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ASSESSMENT OF NEUROPATHOLOGY , AMINO ACID PROFILE AND BIOACCUMULATION FOLLOWING SUB CHRONIC INHALATION OF MANGANESE PHOSPHATE (AS ONE OF GASOLINE COMBUSTION PRODUCTS)IN MALE SPRAGUE -DAWLEY RATS.

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SUMMARY

The increased incidence of inhalation exposure to manganese had resulted in increased attention to the potential toxic effects of manganese especially its adverse neurotixuc effects.

The central nervous system represents an important target for manganese (Mn) intoxication that may cause neurological symptoms similar to Parkinson's disease in human.

The aim of this work was to investigate the exposure -response relationship of bioaccumulation, neurophathology and neurobehavioral damage following sub chronic inhalation exposure to Mn phosphate. For this purpose 45 Sprague-Dawley rats were divided into 3 groups (fifteen each), G1 were kept as a control, and G2 and G3 were exposed to managanese phosphate inhalation in a dose of 300 and 3000 microg/m3, 6 hours/day, 5 days /week, for 12 consecutive weeks.

At the ends of the experimental period, rats were exanguinated, the brain of all rats were rapidly collected, weighted, and part was taken for determination of manganese level while the other part used for histopathological examination. There was a significant increase in manganese level in the blood and brain samples of the treated groups, this increase was dose dependant. While brain specimens showed focal glial cells proliferation, various degrees of neuronal degeneration, prominent astrocytic nodules.

Our results reinforce the hypothesis of the neurotoxic effects of manganese after its sub chronic exposure and increased the attention of the dangerous effects resulted from the addition of MMT to unleaded gasoline.

INTRODUCTION

Manganese (Mn) is an essential trace element and also has significant toxicity after excessive exposure. Manganese plays an important role in a number of physiological processes as a constituent of some enzymes and an activator of other enzymes (Nilson, 1999). It has a catalytic properties in several enzymatic reactions as, manganese superoxide dismutase, an impotant antioxidant, and pyrovate carboxylase, essential in energy metabolism (Leach and Harris, 1997).

Toxicity problems of manganese had taken a great attention since nintys due to its increased level in the environment. Manganese (Mn) is ubiquitous in ambiant air due to both industerial and crustal sources. It is also a component of the octane-enhancing fuel additive methyl cyclopentadienyl manganese tricarbonyle (MMT), (Vitarella et al , 2000).

Increased Mn concentration in the ambient air will increase the incidence of its gain access to the brain and subsequently its neurotoxic problems. Not only this, but also, human and wild life exposure to Mn has increased due to MMT use which results in the accumulation of Mn in plants and surface water deposition.

MMT is an organic manganese-containing compound that added to unleaded gasoline. The combustion of MMT by Automobile engine results in the formation of Mn particles including Phosphate, sulfate, and oxide forms.

Addition of MMT to gasoline results in low level, continuous releases of Mn -containing particles to air, soil, and water. Unlike, ingested and inhaled Mn, transported directly to the brain before its metabolization in the liver (Davis, 1998).

The use of the additive MMT in unleaded gasoline has resulted in increased attention to the potential toxic effect to Mn. (Salehi et al, 2001), especially with the increase in its incidence of inhalation exposure.

Though, Mn is an abundant element in the earth's crust, levels of the exchangeable Mn , the form available for uptake into plants, are orders of magnitude lower than total Mn in the soil. There is a relationship between MMT use and Mn contamination in soil (Keen et al, 1999).

It has been suggested that the Mn combustion products could cause neurological symptoms similar to Parkinsonís disease in human (Normandian et al,2002), and subsequently marked brain pathology.

Hypothetically, people with chronic liver diseases may be more sensitive to the adverse neurotoxic effects of Mn as a result of hepatic encephalopathy. (Davis, 1998).

The reversibility of neurological damage due to Mn is unpredictable. Pal et al, 1999, reported that once neurotoxic effects from Mn exposure are openly expressed at the clinical levels, the damage to the central nervous system is essentially irreversible and may in some cases be progressive.

Newborns and people with chronic liver diseases are at a high risk for manganese toxicity.

It has been known that infants and youngs absorb the ingested Mn more than the adults, while excrete less. And because of the immature blood-brain barrier (BBB) of youngs, their CNS is less well protected from blood- born Mn than that of adults.(Weiss, 2000). While, Mn is eliminated from the body mainly in bile. Thus, impaired liver function may lead to decreased Mn excretion (Keen et al, 1999 and Pal et al, 1999).

A line of evidence suggests that the exposure to many of the gasoline components leading to accumulation of those compounds in the brain regions resulting in profound neurotoxic effects on the function and structures of these areas Satriotomo et al, 1999.

The objectives:

The present work was initiated in order to provide an integrated evaluation of the bioaccumulation, neuropathology, and free amino acid profile following chronic inhalation exposure to manganese phosphate as a gasoline combustion product in male Sprague-Dawley rats.

MATERIALS AND METHODS

1- Experimental animals:

Forty-five male Sprague-Dawley rats were obtained from animal house belonging to NAMERO (Egypt). Animals were transported to the laboratory and kept for two weeks for adaptation to the laboratory conditions prior to the initiation of the experiment.

2- Experimental design:

Animals were grouped into three groups, the first group (G1) was kept as a control (15 rats). The remaining rats were divided into 2 treated groups (G2 and G3), and fifteen rats each.

Rats of groups 2 and 3 were exposed to a dynamic system applied in the present work for the purpose of Mn phosphate inhalation.

Animals of G2 and G3 were exposed 6hrs/day, 5days/week for 12 consecutive weeks to 300 and 3000 microg/m(3) Mn phosphate (Mn5 (Po4)[Po2(OH]2.4H2o)respectively and compared to the control.

Briefly animals received the inhalant particles in an inhalation chamber after passing in an evaporizer then through a rubber tube to the inhalation chamber.

At the end of the experimental period, blood samples were collected from each animal in a clean sterile tube for serum separation and determination of blood-Mn concentration. Then animals were sacrificed by exsanguinations, the brain was dissected out and weighted for the calculation of relative brain/body weight.

3- Biochemical assay:

3-a- Determination of manganese concentration in brain tissue and blood:

Mn concentration and blood was determined by Zeenman atomic absorption spectrophotometer 4100ZL Perkin Elmer. Ashes were dissolved in 1M HNO3 and metal concentration was determined according to AOCA, 1980.

3-b- Determination of free brain amino acid content:

Free amino acids content in the brain including both excitatory and inhibitory amino acids was assayed using HPLC (Model A0099-600) with spectra focus optical scanning detector c18 and Bechman column. The analysis was carried out using a gradient of Pico-Tag solvent at 40°C and a flow rate of 1ml/min.

The detection of the separated Pico-Tag amino acids was performed at 254nm.

4- Histopathological studies:

Specimens of brain tissue of both control and treated groups were fixed in 10% buffered neutral formaline and routinely processed for light microscopical examination according to (Bancroft et al, 1996).

Tissue sections were stained by Hematoxyline and Eosin according to (Bancroft et al, 1996).

5- Statistical analysis:

Data were analyzed to detect the differences between means using t-test and analysis variance (ANOVA) according to Snedecor and Cochran (1980).

RESULTS

Studies of the present work revealed that Mn induced marked brain pathology and alterations in the determined biochemical parameters. In addition, animals of G2 and G3 showing marked hyper excitability, irritability after 5 weeks of inhalation especially in G3.

1- Effect of the inhaled manganese phosphate on body weight and brain weight of rats:

Concerning the effect of the inhaled particles on body weight and brain weight of the treated groups comparing to the control, it is presented in table (1).

From that table it was clear that; there was a significant decrease in body weight gain in the treated groups especially G3 ($p<0.001$) comparing to the control group.

While brains of treated groups' revealed highly significant weight increase in G3 comparing to the control animals.

Table (1): Showing the effect of managense phosphate inhalation on body weight and relative brain weight of male Sprague Dawley rats:

	Control	Group 1.	Group 2.
Body weight prior to exposure.	170 ± 3.17	170 ± 1.80	172 ± 2.6
Body weight after exposure.	186 ± 4.16	163 ± 1.31***	161 ± 2.48***
Relative brain weight.	0.72 ± 0.02	0.79 ± 0.01**	0.83 ± 0.03***

* P<0.05, ** P<0.01 *** P<0.001

Table (2): Showing manganese concentration (micro/g) in the brain tissue of the treated and control rats groups:

Animal Groups	MN Concentration in the brain (Microg/g)	MN Concentration blood (Microg/g)
Group 1 (Gp1)	0.64	0.02
Group 2 (Gp2).	1.28**	0.03*
Group 3 (Gp3).	2.047***	0.05**

* P<0.05, ** P<0.01 *** P<0.001

Table (3): Showing the effect of Mn phosphate inhalation on the free amino acids content in the brain of the treated and and control groups (mg/100 g tissue)

Amino Acid	Control group	Group 1. .	Group 2.
Glutamic acid.	165.51 ±56.92	227.01 ± 24.87**	343 ± 30.93***
Aspartic acid	82.54 ± 24.74	83.52 ± 10.34	94.39 ± 9.14
Phenyalanine	2.50 ± 1.49	13.11 ± 5.55*	77.44 ± 21.8***
Tyrosine.	5.93 ± 1.07	14.81 ± 2.25**	54.99 ± 2.16***

* P<0.05, ** P<0.01 *** P<0.001

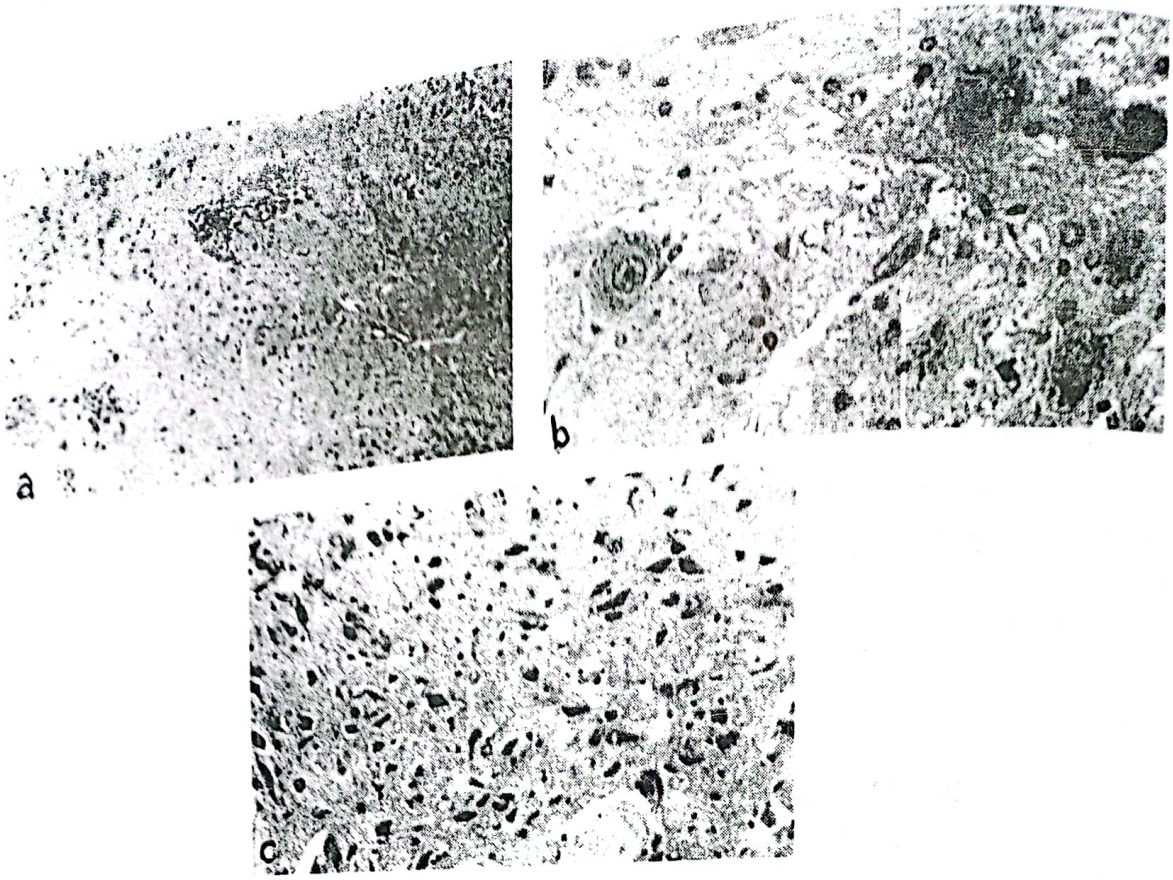

Fig.(1): a- Cerebral cortex of rat of G2 showing multiple glial nodules. b and c - Cerebral cortex of rats of G3 showing; b- Degenerated neurons with central and peripheral chromatolysis with demylination of nerve fibrils. c- Marked microgliosis in the vicinity of the degenerated neurons.
(H & E X100 and 200).

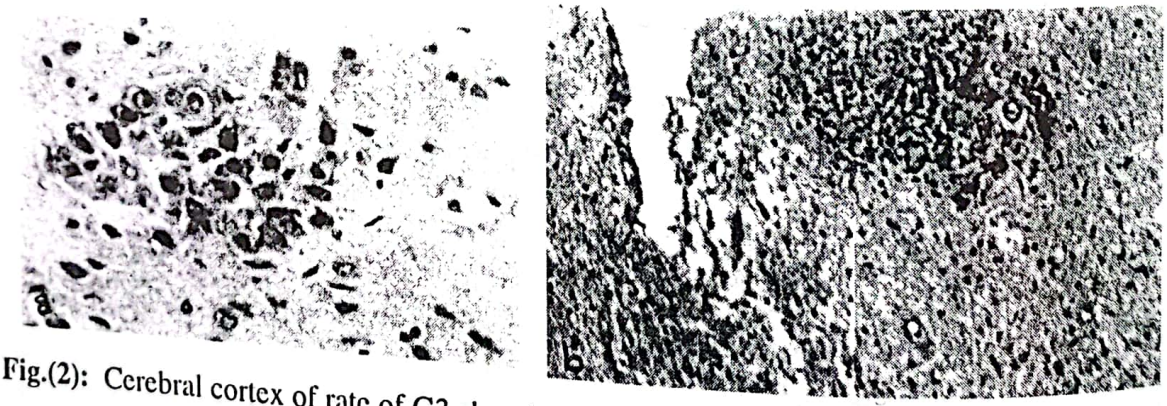

Fig.(2): Cerebral cortex of rate of G3 showing; ; a- several types of neuronal damage with swollen and enlarged astrocytes . b-Focal area of malacia impacted by astrocytes and neutrophilic dust. (H & E, X 200).

2- Results of biochemical assays:

2- a- Manganese concentration in the brain and blood:

Increased Mn concentration was observed in brain tissue of the treated groups in a dose dependant manner as it is presented in table (2). On the other hand, blood manganese level showed also a significant increase in G2 and G3 comparing to its blood level in the control group.

2- b- Values of the amino acids content in the brain:

The values of the measured amino acids concentrations showed great alterations in G2 and G3 in comparison with the control values as presented in table (3).

3- Results of histpathological examination:

Brain sections from animals of the treated groups revealed marked tissue alterations which were dose dependant .Those changes characterized by cerebral focal gliosis that characterized by multiple glial cells aggregations (Fig.1, a) accompanied with variable degrees of neuronal degeneration especially in G2.

While, the most conspicuous lesions in G3, were the wide spread basophilic appearance of the cytoplasm of the degenerated and necrotic neurons together with peripheral and central chromatolysis of the cytoplasmic elements. In addition, demylination was a constant finding observed here and there in the brain tissue (Fig.1, b).

Marked microgliosis was clearly noticed in the vicinity of the damaged neurons in the cerebral cortex (Fig.1, c). On the other hand, astrocytic nodules that characterized by aggregation of large number and size of astrocytes were observed in the cerebral cortexes of 9 animals in G3 (Fig.2, a).

Moreover, the strangest lesion was the appearance of focal neutrophilic dust cells of living and dead neutrophiles with several areas of encephalomalacia in the cerebral cortexes of 3 cases of G3 (Fig. 2, b).

DISCUSSION

The present study revealed that exposing rats to Mn phosphate induced sever histopathological changes in the brain and caused marked alterations in the free amino acids levels with an increase in Mn concentration in brain tissue.

The use of the inhalation route for Mn in this work resulted in its gain access to the brain with great alterations. Mn migrates to the brain either directly through the intranasal route or more slowly across the BBB.

Intranasal exposure of Mn provides direct access to the brain, bypassing the blood- brain barrier (BBB) as it binds to transferrier (carrier protein), then within the brain, Mn accumulates primarly in the astrocytes, perhaps related to the role of Mn in regulating the astrocyte-speceific enzymes (Glutamate synthetase), (Mergler et al,1999, and Dorman et al,2004).

Tjalve and Henriksson, 1999, has mentioned that, Mn uptake from the nasal mucosa to the brain is ready via the olfactory nerve which is another way of reaching the brain.

Moreover, another important issue is that, Mn uptake to the brain is slow and its clearance from the brain is also slow (half-life of about 150 days) (Mergler et al,1999). All of this increased the threat from Mn induced neurotoxicity.

As it is shown from the present work, Mn concentration was significantly increased ($p< 0.001$) in the brain tissue of the treated rats , This increased Mn concentration was dose dependant, which may be a reflect of its transport via the above mentioned two ways and accumulates there. A result which is in agreement with that of Dorman et al, 2002 , Normandian et al , 2002 and Salehi et al, 2001.

Our currant investigation demonstrated that the levels of the major excitatory amino acids, aspartic and glutamic acids in the brain were increased and were significantly higher in animals of G3.

These increased levels may be due to their release from the degenerated cells as indicated in the present histopathological result. This assumption is supported by the work of Cunningham et al, 1994, who reported that the elevation of the excitatory amino acids neurotransmitters is accompanied with cellular abnormalities and are usually indicative of brain damage and neurodegenerative disorders.

Furthermore, the marked degeneration of the brain neurons observed in our study could have caused the release of glutamate and aspartate from the injured cells which would induced even more damage to the adjacent cells in a positive feed back way. This is supported by the opinion of Manev et al, 1990 who reported that excessive production of glutamate causes toxic effects on neurons.

In addition, as mentioned before, the increased Mn concentration accumulates in astrocytes which showed great increase in its number and size on microscopical examination, and consequently increase glutamate-synthatase enzyme. The later is reflected on increase glutamate and aspartate levels. This comes in accordance with Thomas, 1995 who mentioned that astrocytes rep-

resent a good source of glutamate in the brain and their increase in number or swelling results in further release of glutamate.

A significant additional support to the concept of the relationship between nerve cell injury and increased brain levels of excitatory amino acids is that, the increase in aspartic and glutamic acids was found co-localized with partially degenerated areas in the brain under the effect of dexamethazone in a study conducted by Berkman et al, 1998.

Likewise glutamate was reported to cause further release of neuroactive compounds and excitatory amino acids (Teichberg, 1991).

Regarding, phenylalanine and tyrosine which represent the precursors of the two most important neuroactive catecholamines, dopamine and noradrenaline, both were increased. This increase may be attributed to increased stimulation for their synthesis in order to provide an enough supply for the important catecholamines, especially that increased exposure to Mn causes antioxidation of catecholamines (Aschner et al, 2003).

Histological examination of brain sections of G2and G3 revealed varying degrees of neuronal degeneration and necrosis together with glial nodules and a surprising finding of neutrophilic dust in the cerebral cortex. Those finding are agreed with those observed by Salehi et al, 2001 and Normandin et al, 2002.

The last mentioned neuronal changes may be resulted from the increased cytotoxic free radicals produced by Mn, which comes in harmony with Keen et al, 1999, who mentioned that, there is evidence that Mn increased the production of cytotoxic free radicals. In addition, the determined increased levels of glutamate and aspartate may indirectly have a damaging effect on the neurons in the vicinity.

Concerning, the increased number of neuroglial cells represented by glial nodules in the present histopathological examination, it may be attributed to their intimate functional relationships with neurons in providing both mechanical and metabolic support; this is agreed with (Burkih et al, 1996).

While, the great response of astrocytes as astrocytic nodules could be attributed to the last mentioned relationship between astrocytes and Mn.

Satriotomo et al, 1999, suggested that, the neuroadaptive response of astrocytes could occur before the neurotoxic effects emerge on neurons of the suprachiasmatic nucleus.

The surprising observation of nutrophilic dust in the cerebral cortex of 3 rats may be attributed to that, the high doses of Mn may induce a possible inflammatory reaction which may subside later on, or may be attributed to decreased immunity of those rats under the experimental toxicity allowing neutrophile accumulation.

Some authors reported neutrophile invasion in the brain in their studies on some toxic materials as (Nishino et al, 1995) on 3- nitropropionic acid in rats, (Clegg and Van Gemert, 1999) on chlorpyifos toxicity in rats.

IN CONCLUSION

Our results gave a coherent picture on the effects of Mn exposure on the brain and neuronal behavior, which increased the threat from the increased Mn exposure in the surrounding ambient air, soil and water. One of the major Mn sources in the ambient air is the combustion of MMT in gasoline which recommended stopping its addition.

The authors opinion go parallel with Davis,1998, Zayed et al, 1999, and Aschner, 2000, concluded that, there does no appear to be any public health benefit from adding MMT to fuel, the advantages of MMT use to be only limited to those that resulted from octane enhancement (automobile performance).

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