Original Article

Cold Stress Offered Modulation on Chlorpyrifos Toxicity in Aging Rat Central Nervous System

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ABSTRACT

The adverse effects produced by chlorpyrifos (CPF) or cold stress alone in humans and animals are well documented, but there is no information available relating to the consequences of their co- exposure in an age-related manner. In this study, effects of sublethal doses of CPF were carried out *in vivo,* for 48 h to assess the biochemical perturbations in relation to interactions with cold stress (15°C and 20°C) in different age group rat CNS. A positive interaction of CPF with age of animal and cold exposure was observed resulting in marked decrease in the activity levels of AChE ($P<0.05$), ChAT ($P<0.05$), Na^+, K^+-ATPase ($P<0.05$), Ca^{2+}-ATPase ($P<0.05$), and Mg^{2+}-ATPase ($P<0.05$). The ANOVA and posthoc analysis showed that regulatory enzymes decreased significantly ($P<0.05$) on CPF exposure. Overall, the effect of co-exposure was appreciably different from either of the exposures. Synergistic interaction of CPF and cold stress at 15°C showed higher inhibition in comparison with CPF and cold stress alone and together at 20°C. Further, this study reveals that young animals are significantly vulnerable and sensitive than adults.

Key words: Acetylcholinesterase, ATPases, ChAT, chlorpyrifos, CNS, cold stress, interactive effects

INTRODUCTION

Chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothionate, CPF) Is a synthetic chlorinated organo phosphate (OP), nonsystemic, broad-spectrum insecticide, and acaricide, widely used in agriculture and public health. It is continued to be used worldwide, largely because of its greater stability, persistence, and less toxic to cause delayed peripheral neuropathies in mammals.[1]

The primary toxic action of chlorpyrifos (CPF) is the inhibition of acetylcholinesterase (AChE)[2] causing cholinergic over stimulation, autonomic and neuromuscular dysfunction, and at high doses, results in coma and cell death.[3] CPF also elicits cholinergic toxicity by inhibiting the ChAT activity.[4] CPF exposure affect mammalian brain through a variety of mechanisms beyond its shared property of cholinesterase inhibition.[5,6] In addition, intracellular signaling gets affected during or following CPF exposure altering the function of multiple ATPases.[2]

Cold stress has detrimental effect(s) on organism by altering cellular homeostasis and plays a considerable role either in accelerating or modifying the toxic mechanisms. Cold exposure leads to oxidative stress[7] and also perturbs brain AChE[8] and ChAT[9] activities. Our previous studies have shown that exposure to CPF and cold stress alone and together causes oxidative stress.[10]

CPF is among the OP pesticides known to have adverse impact on the developing brain of children as well as laboratory animals.[11] Public 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.

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Understanding the potential age-related sensitivity is especially important in light of data suggesting greater exposure of children to pesticides.[12] 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.[13,14] The limited data available suggest some differential effects on developing as well as maturing animals on exposure to CPF.[13] However, little information is available to explore the interactive effects of CPF – cold stress exposure and neurochemical outcomes in rats in an age-related manner.[10] Keeping in view the lacuna in the literature and the role of cold stress in accelerating quantum of toxicity, this study was undertaken and focused on adenosine triphosphatases (ATPases) such as Na^+, K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase, biomarker AChE and ChAT in discrete regions of brain and spinal cord of neonatal, juvenile, and adult age group rats.

MATERIALS AND METHODS

Chemicals

A commercial grade having 20% W/V of CPF in organic solvent dimethyl sulfoxide (DMSO) marketed as Darsban was procured from Lupin Agrochemicals Pvt. Ltd, Bharuch, Gujarat (India) was used in this study. Acetyl CoA, Acetyl thio-choline and ATP were purchased Sigma-Aldrich, other chemicals (AR grade) from BDH.

Animals

Male albino rats, Wistar strain (*Rattus norvegicus albinus*) of different age groups, namely 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±1°C) for 1 week prior to commencement of the experiment. They were maintained on standard rodent pellet 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 design

Rats were divided into six groups: Group I – control animal group was kept at the laboratory room temperature (28±1°C); Group II – exposed to CPF at the laboratory temperature, 28±1°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 6. The neonatal and juvenile animals were kept along with the dam and other littermates in order to avoid additional stress. The group II, V, and VI animals were treated with sub lethal 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[15] 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/2-h dark cycle. 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.

In vivo biochemical assays

Acetylcholine esterase (AChE, EC 3.1.1.7)

The acetylcholinesterase (AChE) activity was determined according to the method of Ellman *et al*.[16] The reaction mixture, containing 3 mL of 0.1 M phosphate buffer (pH 8.00) having 20 µL of 0.075 M acetyl thiocholine iodide and 0.1 mL of 0.01 M DTNB was incubated at room temperature for 10 min. Further, 0.1 mL of tissue homogenate was added and mixed by inversion. The color absorbance was monitored for 5 min at 412 nm in spectrophotometer at intervals of 0.5 min and linearity was checked.

Choline acetyl transferase (ChAT, EC 2.3.1.195)

The ChAT activity was estimated by the method of Morris.[17] A final volume of 1 mL of incubation medium (pH 7.5) consisted of 12 mM choline chloride, 300 mM KCl, 0.26 mM eserinesulphate, 40 mM phosphate citrate buffer pH 7.5, 0.35 mM acetyl CoA and homogenate. After the period of incubation for 15 min at 37°C the Ach content was estimated by the colorimetric method of Hestrin (1949).

Adenosine-tri-phosphatases (ATPases, EC 3.6.1.3)

The activity of adenosine-tri-phosphatases (ATPases) such as Na^+, K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase were measured as per the method given by Yamaguchi *et al*.[18] and liberated inorganic phosphate was estimated by adopting the method of Fiske and Subbarow.[19] Total protein concentration was measured following the method of Lowry *et al*.[20] using bovine serum albumin as the standard. The final volume of ATPase assay was 1.5 mL that included 500 µg of tissue and 3 mM ATP (Sigma). The total ATPase activity was measured in an incubation medium containing

25 mM Tris-HCl pH 7.4, 100 mM NaCl, 300 mM KCl, 3 m*M* $MgCl_2$ and in the presence and absence of 3 mM Ouabain at 37°C. After 5 min of preincubation, the reaction was initiated by adding Tris-ATP. The reaction was stopped after 12 min by adding 1mL of 10% TCA followed by 1mL of 2.5% ammonium molybdate and 0.1 mL of reducing solution (ANSA) and the volume was made up to 10 mL using distilled water and optical density was read at 660 nm. Ouabain sensitive Na^+-K^+-ATPase activity was taken as the difference between total inorganic phosphate released in the absence of Ouabain and inorganic phosphate released in the presence of 3 m*M* Ouabain. The specific activity of ATPase was expressed as μmoles of Pi liberated/mg protein/min.

For the Mg^{2+} ATPase activity measurement the incubation medium contained 25 mM Tris-HCl pH 7.4, 0.32*M* buffered sucrose, 3 m*M* $MgCl_2$ and 0.1 mM Ouabain. The final volume was 1.5 mL that included 3 mM ATP and 500 μg of homogenate.

To assay the Ca^{2+} ATPase activity, the incubation medium consisted of 2 m*M* $CaCl_2$, 25 mM Tris-HCl, 0.2 *M* sucrose and 0.1 mM Ouabain was used. The final volume of incubation medium was 1.5 mL and included 500 μg of homogenate and 3 mM ATP.

Statistical analysis

The results are expressed as mean±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 version. Posthoc testing was performed for intergroup comparisons using the Bonferroni test at the 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 this study are depicted region wise in [Tables 1–4].

Cerebral cortex

Table 1 presents the data on individual and interactive effects of CPF and cold stress in different age group of rats in cerebral cortex. The three-way ANOVA indicated an interaction between age and cold exposure resulting in statistically decreased activity levels of AChE, ChAT, Na^+,K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+} ATPase. Moreover, interaction of CPF and cold exposure at 15°C presented higher inhibition of the activity level of AChE (P<0.05), ChAT (P<0.05) and all the three ATPases (P<0.05) when compared to CPF and cold stress alone and together at 20°C. Comparatively, neonatal and juvenile

Table 1: Effects of CPF, cold stress, and their exposure on AChE and different ATPase activities in the cerebral cortex of different age group rats

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
AChE (μmole of ACh hydrolysed/min/mg protein)						
Neonatal	52.48 ±1.30^{and}	16.30±1.28*$^{¥}$ (-68.94)	20.60±1.18* $^{¥}$ (-60.74)	28.90±2.06*$^{and¥}$ (-44.93)	8.96±0.77*$^{and¥}$ (-82.92)	11.87±1.37*$^{¥}$ (-77.38)
Juvenile	48.97±2.14^{and}	35.82±1.73*† (-26.85)	39.53±2.49*^{and†} (-19.07)	41.63±1.94*^{and†} (-14.98)	18.27±1.58*^{and†} (-62.69)	22.41±1.55*^{and†} (-54.23)
Young adult	63.04±2.81^{and}	41.39±2.76*$^{†¥}$ (-34.34)	39.37±2.59*† (-37.54)	62.41±3.17$^{†and¥}$ (-0.99)	34.56±0.96*$^{and†¥}$ (-45.17)	36.30±0.78*$^{and†¥}$ (-42.41)
ChAT (μmole Ach formed/h/mg protein)						
Neonatal	1.19±0.30^{and}	0.43±0.02*$^{¥}$ (-63.87)	0.51±0.03*$^{¥}$ (-57.14)	0.76±0.02*$^{and¥}$ (-36.13)	0.23±0.02*$^{¥}$ (-80.67)	0.30±0.02*$^{¥}$ (-74.76)
Juvenile	1.45± 0.07^{and}	0.86±0.04*† (-40.69)	0.94±0.04*† (-35.17)	1.18±0.06*^{and†} (-18.62)	0.47±0.04*^{and†} (-67.59)	0.69±0.03*^{and†} (-52.42)
Young adult	2.34±0.08^{and}	1.91±0.03*$^{†¥}$ (-18.38)	2.15±0.04*$^{and†¥}$ (-8.12)	2.38±0.03$^{and†¥}$ (1.71)	1.47±0.05*$^{and†¥}$ (-37.17)	1.78±0.03*$^{and†¥}$ (-23.93)
Na+K+ATPase (μmole of Pi released/h/mg protein)						
Neonatal	4.52±0.33	4.03±0.18$^{¥}$ (-10.84)	6.23±0.19*$^{and¥}$ (+46.77)	7.34±0.45*$^{and¥}$ (+77.53)	2.86±0.40*$^{and¥}$ (-36.65)	3.37±0.18*$^{and¥}$ (-25.44)
Juvenile	6.44±0.63^{and}	3.42±0.27*† (-46.98)	4.38±0.37*^{and†} (-31.98)	4.47±0.16*^{and†} (-30.59)	0.73±0.36*^{and†} (-85.85)	0.98±0.31*^{and†} (-84.78)
Young adult	5.16±0.17^{and}	5.03±0.30*$^{†¥}$ (-2.58)	5.44±0.32*$^{†¥}$ (+5.42)	6.09±0. 63$^{†and¥}$ (+18.02))	2.21±0.20*$^{and†¥}$ (-57.17)	4.22±0.14*$^{and†¥}$ (-18.21)
Ca2+ATPase (μ mole of Pi released/h/mg protein)						
Neonatal	5.68±0.19^{and}	4.50±0.28* $^{¥}$ (-20.88)	4.55±0.34*$^{¥}$ (-19.90)	4.59±.0.29* $^{¥}$ (-19.26)	1.18±0.08 *^{and} (-76.66)	4.04±0.19* $^{¥}$ (-28.88)
Juvenile	1.49±0.30	1.48±0.28 † (-0.40)	1.71±0.03 † (+14.76)	2.36±0.37*^{and†} (+58.38)	1.39±0.38 (-6.71)	1.47±0.23 † (-1.34)
Young adult	9.24±0.05^{and}	7.94±0.40*$^{†¥}$ (-14.25)	14.64±0.20*$^{†¥}$ (+58.09)	15.6±0.46*$^{and†¥}$ (+68.46)	4.43±0.34*$^{and†¥}$ (-51.94)	4.59±0.44*$^{and¥}$ (-50.43)
Mg2+ATPase (μ mole of Pi released/h/mg protein)						
Neonatal	3.16±0.80^{and}	1.67±0.12* $^{¥}$ (-47.15)	2.10±0.50* $^{¥}$ (-33.54)	2.16±0.13* $^{¥}$ (-31.64)	0.87±0.1* ^{and} (-72.46)	1.64±0.25* $^{¥}$ (-48.03)
Juvenile	2.52±0.09	2.05±0.30† (-18.65)	3.36±0.31* ^{and†} (+33.57)	7.12±0.10*^{and†} (+182.57)	0.89±0.08*^{and} (-64.68)	1.88±0.04^{and†} (-25.39)
Young adult	20.32±0.15	16.08±0.25 $^{†¥}$ (-0.21)	26.17±1.76*$^{and†¥}$ (+28.78)	28.04±1.6*$^{and†¥}$ (+37.99)	10.34±0.10 *$^{and†¥}$ (-49.11)	13.30±0.15*$^{and†¥}$ (-34.55)

Results are presented as mean±S D, n=6. values are in the parenthesis indicate percentage change over controls. Significantly different from control: *P<0.05; Significantly different from CPF: ^{and}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; "-" indicates % decrease over control, "+" sign indicates % increase over control

Table 2: Effects of CPF, cold stress, and their exposure on AChE and different ATPase activities in the cerebellum of different age group rats

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
AChE (µ mole of ACh hydrolysed/min/mg protein)						
Neonatal	48.36±2.46^{and}	18.47±1.27*$^{¥}$ (-61.80)	20.73±1.12* (-57.13)	34.32±1.61*$^{¥}$ (-29.03)	5.22±0.75 *^{and} (-89.20)	7.48±1.22*^{and} (-84.53)
Juvenile	47.26±2.69^{and}	12.24±1.41*† (-74.10)	19.12±1.52*^{and} (-59.54)	28.16±3.01*^{and†} (-40.41)	5.28±0.83*^{and} (-88.82)	9.42±1.38* (-80.06)
Young adult	97.41±3.55^{and}	46.01±2.26*$^{†¥}$ (-52.76)	53.75±1.89*^{and†} (-44.82)	78.08±3.85*$^{and†¥}$ (-19.84)	34.36±1.78*$^{and†¥}$ (-64.72)	43.03±2.14*$^{†¥}$ (-55.82)
ChAT (µmole Ach formed/hr/mg protein)						
Neonatal	1.34±0.30^{and}	0.46±0.04*$^{¥}$ (-65.67)	0.79±0.04*$^{and ¥}$ (-41.04)	0.84±0.03*$^{and¥}$ (-37.31)	0.18±0.02*$^{and¥}$ (-86.56)	0.28±0.03*$^{and¥}$ (-79.1)
Juvenile	1.47±0.05^{and}	0.99±0.06*† (-32.65)	1.07±0.02*† (-27.21)	1.19±0.06*^{and†} -(25.85)	0.61±0.04*^{and†} (-58.50)	0.83±0.03*^{and†} (-43.54)
Young adult	2.48±0.04^{and}	2.30±0.03*$^{†¥}$ (-7.26)	2.39±0.03$^{†¥}$ (-3.63)	2.59±0.03*$^{and†¥}$ (4.44)	1.35±0.04*$^{and†¥}$ (-45.56)	1.79±0.10*$^{and †¥}$ (-27.82)
Na+K+ATPase (µ mole of Pi released/hr/mg protein)						
Neonatal	3.50±0.42^{and}	2.85±0.30*$^{¥}$ (-18.46)	6.29±0.28*$^{and¥}$ (+79.73)	6.56±0.40*$^{and¥}$ (+87.42)	2.06±0.08*$^{and¥}$ (-41.14)	2.37±0.33*$^{and¥}$ (-32.28)
Juvenile	3.18±0.26^{and}	2.97±0.25*† (-6.69)	3.18±0.27^{and†} (-0.09)	3.45±0.30^{and†} (+8.38)	0.35±0.09*^{and†} (-89.00)	1.19±0.07*$^{and †}$ (-62.58)
Young adult	5.28±0.23^{and}	3.32±0.14*$^{†¥}$ (-37.06)	5.33±0.28$^{and†¥}$ (+0.94)	9.06±0.17*$^{and†¥}$ (+71.59)	2.21±0.35*$^{and†¥}$ (-58.18)	2.82±0.33*$^{and†¥}$ (-46.59)
Ca2+ATPase (µ mole of Pi released/hr/mg protein)						
Neonatal	3.26±0.15	3.08±0.32$^{¥}$ (-5.52)	3.09±0.07$^{¥}$ (-5.03)	4.70±0.25$^{¥}$ (+44.26)	0.68±0.34*^{and} (-79.14)	2.29±0.16*$^{¥}$ (-29.75)
Juvenile	0.82±0.04	0.85±0.17† (+2.89)	2.56±0.33*^{and†} (+209.17)	3.34±0.14*^{and†} (+303.74)	0.12±0.01*^{and} (-84.90)	0.27±0.01*^{and†} (-66.42)
Young adult	16.34±0.28^{and}	7.65±0.65*$^{†¥}$ (-58.18)	8.99±0.30*$^{and†¥}$ (-44.99)	18.59±0.23*$^{and†¥}$ (+13.74)	4.39±0.33*$^{and†¥}$ (-73.11)	5.45±0.46*$^{and†¥}$ (-66.64)
Mg2+ATPase (µ mole of Pi released/hr/mg protein)						
Neonatal	1.92±0.28^{and}	1.39±0.27* (-0.27)	2.50±0.33*$^{and¥}$ (+30.20)	2.53±0.20*$^{and ¥}$ (+31.77)	1.20±0.16*$^{¥}$ (-37.50)	1.23±0.16*$^{¥}$ (-35.93)
Juvenile	4.25±0.18^{and}	1.81±0.39* (-59.95)	3.41±0.31*$^{and †}$ (-13.69)	5.51±0.24*^{and†} (+29.83)	0.62±0.21*^{and} (-84.70)	0.83±0.03*^{and†} (-80.35)
Young adult	32.42±0.31^{and}	10.46±0.33$^{*†¥}$ (-67.71)	22.25±0.25$^{*and †¥}$ (-31.35)	34.25±0.21$^{*and†¥}$ (+5.64)	1.47±0.26$^{*and†¥}$ (-95.44)	4.06±0.31$^{*and†¥}$ (-87.45)

Results are presented as mean±S D, *n*=6. values are in the parenthesis indicate percentage change over controls. Significantly different from control: *P<0.05; Significantly different from CPF: ^{and}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; "-" indicates % decrease over control, "+" sign indicates % increase over control

Table 3: Effects of CPF, cold stress, and their exposure on AChE and different ATPase activities in the medulla oblongata of different age group rats

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
AChE (µ mole of ACh hydrolysed/min/mg protein)						
Neonatal	70.35±3.11^{and}	21.43±1.07*$^{¥}$ (-69.53)	25.75±1.89*$^{and¥}$ (-63.39)	57.74±2.66*$^{and¥}$ (-17.92)	11.61±0.96*$^{and¥}$ (-83.49)	16.13±1.17* $^{and¥}$ (-77.07)
Juvenile	107.30±3.29^{and}	26.43±1.83*† (-75.36)	34.12±1.56*^{and†} (-68.20)	38.77±2.46*^{and†} (-63.86)	16.29±1.37*^{and†} (-84.81)	18.73±1.94*^{and†} (-82.54)
Young adult	140.98±5.81^{and}	62.63±3.22*$^{†¥}$ (-55.57)	83.70±4.66*$^{and†¥}$ (-40.62)	131.88±5.13*$^{and†¥}$ (-6.45)	25.09±0.74*$^{and†¥}$ (-82.20)	47.27±3.52*$^{and†¥}$ (-66.47)
ChAT (µmole Ach formed/h/mg protein)						
Neonatal	1.40±0.12^{and}	0.49±0.04*$^{¥}$ (-65.00)	0.48±0.03*$^{¥}$ (-65.71)	0.89±0.03*$^{and¥}$ (-36.43)	0.20±0.02*$^{and ¥}$ (-85.71)	0.27±0.03*$^{and¥}$ (-80.71)
Juvenile	1.49±0.09^{and}	1.03±0.10*† (-30.87)	1.14±0.03*† (-23.49)	1.31±0.05*^{and†} (-12.08)	0.64±0.05*^{and†} (-57.05)	0.88±0.03*^{and†} (-40.94)
Young adult	2.38±0.03^{and}	2.09±0.04*$^{†¥}$ (-12.18)	2.10±0.05*$^{†¥}$ (-11.76)	2.29±0.04*$^{and†¥}$ (-3.78)	1.42±0.03*$^{and†¥}$ (-40.34)	1.83±0.03*$^{and†¥}$ (-25.11)
Na+K+ATPase (µ mole of Pi released/h/mg protein)						
Neonatal	4.25±0.35^{and}	2.92±0.22*$^{¥}$ (-31.29)	3.54±0.28* (-16.72)	4.14±0.30 $^{¥}$ (-2.59)	1.58±0.31*$^{¥}$ (-62.83)	1.71±0.07* $^{¥}$ (-59.58)
Juvenile	6.90±0.13^{and}	2.89±0.35*† (-58.16)	3.25±0.18*^{and} (-52.84)	6.22±0.20*^{and†} (-9.87)	1.17±0.08*^{and†} (-82.97)	2.42±0.32*^{and†} (-64.92)
Young adult	3.33±0.22^{and}	2.46±0.350* (-26.12)	3.74±0.16*$^{and¥}$ (+12.31)	7.07±0.27*$^{and†¥}$ (+112.31)	1.12±0.06*$^{and †}$ (-66.36)	2.25±0.45*$^{and¥}$ (-32.43)
Ca2+ATPase (µ mole of Pi released/h/mg protein)						
Neonatal	5.33±0.23^{and}	3.30±0.10*$^{¥}$ (-38.08)	3.62±0.28*$^{and ¥}$ (-32.02)	4.42±0.12*$^{¥}$ (-17.10)	2.24±0.32*$^{and¥}$ (-57.97)	2.80±0.12*$^{and¥}$ (-47.38)
Juvenile	1.25±0.15	1.09±0.31† (-12.80)	2.14±0.10*^{and†} (+70.91)	2.56±0.09*^{and†} (+103.98)	0.22±0.12*^{and†} (-82.47)	0.88±0.02† (-29.24)
Young adult	23.32±0.22^{and}	13.96±0.11*$^{†¥}$ (-40.13)	16.51±0.31*$^{and†¥}$ (-29.19)	17.23±0.18*$^{and†¥}$ (-26.13)	5.12±0.42*$^{and†¥}$ (-78.04)	8.01±0.4*$^{and†¥}$ (-50.96)
Mg2+ATPase (µ mole of Pi released/h/mg protein)						
Neonatal	1.80±0.11	1.60±0.18$^{¥}$ (-1.23)	1.67±0.24$^{¥}$ (-7.22)	1.82±0.09$^{¥}$ (+12.34)	0.92±0.02*$^{and¥}$ (-42.83)	1.47±0.27 $^{¥}$ (-18.33)
Juvenile	3.09±0.07	2.69±0.49† (-13.16)	5.02±0.63*^{and†} (+62.45)	9.19±0.16* ^{and†} (+196.64)	0.47±0.15*^{and†} (-84.78)	1.16±0.08*^{and†} (-62.52)
Young adult	17.24±0.16^{and}	15.48±0.39*$^{†¥}$ (-10.17)	16.32±0.15*$^{and†¥}$ (-5.33)	19.31±0.24*$^{and†¥}$ (+110.59)	6.06±0.22*$^{and†¥}$ (-64.80)	12.84±0.32*$^{and†¥}$ (-25.50)

Results are presented as mean±S D, *n*=6. values are in the parenthesis indicate percentage change over controls. Significantly different from control: *P<0.05; Significantly different from CPF: ^{and}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; "-" indicates % decrease over control, "+" sign indicates % increase over control

Table 4: Effects of CPF, cold stress and their exposure on AChE and different ATPase activities in the spinal cord of different age group rats

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
AChE (µ mole of ACh hydrolysed/min/mg protein)						
Neonatal	71.27±2.85^{and}	23.26±1.63*$^{¥}$ (-67.36)	42.83±1.77*$^{and¥}$ (-39.90)	62.69±2.12*$^{and¥}$ (-12.03)	14.41±1.44*$^{and¥}$ (-79.78)	20.19±1.76*$^{¥}$ (-71.67)
Juvenile	39.65±2.62^{and}	28.39±2.07*† (-28.39)	31.15±2.16*^{and†} (-21.43)	33.80±1.09*^{and†} (-14.75)	10.26±1-64*^{and†} (-74.12)	23.24±2.0*^{and†} (-41.38)
Young adult	75.28±4.10^{and}	50.04±3.44*$^{†¥}$ (-33.52)	60.66±2.98*$^{and†¥}$ (-19.42)	119.61±2.16*$^{and†¥}$ (+58.88)	15.71±1.61*$^{and†¥}$ (-79.13)	20.31±2.26*$^{and†¥}$ (-73.02)
ChAT (µmole Ach formed/hr/mg protein)						
Neonatal	1.05±0.06^{and}	0.45±0.03*$^{¥}$ (-57.14)	0.48±0.02*$^{and¥}$ (-54.29)	0.61±0.05*$^{¥}$ (-41.90)	0.19±0.02*$^{and¥}$ (-81.90)	0.32±0.03*$^{and¥}$ (-69.52)
Juvenile	1.58±0.08^{and}	0.82±0.07*† (-48.10)	0.96±0.09*† (-39.24)	1.31±0.26*^{and†} (-17.09)	0.58±0.05*^{and†} (-63.29)	0.63±0.04*† (-60.13)
Young adult	2.41±0.07^{and}	1.94±0.03*$^{†¥}$ (-19.50)	2.14±0.05*$^{†¥}$ (-11.20)	2.34±0.04$^{and†¥}$ (-2.90)	1.37±0.07*$^{and†¥}$ (-43.15)	1.84±0.06*$^{and†¥}$ (-23.65)
Na+K+ATPase (µ mole of Pi released/hr/mg protein)						
Neonatal	4.67±0.31^{and}	1.64±0.22*$^{¥}$ (-64.84)	2.28±0.15*$^{¥}$ (-51.19)	3.61±0.19* (-22.83)	0.94±0.04*$^{¥}$ (-87.79)	1.54±0.35*$^{and¥}$ (-86.97)
Juvenile	4.12±0.08^{and}	0.89±0.05*† (-78.35)	2.94±0.44*^{and†} (-28.55)	3.40±0.46*^{and} (-17.59)	0.63±0.13*^{and†} (-108.91)	1.97±0.24*^{and†} (-84.68)
Young adult	4.51±0.16^{and}	2.99±0.22*$^{†¥}$ (-33.70)	4.10±0.32*$^{†¥}$ (-9.11)	5.56±0.26*$^{and†¥}$ (+23.28)	1.83±0.48*^{and†} (-59.42)	3.33±0.22*$^{and†¥}$ (-26.16)
Ca2+ATPase (µ mole of Pi released/hr/mg protein)						
Neonatal	4.70±0.32^{and}	4.17±0.07*$^{¥}$ (-11.14)	4.21±0.21*$^{¥}$ (-10.40)	4.52±0.36$^{¥}$ (-3.76)	2.94±0.11*$^{and¥}$ (-37.31)	3.47±0.29*$^{and¥}$ (-26.00)
Juvenile	1.13±0.14^{and}	0.57±0.05*† (-49.)	0.97±0.02*† (-14.16)	1.16±0.13^{and†} (+2.73)	0.23±0.03*^{and†} (-79.55)	0.37±0.04*† (-67.25)
Young adult	6.33±0.13^{and}	4.42±0.24*$^{¥}$ (-30.30)	7.83±0.20*$^{and†¥}$ (+23.63)	9.00±0.15*$^{and†¥}$ (+41.25)	3.20±0.20*$^{and†¥}$ (-49.51)	3.49±0.26*$^{and¥}$ (-44.86)
Mg2+ATPase (µ mole of Pi released/hr/mg protein)						
Neonatal	3.41±0.21^{and}	1.42±0.35*$^{¥}$ (-58.35)	1.80±0.03*$^{¥}$ (-47.21)	2.65±0.12*$^{and¥}$ (-22.28)	0.84±0.42* (-70.39)	1.09±0.33*$^{¥}$ (-59.23)
Juvenile	6.92±0.22^{and}	4.16±0.10*† (-39.88)	4.20±0.18*† (-39.19)	4.67±0.20*^{and†} (-32.42)	1.55±0.41*^{and} (-75.78)	1.94±0.21*^{and†} (-71.84)
Young adult	6.40±0.39^{and}	5.24±0.14*$^{†¥}$ (-18.13)	7.16±0.09*$^{†¥}$ (+11.87)	10.71±0.48*$^{†¥}$ (+67.34)	3.03±0.14* $^{†¥}$ (-52.65)	3.79±0.38* $^{†¥}$ (-40.78)

Results are presented as mean±S D, *n*=6. values are in the parenthesis indicate percentage change over controls. Significantly different from control: *P<0.05; Significantly different from CPF: ^{and}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; "-" indicates % decrease over control, "+" sign indicates % increase over control

animals presented higher sensitivity (P<0.05) than adult animals (P<0.05) [Table S-1].

Cerebellum

In cerebellum, interactive effects were more pronounced in neonatal and juvenile age groups exposed at 15°C over the adult. However, the three-way ANOVA indicated a marked significant decrease in the activity levels of AChE, ChAT, Na^+,K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase. Comparatively, this region appears to be more vulnerable in neonatal and juvenile animals than young adults, data presented in Table 2 and Table S-2.

Medulla oblongata

The effect of CPF and cold exposure alone and together in medulla oblongata are presented in Table 3. The three-way ANOVA indicated a significant effect of CPF on the activities of regulatory enzymes namely AChE, ChAT, and ATPases (P<0.05), and found interaction between age and cold stress. Neonatal and juvenile animals presented significantly decreased activity levels of regulatory enzymes. Comparatively, this region appears to be more vulnerable in young animals [Table S-3].

Spinal cord

Interactive effects of CPF and cold stress in spinal cord are presented in Table 4. The three-way ANOVA indicated an interaction between age and cold exposure resulting in decreased activities of enzymes like AChE, ChAT, Na^+,K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase. Exposure to 15°C modified the CPF toxicity by accelerating the inhibition of regulatory enzymes [Table S-4].

DISCUSSION

Biochemical markers are increasingly used in ecological risk assessment of the ecosystem to identify the incidence and effect of pesticides. Assessment of deleterious effects induced by concurrent exposure to chlorpyrifos and cold stress is of great concern to find out toxicological consequences arising as a result of their interaction and such information is essential for the comprehensive management of pesticide-induced untoward effects in nontarget animals. We have previously investigated the role of oxidative stress in the neuronal injury caused by interactive exposure to CPF and cold stress in rats in an age-related manner that is characterized by increased production of ROS (reactive oxygen species), sharp reduction in antioxidant defense and altered cellular redox status.[10]

AChE, a sensitive marker of neurotoxicity that plays an important role in neurotransmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine to

Table S-1: Percent change over CPF treated group as a result of interactive effects of AChE, ChAT, and cold stress exposure on AChE and different ATPase activities in the cerebral cortex of different age group rats

Parameters	CPF	CPF+ cold stress at 15°C	CPF+ cold stress at 20°C
AChE (µmole of ACh hydrolysed/ min/mg protein)			
Neonatal	16.30±1.28	8.96±0.77 (−45.03)	11.87±1.37 (−27.17)
Juvenile	35.82±1.73	18.27±1.58 (−48.99)	22.41±1.55 (−37.43)
Young adult	41.39±2.76	34.56±0.96 (−16.50)	36.30±0.78 (−12.29)
ChAT (µmole Ach formed/h/mg protein)			
Neonatal	0.43±0.02	0.23±0.02 (−46.51)	0.30±0.02 (−30.23)
Juvenile	0.86±0.04	0.47±0.04 (−45.34)	0.69±0.03 (−19.77)
Young adult	1.91±0.03	1.47±0.05 (−23.04)	1.78±0.03 (−6.80)
Na+K+ATPase (µmole of Pi released/h/mg protein)			
Neonatal	4.03±0.18	2.86±0.40) (−29.03)	3.37±0.18 (−16.37)
Juvenile	3.42±0.27	0.73±0.36 (−78.65)	0.98±0.31(−71.34)
Young adult	5.03±0.30	2.21±0.20 (−56.06)	4.22±0.14 (−16.10)
Ca2+ATPase (µmole of Pi released /h/mg protein)			
Neonatal	4.50±0.28	1.18±0.08 (−73.77)	4.04±0.19 (−10.22)
Juvenile	1.48±0.28	1.39±0.38 (−6.08)	1.47±0.23 (−0.67)
Young adult	7.94±0.40	4.43±0.34 (−44.20)	4.59±0.44 (−42.19)
Mg2+ATPase (µ mole of Pi released /hr/mg protein)			
Neonatal	1.67±0.12	0.87±0.1 (−47.90)	1.64±0.25 (−1.79)
Juvenile	2.05±0.30	0.89±0.08 (−56.58)	1.88±0.04 (−8.29)
Young adult	16.08±0.25	10.34±0.10 (−35.69)	13.30±0.15 (−17.28)

Results are presented as mean ± S D, *n* = 6. Values in parenthesis indicate % change over CPF. '−'sign indicates decrease and '+' sign indicates increase.

Table S-2: Effects of CPF, cold stress and their exposure on AChE, ChAT, and different ATPase activities in the cerebellum of different age group rats

Parameters	CPF	CPF+ cold stress at 15°C	CPF+ cold stress at 20°C
AChE (µmole of ACh hydrolysed/min/mg protein)			
Neonatal	18.47±1.27	5.22±0.75 (−71.73)	7.48±1.22 (−59.50)
Juvenile	12.24±1.41	5.28±0.83 (−56.86)	9.42±1.38 (−23.03)
Young adult	46.01±2.26	34.36±1.78 (−25.32)	43.03±2.14 (−6.47)
ChAT (µmole Ach formed/h/mg protein)			
Neonatal	0.46±0.04	0.18±0.02 (−60.86)	0.28±0.03 (−39.13)
Juvenile	0.99±0.06	0.61±0.04 (−38.38)	0.83±0.03 (−16.16)
Young adult	2.30±0.03	1.35±0.04 (−41.30)	1.79±0.10 (−6.47)
Na+K+ATPase (µmole of Pi released/h/mg protein)			
Neonatal	2.85±0.30	2.06±0.08 (−27.71)	2.37±0.33 (−16.84)
Juvenile	2.97±0.25	0.35±0.09 (−88.21)	1.19±0.07 (−59.93)
Young adult	3.32±0.14	2.21±0.35 (−33.43)	2.82±0.33 (−15.06)
Ca2+ATPase (µmole of Pi released /h/mg protein)			
Neonatal	3.08±0.32	0.68±0.34 (−77.92)	2.29±0.16 (−25.64)
Juvenile	0.85±0.17	0.12±0.01 (−85.88)	0.27±0.01 (−68.23)
Young adult	7.65±0.65	4.39±0.33 (−42.61)	5.45±0.46 (−28.75)
Mg2+ATPase (µ mole of Pi released /h/mg protein)			
Neonatal	1.39±0.27	1.20±0.16 (−13.66)	1.23±0.16 (−11.51)
Juvenile	1.81±0.39	0.62±0.21 (−65.74)	0.83±0.03 (−54.14)
Young adult	10.46±0.33	1.47±0.26 (−85.94)	4.06±0.31 (−61.18)
	1.20±0.16 (−13.66)	1.23±0.16 (−11.51)	

Results are presented as mean ± S D, *n* = 6. Values in parenthesis indicate % change over CPF.

choline and acetate.[21] It is clear from this study that CPF exerts an imbalance in neurochemical homeostasis resulting in AChE inhibition. The inhibition of the AChE activity disturbs the metabolic and neuronal activities and also causes deformities of the cell membrane.[22] The decreased AChE activity was observed following CPF exposure in this study is in agreement with the earlier report[2] and such a decrease leads to over accumulation of acetylcholine

Table S-3: Effects of CPF, cold stress and their exposure on AChE, ChAT, and different ATPase activities in the medulla oblongata of different age group rats

Parameters	CPF	CPF+ cold stress at 15°C	CPF+ cold stress at 20°C
AChE (µmole of ACh hydrolysed/ min/mg protein)			
Neonatal	21.43±1.07	11.61±0.96 (−45.62)	16.13±1.17 (−24.73)
Juvenile	26.43±1.83	16.29±1.37 (−38.37)	18.73±1.94 (−29.13)
Young adult	62.63±3.22	25.09±0.74 (−59.99)	47.27±3.52 (−24.52)
ChAT (µmole Ach formed/h/mg protein)			
Neonatal	0.49±0.04	0.20±0.02 (−59.18)	0.27±0.03 (−44.90)
Juvenile	1.03±0.10	0.64±0.05 (−37.86)	0.88±0.03 (−14.56)
Young adult	2.09±0.04	1.42±0.03 (−32.05)	1.83±0.03 (−12.44)
Na+K+ATPase (µmole of Pi released/h/mg protein)			
Neonatal	2.92±0.22	1.58±0.31 (−45.89)	1.71±0.07 (−41.44)
Juvenile	2.89±0.35	1.17±0.08 (−59.52)	2.42±0.32 (−16.26)
Young adult	2.46±0.350	1.12±0.06 (−54.47)	2.25±0.45 (−8.54)
Ca2+ATPase (µmole of Pi released /h/mg protein)			
Neonatal	3.30±0.10	2.24±0.32 (−32.12)	2.80±0.12 (−15.15)
Juvenile	1.09±0.31	0.22±0.12 (−79.81)	0.88±0.02 (−19.26)
Young adult	13.96±0.11	5.12±0.42 (−63.32)	8.01±0.4 (−42.62)
Mg2+ATPase (µ mole of Pi released /hr/mg protein)			
Neonatal	1.60±0.18	0.92±0.02 (−42.50)	1.47±0.27 (−8.12)
Juvenile	2.69±0.49	0.47±0.15 (−82.52)	1.16±0.08 (−56.87)
Young adult	15.48±0.39	6.06±0.22 (−60.85)	12.84±0.32 (−17.05)
		1.20±0.16 (−13.66)	1.23±0.16 (−11.51)

Results are presented as mean ± S D, *n* = 6. Values in parenthesis indicate % change over CPF. '−'sign indicates decrease and '+' sign indicates increase.

Table S-4: Effects of CPF, cold stress and their exposure on AChE, ChAT, and different ATPase activities in the spinal cord of different age group rats

Parameters	CPF	CPF+ cold stress at 15°C	CPF+ cold stress at 20°C
AChE (µmole of ACh hydrolysed/ min/mg protein)			
Neonatal	23.26±1.63	14.41±1.44 (−38.04)	20.19±1.76 (−13.20)
Juvenile	28.39±2.07	10.26±1−64 (−63.86)	23.24±2.0 (−18.14)
Young adult	50.04±3.44	15.71±1.61 (−68.60)	20.31±2.26 (−59.41)
ChAT (µmole Ach formed/h/mg protein)			
Neonatal	0.45±0.03	0.19±0.02 (−57.77)	0.32±0.03 (−28.88)
Juvenile	0.82±0.07	0.58±0.05 (−29.26)	0.63±0.04 (−23.17)
Young adult	1.94±0.03	1.37±0.07 (−2938)	1.84±0.06 (−5.15)
Na+K+ATPase (µmole of Pi released/h/mg protein)			
Neonatal	1.64±0.22	0.94±0.04 (−42.68)	1.54±0.35 (−6.10)
Juvenile	0.89±0.05	0.63±0.13 (−29.21)	1.97±0.24 (+121.34)
Young adult	2.99±0.22	1.83±0.48 (−38.79)	3.33±0.22 (+11.37)
Ca2+ATPase (µmole of Pi released /h/mg protein)			
Neonatal	4.17±0.07	2.94±0.11 (−29.50)	3.47±0.29 (−16.79)
Juvenile	0.57±0.05	0.23±0.03 (−59.65)	0.37±0.04 (−35.09)
Young adult	4.42±0.24	3.20±0.20 (−27.60)	3.49±0.26 (−21.04)
Mg2+ATPase (µ mole of Pi released /h/mg protein)			
Neonatal	1.42±0.35	0.84±0.42 (−40.85)	1.09±0.33 (−23.24)
Juvenile	4.16±0.10	1.55±0.41 (−62.74)	1.94±0.21 (−53.37)
Young adult	5.24±0.14	3.03±0.14 (−42.18)	3.79±0.38 (−27.67)
		1.20±0.16 (−13.66)	1.23±0.16 (−11.51)

Results are presented as mean ± S D, *n* = 6. Values in parenthesis indicate % change over CPF. '−'sign indicates decrease and '+' sign indicates increase.

at the post synaptic site leading to over sensitization of receptors and may cause neurodegeneration. The ChAT activity provides insight into the potential rate of ACh biosynthesis and is often used as a prototype marker for functional cholinergic neurons. The decreased ChAT activity observed in this study on exposure to CPF could be through a compensatory mechanism and also related to changes in high affinity for choline uptake as a result

of oxidative stress. Similar to the effect on ChAT in this study, Slotkin *et al*.[4] reported reductions in the ChAT activity on post natal day 30 and 60 in rats following subcutaneous exposure.

Membrane bound neuronal ATPases have a central role in performing physiological functions of cells as energy transducers by coupling the chemical reactions of ATP hydrolysis. The decreased Na^+, K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase activity observed in this study may be due to the impairment in the energy metabolism,[23] this attributes to the lipophilic nature of the CPF and its direct action on the enzymes in general. The synthesis of ATP by phosphorylation of ADP is mainly associated with glycolysis and biological oxidation involving the citric acid cycle and electron transport systems;[24] thus, the decreased ATPase activity observed in this study through the lipophilic action of CPF seems to be possible cause. It is tempting to speculate from the earlier studies of Anugya Mehta *et al*.[2] as well as this study that decreased the ATPase activity may be due to the altered membrane structure and function as a consequence of AChE inhibition and oxidative stress[10] rather than the direct effect of CPF on the ATPase.

In this study, different age group of rats were exposed to 15°C and 20°C temperatures and results have shown decreased activity levels of AChE and ChAT that may lead by the production of free radicals. Further, aforesaid biochemical changes are more apparent at 15°C compared to 20°C. It is to be noted that the cold stress can cause an increase in the oxidative stress as previously reported[10] that might have triggered the suppression of Na^+, K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase on exposure to cold stress. In addition, cold stress activate hypothalamic-pituitary-adrenal axis, subsequently release corticosteron from the adrenal cortex into the bloodstream[25] that in turn accelerates the generation of free radicals.[26]

In developing animals, activity levels of AChE, ChAT, Na^+, K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase decreased markedly compared to adult age group as found in this study indicates vulnerability of CNS to toxic potential of CPF and cold stress. A decreased sensitivity was observed in developing animals than the adult group primarily because of lower level of detoxifying enzymes. The greater neonatal sensitivity was observed in this study primarily due to the lack of complete metabolic competence.[27] Young animals were unique because of the immature nervous system which responded differentially to the exposure of CPF with its kinetic ability (absorption, distribution, metabolism, and excretion). Perhaps young rat brain as a whole appears to be susceptible to injury by free radicals; the cerebellum and medulla oblongata are observed to be more prone to oxidative damage as indicated from our recent study.[10]

OP pesticides pose the threat of hypothermia, because they amplify body temperature decline that accompanies cold exposure.[28] In this study, when different age group of animals exposed to CPF toxicity at the temperatures of 15°C and 20°C, their interaction exacerbated resulting in a synergistic action and substantially modified the toxicity in cerebral cortex indicating potential effect of CPF and the quantum of synergistic interaction was most apparent at 15°C. In line with our findings, earlier studies[8] 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 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 lipidperoxidation because of their interaction[10] and brought changes in the activities of marker enzymes studied.

In conclusion, present findings have importance in understanding modulatory effect of CPF brought by cold stress and age. The synergistic interaction of CPF and cold stress cause biochemical alterations in CNS of different age group rats in a region specific manner. Furthermore, results reveal that young animals are markedly more sensitive than adults.

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