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Cinnamaldehyde Induces Behavioral and Biochemical Changes in the Male Albino Wistar Rat

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Abstract

Cinnamaldehyde (CNMA) is a widely consumed food flavoring. In addition, it is also used as an antiseptic and an antiallergic drug and as a tonic in traditional Chinese medicines. Certain behavioral parameters were carried out in this study to evaluate the effect of CNMA on rats. Rats were given CNMA orally by gavage at the dose levels of 2.14, 6.96, 22.62 and 73.5mg/kg body weight/day for the period of 10, 30 or 90 days. Only the group of rats treated with CNMA at the dose of 73.5 mg/kg body weight/day for 90 days showed significant changes in the olfactory discrimination, auditory startle response and negative geotaxis behavior. No treatment related impairment of cliff avoidance behavior was noted. Thus, CNMA has an effect on the neuromuscular system. This is evident from changes in acetyl cholinesterase (AChE) and creatine kinase (CK) activities in the serum of these rats. Further examination of non-enzymatic and enzymatic antioxidants and also thiobarbituric acid reactive substances (TBARS) in the serum showed CNMA induced oxidative stress that resulted in the behavioral changes of these rats. From this study, we can conclude that the oxidative stress induced by CNMA has an effect on rat behavior and its effect is time and dose dependent.

Keywords: Behavior, cinnamaldehyde, food flavor, rat, serum enzymes.

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1. Introduction

Cinnamaldehyde (CNMA) is a pale yellow liquid with a warm, sweet, spicy odor and pungent taste reminiscent of cinnamon. It occurs naturally in the leaves and twigs of various species of the genus *Cinnamomum*. CNMA is commercially prepared by the condensation reaction of benzaldehyde and acetaldehyde.[1] It is used in foods, beverages, medicinal products, perfumes, cosmetics, soaps, detergents, creams, and lotions. CNMA is often used as an antiseptic and an antiallergic drug and as a tonic in traditional Chinese medicines.[2] It has been used as an animal repellent, an insect attractant, and an antifungal agent.[3] It is used effectively in air freshners where odor neutralization can be accompanied by reaction with sulfur and nitrogen malodorants.[1]

CNMA is chemically related to toxicologically more active compounds like acrolin and crotanaldehyde.[4] Besides CNMA, acute toxicity of other major components of *Cinnamomum* - cinnamic acid (5000 mg/kg body weight) and cinnamyl alcohol (4000 mg/kg body weight) was also noticed in rats. [5] CNMA in tooth paste is suggested to be the reason for allergic contact dermatitis and allergic contact stomatitis.[6] North American Contact Dermatitis Research Group suggested that CNMA might be a frequent cause of allergic reactions to perfumes. [7] Thus, concern about the safety of CNMA in general has been raised. The WHO

which established a temporary acceptable daily intake (ADI) of 0.7mg/kg body weight for CNMA in 1984 was unable to extend this level because of inadequate toxicity data and later, the committee suggested that the metabolic and pharmacokinetic data be revised. [8]

Behavioral toxicology has assumed a role of increasing importance in the evaluation of food chemicals in recent years before it is recommended for human use.[9] We have previously studied CNMA induced oxidative stress [10] and oxidative stress mediated changes in the hematological profiles[11] and also the histological architecture of kidney of rats.[12] In this study, we measured behavioral and biochemical changes in order to assess the possible adverse effects of CNMA in rats.

2. Materials and Methods

2.1. Materials

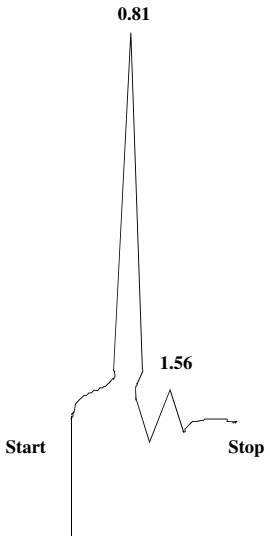
Food grade CNMA was purchased from Basil & Co., Madras, India. All other chemicals for biochemical studies were obtained from Sigma Chemical Co., St. Louis, USA and SD Fine Chemicals Ltd., Bombay, India. The materials required for behavioral measurements were procured from the Unit of Neurobiology, Department of Zoology, School of Life Sciences, University of Madras.

2.2. Analysis of Purity of CNMA

The purity of CNMA was analysed by gas chromatography (Hewlett Packard 5890) using the standard protocol at the Dept. Chemistry, IIT, Chennai, India. Carbowax 20 M column of diameter 1/8 inch was used in this process. Column and injection temperature were at 140˚ C and 150˚ C respectively. Detector temperature was recorded as 480˚C with N_2 as carrier gas and H_2 –air mixture as fuel. By this process, the purity of food grade CNMA was observed to be 98 % which is shown in *Fig. (1)*.

2.3. Animals, Experimental Diet and Treatment

Weaning male albino rats, *Rattus norvegicus* derived from the Wistar strain, weighing 100± 10g were obtained from FIPPAT, Padappai, Chennai, India. The rats were housed in well ventilated plastic cages. They were acclimatized to laboratory conditions for one week



	Area %	AR / HT	Area%
Cinnamaldehyde	0. 81	0.071	97.989
Cinnamic acid	1.56	0.257	2.011

Fig. (1). Analysis of purity of CNMA by gas chromatography.

before the experiment began. Animals were given pelleted diet for laboratory rats (Lipton India Ltd., Bangalore, India) and water, *ad libitum.* Composition of pelleted diet: Crude protein (21%), Ether extract (5%), Crude fiber (4%), Ash (8%), Calcium (1%), Phosphorous (0.6%), Nitrogen free extract (53%), Vitamins A, C & D, Vitamin E, Vitamin K, Vitamin B12, Thiamine, Riboflavin, Pantothenic acid, Niacin, Choline chloride, Folic acid, all minerals and trace elements (Technical Information Bulletin, Lipton India Ltd., Bangalore, India).

Since human exposure to CNMA occurs through ingestion, it was given orally by gavage to the test rats at the dose levels of 2.14, 6.96, 22.62 and 73.5mg/kg body weight/day for 10, 30 and 90 days. The low dose level (2.14mg/kg body weight/day) approximates the estimated human exposure to CNMA[4]. The other dose levels are in geometric proportion. The dose level 73.5mg/kg body weight is 100 times more than the acceptable daily intake (ADI) level of cinna-

maldehyde-0.7mg/kg body weight. This dose was chosen based on the report of Pearson's standard, [13] that is, the animals must be fed with the additives in amounts at least 100 times greater than that is likely to occur in the human diet. The safety factor of 100 allows for differences between test species and humans (10 fold) and differences between individuals (10 fold).[14] We followed the same dose level principles to evaluate CNMA induced oxidative stress in our previous studies.[15] All experiments were done under the Ethical Guidelines for Animal Experiments in the University of Madras.

2.4. Behavioral Measurements

At the end of each experimental period, behavioral measurements were carried out to assess the effect of CNMA. Olfactory function was tested as described by Voorhees *et al.* [16] Rats were placed on a board covering half each of two cages placed one after another. One cage was covered with the home bed covering with which the animals were familiar and the other was covered with fresh (unused) husk. The time required to enter the home bed covering cage was recorded with a stop watch. The time was recorded in terms of seconds. Auditory startle response was tested as described by Voorhees.[17] Auditory startle response was elicited by a stimulus using a child's toy clicker. The rat was placed on a flat surface. The response of the rat to the stimuli was a jerk of the head and legs essentially synchronous with the sharp click. Presence or absence of an auditory startle response was determined for 3 clicks (or 3 trials). Cliff avoidance reaction was measured following the method of Adams.[18] A rat was placed on a table edge with the forepaws and nose over the edge. The amount of time required to complete backing and turning away from the edge was recorded. Each rat was tested in one trial. The number of rats with successful responses within 30 seconds was recorded. Negative geotaxis behavior was measured according to the method of Voorhees *et al.*[16] Rats were placed facing downward on a 25° inclined plane and the time (in seconds) to rotate through 180° to a head-up orientation was noted.

2.5. Body Weight and Food Intake

Body weight and food intake were measured at the end of the experimental period (*Table 1*).

2.6. Biochemical Assays

Protein content was measured by the method of Lowry *et al.*[19] using bovine serum albumin as a standard. Acetylcholinesterase (AChE; EC 3.1.1.7) was assayed using acetylthiocholine as substrate by the method of Ellman *et al.*[20] and the released thiocholine was measured spectrophotometrically at 412 nm. Creatine Kinase (CK; EC 2.7.3.2) was measured by the method of Okinaka *et al.* [21] CK catalyses the transfer of a phosphoryl group from phosphocreatinine to ADP to form ATP.

The activity of Catalase (CAT; EC 1.11.1.6) was assayed by the method of Sinha. [22] In this assay, the chromic acetate formed from dichromate was measured at 610 nm in a Hitachi 320 spectrophotometer. Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of Misra and Fridovich. [23] The amount of enzyme required to produce 50% inhibition of epinephrine to adrenochrome transition was taken as one unit. Glutathione peroxidase (GPX; EC 1.11.1.9) was measured by the method of Rotruck *et al.*[24] with some modifications. The glutathione formed while in the reaction reacts with the DTNB forming a compound which has an absorbance maxima at 412 nm.

The method of Okhawa *et al.* [25] was adopted for determining the level of thiobarbituric acid reactive substances (TBARS). This method is based on the principle that the compound formed between the aldehyde products formed during lipid peroxidation and TBARS absorbs maximally at 535 nm. Ascorbic acid was measured by the method of Omaye *et al.* [26] Ascorbic acid is oxidized by copper to form dehydro ascorbic acid and diketo glutaric acid. These products react with 2, 4 – dinitrophenyl hydrazine (DNPH) to form a compound, which in strong H_2SO_4, undergoes

Table 1. Effect of Cinnamaldehyde (73.5 mg/ kg body weight / day) on serum parameters of rats treated for 90 days. Values are Mean ± SD for 6 animals in each group

Serum parameters	Control	Test	P - value
Marker Enzymes (U / L)			
Acetyl cholinesterase	6747.2 ± 299.0	5012.0 ± 428.3	p < 0.001
Creatine kinase	169.9 ± 19.0	266.3 ± 24.3	p < 0.001
TBARS (mmol / L)	1.9 ± 0.2	3.7 ± 0.2	p < 0.001
Non enzymatic antioxidants (μg / ml)			
GSH	6.62 ± 0.92	2.20 ± 0.46	p < 0.001
Ascorbic acid	9.82 ± 0.82	3.74 ± 0.62	p < 0.001
α - tocopherol	9.32 ± 1.37	3.30 ± 0.40	p < 0.001
Enzymatic antioxidants (U / ml)			
Catalase	9.79 ± 0.76	19.5 ± 0.58	p < 0.001
Superoxide desmutase	0.53 ± 0.06	1.28 ± 0.15	p < 0.001
Glutathione peroxidase	11.44 ± 1.64	20.52 ± 2.64	p < 0.001

rearrangement to form a product with absorbance maximum at 520 nm. α-tocopherol was measured by the method of Desai. [27] Tocopherol reduces ferric to ferrous ions which will form a pink colored complex with bathophenanthraline. The method of Moron *et al.* [28] was followed to determine the total reduced glutathione. When glutathione reacts with dithiobis nitrobenzoic acid (DTNB), a compound is formed. This compound absorbs maximally at 412 nm.

2.7. Statistical Analysis

The statistical analyses were carried out by using the statistical software SPSS for PC 4.01 (Statistical Package for Social Sciences). Comparisons between duration and dose levels and also their interactions were performed with the two way analysis of variance. Duncan's multiple range tests with one way analysis was used to identify significant differences in body weight of the individual groups. Body weight, food intake, serum enzymes, serum antioxidants and TRARS were measured by means of Students *t* test. The minimum acceptable level of significance was p<0.05.

3. Results

Significant changes in olfactory discrimination (p< 0.001), auditory startle response (p< 0.001) and negative geotaxis (p< 0.001) were observed in rats treated with CNMA at the dose level of 73.5 mg/kg body weight/day for 90 days when compared with the normal behavior of the control rats. No significant change in cliff avoidance behavior reaction was observed in the above CNMA treated rats when compared with the normal behavior of the controls. The other groups of rats (rats treated with CNMA at the dose level of 2.14, 6.96, 22.62 and 73.5 mg/kg body weight/day for 10 and 30 days and the group of rats treated with CNMA at the dose level of 2.14, 6.96 and 22.62 mg/kg body weight/day for 90 days) did not show any change in any of the parameters (*Fig. 2*). In the ANOVA, the interaction effect of duration by dose level on olfactory discrimination (F=7.02, p< 0.001) and negative geotaxis (F= 41.8, p< 0.001) was significant.

We measured body weight, food intake, acetyl cholinesterase activity, creatinine kinase activity, TBARS and serum antioxidant levels only in the rats which demonstrated changes in olfactory discrimination, auditory startle response and negative geotaxis behavior. The following changes were observed in these rats (rats treated with CNMA at the dose of 73.5 mg/kg body weight/day for 90 days):

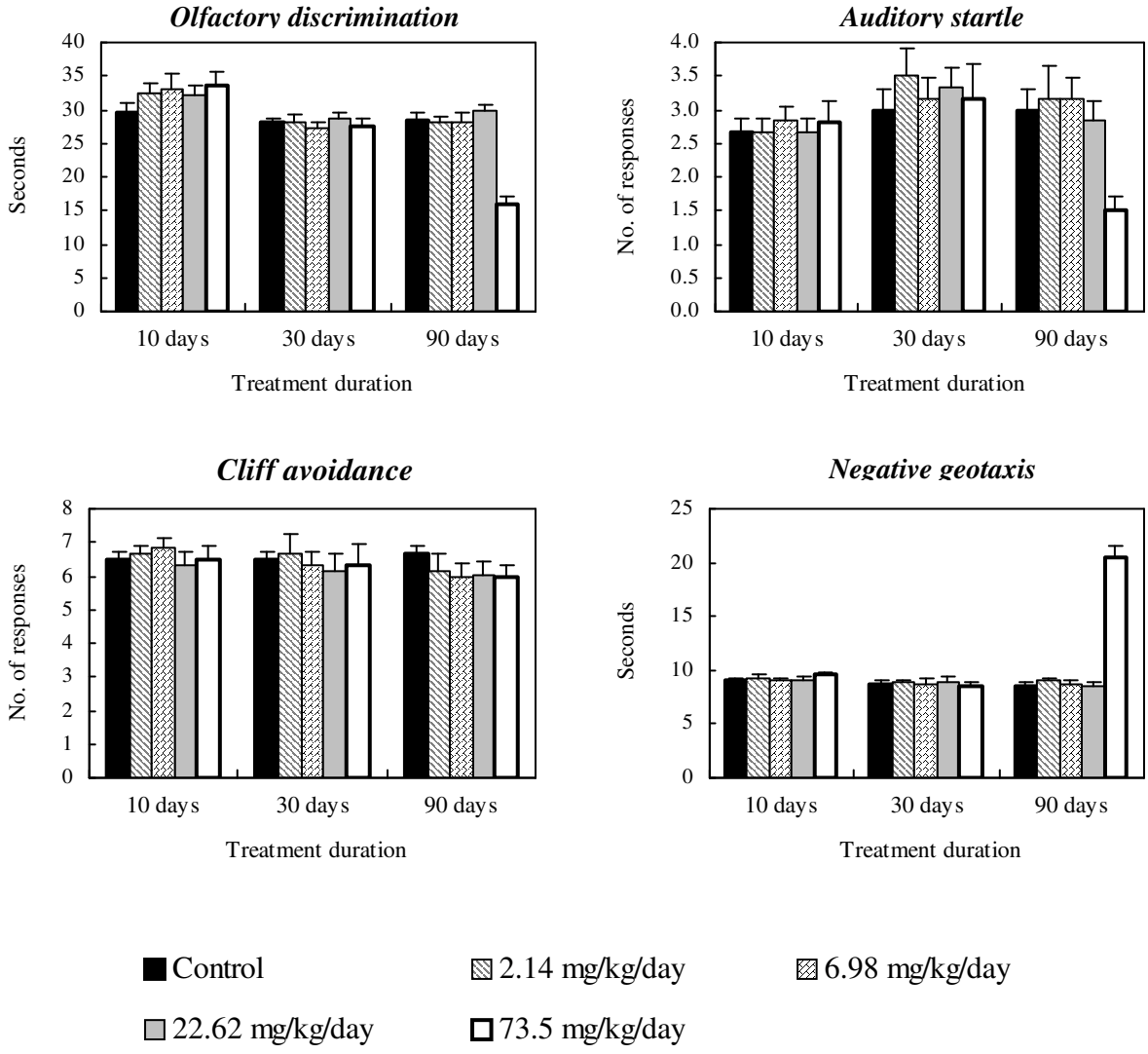


Fig. (2). Effect of Cinnamaldehyde (0, 2.14, 6.96, 22.62 and 73.5 mg/kg body weight/day) on behavioral parameters of rats treated for 10, 30 and 90 days. Values are Mean ± SD for 6 animals in each group. Significant changes in olfactory discrimination (p< 0.001), auditory startle (p< 0.001) and negative geotaxis (p< 0.001) and no significant change in cliff avoidance behavior were observed in rats treated with CNMA at the dose level of 73.5mg/kg body weight/day for 90 days when compared with the normal behavior of the control rats. ANOVA showed that the interaction effect of duration by dose level on olfactory discrimination (F=7.02, p< 0.001) and negative geotaxis (F= 41.8, p< 0.001) was significant.

body weight and food intake were decreased (p<0.001) (*Fig. 3 and Fig. 4*); serum AChE level was decreased (p<0.001); serum CK was increased (p<0.001) and TBARS in the serum were increased (p<0.001) when compared with that of the controls. The non enzymatic antioxidants - ascorbic acid (p<0.001), α-tocopherol (p<0.001) and reduced glutathione (p<0.001) - were decreased while the antioxidant enzymes – catalase (p<0.001), superoxide dismutase (p<0.001) and glutathione

peroxidase ($p<0.001$) - were increased in the serum of test rats (*Table 1*).

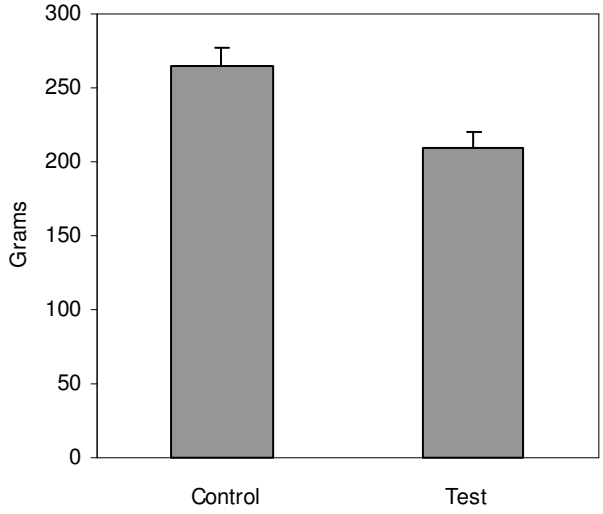


Fig. (3). Effect of Cinnamaldehyde (73.5 mg/kg body weight/day) on body weight of rats at the end of 90 days. Values are Mean ± SD for 6 animals in each group.

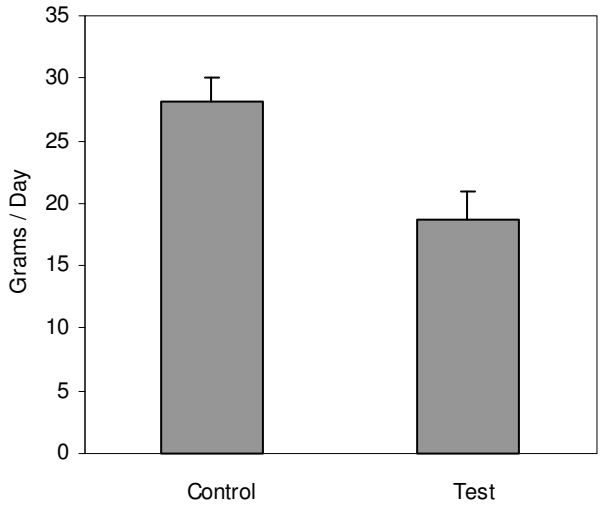


Fig. (4). Effect of Cinnamaldehyde (73.5 mg/kg body weight/day) on food intake of rats at the end of 90 days. Values are Mean ± SD for 6 animals in each group.

4. Discussion

In order to evaluate the effect of CNMA, we carried out different behavioral experiments in this study. Changes in olfactory discrimination, auditory startle response and negative geotaxis behavior were observed only in the group of rats treated with CNMA at the dose of 73.5mg/kg body weight/day for 90 days. No significant change in the cliff avoidance behavior reaction was observed in these rats. Animal's response to odors can be observed by the olfactory discrimination test. Recent studies have shown the relative change in olfactory discrimination behavior in mice fed with aliphatic aldehydes. [29] Auditory discrimination has been used to detect partial hearing loss. Loquet *et al.* [30] studied styrene induced auditory dysfunction in rats. Since CNMA has no effect on the cliff avoidance reaction, it reveals that the cognitive-behavioral function (the process of thought) is not altered in these rats. Cliff avoidance reaction is less sensitive to many of the toxicants. [31, 32] The sensory motor co-ordination is generally assessed by studying the negative geotaxis behavior. Studies of Malek *et al.* [33] show that formaldehyde has an impact on locomotor activity in rats. Chronic ethanol intoxication has an effect on the open field behavior, on antioxidant enzyme activities, and the degree of lipid peroxidation. [34] It is revealed from this study that CNMA has an effect on central nervous system. This is evident from the decreased serum AChE activity in the test rats. AChE is considered a biological marker for adverse toxicity [35] and neurotoxicity. [36] Previous reports show that acetaldehyde inhibits AChE and enhances CK activity in the serum.[37, 38] CK is an indicator of neuromuscular or skeletal muscle injury.[39] Acetaldehyde also affects mammalian neuromuscular transmission. [40]

Decreased body weight and food intake were also observed in these rats (rats treated with CNMA at the dose level of 73.5 mg/kg body weight/day for 90 days). Decrement in body weight is an expression of general toxicity or decreased food consumption.[41] Hebert *et al.* [42] observed dose related decrease in the terminal body weight of CNMA treated rats. Serum of the test rats (rats treated with CNMA at the dose level of 73.5 mg/kg body weight/day for 90 days) showed imbalance in the antioxidant status. Our group has previously observed CNMA induced peroxidant state in

the rat liver [43] and kidney.[15] The increase in the level of thiobarbituric acid reactive substances in the serum of test rats is due to the onset of lipid peroxidation. Niknahad *et al.* [44] also studied CNMA induced lipid peroxidation. During free radical scavenging action, ascorbic acid is suggested to be transformed into semidehydro ascorbate; reduced glutathione is required for the conversion of semidehydro-ascorbate back to ascorbate.[45] The reduction in the level of reduced glutathione affects the conversion of semidehydro ascorbate and thus the level of ascorbic acid is lowered in the serum of CNMA treated rats. Wang *et al*.[46] studied the interaction of ascorbic acid and glutathione forms which, under oxidative stress. Decrease in the level of vitamin E is due to increase in the level of lipid peroxides.[47] While in free radical scavenging action, the enzymes such as superoxide dismutase, glutathione peroxidase and catalase are involved in the direct elimination of active oxygen species.[48] In this study, superoxide dismutase is elevated in the serum of test rats and hence there is an increase in the production of reactive oxygen species and hydrogen peroxide. The increase in the activity of catalase and glutathione peroxidase is to suppress the level of hydrogen peroxide, which is cytotoxic. [49]

Thus, CNMA induced lipid peroxidation and oxidative stress may affect the neuromuscular coordination in these rats, which result in changes of behavioral parameters. Free radicals mediated neuromuscular degenerative diseases were studied by Davies and Shringarpure [50] and Milatovic *et al.* [51] HaMai *et al.* [52] studied free radicals induced neuromotor symptoms. Earlier report [53] revealed that the bioavailability of CNMA administered by the oral route to the rats was low in blood since all of the CNMA absorbed must pass through liver before being distributed to the whole system and the low level CNMA that remained in the blood was retained for a long time. Since CNMA has to pass through the hepatic first phase metabolism, prolonged exposure and the concentration of CNMA have an impact on its effect on the neuromuscular system. We also noticed CNMA induced antioxidant imbalance and histopathological changes (hemorrhage, necrosis, presence of hyaline casts etc.) of different regions of brain and significant increase in the level of AChE in brain (different regions) and muscle (unpublished data). Our group already studied CNMA induced oxidative stress in the liver [10] and kidney [15] and also CNMA induced oxidative stress mediated alterations in the hematological profiles of CNMA treated rats [11] at the dose level of 73.5 mg/kg body weight/day for 90 days. In addition, we demonstrated that CNMA induced lipid peroxidation resulted in various histological changes of kidney – congestion of glomerular capillaries, mild degenerative changes with appearance of fibrin casts in tubules and occurrence of high mitotic activity in the kidney of rats treated with CNMA at the above dose level (73.5 mg/kg body weight/day for 90 days). [12]

In this study, we conclude that CNMA induced lipid peroxidation results in the behavioral parameters of rats. The effect of CNMA is time and dose dependent. We already suggested that the dose level of 73.5 mg/kg body weight/day approximates the WHO suggested ADI level of CNMA for human (ref: materials and methods section) and hence the WHO suggested ADI level should be lowered.[15] Our findings contribute to a growing body of evidence that widespread food additives may have greater behavioral toxicity than previously understood. Although artificial food colorings are increasingly recognized as and regulated as behavioral toxicants, [54, 55] researchers and policy makers may need to pay more attention to the possible behavioral toxicity of food flavorings.

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