

Oxidative Macromolecular Alterations in the Rat Central Nervous System in Response to Experimentally Co-Induced Chlorpyrifos and Cold Stress: A Comparative Assessment in Aging Rats

P. Mahaboob Basha · Annappa Poojary

Received: 10 July 2011 / Revised: 10 September 2011 / Accepted: 22 September 2011 / Published online: 13 October 2011
© Springer Science+Business Media, LLC 2011

Abstract Reactive oxygen species are generated as a result of a number of physiological and pathological processes which can promote multiple forms of oxidative damage including protein oxidation, and thereby influence the function of a diverse array of cellular processes. In our previous study we have reported that co-exposure to chlorpyrifos and cold stress in aging rats markedly influence the toxic outcome as a result of oxidative stress. In the present study, key neurochemical/enzymes were measured in order to evaluate the macromolecular alterations in response to experimentally co-induced chlorpyrifos and cold stress (15 and 20°C) either concurrently or individually in vivo for 48 h in discrete regions of brain and spinal cord of different age group rats. CPF and cold stress exposure either individually or in combination substantially increased the activity/levels of protein carbonyls, AST, ALT and decreased protein thiols, DNA, RNA and total proteins in discrete regions of CNS. Overall, the effects of co-exposure were appreciably different from either of the exposures. However, synergistic-action of CPF and cold stress at 15°C showed higher dyshomeostasis in comparison with CPF and cold stress alone and together at 20°C indicating the extent of oxidative macromolecular

damage in discrete regions of brain and spinal cord. Furthermore, the present study demonstrates that macromolecular oxidative damage is highly pronounced in neonates and juveniles than the young adults.

Keywords Interactive toxicity · Chlorpyrifos · Cold stress · Macromolecular changes

Introduction

A foreign body in the form of a chemical stress like insecticide or physical stress like cold or their interaction is sufficient enough to give a jolt either mild or severe in functioning potential of central nervous system (CNS). The CNS is especially sensitive to free radical oxidative damage as it contain post mitotic cells, high levels of iron, easily oxidizable fatty acids, low antioxidant defence system and use large amount of oxygen which renders the tissue susceptible to oxygen radicals [1]. Moreover, the heterogeneity of developing nervous system, with different cell types and function makes it vulnerable to environmental contaminants than the adult nervous system [2].

Indiscriminate use of pesticide for crop protection and for the control of vector borne disease(s) caused widespread harmful effects in human and other non-target biosystem. Chlorpyrifos (O,O¹-diethyl O-3,5,6-trichloro-2-pyridinyl phosphorothioate, CPF) is a synthetic chlorinated OP insecticide utilized extensively in agriculture and for residential pest control throughout the world under the registered trademarks {LORSBAN Insecticide and DURSBAN Insecticide}. Despite the restrictions imposed on its use, CPF continues to be one of the most commonly used and extensively studied OP insecticides. Although, the mechanism of CPF toxicity involves acetylcholinesterase (AChE)

Electronic supplementary material The online version of this article (doi:10.1007/s11064-011-0617-9) contains supplementary material, which is available to authorized users.

P. M. Basha (✉)
Department of Zoology, Bangalore University,
Bangalore 560 056, India
e-mail: pmbashabub@rediffmail.com

A. Poojary
Department of Zoology, Government College for Boys,
Kolar 563 101, India
e-mail: annu1967@gmail.com

inhibition, other mechanisms unrelated to AChE inhibition, including the induction of oxidative stress, have been implicated [3]. As a lipophilic molecule, CPF easily passes through the cells into the cytoplasm [4] and induce damage to the cellular molecules [5]. Oxidative modifications of proteins in vivo may affect a variety of cellular functions involving receptors, signal transduction mechanisms, transport systems and enzymes [6]. Reactive oxygen species (ROS = free radicals) can directly affect the conformation and/or activities of all sulfhydryl containing molecules, by oxidation of their thiol moiety [7]. The combined effect of these ROS-triggered cellular changes may eventually lead to cellular dysfunction and ultimate destruction.

Cold stress, a physical environmental stressor, cause detrimental effect(s) on organism by altering cellular homeostasis and plays a considerable role either in accelerating or modifying the toxic mechanisms. Exposure to extreme cold stress can lead to wind-chill, frostbite, and hypothermia [8] and many other harmful effects [9]. Cold stress can disrupt the balance in an oxidant/antioxidant system and cause oxidative damage to several tissues by altering antioxidant status, protein oxidation and lipid peroxidation [10]. Earlier studies of Maguire and Williams [11] reported that a cold stress enhances brain acetylcholinesterase (AChE) inhibition induced by chlorpyrifos in Northern bobwhites.

The effects of single stressor, either cold stress or CPF toxicity are well known. In this study attention has been drawn not only to assess the effects of CPF but also to its interaction with cold stress. Since co-exposure can result in antagonistic or synergistic effects, studies on these interactions will be of immense use to understand the realistic situation in the field especially on non-target organisms. Studies made in our laboratory have reported that increased lipid peroxidation and decreased antioxidant status causing architectural damage to the membranes of nervous system of rat in an age-related manner during co-exposure with CPF and cold stress [12]. Cold stress is an important contributor to enhance the neurotoxicity of CPF.

CPF is among the OP pesticides known to have adverse impact on the developing brain of children as well as laboratory animals [13]. Several other studies using different routes of CPF administration have reported greater sensitivity in younger rats [14, 15] and the mechanism(s) for the greater susceptibility in young rats is not well defined. Laboratory evidences, obtained largely through the use of rodents, suggest that acute or chronic exposure to CPF and/or its metabolic product(s) may overtly injure the central nervous system (CNS) or produce marked changes in neuronal function that persist even after exposure has ceased, particularly during the early postnatal period [16].

A major area of biomedical and societal concern regarding age-related sensitivity to the effects of pesticides

led to the passage of the Food quality Protection Act in 1996, which mandated greater assurance of protection for the young. Understanding potential age-related sensitivity is especially important in light of data suggesting greater exposure of children to pesticides [17]. While the actions of OP pesticides have been widely studied for decades, almost all such studies have used only adult laboratory animals and there are few data concerning relative sensitivity in the young [18, 19]. The limited data available suggest some differential effects on developing as well as maturing animals on exposure to CPF [18]. In spite of the extensive use of CPF in crop protection and in the household, information related to its interaction with cold stress, and resulted effects on health with particular reference to neurotoxicity are scarcely available. Thus, the mechanism of CPF and cold stress interaction induced CNS dysfunction remains to be elucidated. Keeping in view the lacuna in the literature and the role of cold stress in aggravating quantum of toxicity, the present study was therefore initiated to determine an array of responses of interactive effects of CPF and cold stress in an age-related manner in discrete regions of rat CNS.

Materials and Methods

Chemicals

A commercial grade OP compound having 20% w/v of CPF in organic solvent dimethyl sulphoxide (DMSO) marketed as Darsban was procured from Lupin Agrochemicals Pvt. Ltd., Bharuch, Gujarat (India) was used in this study. The commercial formulation was selected because it would reflect the realistic situation in the field and this product is extensively used for agricultural practices in India. The other chemicals were purchased from BDH and Sigma–Aldrich.

Animals

Male albino rats, Wistar strain (*Rattus norvegicus albinus*) of different age groups viz., neonatal (7-day old, 10–12 g wt), juvenile (21-day old, 35–40 g wt) and adult (90-day old, 160–180 g wt) were used throughout the experiment. Rats were procured from Sri Raghavendra enterprises, Bangalore and acclimatized to laboratory conditions (12 h dark/light cycle at $28 \pm 1^\circ\text{C}$) for 1 week prior to commencement of the experiment. They were maintained on standard rodent pellet diet and tap water ad libitum; in accordance with the guidelines of National Institute of Nutrition, ICMR, Hyderabad, and experimental protocol was approved by the Institutional animal ethical committee, Bangalore University, Bangalore.

Experimental Protocol

Rats were divided into six groups: Group I—control animal group was kept at the laboratory room temperature ($28 \pm 1^\circ\text{C}$); Group II—exposed to CPF at the laboratory temperature, $28 \pm 1^\circ\text{C}$; Group III—exposed to cold stress at 15°C ; Group IV—exposed to cold stress at 20°C ; Group V—exposed to CPF plus cold stress at 15°C ; Group VI—exposed to CPF plus cold stress at 20°C . The number of animals in each group was six. The neonatal and juvenile animals were kept along with the dam and other littermates in order to avoid additional stress. We chose to administer CPF via subcutaneous injection in dimethyl sulfoxide (DMSO), a vehicle appropriate for water-insoluble agents and already known not to affect the corresponding measures of brain development [20]. The group II, V and VI animals were treated with sublethal doses ($1/3$ of LD_{50}) of CPF subcutaneously in a volume of 1 ml/kg body weight dissolved in DMSO. The group I, III and IV animals were injected with plain DMSO (vehicle controls). The 48 h LD_{50} of CPF (EC 20%) in rats by subcutaneous route was assessed by probit analysis method [21] and observed values were found to be 15, 242 and 510 mg per kg body weight for 7-day old, 21-day old and 90-day old age groups, respectively.

To induce cold stress, rats were housed in an acute cold stress apparatus (Colton BOD incubator) for 48 h on a 12-h light/12-h dark cycle with a built-in heater and cooler that could be controlled by self-timer. Rats were sacrificed by cervical dislocation 48 h after respective treatments and discrete regions like cerebral cortex (CC), cerebellum (CB), medulla oblongata (MO) and spinal cord (SC) were quickly separated and washed in ice cold 0.9% saline. The tissue homogenates were made by using appropriate buffer and supernatant was stored at a temperature of -20°C and used for biochemical assays.

Biochemical Methods

The biochemical estimations were performed spectrophotometrically using Jenway-6405(UV/VIS) Spectrophotometer by the following methods viz., Deoxy ribose nucleic acid (DNA) by Schneider [22], Ribose nucleic acid (RNA) by Munro [23], Proteins by Lowry et al. [24] Protein oxidation (Protein carbonyls) by Levine et al. [25], Protein thiols by Reznick and Packer [26], and Cellular damages were evaluated by measuring Alanine-aminotransferase (ALT) and Aspartate-aminotransferase (AST) activity levels by Reitman and Frankel [27].

Statistical Analysis

The results are expressed as mean \pm standard deviation (SD) of six observations ($n = 6$) in each group.

Differences between treatment groups of same age group were assessed by one-way analysis of variance (ANOVA) using the SPSS software package for windows version 15.0. *Post hoc* testing was performed for inter-group comparisons using Bonferroni test at probability (P) value <0.05 level of significance. To analyze the interactive effects, three-way ANOVA was carried out and tested by Duncan's test for multiple comparisons to define the nature of the effect.

Results

The results of the present study are depicted in Figs. 1, 2, 3, 4 and Tables 1, 2.

Neuro-Somatic Index

Individual and co-exposures of CPF and cold stress resulted decline in body weights followed by brain and spinal cord weights indicating reduction in the neuro-somatic index in all age groups of rats studied (Table 1). Similarly CPF and cold stress exposure individually and concurrently resulted in a lower RNA/DNA ratio in all age groups of rat CNS regions while protein/DNA ratio found to be more in juveniles followed by neonates than young adults (Table 2), further results indicate that DNA concentration per gm of tissue decreased with age, from neonatal to adult.

Cerebral Cortex

Figure 1 presents data on individual and interactive effect of CPF and cold stress in rat cerebral cortex of different age group rats. The levels of DNA, RNA, total protein and protein thiols were significantly decreased, and protein carbonyl content and activity level of AST and ALT were markedly elevated. Moreover, interaction of CPF and cold exposure at 15°C exhibited pronounced change when compared to CPF and cold stress alone and together at 20°C . Comparatively, neonatal and juvenile animals showed higher sensitivity ($P < 0.05$) than adult animals ($P < 0.05$).

Cerebellum

In comparison with control values, the levels of DNA, RNA, total protein and protein thiols were statistically decreased, while the levels of protein carbonyl, AST and ALT were dramatically increased in different age group rats exposed to CPF or cold stress. However, interactive effects were more pronounced in neonatal and juvenile age groups exposed at 15°C over the adult. Comparatively, this

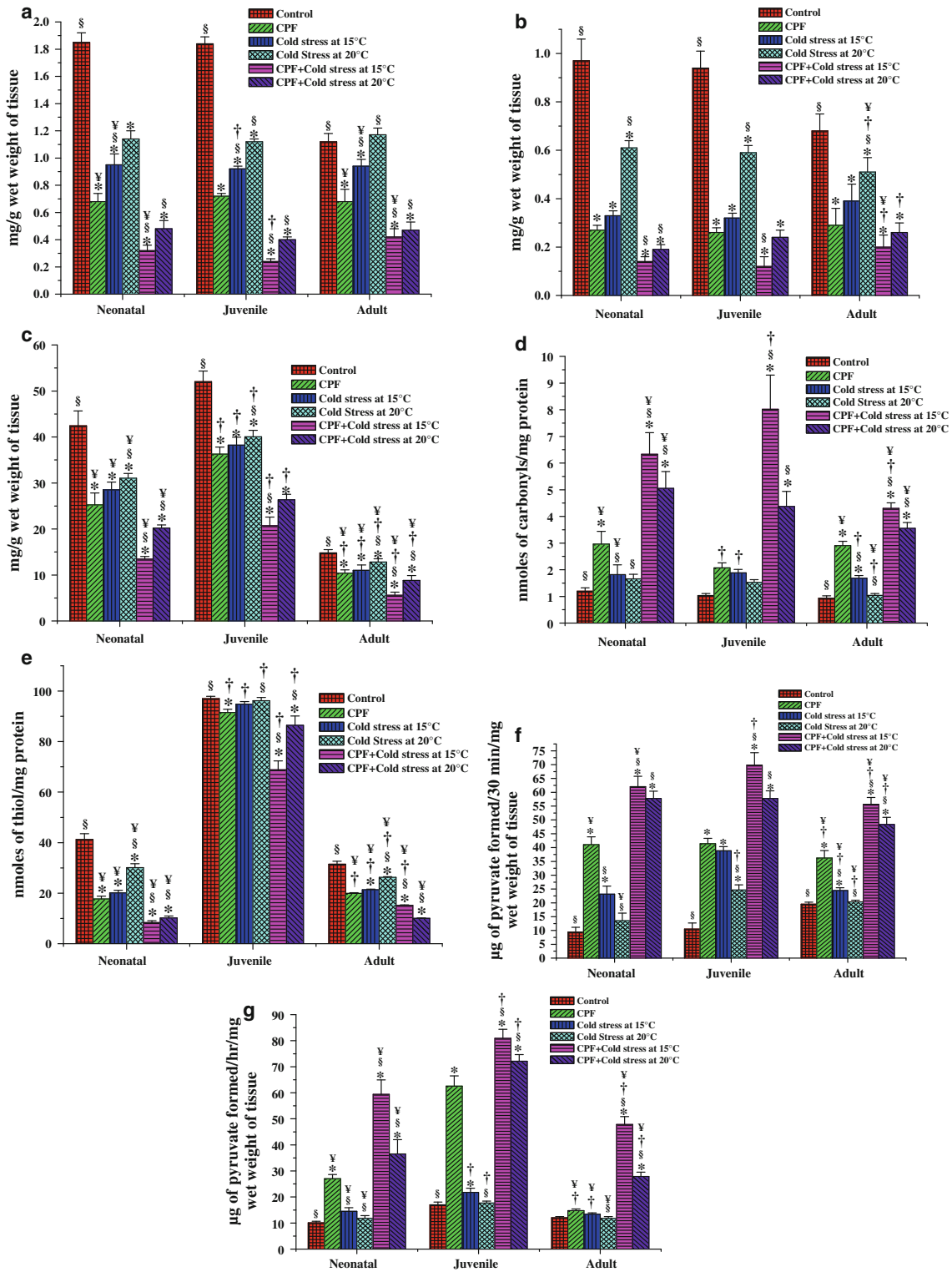


Fig. 1 Effects of CPF, cold stress and their co-exposure on protein oxidation parameters in the cerebral cortex of different age group rats: **a** DNA content; **b** RNA content; **c** Total protein; **d** Protein carbonyl content; **e** Total protein thiol; **f** ALT activity; **g** AST activity. Results are presented as mean \pm SD, $n = 6$. Significantly different from

control: * $P < 0.05$; significantly different from CPF: & $P < 0.05$; significantly different from neonatal in corresponding treatment groups: † $P < 0.05$; significantly different from juvenile in corresponding treatment groups: ‡ $P < 0.05$; significantly different from adult in corresponding treatment groups: § $P < 0.05$

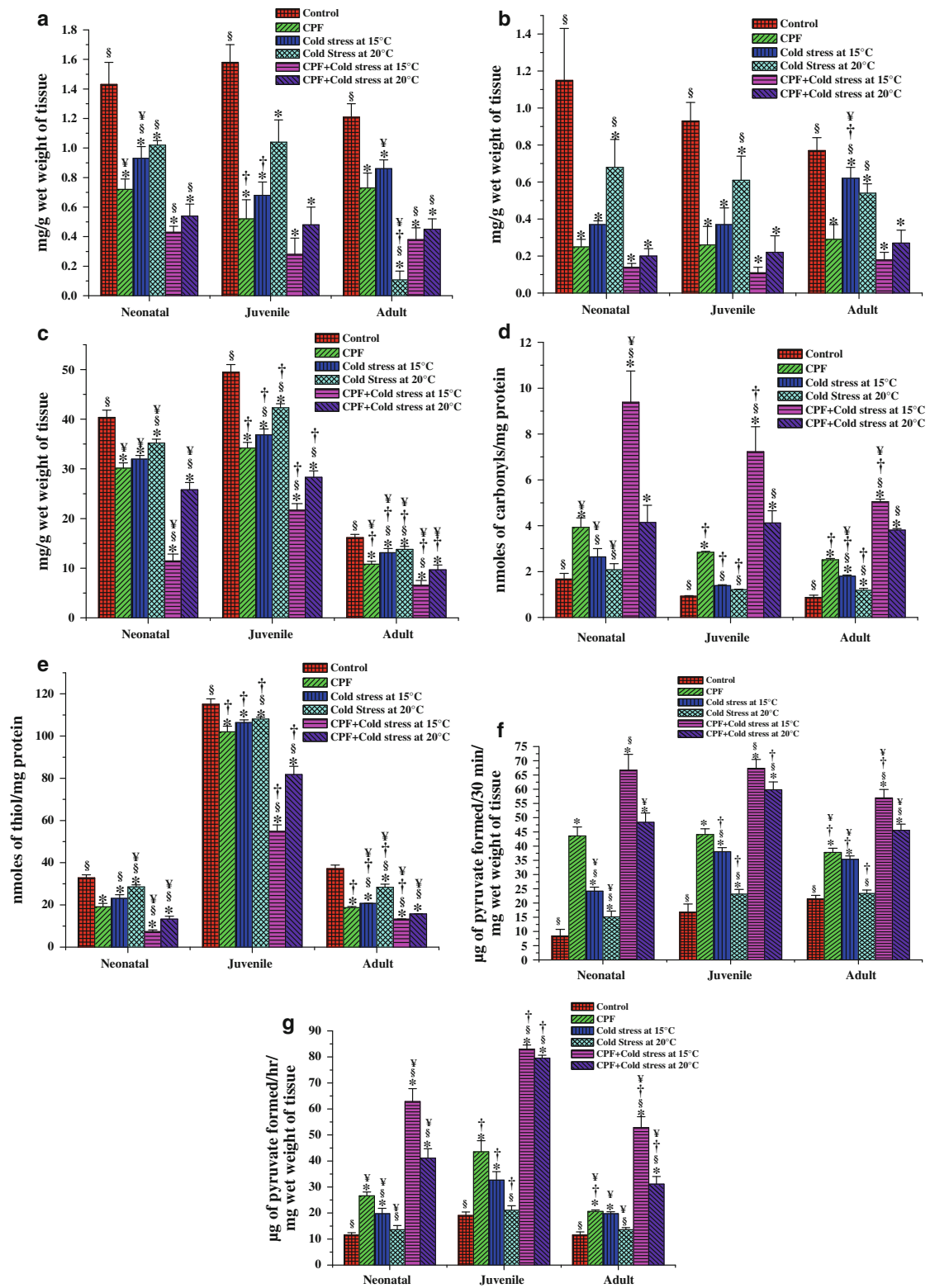


Fig. 2 Effects of CPF, cold stress and their co-exposure on protein oxidation parameters in the cerebellum of different age group rats: **a** DNA content; **b** RNA content; **c** Total protein; **d** Protein carbonyl content; **e** Total protein thiol; **f** ALT activity; **g** AST activity. Results are presented as mean ± SD, n = 6. Significantly different from

control: **P* < 0.05; significantly different from CPF: &*P* < 0.05; significantly different from neonatal in corresponding treatment groups: †*P* < 0.05; significantly different from juvenile in corresponding treatment groups: ‡*P* < 0.05

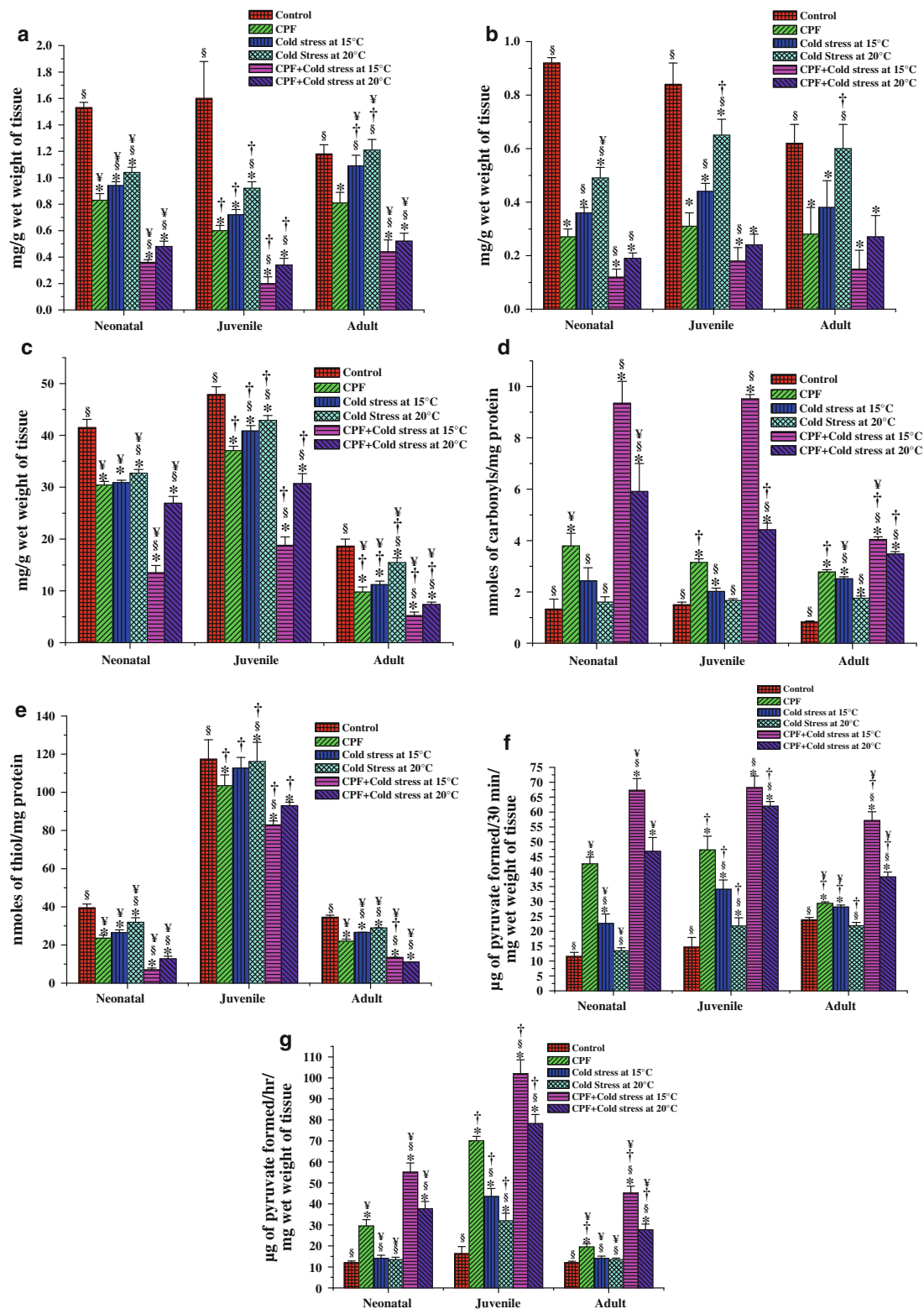


Fig. 3 Effects of CPF, cold stress and their co-exposure on protein oxidation parameters in the medulla oblongata of different age group rats: **a** DNA content; **b** RNA content; **c** Total protein; **d** Protein carbonyl content; **e** Total protein thiol; **f** ALT activity; **g** AST activity. Results are presented as mean \pm SD, $n = 6$. Significantly different

from control: * $P < 0.05$; significantly different from CPF: & $P < 0.05$; significantly different from neonatal in corresponding treatment groups: † $P < 0.05$; significantly different from juvenile in corresponding treatment groups: ‡ $P < 0.05$

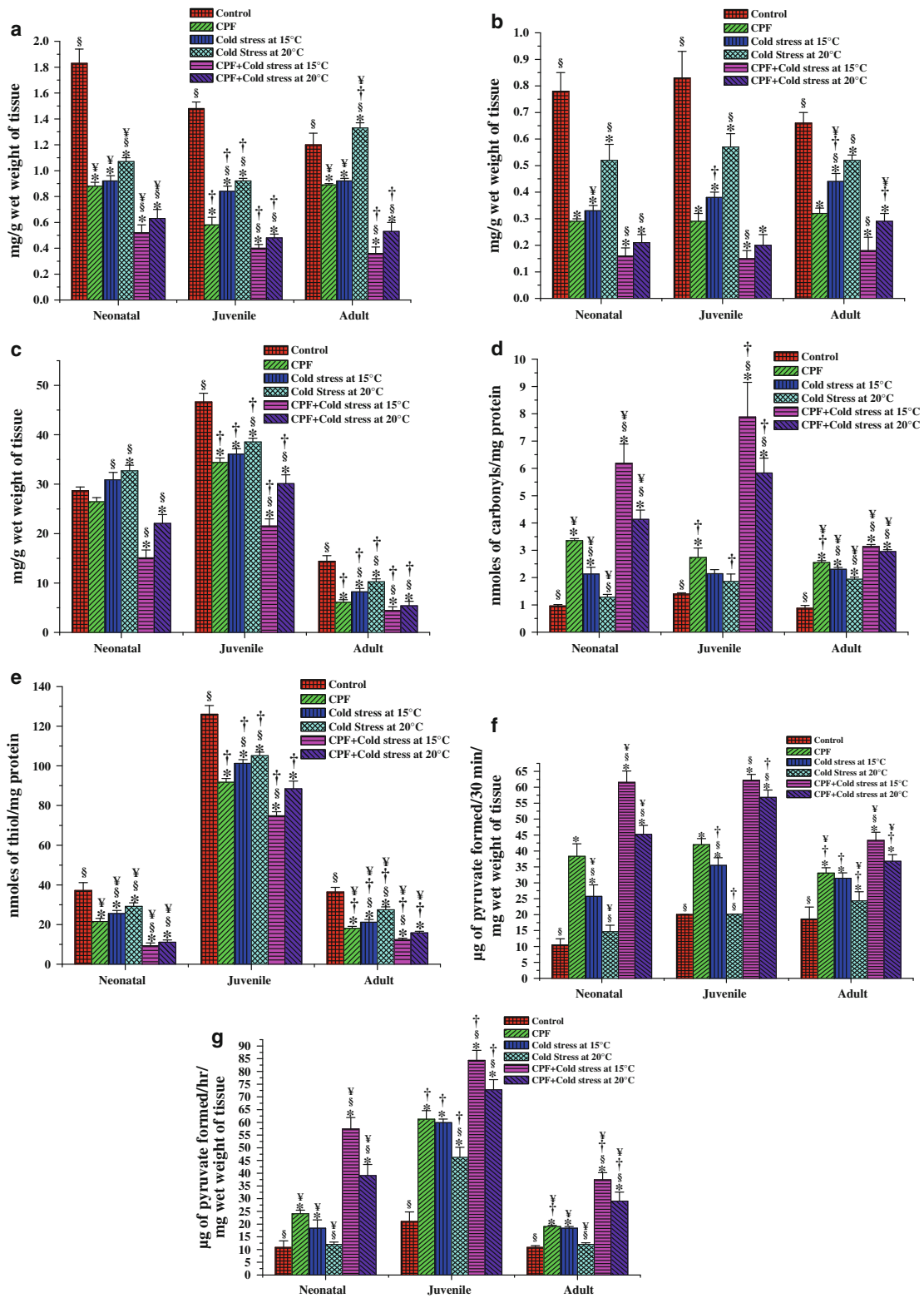


Fig. 4 Effects of CPF, cold stress and their co-exposure on protein oxidation parameters in the spinal cord of different age group rats: **a** DNA content; **b** RNA content; **c** Total protein; **d** Protein carbonyl content; **e** Total protein thiol; **f** ALT activity; **g** AST activity. Results are presented as mean ± SD, n = 6. Significantly different from

control: * $P < 0.05$; significantly different from CPF: & $P < 0.05$; significantly different from neonatal in corresponding treatment groups: † $P < 0.05$; significantly different from juvenile in corresponding treatment groups: ‡ $P < 0.05$

region appears to be most vulnerable in neonatal and juvenile animals than young adults (data presented in Fig. 2).

Medulla Oblongata

The effect of CPF and cold exposure alone and together in medulla oblongata are shown in Fig. 3. The three-way ANOVA indicated a remarkable significant decrease in the levels of DNA, RNA, total protein and protein thiol, and significant elevations in the level of protein carbonyl, AST and ALT in neonatal, juvenile and young adult rats. Synergistic interaction of CPF and cold stress at 15°C showed marked macromolecular perturbations in comparison with CPF and cold stress alone and together at 20°C. Comparatively, this region appears to be more vulnerable in young animals.

Spinal Cord

Interactive changes in spinal cord are shown in Fig. 4. In comparison with control values, the levels of nucleic acids, total protein and protein thiols were drastically decreased, while the levels of protein carbonyl, AST and ALT were statistically increased in different age group rats exposed to CPF and cold stress. However, interactive effects were more pronounced in neonatal and juvenile age groups exposed at 15°C over the adult.

Discussion

Pesticides are known to alter protein and nucleic acid metabolism [28] and OP insecticides have an effect in addition to their specific inhibition of cholinesterase enzyme. Our previous study demonstrated that developing animals were more susceptible to CPF, cold stress; their interaction as a result of oxidative stress showed to be one of the decisive pathologic mediating factor(s) in CPF toxicity [12]. The generated data in the present study was concentrated on changes in indices of protein oxidation, a major event of oxidative stress, changes in transaminases and nucleic acid macromolecular alterations.

Protein Carbonyls

Oxidative damage to proteins by ROS can result in cleavage of the polypeptide backbone, their cross-linking, and thereby modify the side chains of amino acids that may be a more critical to cause damage to lipids, because enzyme inactivation can have rapid and supra-stoichiometric effects [29]. Protein carbonyl content (PCC) [aldehyde or ketone] is a widely used marker to assess the

presence of oxidative stress in physiological and pathological conditions [30]. Our results clearly show that toxic insults induced a significant increase in protein carbonyl level in discrete CNS areas and oxidized protein accumulates in neuronal tissues and eventually resulting in age-associated brain dysfunction. An increase observed in protein carbonyl levels in the discrete regions of CNS is in accordance with earlier reported works where augmented carbonyl level formation observed upon several pollutant exposures [31].

Protein Thiols

Protein thiols are frequent sites for post translational modification mediated by oxidative stress [32] observed in a wide variety of disorders including aging, and pesticide neurotoxicity [33]. In line with the previous studies we found decrease in protein thiol in the discrete regions of CNS studied due to CPF administration, which may be due to increased degradation of protein or increased consumption of antioxidant in stress environment which confirm the role of OPs in disruption of body's total antioxidant capacity [34, 35]. These findings indicate the efficiency of S-thiolation as a mechanism of antioxidant defence with pathological state that creates an increased risk of irreversible oxidation of—SH groups of proteins [36].

Transaminases

AST is present in rat cerebral homogenate at about the same concentrations as in liver suggests its importance in the brain amino acid pool homeostasis [37, 38]. Though ALT is indicated to be distributed in both mitochondrial and soluble fractions of the brain, unlike GOT, it is less active in brain, however, Matthews et al. [39] reported that both the enzymes degrade glutamate and GPT is able to reduce toxic (500 µM) levels of glutamate into the physiologic (<20 µM) range. The metabolism of amino acids is indicated by activities of specific transaminases, which were altered in response to CPF exposure. Further, increased aminotransferase activities may alter the levels of amino acid neurotransmitters in the nervous system. The transaminases are implicated to have a role in protein synthesis, are seriously affected by CPF. In the present study, a significant increase in the AST/ALT activities was observed. This result go hand-to-hand with Abdul Naveed et al. [40] who reported increased levels of brain AST and ALT enzymes in the fish treated with OP pesticide. Similar results showed the same trend in the activity level of AST/ALT caused by OP pesticides had been also reported in rabbit [41] and rat [42]. Indeed, it is believed that CPF causes damage to the nervous tissue probably through the induction of oxidative stress [12]. Although transaminase

Table 1 Comparison of mean body weight, mean absolute weight of the neurosomatic index in control and experimental groups at 48 h of exposure

Parameters	Control	CPF	Cold stress at 15°C	Cold stress at 20°C	CPF + cold stress at 15°C	CPF + cold stress at 20°C
Neonatal						
Mean body weight at the end of experiment (g)	10.90 ± 0.58 ^{&}	10.14 ± 0.20*	10.20 ± 0.11* (-6.42)	10.45 ± 0.18 (-4.13)	10.00 ± 0.29* (-8.26)	10.10 ± 0.09* (-7.34)
Absolute weight of the brain + spinal cord [CNS] (g)	1.45 ± 0.06 ^{&}	1.24 ± 0.01*	1.27 ± 0.02* (-12.41)	1.32 ± 0.02* ^{&} (-8.97)	1.20 ± 0.01* (-17.24)	1.22 ± 0.02* (-15.86)
Neurosomatic index	13.30	12.22	12.45	12.63	12.00	12.07
Juvenile						
Mean body weight at the end of experiment (g)	40.10 ± 0.11 ^{&}	38.60 ± 0.24*	39.00 ± 0.33* (-2.74)	39.45 ± 0.32* ^{&} (-1.62)	38.10 ± 0.20* (-4.99)	38.35 ± 0.63* (-4.36)
Absolute weight of the brain + spinal cord [CNS] (g)	2.20 ± 0.02 ^{&}	1.99 ± 0.01*	2.08 ± 0.01* (-5.45)	2.12 ± 0.01 ^{&} (-3.64)	1.90 ± 0.02* (-13.64)	1.94 ± 0.01* (-11.82)
Neurosomatic index	5.49	5.15	5.33	5.37	4.99	5.06
Young adult						
Mean body weight at the end of experiment (g)	230.70 ± 0.96 ^{&}	225.00 ± 0.63* (-2.47)	225.00 ± 0.32* (-2.47)	229.00 ± 0.70 ^{&} (-0.74)	220.00 ± 0.44* ^{&} (-4.63)	222.00 ± 0.89* ^{&} (-3.77)
Absolute weight of the brain + spinal cord [CNS] (g)	2.90 ± 0.05 ^{&}	2.62 ± 0.01*	2.66 ± 0.01* (-8.28)	2.69 ± 0.01* ^{&} (-7.24)	2.49 ± 0.03* ^{&} (-14.13)	2.56 ± 0.02* ^{&} (-11.72)
Neurosomatic index	1.25	1.16	1.18	1.24	1.13	1.14

Results are presented as mean ± SD, n = 6. Values are in the parenthesis indicate percentage change over controls
 Significantly different from control: * $P < 0.05$. Significantly different from CPF: [&] $P < 0.05$

Table 2 Effect of CPF, cold stress and their co-exposure on protein/DNA and RNA/DNA ratio in the (a) cerebral cortex, (b) cerebellum, (c) medulla oblongata, (d) spinal cord of different age group of rats at 48 h of exposure

Treatments	Age (days)	Protein/DNA	RNA/DNA
<i>(a) Cerebral cortex</i>			
Control	7 (neonatal)	22.95	0.52
	21 (juvenile)	28.29	0.51
	90 (adult)	13.21	0.60
CPF	7 (neonatal)	37.13 (+61.79)	0.39 (−25.00)
	21 (juvenile)	50.41 (+78.19)	0.36 (−0.29)
	90 (adult)	15.32 (+15.97)	0.42 (−30.00)
Cold stress at 15°C	7 (neonatal)	30.00 (+30.72)	0.35 (−32.69)
	21 (juvenile)	41.55 (+46.87)	0.35 (−31.37)
	90 (adult)	11.72 (−11.28)	0.41 (−31.67)
Cold stress at 20°C	7 (neonatal)	27.24 (+18.69)	0.53 (+1.92)
	21 (juvenile)	35.76 (+26.41)	0.53 (+3.92)
	90 (adult)	10.94 (−17.18)	0.44 (−26.67)
CPF + cold stress at 15°C	7 (neonatal)	42.03 (+83.13)	0.44 (−15.38)
	21 (juvenile)	86.25 (+204.87)	0.50 (−1.96)
	90 (adult)	13.33 (+0.91)	0.48 (−20.00)
CPF + cold stress at 20°C	7 (neonatal)	42.08 (+83.35)	0.40 (−23.08)
	21 (juvenile)	65.88 (+132.87)	0.60 (+17.64)
	90 (adult)	18.72 (+41.71)	0.55 (−8.33)
<i>(b) Cerebellum</i>			
Control	7 (neonatal)	28.22	0.80
	21 (juvenile)	31.33	0.59
	90 (adult)	13.36	0.63
CPF	7 (neonatal)	41.88 (+48.41)	0.34 (−57.50)
	21 (juvenile)	65.77 (+109.93)	0.50 (−15.25)
	90 (adult)	14.79 (+11.45)	0.40 (−36.51)
Cold stress at 15°C	7 (neonatal)	34.35 (+21.72)	0.40 (−50.00)
	21 (juvenile)	54.19 (+72.96)	0.54 (−8.47)
	90 (adult)	15.23 (−13.39)	0.72 (−10.00)
Cold stress at 20°C	7 (neonatal)	34.51 (+22.29)	0.67 (−16.25)
	21 (juvenile)	40.30 (+28.63)	0.59 (0.00)
	90 (adult)	125.45 (+846.07)	4.91 (−679.36)
CPF + cold stress at 15°C	7 (neonatal)	26.63 (−5.63)	0.32 (−60.00)
	21 (juvenile)	77.50 (+147.37)	0.39 (−33.89)
	90 (adult)	17.37 (+30.01)	0.47 (−25.40)
CPF + cold stress at 20°C	7 (neonatal)	47.78 (+69.31)	0.37 (−53.75)
	21 (juvenile)	58.96 (+88.19)	0.45 (−23.73)
	90 (adult)	21.49 (+60.85)	0.60 (−4.76)
<i>(c) Medulla oblongata</i>			
Control	7 (neonatal)	27.12	0.60
	21 (juvenile)	29.94	0.53
	90 (adult)	15.77	0.53
CPF	7 (neonatal)	36.69 (+35.28)	0.33 (−45.00)
	21 (juvenile)	61.75 (+106.24)	0.52 (−1.88)
	90 (adult)	12.04 (−23.65)	0.35 (−33.96)
Cold stress at 15°C	7 (neonatal)	32.87 (+21.20)	0.38 (−36.66)
	21 (juvenile)	56.74 (+89.51)	0.61 (+15.09)
	90 (adult)	10.28 (−34.81)	0.35 (−33.96)
Cold stress at 20°C	7 (neonatal)	31.44 (+15.92)	0.47 (−21.66)

Table 2 continued

Treatments	Age (days)	Protein/DNA	RNA/DNA
CPF + cold stress at 15°C	21 (juvenile)	46.63 (+55.74)	0.71 (+33.96)
	90 (adult)	12.43 (−21.18)	0.50 (−5.66)
	7 (neonatal)	37.50 (+38.27)	0.33 (−45.00)
	21 (juvenile)	94.00 (+213.96)	0.90 (+69.81)
	90 (adult)	11.91 (−24.48)	0.34 (−35.84)
	7 (neonatal)	56.04 (+106.63)	0.40 (−33.33)
CPF + cold stress at 20°C	21 (juvenile)	90.29 (+232.93)	0.71 (+33.96)
	90 (adult)	14.13 (−10.39)	0.52 (−1.88)
<i>(d) Spinal cord</i>			
Control	7 (neonatal)	15.68	0.43
	21 (juvenile)	31.52	0.56
	90 (adult)	12.02	0.55
CPF	7 (neonatal)	30.05 (+91.64)	0.32 (−25.58)
	21 (juvenile)	59.28 (+88.87)	0.50 (−10.71)
	90 (adult)	6.10 (+97.04)	0.35 (−36.36)
Cold stress at 15°C	7 (neonatal)	33.58 (+114.16)	0.36 (−16.27)
	21 (juvenile)	42.94 (+36.23)	0.45 (−19.64)
	90 (adult)	8.91 (−25.87)	0.48 (−12.72)
Cold stress at 20°C	7 (neonatal)	30.56 (+94.89)	0.49 (+13.85)
	21 (juvenile)	41.90 (+32.93)	0.62 (+10.71)
	90 (adult)	7.70 (−35.94)	0.39 (−29.09)
CPF + cold stress at 15°C	7 (neonatal)	29.04 (+85.20)	0.31 (−27.90)
	21 (juvenile)	53.88 (+70.93)	0.38 (−32.14)
	90 (adult)	12.31 (+2.41)	0.50 (−9.09)
CPF + cold stress at 20°C	7 (neonatal)	35.00 (+123.21)	0.33 (−23.25)
	21 (juvenile)	62.71 (+98.95)	0.42 (−25.00)
	90 (adult)	10.15 (−15.55)	0.55 (0.00)

Values are in the parenthesis indicate percentage change over controls

“−” indicates % decrease over control, “+” sign indicates % increase over control

reactions are reversible, the equilibrium of the AST and ALT reactions favours the formation of aspartate and alanine respectively, leading to excitotoxicity [43]. In this study the regional variation of ALT/AST activities was prominent on CPF exposure but the response to cold stress and CPF toxicated insult showed exacerbation in the AST/ALT levels. Three factor ANOVA with replication showed that CPF exposure contributed significantly on the changes of the ALT activity in all the CNS regions, but the interactions of impact of CPF and Cold exposure were significant in all age groups at 15°C. These observations support region-wise specific sensitivity to toxic insults as indicated in transaminases. Thus, it may be suggested that co-exposure of CPF and cold stress cause region specific alteration in glutamate metabolism. Hence, altered amino acid distribution pattern within the CNS regions might be expected in response to exposures of CPF and cold stress.

Nucleic Acids

Nucleic acids are the most sensitive indices to access the extent of cellular damage upon the impact of pesticide

because of the relative constancy of nucleic acid per somatic cell nucleus [44]. The reduction observed in the level of RNA, DNA and protein concentrations may be due to inhibition of their syntheses and/or enhancement of degradation in the cells and such reductions are in accordance with earlier report on OP pesticide [28]. It may be suggested that CPF affects the turnover of RNA and protein thereby changing the levels of macromolecular constituents in the neuronal tissues of the rat. CPF exposure resulted in a lower RNA/DNA ratio in all age groups of rat CNS regions. Further results indicate that DNA concentration per gm of tissue decreases with age, from neonatal to adult. The data indicate that cold stress also results in less protein and DNA. RNA/DNA ratio used as stress indicator and generated data (Table 2) suggests that neuronal tissues of younger age group animals affected more than young adult. Protein/DNA ratio used as an index of cell size and in comparison, the cell size was found to be more in juveniles followed by neonates than young adult indicating higher macromolecular content in developing rats. Decline observed in nucleic acids and proteins may also be due to DNA damage caused by the free radicals and

inhibition of RNA by direct interaction of ROS. Oxygen radicals can attack proteins, nucleic acids and lipid membranes, thereby disrupting cellular functions and integrity. Therefore, the oxidative changes appear to be related to cellular alterations.

Cold Stress

Brain is the organ most vulnerable to thermal fluctuations, since most of the physiological acclimation responses are initiated by the CNS [45]. Cold shock cause severe pathologies in the mammalian brain [46]. The present finding that activity levels of transaminases, protein carbonyl levels, protein thiols, nucleic acids and protein content were reduced in cold stress exposed group seems to be correlated with brain tissue damage induced by free radicals likely formed by acute cold stress [12]. These findings run in agreement with previous reports of increased levels of protein carbonyls [47, 48] and decreased level of RNA content [49] in tissues of animals exposed to cold stress. The increased protein oxidation may be a result of the decreased GSH concentration, which would otherwise protect proteins against the oxidation of the sulfhydryl groups [50].

Influence of Age

The present results reveal that young animals are markedly more sensitive and vulnerable than young adults. Age-related differences in susceptibility have been attributed in general to the following differences in the young immature nervous system, poor development of metabolizing/detoxifying enzymes, weak kinetic ability, differences in permeability of membranes, distribution of fat and overall excretory functions in the very young when compared with adults [51]. The brain is heterogeneous both metabolically as well as structurally. This heterogeneity not only allow various parts to perform the specific functions, but also permits the same metabolite to be used for different functions [52]. It is inevitable that metabolic variations that occur are due in part to preferential uptake or forced accumulation of CPF into particular cells or their processes. Further the same metabolites are proportionately different in their relative distribution among cellular structure. In addition, the mitochondrial population of brain is apparently heterogeneous in size as well as enzyme compliment [52]. The mechanism behind regional alterations could lead to degeneration or loss of neurons in vulnerable area of anatomical resolution which are supposed to carry designated function. In this study perhaps young rat, CNS as a whole appears to be susceptible to oxidative damage; the medulla oblongata and cerebellum is observed to be more prone to oxidative

macromolecular alterations which may be due to pronounced oxidative stress as indicated from our recent study [12]. It is known that the differential sensitivity to CPF neurotoxicity in different brain regions is due to preferential CPF metabolites accumulation, also due to alteration of biochemical or cellular process that are uniquely associated with or greatly enhanced in a particular region.

Impact of Cold Stress on CPF Toxicity

OP pesticides pose the threat of hypothermia, because they amplify body temperature decline that accompanies cold exposure [53]. In the present study, when different age group animals exposed to CPF toxicity at the temperatures of 15 and 20°C, their interaction exacerbated resulting in a synergistic action and substantially modified the toxicity in discrete CNS regions indicating potential effect of CPF and the quantum of synergistic interaction was most apparent at 15°C. In line with our findings, earlier studies [11] have reported that concomitant exposure of animals to CPF and cold stress exaggerates the neurotoxicity by provoking the AChE inhibition. The co-exposure of CPF and cold stress induced severe hypothermia and may retard the detoxification mechanisms of CPF resulting in its longstanding effect. Further, toxic insult of CPF and cold stress either alone or concurrently induced AChE inhibition might have triggered pronounced lipid peroxidation because of their interaction and the production of ROS in the tissues exceeds the ability of the antioxidant system to eliminate them, oxidative stress ensues [12]. Because of the high reactivity of ROS, most cellular components are likely to be targets of oxidative damage; lipid peroxidation, protein oxidation, GSH depletion, DNA single strand breaks, are all initiated by ROS in excess. As a whole, these events ultimately lead to cellular dysfunction and injury [54].

In conclusion, CPF and cold stress exposure either individually or in combination substantially increased the activity/levels of protein carbonyls, AST, ALT and decreased protein thiols, DNA, RNA and total proteins in discrete regions of CNS. Overall, the effects of co-exposure were appreciably different from either of the exposures. However, synergistic-action of CPF and cold stress at 15°C showed higher dyshomeostasis in comparison with CPF and cold stress alone and together at 20°C indicating the extent of oxidative macromolecular damage in discrete regions of brain and spinal cord. Furthermore, the present study demonstrates that macromolecular oxidative damage is highly pronounced in neonates and juveniles than the young adults. Indeed, this is an area of research whose results certainly have great potential to be translated in important advances in public health.

Acknowledgments This study was supported by University Grant Commission (UGC), south western regional office, PK block, palace road, Bangalore-560009, India under the Faculty Improvement Programme (FIP) for second author.

References

- Donnelly DF, Carroll JL (2005) Mitochondrial function and carotid body transduction. *High Alt Med Biol* 6:121–132
- Tilson HA (1998) Developmental neurotoxicology of endocrine disruptors and pesticides: identification of information gaps and research needs. *Environ Health Perspect* 106:807–811
- Ambali SF, Abubakar AT, Shittu M, Yaqub LS, Anafi SB, Abdullahi A (2010) Chlorpyrifos-induced alteration of hematological parameters in Wistar rats: ameliorative effect of zinc. *Res J Environ Toxicol* 4:55–66
- Uzun FG, Demir F, Kalender S, Bas H, Kalender Y (2010) Protective effect of catechin and quercetin on chlorpyrifos induced lung toxicity in male rats. *Food Chem Toxicol* 6:1714–1720
- Ncibi S, Ben Othman M, Akacha A, Krifi MN, Zourgui L (2008) *Opuntia ficus indica* extract protects against chlorpyrifos induced damage on mice liver. *Food Chem Toxicol* 46:797–802
- Kerr LD, Inoue JI, Verma IM (1992) Signal transduction: the nuclear target. *Curr Opin Cell Biol* 4:496–501
- Webster KA, Prentice H, Bishopric NH (2001) Oxidation of zinc finger transcription factors: physiological consequences. *Antioxid Redox Signal* 3:535–548
- Rajman M, Jurani M, Lamosova D, Macajova M, Sedlackova M, Kostal L, Jezova D, Vyboh P (2006) The effects of feed restriction on plasma biochemistry in growing meat type chickens (*Gallus gallus*). *Comp Biochem Phys A* 145:363–371
- Gordon CJ, Grantham TA, Yang Y (1997) Hypothermia and delayed fever in the male and female rat exposed to chlorpyrifos. *Toxicology* 118:149–158
- Sahin E, Gumuslu S (2004) Cold-stress-induced modulation of antioxidant defence: role of stressed conditions in tissue injury followed by protein oxidation and lipid peroxidation. *Int J Biometeorol* 48:165–171
- Maquire CC, Williams BA (1996) Response of thermal stressed bobwhite to organophosphorus exposure. *Rev Environ Health* 11(3):101–117
- Basha PM, Poojary A (2011) Chlorpyrifos induced region specific vulnerability in rat CNS and modulation by age and cold stress: an interactive study. *Neurochem Res* 36:241–249
- Rauh VA, Garfinkel R, Perera FP, Andrews HF, Hoepner L, Barr DB, Whitehead R, Tang D, Whyatt RW (2006) Impact of prenatal chlorpyrifos exposure on neurodevelopment in the first 3 years of life among inner-city children. *Pediatrics* 118:1845–1859
- Whitney KD, Seidler FJ, Slotkin TA (1995) Developmental neurotoxicity of chlorpyrifos: cellular mechanisms. *Toxicol Appl Pharmacol* 134:53–62
- Atterberry TT, Burnett WT, Chambers JE (1997) Age-related differences in parathion and chlorpyrifos toxicity in male rats: target and non-target esterase sensitivity and cytochrome P450-mediated metabolism. *Toxicol Appl Pharmacol* 147:411–418
- Slotkin TA, Cousins MM, Tate CA, Seidler FJ (2001) Persistent cholinergic presynaptic deficits after neonatal chlorpyrifos exposure. *Brain Res* 902:229–243
- Boon PE, Van der Voet H, Van Raaij MT, Van Klaveren JD (2008) Cumulative risk assessment of the exposure to organophosphorus and carbamate insecticides in the Dutch diet. *Food Chem Toxicol* 46:3090–3098
- Zheng Q, Olivier K, Won YK, Pope CN (2000) Comparative cholinergic neurotoxicity of oral chlorpyrifos exposures in pre-weanling and adult rats. *Toxicol Sci* 55:124–132
- Moser VC, Chanda SM, Mortensen SR, Padilla S (1998) Age- and gender-related differences in sensitivity to chlorpyrifos in the rat reflect developmental profiles of esterase activities. *Toxicol Sci* 46:211–222
- Slotkin TA, Levin ED, Seidler FJ (2006) Comparative developmental neurotoxicity of organophosphate insecticides: effects on brain development are separable from systemic toxicity. *Environ Health Perspect* 114:746–751
- Finney DJ (1971) Probit analysis, 3rd edn. Cambridge University Press, London, p 333
- Schneider WC (1957) Determination of nucleic acids in tissues by pentose analysis. In: Colowick SP, Kaplan NO (eds) *Methods in enzymology*, vol 3. Academic press, New York, pp 680–684
- Munro HN (1966) The determination of nucleic acids. *Methods Biochem Anal* 14:113–176
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 186:464–478
- Reznick AZ, Packer L (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol* 233:357–363
- Reitman S, Frankel S (1957) A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol* 28:56–63
- Zahrán MM, Abdel-Aziz KB, Abdel-Raof A, Nahas EM (2005) The effect of subacute doses of organophosphorus pesticide, nuvacron, on the biochemical and cytogenetic parameters of mice and their embryos. *Res J Agric Biol Sci* 1(3):277–283
- Davies MJ (2005) The oxidative environment and protein damage. *Biochim Biophys Acta* 1703:93–109
- Prevodnik A, Gardestrom J, Lilja K, Elfving T, McDonagh B, Petrovic N, Tedengren M, Sheehan D, Bollner T (2007) Oxidative stress in response to xenobiotics in the blue mussel *Mytilus edulis* L.: evidence for variation along a natural salinity gradient of the Baltic Sea. *Aquat Toxicol* 82:63–71
- Almroth CB, Sturve J, Berglund A, Forlin L (2005) Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. *Aquat Toxicol* 73:171–180
- Chai YC, Hendrich S, Thomas JA (1994) Protein S-thiolation in hepatocytes stimulated by t-butyl hydroperoxide, menadione, and neutrophils. *Arch Biochem Biophys* 310:264–272
- Mallis RJ, Hamann MJ, Zhao W, Zhang T, Hendrich S, Thomas JA (2002) Irreversible thiol oxidation in carbonic anhydrase III: protection by S-glutathiolation and detection in aging rats. *Biol Chem* 383:649–662
- Soltaninejad K, Shadnia S, Afkhami-Taghipour M, Saljooghi R, Mohammadirad A, Abdollahi M (2007) Blood-glucuronidase as a suitable biomarker at acute exposure of severe organophosphorus poisoning in human. *Hum Exp Toxicol* 26:963
- Ahmed MM, Zaki NI (2009) Assessment the ameliorative effect of pomegranate and rutin on chlorpyrifos-ethyl-induced oxidative stress in rats. *Nature and Science* 7:49–61
- Iciek M, Chwatko G, Lorenc-Koci E, Bald E, Wlodek L (2004) Plasma levels of total, free and protein bound thiols as well as sulfane sulfur in different age groups of rats. *Acta Biochim Pol* 51:815–824
- Streecher HJ (1970) Transaminases. In: Lajtha A (ed) *Handbook of neurochemistry*, vol III. Plenum Press, New York, pp 173–192

38. Beal MF, Brovillet E, Jenkins B, Henshaw R, Rosen B, Hyman BT (1993) Age-dependent striatal excitotoxic lesion produced by the endogenous mitochondrial inhibitor malonate. *J Neurochem* 61:1147–1150
39. Matthews CC, Zielke HR, Wollack JBPS (2000) Fishman enzymatic degradation protects neurons from glutamate excitotoxicity. *J Neurochem* 75:1045–1052
40. Naveed A, Venkateshwarlu P, Janaiah C (2010) Impact of sub-lethal concentration of triazophos on regulation of protein metabolism in the fish *Channa punctatus* (Bloch). *Afr J Biotechnol* 9:7753–7758
41. Elias Salih MA (2010) Toxic effect of dimethoate and diazinon on the biochemical and hematological parameters in male rabbits. *Jordan J Biol Sci* 3:77–82
42. Attar AM (2010) Physiological and histopathological investigations on the effects of α -lipoic acid in rats exposed to Malathion. *J Biomed Biotechnol*, Article ID 203503, 8 p. doi: [10.1155/2010/203503](https://doi.org/10.1155/2010/203503)
43. Moss DW, Henderson AR (1999) *Clinical enzymology*. Harcourt Brace and Company Asia Pvt. Ltd., Singapore
44. West ES, Todd WR, Mason HS, Bruggen JT (1970) *Textbook of biochemistry*, 14th edn. The MacMillan Company, Collier-Mac Millan Ltd., London, p 828
45. Crawshaw L, Grahn D, Wollmuth L, Simpson L (1985) Central nervous regulation of body temperature in vertebrates: comparative aspects. *Pharmacol Ther* 30:19–30
46. Chan PH, Yang GY, Chen SF, Carlson E, Epstein CJ (1991) Cold-induced brain edema and infarction are reduced in transgenic mice over expressing CuZn-superoxide dismutase. *Ann Neurol* 29:482–486
47. Tsiakitzis K, Kourounakis AP, Tani E, Rekka EA, Kourounakis PN (2005) Stress and active oxygen species—effect of α -tocopherol on stress response. *Arch Pharm Chem Life Sci* 338:315–321
48. Sahin E, Gumuslu S (2004) Cold-stress-induced modulation of antioxidant defence: role of stressed conditions in tissue injury followed by protein oxidation and lipid peroxidation. *Int J Biometeorol* 48:165–171
49. Wang JW, Xu SW (2008) Effects of cold stress on the messenger ribonucleic acid levels of corticotrophin-releasing hormone and thyrotropin-releasing hormone in hypothalami of broilers. *Poult Sci* 87:973–978
50. Slusser SO, Grotyohann LW, Martin LF, Scaduto RC Jr (1990) Glutathione catabolism by the ischemic rat kidney. *Am J Physiol* 258:1546–1553
51. Borghoff SJ, Birnbaum LS (1985) Age-related changes in glucuronidation and deglucuronidation in liver, small intestine, lung and kidney of male Fischer rats. *Drug Metab Dispos* 13:62–67
52. Leong SF, Lai JC, Lim L, Clark JB (1984) The activities of some energy-metabolising enzymes in nonsynaptic (free) and synaptic mitochondria derived from selected brain regions. *J Neurochem* 42:1306–1312
53. Rattner BA, Franson JC (1984) Methyl parathion and fenvalerate toxicity in American Kestrels: acute physiological responses and effects of cold. *Can J Physiol Pharmacol* 62:787–792
54. Marks DB, Marks AB, Smith CM (1996) Oxygen metabolism and toxicity. In: Williams Wilkins (ed) *Basic medical biochemistry: a clinical approach*. Lippincott Williams and Wilkins, Baltimore, pp 327–340