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ALLEVIATION OF CHLORPYRIFOS-INDUCED DELAYED NEUROTOXICITY WITH VITAMIN E AND PHENYTOIN IN HENS.

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ABSTRACT

The objective of the study was to see if oxidative damage is involved in organophosphorus-induced delayed neurotoxicity (OPIDN) and its subsequent alleviation through antioxidants. A total of 72 hens aged 56 weeks were divided into 4 groups (n=18).Group 1 sham, Groups 2, 3 and 4 were administered chlorpyrifos (CPS) @ 350mg/kg BW s.c. in divided doses over a period of 24 hrs. To prevent death due to cholinergic toxicity, atropine and 2-PAM were administered. In group 3, vit. E was administered @ 50mg/kg p.o 10 days prior to administration of CPS and in group 4 phenytoin @ 50mg /kg p.o.was administered for 5 days prior to CPS. Signs of OPIDN were scored. Acetyl cholinesterase (AchE) activity in distal spinal cord served as a biomarker of exposure. Sciatic nerve total calcium was estimated, since Ca^{2+} is implicated in generation of ROS and in phosphorylation of proteins. Thiobarbituric acid reacting substances (TBARS), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase were estimated to assess lipid peroxidation. CPS produced signs of OPIDN that were reversed on 10th day. There was a decline in AChE activity. Oxidative stress with CPS was manifested by a significant rise in TBARS, increased activity of SOD, catalase and decline in glutathione levels. The above parameters were significantly lowered with prior administration of vitamin E. Phenytoin offered mild protection to the birds. Hence, it is concluded that oxidative stress could be one of the mechanisms of OPIDN and antioxidants might provide a viable therapeutic regimen for alleviation of OPIDN.

KEY WORDS: Chlorpyrifos, phenytoin, OPIDN,oxidative stress, ROS, Vit E,

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INTRODUCTION

Organophosphorus compounds are known for their non-selective toxicity. The toxicity is wide and varied ranging from acute cholinergic crisis, intermediate, delayed neuropathy and chronic neuro behavioural abnormalities[1]. The mechanism of acute toxicity and delayed neuropathy differ in the sense that cholinesterase is inhibited in the former where as inhibition of NTE is said to be involved in the later[2]. NTE has no known physiological function and NTE knockout mice too showed delayed neuropathy implying that more than NTE, other mechanisms could play a role in development of OPIDN[3,4,5] . In the recent past, many studies have defined the role of free radicals in varied forms of OP toxicity more so of the delayed and the chronic types. Added to the above, increased intracellular Ca^{2+} causes phosphorylation of cytoskeletal proteins actin and tubulin results in peripheral axonopathy[6,7]. Based on this background, Vit E and phenytoin, a known inhibitior of kinase phosphorylation[8,9]were studied in alleviating the OPIDN produced by a lipophilic OP pesticide CPS in the only experimental animal model, the adult hen[10,11].

MATERIALS & METHODS

i. Chemical Reagents

Chlorpyrifos 99% (Tech) was obtained from National Plant Protection Training Institute (NPPTI), India. Phenytoin (Eptoin) was procured from Kare Labs, Goa. Other chemicals and reagents were obtained from Sisco Research Laboratories, Mumbai, India.

ii. Animals

White leghorn layers aged 56 weeks old (weighing 1.0 – 1.2 kg) were procured from a commercial poultry farm, Hyderabad, Andhra Pradesh. The animals used in this study were approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The birds were housed in the deep litter system at a temperature of 28 + 2°C and relative humidity of 50%.

iii. Experimental procedure

A total of 72 layers were randomly divided into 4 groups consisting of 18 in each group.
The duration of the study was 21 days. All the groups were maintained as per the following treatment schedule.

Group 1: Control. Treated with vegetable oil(solvent)
Group-2: CPS @ 350mg/kg BW s.c. three times a day in divided dose,
Group-3: CPS + Vit.E@ 50mg/bird/day orally for 10 days prior to administration of CPS
Group-4: CPS + Phenytoin @ 50mg/bird/ two times a day/orally/ 4 days prior to administration of CPS.

iv. Collection and preparation of samples

Spinal cord and sciatic nerve were collected at the end of 21 days. The spinal cord was collected in 10% Tris HCl (Ph 7.4) and was homogenized and centrifuged at 10000 g for 15 min and the supernatant was analyzed for thiobarbituric acid reacting substances (TBARS), GSH, SOD, catalase and acetyl cholinesterase. Sciatic nerve was preserved in 10% neutral buffered formalin for histopathological investigations. Sciatic nerve was collected for estimation of total nerve calcium by flame photometry.

v. Clinical Evaluation

Treated hens were examined daily to detect abnormalities in gait or posture. Muscarinic signs such as weakness, salivation and diarrhoea were observed to assess acute cholinergic toxicity. Signs of OPIDN were scored[12]as:
Stage 1; Leg weakness, reluctance to walk, stiffness.
Stage 2: Mild ataxia, change in gait.
Stage 3: severe leg weaknesses, falling on floor.

Stage 4: Unable to stand, curled toes, balancing on wings (typical signs of OPIDN).

vi. Acetyl Cholinesterase activity (AchE) activity

Acetyl Cholinesterase activity was measured by as per the procedure described by[13] . It is a specific esterase that catalyses the hydrolysis of a neurotransmitter acetyl choline. The acetyl group reacts with alkaline hydroxylamine to form acetyl hydroxamate, which then reacts with ferric chloride in acid medium to form a coloured complex that can be read at 540nm.

vii. Estimation of total nerve calcium (Flame Photometry)

Total nerve calcium was estimated by flame photometry following acid digestion with concentrated nitric acid and perchloric acid in 4:1 ratio.

viii. Measurement of Thiobarbituric acid reacting substances(TBARS)

Brain TBARS levels were measured as per the procedure described by[14]. 500 µl of supernatant from the homogenate, 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid were taken in a tightly Stoppard tube. The tube was heated to boiling temperature for 45 min. cool the tube and the contents were centrifuged. The supernatant was read at 532 nm against blank. The concentration of test samples was obtained using molar extinction coefficient of MDA.

ix. Measurement of Glutathione (GSH)

Brain GSH levels were measured as per the procedure described by[15]. 100 µl of 25% trichloroacetic acid was added to 400 µl of homogenate, centrifuged and collected supernatant and used as sample. To 2.0 ml of 0.6 mM 5-5' dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate (pH 8), 0.1 ml of sample and 0.9 ml of 0.2 M phosphate buffer was added and the absorbance was read at 412 nm against a reagent blank. The standards (0.05-5 mg/ml) were also treated in the same way.

x. Estimation of Superoxide dismutase (SOD)

Superoxide dismutase measured as per procedure described by[16] .Three test tubes labeled as Blank (B), Standard (S) and Test (T). 0.65 ml of PBS, 30µl of MTT was added in all three test tubes. Then 10µl of sample added to Test and 75µl of pyrogallol was added to Test and Standard Then mixed well and incubated at room temperature. Exactly after 5 min, added 0.75 ml of DMSO to the test, standard and blank. Then measured the absorbance of the purple colour formed at 570nm.

xi. Estimation of Catalase

Catalase measured as per procedure described by[17]. To assay mixture containing 0.4 ml of 0.2 M H_2O_2 and 0.5 ml of 0.01 M phosphate buffer (pH 7), 0.1 ml of haemolysate was added and mixed well. Into this, 2ml of dichromate acetic acid solution was blown exactly after 60 sec. Then kept in boiling water bath for 10 min. Read the absorbance of greencoloured chromic acetate at 570 nm against reagent blank containing 0.4 ml of 0.2 M H_2O_2 and 0.5 ml of 0.01 M phosphate buffer (pH 7).

xii. Estimation of proteins

Proteins measured as per procedure described by[18] .100 µl of sample was made up to 1.0 ml with distilled water. To this, 5 ml of freshly prepared alkaline copper sulphate solution (a mixture of 50 ml of 2% sodium carbonate in 0.1 N sodium hydroxide and 1.0 ml of 0.5% copper sulphate in 1% potassium sodium tartrate) was added and kept for 10 min at room temperature. 0.5 ml of Folin-ciocalteu reagent was added and allowed to stand at dark for 30 min. The resultant blue colour was read at 660 nm against distilled water blank. Known concentrations of bovine serum albumin

ranging from 5 -50 mg/ml added in the place of sample was used as standard.

xiii. Histopathology

Tissue pieces of sciatic nerve were collected from the birds that were sacrificed on 3rd, 7th, 10th and 14th day and fixed in 10% neutral buffered formalin (NBF) for histopathological studies. The fixed tissues were processed and stained with Hematoxylin and Eosin (H & E) stain as described by [19] and special staining Woelcke's method by[20].

xiv. Analysis of variance

Results were expressed as mean ± S.E. One-way analysis of variance (ANOVA) by SPSS (Statistical Package for Social Sciences) (Ver. 10.00) followed by Duncan test was used to analyze the results with $p < 0.05$ considered significance.

RESULTS

i. Clinical Signs

The maximum number of birds affected in group 2 exhibited stage IV signs. In group 3, minimum number of animals were affected and showed very feeble symptoms. Whereas in group 4, stage II signs were observed (Table 1 & Fig 1, 2, 3, 4).

Figure 1
Photograph showing bird suffering from Acute OP toxicity

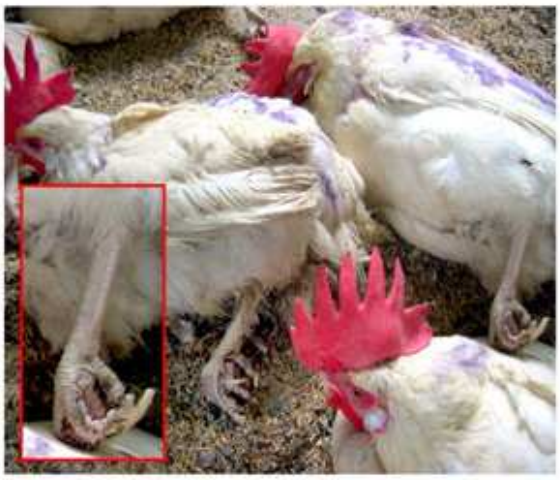

Figure 2
Photograph showing group 2 bird with curled toes on 6th day (a characterstic sign of OPIDN)

Figure 3
Photograph showing group 2 bird balancing on wings

Figure 4
Photograph showing birds of group 2 and group 3 (arrow)

ii. Acetyl Cholinesterase (AChE)

Group 1 did not showed any significant (P > 0.05) difference in AChE levels throughout the experiment. Group 2 showed significant (P < 0.05) inhibition of choline esterase on 3rd day (3.1728 ± 0.9884) and 7th day (5.18 ± 0.2118) when compared to group 1. The values did not differ significantly on 10th and 14th day (5.8175 ± 0.1545; 5.95 ± 0.7246). Groups 3 and 4 did not show any significant difference in enzyme levels in comparison to group 2 throughout the experiment (Table 2).

iii. Total Nerve Calcium (Ca2+)

Calcium levels in group 2 revealed a significant (P < 0.05) increase (38.6013 ± 0.7826; 37.1683 ± 0.8873; 33.49 ± .1366 ; 34.1225 ± 0.906) when compared to group 1. Group 3 revealed no significant difference in comparison to group 2. The total nerve calcium in group 4 was significantly (P < 0.05) lower when compared to groups 2 and 3 on 3rd and 7th day (Table 2).

iv. TBARS

In group 2, the MDA concentration significantly (P < 0.05) decreased on 14th day (2.2430 ± 0.4409) when compared with the concentration on 3rd day. Group 3 exhibited a significant (P < 0.05) increase in MDA concentration when compared to group 2 throughout the experiment. The results were) similar on 7th and 10th day but MDA concentration was significantly (P < 0.05) declined on 14th day and was comparable to group 1 value on 14th day. In group 4 MDA concentration differed but non-significantly when compared with group 2 on all the days of estimation (Table 2).

v. Glutathione

Glutathione levels were significantly (P < 0.05) reduced in groups 2 and 4 compared to control throughout the experiment. In group 3, administration of Vit E + OP caused a significant (P< 0.05) increase in glutathione levels on comparison with group 2 throughout the experiment (Table 2).

vi. SUPER OXIDE DISMUTASE (SOD)

SOD activity in group 2 was significantly (P < 0.05) increased (15.4793±0.7609; 12.867 ± 0.6278; 10.6905±0.6031; 10.203 ± 0.6307)

throughout the experiment. In group 3, a significant (P < 0.05) fall in enzyme activity was observed in comparison with group 2. Group 4 exhibited a significant (P < 0.05) increase in SOD activity that was in tune with group 2 values throughout the experiment (Table 2).

vii. Catalase (CAT)

CAT activity was significantly (P < 0.05) increased in group 2 (4.5075±0.3062; 4.02 ±

0.9857; 3.5775 ± 0.1283; 3.0425 ± 0.2527) compared with group 1 throughout the experiment. In group 3, a significant ($P < 0.05$) fall in enzyme activity was seen in comparison to group 2. No significant difference in enzyme activity was observed between group 4 and group 2 throughout the experiment (Table 2).

viii. Histopathology

The histopathology of Sciatic nerve revealed axonal degeneration and subsequent wallerian degeneration of myelin in group 2 (Fig 5 & 6) and group 4. However, the above noted changes were mild in group 3 (Fig 7 & 8).

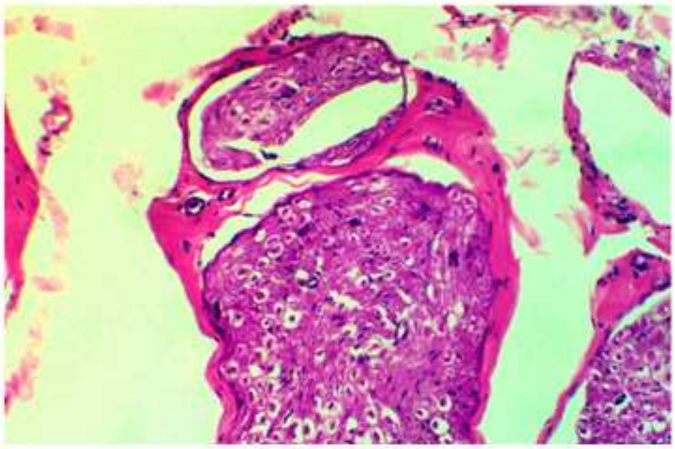

Figure 5
Microphotograph of sciatic nerve showing myelinated nerve fibres. Woelcke's x 500 (Group 2)

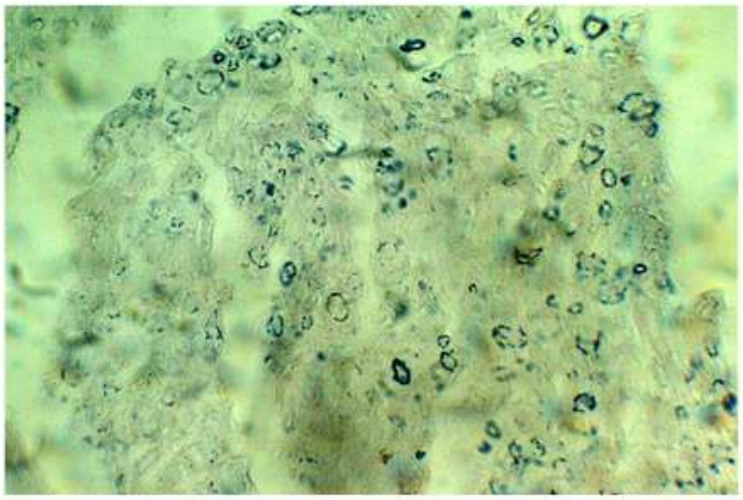

Figure 6

Micro photograph of sciatic nerve showing mild degenerated myelin sheath. Woelcke's method x 500 (Group-2)

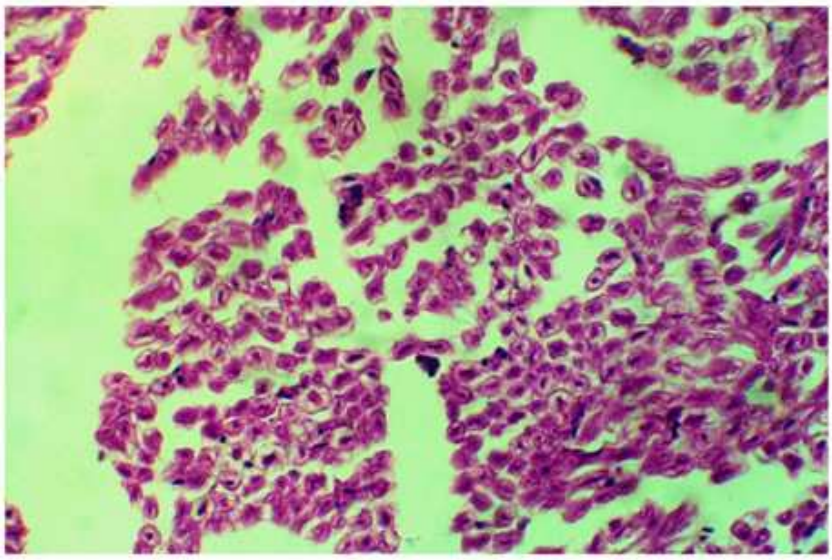

Figure 7

Micro photograph of sciatic nerve showing myelinated nerve fibres. H & E x 500 (Group 3)

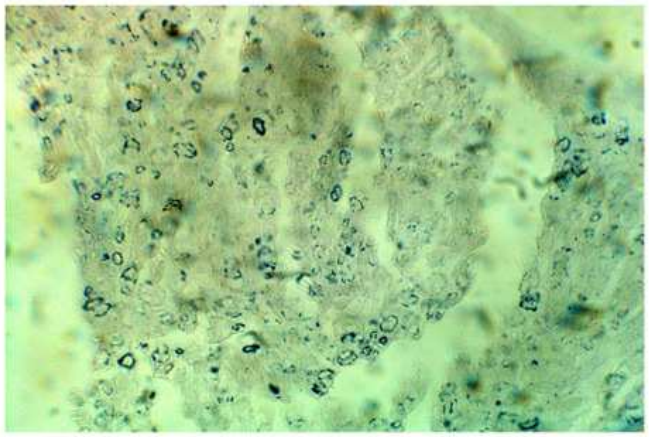

Figure 8
Micro photograph of sciatic nerve showing myelinated nerve fibres. Woelcke's x 500 (Group 3)

DISCUSSION

Organophosphorus compounds are known to produce delayed neurotoxicity in hens. Hence, administration of CPS @ 350 mg/kg s.c revealed classical signs of OPIDN. Group 2 birds exhibited signs of ataxia, incoordination of gait, curling of toes, twisting of neck, torticollis, balancing on wings, paresis, and prostration which were pronounced from 4th to 10th day followed by gradual recovery. The recovery might have been due to reversible leg weakness and ataxia shown by chlorpyrifos, which was classified as OPIDN[12]. In group 3, Birds showed extensive improvement in signs of OPIDN, body weight gain and egg production due to administration of Vit E, a known anti-oxidant. Phenytoin group also showed improvement in external signs compared to OP control. CPO a metabolite of CPS is the most potent among known inhibitors of AChE[2,3]. AChE levels in posterior part of spinal cord were significantly ($p<0.05$) inhibited in group 2 by 50% on 3rd and 15% of control on 7th day indicating time dependent, yet adequate regeneration of the enzyme, whereas on 10th and 14th day no significant change was observed. 50% inhibition of AChE on 3rd day could have been due to limited protection offered by atropine sulphate and the normal AChE levels at 14th day may have resulted due to recovery of the enzyme[3,21]. Groups 3 and 4 did not differ significantly with control. AntiChE's cause induction of presynaptic repetitive discharge leading to augmented post junctional response[22] due to entry of Ca^{2+} into motor nerve terminals followed by excessiveneuro transmitter release[23]resulting in accumulation of ACh, which would, centrally, release glutamate causing an excessive inflow of Ca^{2+}leading to generation of ROS, peroxynitrite radical and activation of protein kinases[24] . The end result would be the degeneration of the terminal axon. Hence, total nerve Ca^{2+} in sciatic nerve was estimated. In group 2 there was a significant ($P<0.05$) increase in total Ca^{2+}in comparison to control indicating the effect of cholinesterase inhibitors in increasing Ca^{2+} levels through mechanisms discussed above. In group 3 the increase in Ca^{2+} was evident till 7th day and thereafter were non-significant in comparison with group 2 but significant ($P<0.05$) in group 1. In group 4 too, Ca^{2+} was high compared to sham but a significant decrease was noted when compared with group 2. The results are in conformity with the observations with phenytoin [23] and with nifedipine[25] . The findings of this study further confirm the Ca^{2+} antagonistic action of phenytoin by blocking the movement of action across the cell membrane and preventing

repetitive discharges[9]. This was exceedingly evident in the exhibition of OPIDN signs in group 2 and their subsequent improvement in groups 3 and 4. Cholinesterase inhibition leads to oxidative stress by[26,27]. Therefore, anti-oxidant parameters TBARS, GSH, SOD and Catalase were estimated to confirm the role of reactive oxygen species in producing OPIDN. The TBARS values were high in groups 2 and 4. Similar findings were observed with chlorpyrifos in rat brain[28,29]. Vitamin E decreased production of TBARS, which is in agreement with diazinon[30]; dimethoate and malathion[27]. Prior administration of vitamin E for prophylaxis or administration of large doses for treatment seems to be a novel approach.

Phenytoin, an inhibitor of protein phosphorylation[9]administered @ 50 mg per kg for 5 days blocked the entry of Ca++. TBARS level in group 4 were increased; phenytoin might have been bio activated by peroxidase into a free radical itself[31]and could have synergized with CPS. Following treatment with vit.E, the rise in TBARS levels was restored to normal. GSH content fell significantly in group 2 and 4. There was a significant recovery on tenth day compared to third and seventh days. However, in group 3.the glutathione concentration decreased. This decrease was significantly less compared to group 2 but significantly more in comparison with group 1.

It ranged in between group 2 and group 1 indicating the protective action of vitamin E in quenching the free radical and thus decreasing glutathione consumption. Groups 2 and 4 registered an increase in superoxide dismutase and catalase activity indicating increased generation of super oxide anion under the influence of CPS. In contrast, group 3 showed a significant fall in enzyme levels which were below group 2 and high compared to group 1. The finding coincides with vitamin E and dimethoate[27], melatonin, vitamin C and E against CPS ethyl in lungs[32], kidney[33] . The increase in SOD and CAT activity in group 4 could be due to the role of phenytoin as discussed above. Histopathological examination revealed demyelination and degeneration of axons of sciatic nerve in all CPS treated groups. However, the changes were mild in vit E treated group. From the present study, it is concluded that CPS induces reversible OPIDN by increasing intracellular calcium and by impairing antioxidant defenses and thus favoring the progression of stress due to ROS. Vitamin E could offer significant protection by its antioxidant effect. Phenytoin accomplished moderate improvement by combating increased intracellular calcium level.

Table 1
Effect of subcutaneous dose of CPS @350mg/kg on development of clinical signs in adult hens

Group	Treatment	Day of onset	Day of recovery	Total no. of birds (n=18)	Stage I	Stage II	Stage III	Stage IV	Total Affected	Total Unaffected
1	Nil	-	-	18	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	18 (100.00)
2	CPS @350mg/kg	3	10	18	2 (11.11)	0 (0.00)	3 (16.67)	11 (61.11)	16 (88.89)	2 (11.11)
3	Vit.E @50mg/kg + CPS @350mg/kg	3	4	18	8 (44.44)	0 (0.00)	0 (0.00)	0 (0.00)	8 (44.44)	10 (55.55)
4	Phenytoin @50mg/kg + CPS @350mg/kg	3	7	18	9 (50.00)	4 (22.22)	0 (0.00)	0 (0.00)	13 (72.00)	5 (27.77)

Table 2
Effect of subcutaneous dose of CPS @350mg/kg on various parameters

Parameters	Group 1				Group 2				Group 3				Group 4			
Days	**3**	**7**	**10**	**14**	**3**	**7**	**10**	**14**	**3**	**7**	**10**	**14**	**3**	**7**	**10**	**14**
AChE (µM/50µl/10min)	6.11 ± 0.17 de	6.07 ± 0.25 de	6.30 ± 0.21 de	6.30 ± 0.30 de	3.17 ± 0.19 a	5.18 ± 0.42 b	5.81 ± 0.30 cd	5.95 ± 0.14 cde	3.39 ± 0.18 a	5.55 ± 0.39 bc	6.16 ± 0.55 de	6.23 ± 0.36 de	3.24 ± 0.15 a	5.31 ± 0.40 b	5.92 ± 0.23 cde	6.06 ± 0.22 de
Total Nerve Calcium (ppm)	29.66 ± 0.90 a	30.24 ± 0.85 ab	30.21 ± 0.85 ab	31.05 ± 0.73 abc	38.60 ± 0.78 g	37.16 ± 0.88 fg	33.49 ± 0.13 cde	34.12 ± 0.90 de	36.16 ± 0.88 efg	35.70 ± 0.54 ef	32.65 ± 0.99 bcd	32.19 ± 0.93 abcd	32.12 ± 0.85 abcd	31.39 ± 0.61 abcd	30.82 ± 0.92 abc	31.22 ± 0.98 abcd
Glutathione (mg/g of protein)	5.41 ± 0.26 e	5.14 ± 0.87 e	5.22 ± 0.99 e	5.08 ± 0.16 e	2.49 ± 0.50 a	2.59 ± 0.13 a	3.33 ± 0.15 b	3.33 ± 0.10 b	3.60 ± 0.15 b	4.10 ± 0.15 cd	4.53 ± 0.19 d	4.41 ± 0.27 d	2.61 ± 0.11 a	2.61 ± 0.89 a	3.48 ± 0.35 b	3.84 ± 0.13 bc
TBARS (nM/g of protein)	1.20 ± 0.96 a	1.34 ± 0.41 ab	1.26 ± 0.55 ab	1.28 ± 0.36 ab	2.53 ± 0.68 gh	2.62 ± 0.69 h	2.38 ± 0.98 efg	2.24 ± 0.44 ef	1.77 ± 0.94 d	1.73 ± 0.74 d	1.57 ± 0.81 cd	1.44 ± 0.83 bc	2.44 ± 0.51 fgh	2.45 ± 0.39 fgh	2.21 ± 0.93 e	2.19 ± 0.73 e
SOD (U/min/mg of protein)	7.59 ± 0.36 ab	8.42 ± 0.33 abc	7.41 ± 0.45 a	7.49 ± 0.34 ab	15.47 ± 0.76 i	12.86 ± 0.62 gh	10.69 ± 0.60 def	10.20 ± 0.63 de	11.40 ± 0.77 ef g	9.12 ± 0.38 bcd	8.15 ± 0.30 ab	8.46 ± 0.33 abc	14.22 ± 0.34 hi	12.07 ± 0.84 fg	10.90 ± 0.36 ef	9.8 ± 0.29 cde
CAT (µ moles/min/mg of protein)	2.12 ± 0.84 a	2.43 ± 0.84 abc	2.33 ± 0.16 ab	2.24 ± 0.18 a	4.50 ± 0.30 h	4.02 ± 0.9 fgh	3.57 ± 0.12 ef	3.04 ± 0.25 cde	3.84 ± 0.32 fg	3.14 ± 0.98 de	2.95 ± 0.14 bcde	2.42 ± 0.12 abc	4.39 ± 0.39 gh	3.95 ± 0.13 fgh	3.49 ± 0.21 def	2.89 ± 0.13 bc d

Values are Mean + SE (n = 6); One way ANOVA (SPSS) Means with different alphabets as superscripts differ significantly (P<0.05).

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