

Copper-Nicotinate Complex Amelurates Neuro degenerative Cerebral Cortex of Rats

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Abstract: The effects of Copper nicotinate complex (CNC) on various neuropathological changes induced by 4-dimethylaminoazobenzene (DAB) in cortex of cerebral hemisphere of the brain of male rats were studied. Seventy five male rats, were divided into the following groups: Group 1: served as control. Group 2: rats received a daily dose of CNC (1mg/kg body weight) orally for 6 months. Group 3: rats fed on balanced diet containing (0.06 g DAB/100 gm diet daily) for 6 months. Group 4: rats fed on DAB as in group 3 then after one month, rats started to receive CNC as in group 2. After 2and 6 months from the beginning of DAB administration rats were sacrificed. Our histopathological results (group 2) revealed that there were apoptosis, steatosis, necrosis and mega nuclei. Immunohistochemical findings revealed reduced neuron specific enolase (NSE) immunoreactivity. Also our biochemical results stated that both metal transcription factor (MTF-I mRNA) and metallothionein (MT-II mRNA), being more expressed at the 6th month (group 3) when compared with control group. "Group 4" after 6 months showed that the principal cells reappeared in considerable number, besides, some steatosis and necrosis for some cells. Also, NSE expression showed more or less the same positivity of normal group. Regarding our biochemical findings revealed that MTF-I and MT-II (m RNA) were upregulated at group 4 when compared with group 1. Conclusion: These results suggested that DAB caused glial and neuronal injury possibly as a result of elevated oxidative stress and that CNC protected neurons and glial cells.

Key words: Copper-nicotinate Complex • 4-Dimethylaminoazobenzene • Cerebral Cortex • NSE • Metal Transcription Factor and Metallothionein.

INTRODUCTION

Neurodegenerative diseases and aging of the central nervous system involve themes of neural stresses, dysfunction in homeostatic mechanisms and oxidative damage to cellular structure [1]. Brain phosphatidylserine, the major acidic phospholipid, is highly enriched in poly-unsaturated fatty acids accumulation of oxidizable substrates can lead to high rate of lipid oxidation propagation [3]. On the other hand biomolecules as copper is found to be connected with several neurological diseases [4]. As a component of CuZn-superoxide dismutase, it functions in superoxide disproportionation and as a consequence of deficiency oxidative stress and neurodegenerative results [5]. Besides, it is a cofactor

required for dopamine B-hydroxylase which converts dopamine to norepinephrine [6, 7]. In our environment we are exposed to several classes of environmental pollutants, among them "aminoazodyes" one of the "arylamines" a major class that have genotoxic and cytotoxic effects in most living organisms [8]. They exert their action through oxidative DNA induced damage and DNA adduct formation [9]. In addition, deficiency of certain vitamins as nicotinic acid has deleterious effects on brain [10]. Complexation of copper with nicotinic acid was shown to have potentials in medical practices, as controlling lipid peroxidation [11,12], decreasing inflammation and anti-oxidant properties [13]. Dittmann *et al.* [1] demonstrated that the presence of specific protective molecules in the central nervous system

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Metallothioneines (MT) are small cysteine-rich and heavy metal-binding proteins, participate in a variety of protective stress responses [14]. Their expression increases upon exposure to some metals, cytokines and reactive oxygen species (ROS) [15]. Downstream effects of MT over expression include modulation of tumor suppression proteins P-53 and NF- κ B transcription [16], free radical scavenging, this influences cell survival, differentiation and drug resistance [17]. MTs exist in 4 isoforms: I, II, III and IV. MT-I and II are considered as single species due to their high homology and inability of the primary antibody to differentiate between them [18]. In brain they are localized in glial cells and are induced by metals as Hg, Cd, Cu and Zn, cytokines and (ROS). On the other hand MT-III although specific to brain tissue is not easily induced by exposure to such agents. MT-IV is specific to epithelial cells [1]. That is why in our study we used mRNA of MT-II. The most neuroprotection provided is by MT isoforms I and II [19]. They are transcriptionally activated by metal-responsive element binding transcription factor-1 (MTF-1) [20]. This transcription factor also regulates zinc homeostasis and cellular responses to heavy metal toxicity, hypoxia, ionizing radiation and oxidative stress through metal-dependent induction of gene expression [21-24].

Reflection to the pathogenesis process in the brain and identifying the ongoing neuronal degeneration are carried out by the expression of neuron specific enolase (NSE) a glycolytic enzyme [25], that under normal conditions is detected only in neurons and cells of the amine precursor uptake and decarboxylation (APUD) system [26]. A close relationship between NSE release and the severity of traumatic brain injury was proved by Woertge *et al.* [27]. So the present investigation was designed to examine the effects of Cu-nicotinate complex (CNC) on various neuropathological changes induced by 4-Dimethylaminoazobenzene (DAB) in cortex of cerebral hemisphere of the male brain rats.

MATERIALS AND METHODS

Reagents are obtained from Sigma Co. (St. Louis, MO). Rneasy Mini Kit was purchased from Qigen Co. (Valencia, CA, USA), while reverse transcription was performed using the Revert Aid MULVRTKIT (Ferment as Science, Vilnius, Lithuania).

Copper nicotinic acid complex was prepared according to Goher [28].

Seventy five male rats, 2 months old with average weight 70-90 gms were used. Animals were purchased from Medical Research Institute (MRI), Alexandria University. All ethical protocol of MRI animal treatment was strictly followed. The animals were accommodated in appropriate healthy conditions and were divided into the following groups:

Group 1: 15 rats fed on normal balanced diet and served as control.

Group 2: 20 rats received a daily dose of CNC (1mg/kg body weight) orally for 6 months [29]

Group 3: 20 rats fed on balanced diet containing (0.06 g DAB/100 gm diet daily) for 6 months[30].

Group 4: 20 rats fed on DAB as in group 3 then after one month, rats started to receive CNC as in group 2.

After 2 and 6 months from the beginning of DAB administration 6 rats were chosen randomly from groups 2, 3 and 4 beside 5 rats from group 1 were sacrificed.

Methods: Rat brains were removed, washed with saline, blotted with filter papers, then subjected to the following:

- Histopathological study by using H & E stains
- Immunohistochemical study by using Avidin Biotin Streptavidin method for NSE detection [31].
- Biochemical studies by using PCR for the detection of MTF-I relative expression using GAPDH as an internal control [32].
- PCR for the detection of MT-II relative expression using B-actin as an internal control[33]

Image Analysis: By using image optical density software (Leica Q-500) the comparison between all studied groups according to the Mean value of NSE positivity were recorded. P: value for F test (ANOVA) was statistically significant at $p \geq 0.05$. The standard error = 0.038.

Statistical Analysis of the Data: After visualization of the bands with ethidiumbromide, data were analyzed using

UVPDOS-ITLS^{MT} image analyzer and soft ware (UV product, Ltd-Cambridge, UK), that analyzes the band density relative to the internal control.

RESULTS

Histopathological Results: Normal rat sections showed well developed cerebral hemisphere with its normal outer cortex. The cortex is consisted of the outer grey matter and the inner white matter. The histological features revealed the neurons with polygonal (The pyramidal or principal cells) or round cell bodies (Glial cells) embedded in their background flet work (Neuropil). The cells contain large nuclei with condensed chromatin and dense basophilic cytoplasm. The pyramidal cells have conspicuous cytoplasm as well as “owl eye “or “fried eye “nuclei. The neuropil and glial cell occupied most of the grey matter in all sections (Figs. 1 and 2). Treated-copper complex rat sections after 2 months had no significant changes as compared with their normal counterparts. The cells showed dense basophilic cytoplasm and more or less the same number of principal and glial cells that embedded in their neuropil (Fig. 3). 6 months after complex treatment, the only recorded change was a little decrease in principal cells. The cells showed dense basophilic cytoplasm and regular number and shape of glial cells and neuropil respectively (Fig. 4). DAB administered rat sections after 2 months showed faint cytoplasm besides the increased number of the principal cells that have faint nuclei. Some sections, however, showed hyperplastic changes with dense basophilic cytoplasm (Figs. 5 and 6). 6 months rat sections illustrated severe changes where apoptosis with its different stages was noted such as pyknosis, karyorrhexis and karyolysis. In addition, there were steatosis, necrosis and appearance of mega nuclei (Figs. 7 and 8). In this group the alterations extended to the choroid plexus and ependymal cells where their lumens were shrinkage (Fig. 9). Moreover, a dramatic decrease in the number of principal cells was noted (Fig. 10). Treated-copper complex after DAB for 2 months regained the regular number of cells (Fig. 11). 6 months showed light basophilic cytoplasm, reappearance of considered number of both principal and glial cells. However, there were necrosis and steatosis in some cells (Fig. 12).

Immunohistochemical Results: NSE reaction was revealed as brown deposits and color in both neuron cell

bodies and their processes that are dendrites and axons of different histological features. So, normal sections showed moderate expression of NSE (Fig. 13). The reaction was positive in different layers of grey matter (Fig. 14). CNC rat sections after 2 or 6 months revealed the same positivity of NSE as that of their normal counterparts (Figs. 15,16 respectively). The positivity of NSE was decreased to be loss in the treated-DAB sections after 2 months (Fig. 17). After 6 months the positivity showed a dramatic decrease to be very low (Fig. 18). Treated-copper rats after DAB (2 moths) had no significant effect while after 6 months the effect of CNC was pronounced to regain more or less the same positivity of normal group (Figs. 19,20 respectively).

The comparison between the different groups showed that the mean value of NSE positivity was significantly different $p > 0.001$ among all studied groups. Also, the number of NSE positivity in group 3 after 6 months (0.23 ± 0.06) was significantly lower than all other groups {group 1 (0.77 ± 0.06) group 2 (0.28 ± 0.06) and group 4 (0.70 ± 0.03)}.

Biochemical Results

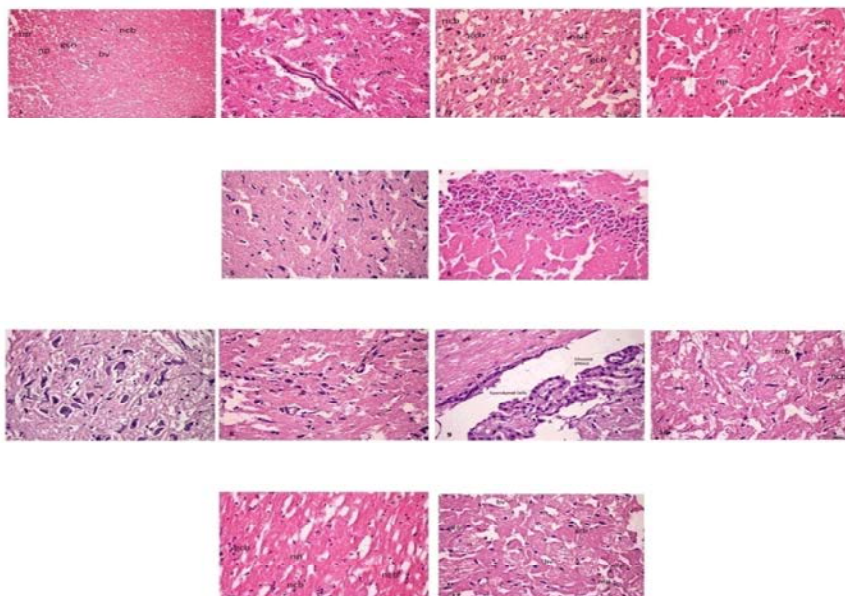
MTF-1 mRNA Expression: At both intervals, the expression was significantly increased in all groups when compared with control animals ($P_1 < 0.04^*$) for all being more significant in group 3 ($P_1 = 0.002^*$).

Treating rats with CNC after DAB (Group 4) caused a significant decrease in m-RNA expression when compared to both groups 2 and 3 but it was higher than control animals ($P_2 = 0.04^*$, $P_3 = 0.004^*$), ($P_2 = 0.03^*$, $P_3 = 0.004$) for 2 and 6 months respectively.

The relative m-RNA expression after 6 months was significantly elevated than that after 2 months for the three treated groups ($P_4 < 0.027^*$, 0.028^* , 0.028^* respectively).

MT-II mRNA Expression: After 2 months the relative expression showed significant elevation in all treated groups when compared with control animals ($P_1 = 0.002^*$) for all. The expression in group 3 was higher than group 2 ($P_2 = 0.006^*$).

On the other hand, in group 4 the relative expression showed non- significant decrease when compared to group 2 ($P_2 = 0.631$), but it was significant when compared to group 3 ($P_3 = 0.01^*$).



- Fig. 1: Cerebral cortex of control rat section showing normal neurons mostly with polygonal shaped and dense nuclei (gcb). Note few neurons with round cell bodies (ncb) and neuropil (np) and blood vessels were also seen. H&E stains Bar=200.
- Fig. 2: Cerebral cortex of control rat section showing that the glial cells gcb, ncb occupied most of the grey matter. H&E stains Bar=50.
- Fig. 3: Cerebral cortex of treated control group CNC after 2 months showing basophilic cytoplasm and more or less the same number of ncb and gcb embedded in their np. H&E stains Bar=50.
- Fig. 4: Cerebral cortex of (group 2) 6 months after CNC showing a little decrease in gcb and ncb with their np and dense basophilic cytoplasm. H&E stains Bar=50.
- Fig. 5: Cerebral cortex of (group 3) 2 months after DAB administration showing faint cytoplasm beside the increased number of ncb with faint nuclei. H&E stains Bar=50.
- Fig. 6: 2 months after DAB administration (group 3) showing hyperplastic changes (H) with dense basophilic cytoplasm. H&E stains Bar=50.
- Figs. (7,8,9 and 10): 6 months after DAB administration (group 3) showing steatosis (S), necrosis (N) and the appearance of mega nuclei (MN). The appearance of pyknosis, (P) karyorrhexis (Kh) and karyolysis (KI) . The alteration extended to choroid plexus and ependymal cells. Note the dramatic decrease in the number of ncb. H&E stains Bar=50.
- Fig. 11: 2 months after CNC treatment (group 4) showing regained the regular number of gcb, ncb and np. H&E stains Bar=50.
- Fig. 12: 6 months after CNC treatment (group 4) showing light basophilic cytoplasm, reappearance of gcb, ncb while there were also S and N. H&E stains Bar=50.

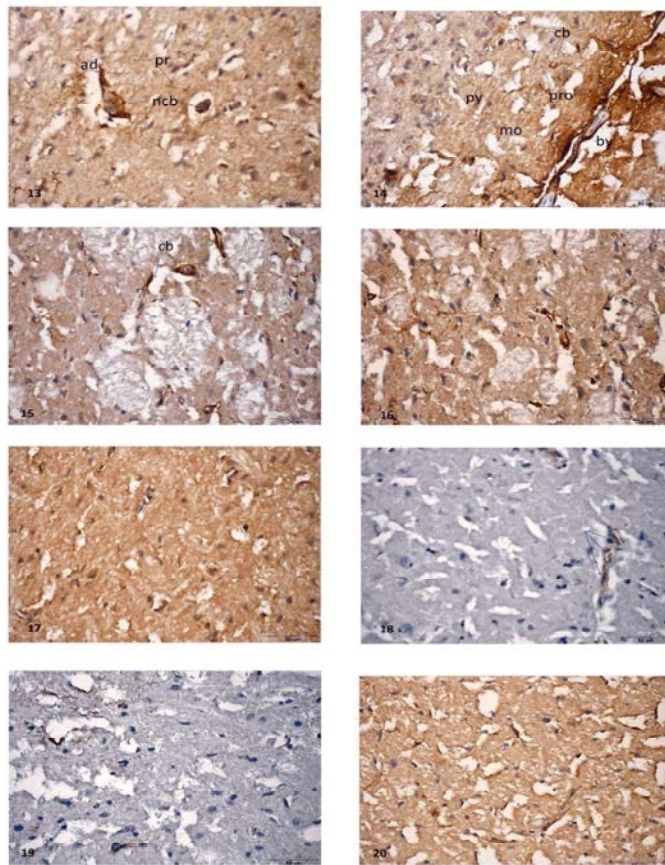
After 6 months, a decline in the expression was noted in group 2 when compared to control animals ($P_1 = 0.485$), while both groups 3 and 4 showed a significant increase when compared to control ($P_1 = 0.002^*$) and when compared to group 2 ($P_2 = 0.004^*$) for both groups.

When rats were treated with the complex and DAB, the relative expression showed

significant increase when compared with group 2

($P_2 = 0.004^*$), but there was a significant increase when compared to group 3 ($P_3 = 0.004^*$).

Comparing the 2 intervals together, group 2 showed significant decrease after 6 month ($P_4 = 0.028^*$) while both groups 3 and 4 showed significant increase ($P_4 = 0.028^*$, 0.046^* respectively).



Figs. 13&14: NSE immunostaining of cerebral cortex of normal rat section showing moderate expression of NSE. Note the reaction positivity in different layers of grey matter. Avidin-Biotin Streptavidin (ABS) method. Bar=50.
 Figs. 15&16: Group 2 after 2&6 months showing moderate positivity of NSE. Avidin-Biotin Streptavidin (ABS) method. Bar=50.
 Figs. 17&18: Group 3 after 2 months showing low positivity of NSE while after 6 months showing a dramatic decrease in NSE positivity respectively. (ABS) method. Bar=50.
 Figs. 19&20: Group 4 after 2 months showing no significant change in the positivity of NSE while after 6 months the reaction showing regain more or less the same positivity of normal group. (ABS) method. Bar=50.

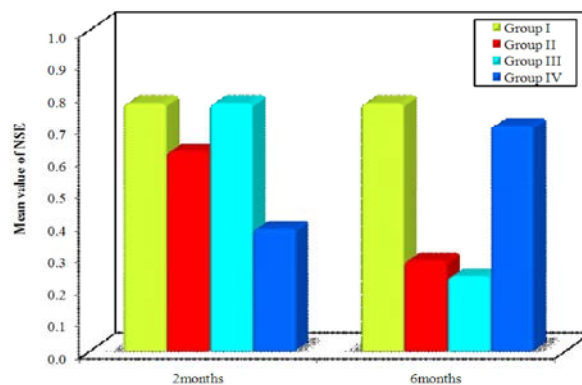


Fig. 21: Bar graph for comparison between the different studied groups according to Mean value of NSE positivity

Table 1: Comparison between the different studied groups according to Mean value of NSE positivity

	Group 1	Group 2		Group 3		Group 4		p
		2months	6months	2months	6months	2months	6months	
Mean Gr								
Min.	0.66	0.58	0.23	0.75	0.17	0.35	0.67	<0.001*
Max.	0.81	0.69	0.37	0.81	0.34	0.47	0.74	
Mean \pm	0.77 \pm	0.62 \pm	0.28 \pm	0.77 \pm	0.23 \pm	0.38 \pm	0.70 \pm	
SD	0.06	0.04	0.06	0.03	0.06	0.05	0.03	
Median	0.78	0.60	0.27	0.77	0.22	0.35	0.69	
p ₁		0.007*	<0.001*	1.000	<0.001*	<0.001*	0.613	
p ₂		<0.001*	<0.001*	<0.001*				
p ₃		0.070	0.174	0.291				

p: p value for F test (ANOVA) for comparing between the different studied group

p₁: p value for Post Hoc test (Scheffé) for comparing between group 1 with different sub groups

p₂: p value for Post Hoc test (Scheffé) for comparing between 2months and 6 months in each group

p₃: p value for Post Hoc test (Scheffé) for comparing between group 1 with group 2, 3 and 4

*: Statistically significant at $p \leq 0.05$

Table 2: Comparison between the expressions of MTF-I m-RNA in the different studied groups

MTF-I	Treated groups				χ^2	p
	Control (G1)	CNC (G2)	DAB (G3)	DAB + CNC (G4)		
2 months						
Min. - Max.	0.27 - 0.58	0.52 - 0.70	0.76 - 0.85	0.40 - 0.59	19.620*	<0.001*
Mean \pm SD.	0.34 \pm 0.12	0.63 \pm 0.07	0.81 \pm 0.03	0.47 \pm 0.07		
SEM.	0.05	0.03	0.01	0.03		
p ₁		0.004*	0.002*	0.041*		
p ₂			0.004*	0.010*		
p ₃			0.004*			
6 months						
Min. - Max.	0.22 - 0.41	0.79 - 0.90	1.36 - 2.0	0.60 - 0.82	20.664*	<0.001*
Mean \pm SD.	0.30 \pm 0.07	0.82 \pm 0.04	1.76 \pm 0.22	0.72 \pm 0.08		
SEM.	0.03	0.02	0.09	0.03		
p ₁		0.002*	0.002*	0.002*		
p ₂			0.004*	0.030*		
p ₃			0.004*			
p ₄	0.075	0.027*	0.028*	0.028*		

χ^2 : chi square for Kruskal Wallis test

p₁: p value for Mann Whitney test for comparing between control with each other treated groups

p₂: p value for Mann Whitney test for comparing between CNC with DAB and DAB + CNC

p₃: p value for Mann Whitney test for comparing between DAB and DAB + CNC

p₄: p value for Wilcoxon signed ranks test for comparing between 2 and 6 months in each group

*: Statistically significant at $p = 0.05$

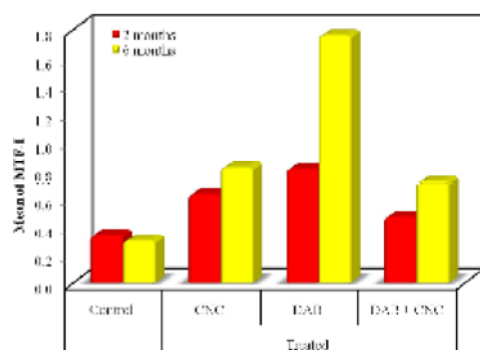


Fig. 22: The bars represent the mean of ratios created by comparing the expression with that of internal control.

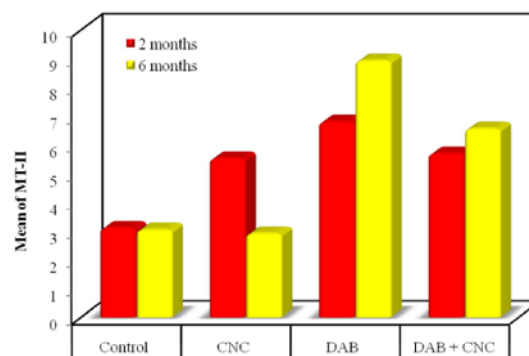


Fig. 23: The bars represent the mean of ratios created by comparing the expression with that of internal control.

Table 3: Comparison between the expressions of MT-II m-RNA in the different studied groups

		Treated groups				
MT-II	Control(G1)	CNC (G2)	DAB (G3)	DAB + CNC (G4)	χ^2	p
2 months						
Min. - Max.	2.66 - 3.50	4.97 - 6.12	6.10 - 7.12	5.01 - 6.21	18.487*	<0.001*
Mean \pm SD.	3.10 \pm 0.30	5.52 \pm 0.42	6.81 \pm 0.37	5.69 \pm 0.47		
SEM.	0.12	0.17	0.15	0.19		
P ₁		0.002*	0.002*	0.002*		
P ₂			0.006*	0.631		
P ₃			0.010*			
6 months						
Min. - Max.	2.64 - 3.52	2.62 - 3.16	8.11 - 9.92	6.02 - 7.59	19.607*	<0.001*
Mean \pm SD.	3.04 \pm 0.28	2.91 \pm 0.20	8.94 \pm 0.62	6.57 \pm 0.62		
SEM.	0.12	0.08	0.25	0.25		
P ₁		0.485	0.002*	0.002*		
P ₂			0.004*	0.004*		
P ₃			0.004*			
P ₄	0.600	0.028*	0.028*	0.046*		

χ^2 :chi square for Kruskal Wallis test

p₁:p value for Mann Whitney test for comparing between control with each other treated groups

p₂:p value for Mann Whitney test for comparing between CNC with DAB and DAB + CNC

p₃:p value for Mann Whitney test for comparing between DAB and DAB + CNC

p₄:p value for Wilcoxon signed ranks test for comparing between 2 and 6 months in each group

*:Statistically significant at $p \leq 0.05$

This Table 2 explained the statistical analysis of relative expression of MTF-I m-RNA in different studied groups at different intervals. This table explained the statistical analysis of relative expression of MT-II mRNA in different studied groups at different intervals.

DISCUSSION

Metal coordination complexes have been rapidly developing in medical practices [34]. Iakovids *et al.* [35] showed that copper complexes showed a diverse of in vitro biological activities. The type of ligand affects and regulat its activity by neutralizing the charge of copper ion, intercalating DNA, interacting with proteins or increasing the lipophilicity of the complexes which facilitates their transport through all membranes. In this aspect, El-Saadani *et al.* [36] concluded that in rats CNC was absorbed by different organs intact without ligand exchange and the uptake by brain and heart was relatively slow. They refered this to be due to that homeostatic mechanisms and utilization of copper, not its storage, is the main event behind the difference in copper uptake and distribution [37].

The results of our study revealed that treating normal rats with CNC "group 2" caused no cytotoxic effects as indicated by the histopathological results, where, the only change recorded was a little decrease in principal cells, but there were regular glial cells and neuropils. Moreover, after 2 and 6 months of CNC administration,

NSE showed the same positivity of their normal counter parts.

Regarding the biochemical findings, we showed that after 2 months of CNC "group 2", the expression of both MTF-1 mRNA and MT-II mRNA were upregulated when compared to normal rats in group I.

It was reported that mouse MT-I and II genes are regulated at the transcriptional level by the metal-response element transcription factor-1(MTF- 1) [21]. MTF-1, itself is induced by metals as Zn and Cu and in turn it will enhance the transcription of MT genes [15].

After 6 months of treatment the expression of MTF-1-m-RNA was more intense than control rats and those treated for 2 months.

On the other hand the expression of MT-II-m-RNA was more or less like that of control animals and less intense than that of 2 months. In this regard, Egli *et al.* [38] reported that in *Drosophila melanogaster*, upon heavy meal accumulation, the activation of MT genes is no longer triggered and this generates a negative feedback on MT gene expression.

Although, as previously mentioned, CNC was slowly up taken by the brain, the prolonged duration of exposure in our study might have let to accumulation.

On the other hand accumulation oxidative damage plays a key role in development of several disease as cancer, arthrosclerosis, arthritis and neurodegeneration [6,7].

DAB is known to exert its cytotoxic effect through oxidative damage and DNA adduct formation. This was shown in our experiment; brain tissue examination revealed that after 2 months of DAB administration, there was increased number of principal cells and hyperblastic changes in some sections. After 6 months, apoptosis, steatosis, necrosis and mega nuclei were observed.

Oteiza *et al.* [39] observed that neuronal death depends on the amount of oxidant degeneration. Apoptotic death is triggered by moderate production of oxidant while high amounts lead to necrosis.

Although Schreiber *et al.* [40] had reported that increased expression of NSE correlates with neurological damage, Ding *et al.* [41] indicated that reduced NSE immune - reactivity reflects neuron loss. Several studies also revealed that neuron injury causes leakage of the enzyme into the extra cellular space, with increased level of the enzyme in serum and cerebrospinal fluid of rats [42,43].

Apoptosis and necrosis noticed in our result confirm neuron loss and explain the decreased immune reactivity of NSE after DAB administration.

DAB administration after 2 and 6 months increased the expression of both MTF-1 mRNA and MT-II mRNA, "group 3" being more expressed at the 6th month compared to control rats. It was shown that the increase in oxidant levels triggers redox - sensitive transcription factors.

MTF-1 regulates oxidative stress either by inducing glutamate-cysteine ligase activity heavy chain (δ GCSHc) which encodes oxidative stress related proteins [44], or by binding to the metal response element of MT genes inducing their expression [45]. In accordance with our results, Dittmann *et al.* [1] suggested a model in which extra cellular MTs provided protection of neurons in the advent of injury or stress. Also it has been reported that MT-I and II expression increased in murine body after brain injury [18].

The neuroprotective role of MT is through its downstream effects as antioxidant properties, due to their high cysteine content [46] and the ability to affect the inflammatory response through altering the toxicity or susceptibility of the tissue [47]. In addition, MTs contain zinc-binding sites that are highly sensitive to redox state, when the cells are exposed to oxidative stress MTs serve as Zn donor, raising free Zn level and stimulating diverse cellular-response signals [48,49], including activation of cell death-inducing pathway in neurons [50].

In a series of experimental studies in our laboratory CNC was chosen to have a growth inhibitory effect

against experimentally induced liver cancer in rats (Unpublished data).

In our study treating rats with the complex one month after DAB "group 4" resulted in regaining the number of regular cells, while after 6 months the principal cells reappeared in considerable number, besides, there were some steatosis and necrosis in some cells. After 6 months NSE expression showed more or less the same positivity of normal group, as a result of reappearance of normal and principal cells. This reflects the possible protective effect of the complex on brain tissue.

Regarding our biochemical findings, MTF-1 m-RNA was upregulated after both 2 and 6 months intervals in group 4 when compared with group I, but less intense than both groups 2 and 3 Dittmann *et al.* [1] stated that after brain injury relief the number of glial cells increases and MTs expression increases.

Regarding MT-II m-RNA it was up regulated after 2 months of group 4 when compared to the control animals but less intense than those of groups 2 and 3 at the same interval. On the other hand after 6 months the expression was more intense than both groups I and 2 but less intense than group 3.

The synergistic effect of both copper and nicotinic acid each proved to have brain neuroprotective effect. CNC was reported to have SOD mimic activity. Beside, the direct action of CNC in inducing MTF-1 mRNA and MT-II mRNA expression both through their downstream effects minimize the cytotoxic effect of DAB on brain tissues.

Our observations may provide an important therapeutic insight to minimize oxidative stress induces neurodegeneration. CNC in our experiment not only possessed cytoprotective activity but it also modulated the cytotoxic action of DAB through modifying the expression of MTF-1 and MT-II- m-RNA.

CONCLUSION

These results indicated that DAB caused glial and neuronal injury, possibly as a result of elevated oxidative stress and that CNC protected neurons and glial cells. Thus, we suggest that CNC treatment attenuated the cortical neurodegeneration observed during DAB administration in rats.

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