c}	2.0	47.2 ± 1.5^{c}	2.0	40.5 ± 0.7^{c}	1.5
C10+S	52.4 ± 6.4^{c}	2.0	45.9 ± 5.2^{c}	2.0	36.2 ± 1.5^{c}	1.5
C5	63.2 ± 2.7^{d}	2.5	58.3 ± 6.4^{d}	2.5	49.5 ± 2.7^{d}	2.0
C5+S	55.5 ± 7.6^{c}	2.5	48.5 ± 5.2^{c}	2.0	37.7 ± 0.9^{c}	1.5

Each value represents mean ± SEM ($n = 5$). CON, control; C5, cold exposure at 5 °C; C10, cold exposure at 10 °C; S, supplement, vitamin C and E; G, gradation of injury; 1.0 = mild (10–25%); 1.5 = mild-to-moderate (26–45%); 2.0 = moderate (46–54%); DG, dentate gyrus, CA1, cornus ammonis 1; CA3, cornus ammonis 3. Significance between groups means was tested by two-way ANOVA followed by Bonferroni's multiple comparison test. Values between groups are represented as superscripts in lower case and considered significant at $p < 0.05$. Means with a common superscript are not significantly different ($p > 0.05$).

number with respect to the unsupplemented rats. The damaged neurons were assessed by increased numbers of acidophilic neurons as identified by very intense cytoplasmic eosinophilia (Fig. 2C) while those on combined supplement showed fewer number of such neurons (Fig. 2E and F).

4. Discussion

Our data on feeding behaviour show that increased food consumption in cold stressed animals is not surprising since enhanced food intake is linked to the energy expenditure necessary for heat production. The principal findings of the present study are threefold.

The first relates ICE induced hyperglycaemic condition in the old rats (Fig. S). The hyperglycemic effect of cold stress is similar to that reported by Larkin et al. (1992) in 26-mo-old male rats that were cold-stressed at 5 °C for 4 h. The hyperglycaemic effect may be related to the diminished ability to oxidise carbohydrates in the cold and the mechanisms can be attributed to the increased plasma glucose levels in cold-stressed rats due to increased plasma glucagon leading to elevated hepatic gluconeogenesis and ketogenesis (Seitz et al., 1981) and this may not be the case in the old rats since the uptake and metabolism of glucose is reduced with age.

Further, the observed hyperglycaemia in the cold stressed rats was accompanied by elevated LDL-C levels suggesting that increased glucose may lead to LDL-C oxidation since glycated LDL is a preferred compound for oxidative modifications (Sobal et al., 2000). The present results on increased LDL-C/TG and LDL-C/HDL-C ratios in animals exposed to cold are indicative of LDL oxidation *in vivo* with the former ratio being suggested as an important predictor of LDL oxidation (Brizzi et al., 2005). Although we have not measured the TCH in the hippocampus, studies by Cutler et al. (2004) have documented increased TCH in old mice at 25 months of age, and have related the increase to similar situations of oxidative stress involving activation of sphingomyelinases resulting in ceramide production and accumulation of TCH, and have shown that α-tocopherol is effective in inhibiting the enzymes. A decrease in HDL in response to cold exposure can be explained by the fact that in rats HDL is the main fraction transporting cholesterol. Additionally, HDL cholesterol regulates the exchange of proteins and lipid between lipoproteins, and thereby inhibits the oxidation of LDL due to its antioxidative property (Das, 2003).

Second, the reduced temperatures showed an increased LPO with a concomitant increase in the H_2O_2 level in the HC; these changes were dependent on the intensity of the cold. The magnitude of the changes in the TBARS level was proportional to the intensity of the cold. Cold stress possibly increases the production of ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\cdot}$) and superoxide anion radicals ($O_2^{-\cdot}$) which in turn causes peroxidation of lipids. The results on increased LPO under cold stress in the rats are consistent with other reports on peripheral tissues (Şahin and Gümüşlü, 2004; Venditti et al., 2004). The increased neuronal damage seen in our study in rats subjected to cold-stress is because of excess H_2O_2 generation in the hippocampus. This is substantiated by earlier studies (Aihara et al., 2001) who have shown that

several ion channels with high thermal sensitivity result in molecular complexity, suggesting that their differential expression in particular neurons in the hippocampus may influence their response to low temperatures and by Naziroğlu and Özgül (2011) who used H_2O_2 in an experimental model of oxidative stress.

Third, the results of this study also revealed that supplementation with vitamins C and E under ICE not only lowered the LDL-C/HDL-C and TCH/HDL-C ratios but also lowered H_2O_2 generation in the HC. Vitamin C and E are antioxidants that prevent LDL oxidation by retarding the generation of ROS (Shariat et al., 2013; Sakuma et al., 2001). The significantly reduced ascorbic acid levels in the HC under ICE, in spite of the rat hepatocyte's ability to synthesise ascorbic acid *de novo* from glucose explains the increased requirement of the vitamin to overcome excess OS. Low concentration of ascorbic acid in rats subjected to ICE explains a plausible interaction of vitamin C with membrane-bound vitamin E reducing the tocopherol radical back to tocopherol. Vitamin E reduction under ICE is known to promote LPO making the cells more susceptible to oxidative injury (Fukui et al., 2001).

The results showing an inverse relation between TBARS and, depletions in vitamin C and E levels in the HC of the cold-stressed rats may explain the increased predisposition for generation of TBARS under stress. It appears that the increased level of H_2O_2 can increase the generation of lipid peroxides and cause greater damage to the neurons. Substantial protection is reported by Gracy (2007) in the hippocampal neurons when treated with α-tocopherol prior to and during H_2O_2 exposure to the oxidative stress. In the present study, the results on reduced levels of vitamin E in the HC of the cold-exposed rats are in accordance with the reports on increased TBARS levels in the brains of cold-stressed animals with a reversal of the depleted levels of vitamin E in treated animals (Celikbilekal et al., 2014). Apart from vitamin E, vitamin C has the ability to protect against LPO by scavenging the ROS, and by a one-electron reduction of lipid hydroperoxyl radicals via the vitamin E redox cycle. We have previously reported that vitamin C with vitamin E significantly lowers OS in the hypothalamus of cold-exposed male rats belonging to different age groups (Manjula et al., 2013).

Our findings on higher LPO at 5 °C than at 10 °C indicate that the acute cold stress that the animal undergoes at lower temperature suggests an increased oxygen consumption, oxidative metabolism, elevated ROS production and heat production. The two temperatures may also suggest step-wise alterations in energy generation and subsequent alterations in the concentration of ROS as the response of organisms to low temperatures depend on the duration of exposure and the intensity of coldness (Blagojević, 2007).

Finally, in the HC, LPO correlated positively with an increase in plasmatic TCH, TG and LDL-C and negatively with HDL-C. Combined treatment with vitamins C and E in the present study attenuated hyperglycemia and lowered oxidative stress in the hippocampus of old rats.

Our findings on ICE-induced hypercholesterolemia and altered lipid profile may be corroborated by the high incidence of metabolic disorders such as hypertension and atherosclerosis in cold-exposed workers. Since exposure of the whole body to ICE is a metabolic stressor and under natural situations ICE is encountered sporadically more often than continuous, old workers who are intermittently exposed to cold areas may be protected from the metabolic and cognitive loss due to OS via supplementation with vitamins C and E.

Acknowledgements

This work was supported by the University Grants Commission, New Delhi (Referral No.F.No. 33-356/2007) to Dr. S. Asha Devi. The financial assistance to Dr. S. Asha Devi under the Promotion of University Research and Scientific Excellence (PURSE)-Department of Science and Technology (DST), New Delhi (No. SR/S9/Z023/2010/38) is also gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuint.2014.05.001.

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