Protective effect of propolis on Manganese chloride (MnCl\textsubscript{2}) neurotoxicity of olfactory bulb in adult male albino rat

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Protective effect of propolis on Manganese chloride (MnCl2) neurotoxicity of olfactory bulb in adult male albino rat

Running title: Manganese and propolis role on olfactory bulb

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Abstract

Manganese (Mn) is widely used for industrial purposes and exposure to high levels of Mn may cause an irreversible brain disease. Propolis is a natural plant product, it acts as a powerful ROS scavenger and improves the neurodegeneration process. In this study forty adult male albino rats were divided randomly into four groups 10 rats each. Group I (control group), group II Manganese Chloride (MnCl2) received 10 mg/kg/day/orally for 4 weeks by intra-gastric tube. Group III (Propolis group) received 50mg/kg/day/orally for 4 weeks by intra-gastric tube and Group IV (MnCl2 +Propolis group) received the same doses with the same duration and route as in groups II and III. Rats were sacrificed after 24 hours of last dose. The olfactory bulbs removed, the right bulb cut to be processed for Haematoxylin and Eosin, immunohistochemical staining and the left cut for electron microscopic studies.

Results revealed that rat olfactory bulb from (MnCl2) group showed darkly stained mitral cells with dark pyknotic nuclei, some show pericellular spaces and vacuolation, dark apoptotic cells in granular cells, neuropil vacuolation and pyknotic astrocyte. Electron microscopic examination showed abnormal granular cell with irregular damaged nuclear membrane, rupture of myelin fiber. Mitral
nerve cell with destructed nucleus, many cytoplasmic vacuoles, swollen rough endoplasmic reticulum, vacuolated Mitochondria and Neuropil. Manganese Chloride + propolis group showed improvement compared to Mn Cl2 group. It was concluded that Propolis can ameliorate the toxic changes of manganese Chloride on rat olfactory bulb.

**Key words:** olfactory bulb, manganese chloride, propolis, rat

**INTRODUCTION**

Odor impairment is associated with preclinical dementia in humans, so it is essential to develop experimental models to evaluate the effects of neuropathology on behavior. The sense of olfaction is critically important for food consumption, maternal, reproductive functions, emotional responses and neuroendocrine regulation. In many species, olfaction plays a very important role in animals’ functions than in humans [16].

Manganese (Mn) is widely used for manufacture purposes, including the production of steel, iron, fertilizers, ceramics, batteries, insecticides, paints, and soaps [7]. Metal and mine workers are frequently exposed to Manganese [24]. It is an essential metal for humans, which disturbs the controlled balance of neurotransmitters release and metabolism. Therefore Mn haemostasis is necessary for all brain function [10]. It acts as an activator or cofactor for many metalloenzymes, as mitochondrial superoxide-dismutase that is an avital enzyme in the suppression of oxidative stress [39]. Mn passed the blood-brain barrier and carried to various brain parts through an axonal transport system [42]. It was stated that exposure to Mn high levels leads to permanent brain disease [45].

Propolis is a resinous substance produced by honeybees from plants. It is composed of resin (50%), wax (30%), pollen (5%) and aromatic oils (10%), flavonoids (quercetin, chrysin, pinocembrin, kaempferol, apigenin, etc.), polyphenolics, beta-steroids, terpenes, vitamins, and minerals. It has relevant therapeutic properties that have been used since ancient times [6]. Its flavonoid component, caffeic acid phenethyl ester (CAPE), possesses several important biological and pharmacological properties including immunomodulatory, antioxidant, anti-inflammatory, anticarcinogenic, antiviral, antimicrobial, neuroprotective effect and anti-diabetic activities [38]. Propolis acts as a potent ROS scavenger. It improves the neurodegeneration process after sciatic nerve injury [37]. Also, it greatly improved the level of acetylcholinesterase (Ache) activity, oxidative stress, and mitochondrial dysfunction in Lead-induced neurotoxicity [15].
Earlier identification of neurodegenerative diseases is a major objective. In preclinical studies testing new neuroprotective approaches in rodent models of neurodegenerative disease, olfactory assessment could be very useful in determining therapeutic potential of compounds and transfer into clinic [27]. So, this study aims to evaluate the protective effect of propolis against Manganese chloride MnCl2 neurotoxicity on the olfactory bulb of adult albino rats.

**MATERIALS AND METHODS:**

**Chemicals**

Manganese Chloride 97% (tetrahydrate) was purchased from ALPHA CHEMIKA, India.

**Preparation of aqueous propolis extract**

Propolis was obtained from honeybee colonies at the apiary of the Faculty of Agriculture at Suez Canal University, Egypt. Propolis freeze until usage, samples were mixed with distilled water, heated and filtered through filter paper. It was freshly prepared to be sure that it contains all its active components given orally by intragastric tube daily at a dose of 50 mg/kg/day for four weeks [15]. Oral administration of propolis is safe up to 5000 mg/kg/day [32].

**Animals**

Forty adult male Sprague Dawley albino rats weighing between 150 and 200 gm purchased from Faculty Veterinary Medicine, Suez Canal University. They kept in a ventilated room in stainless-steel cages at Human Anatomy and Embryology Department, Faculty of Medicine, Suez Canal University. They left two weeks to accommodate, received food, water ad libitum, weighted daily and observed for behavioural changes.

**Experimental design**

Forty adult male albino rats randomly divided into four groups ten rats each:

— **Group I (control group)** divided into two subgroups: Negative control: no treatment was received, and Positive control received 0.5 ml distilled water daily by intra-gastric tube for four weeks.
— **Group II (MnCl2):** Rats received 10 mg/kg/day/orally for four weeks, which dissolved in 1 ml of sterile saline by intra-gastric tube [35].

— **Group III (Propolis group):** Rats received 50mg/kg/day/orally for four weeks by intra-gastric tube [15].

— **Group IV (MnCl2 +Propolis group):** Rats received the same doses, duration, and route as groups II and III.

Rats sacrificed 24 h after the last dose, the olfactory bulbs were collected immediately, the right bulb cut to be processed for H&E, immunohistochemical staining and the left one cut for electron microscopic studies.

**Buried Food Test**

The test is used to confirm ability to smell volatile odour and measures how rapidly an overnight-fasted animal can find familiar food, such as hiding cookies underneath a layer of 3 cm clean bedding in a clean cage. Stopwatch and timer were used for each cage, rat with normal olfaction find the cookies within 1 min. Stop the stopwatch when the rat picks up the cookies. Rat fails to find the cookies after 15 minutes we record 900 sec as its latency score [43].

**Light microscopic study**

Right olfactory bulb was cut and fixed in 10% neutral buffered formalin and processed for light microscopic study. Paraffin sections 5µm thickness were stained with Haematoxylin and Eosin (H&E) and Immunohistochemically staining with Glial fibrillary acidic protein (GFAP) of glial cells (purchased from Lab vision, USA) [33]. Area percent of GFAP immunoreaction were measured using Image J soft [1, 36]

**Electron microscopic study**

The specimens of the left bulbs were divided into small pieces, immersed for two h in 2.5% phosphate-buffered glutaraldehyde solution (pH 7.4) at 4°C, washing with phosphate buffert then post-fixed for one h in 1% buffered osmium tetroxide solution. Ultrathin sections were cut using MT 600-XL RMC ultra-tome, stained with uranyl acetate and lead citrate and examined with JOEL-1010 (Japan) transmission electron microscope at the centre of Mycology and Biotechnology Transmitting Electron unit, Al-Azhar University, Cairo.
Statistical analysis

The morphometric data of each group was statistically analyzed; ANOVA and post-hoc Tukey’s HSD were used to compare the four studied groups: P< 0.05 was considered significant.

RESULTS

No mortality was observed throughout the study. The MnCL2 group showed less active than the other groups. No marked changes in behavior were observed.

Buried Food Test

MnCL2 group showed a statistically significant increase in time compared to control and propolis groups. Mnc12+propolis group showed statistically significant reduction in time compare to Mncl2 group. (Fig 1).

The area percentage of GFAP immuno-stained

the area percentage of the GFAP stained cells in Mncl2 group was statistically significant increase compared with other studied groups (Table 1).

Histopathological study

Control and propolis groups. Olfactory bulb sections of control and propolis groups stained by H &E presented similar heterogeneous architecture and cellular basis, six layers appeared from external to internal: olfactory nerve layer (ONL) contained unmyelinated axons and accumulation of a few organized supporting cells. glomerular layer (GL) showed glomeruli with different size, Juxtaglomerular neurons surrounded by typical glomeruli. external plexiform layer (EPL) consisted of nerve fibers and glial cells. mitral cell layer (MCL) showed widely spaced and broken cell bodies of mitral cells arranged in a single row, they had euchromatic nuclei, prominent nucleoli, and Nissl substance in their cell bodies. internal plexiform layer (IPL) and granular cell layer (GCL). Granular cell layer contains many small granule cells, and Golgi type II cells also noticed (Fig 2a1+a2 and Fig 3a).

Immunohistochemically stained sections of GFAP showed a few positive cells (Fig4 a, table I).
Electron microscopic examination showed normal rounded granule nerve cells with scanty cytoplasm and dark nuclei. Mitral nerve cell with euchromatic nuclei and prominent nucleoli. Abundant cytoplasm containing normal rough endoplasmic reticulum, Mitochondria with normal cristae, dense matrix and electron-dense particles most probably free ribosomes. (Fig 5a, 6a1+a2)

**Manganese Chloride group.** Olfactory bulb of Manganese Chloride group revealed darkly stained mitral cells with dark pyknotic nuclei, some show pericellular spaces, vacuolation, congested blood vessels and dark apoptotic cells in granular cells. Neuropil vacuolation, many pyknotic astrocytes, and Golgi type II cells were also present (Fig 2b and Fig 3b). Immunohistochemically stained sections for GFAP showed a positive reaction in all layers of the olfactory bulb (Fig 4b, table I).

Electron microscopic examination showed abnormal granular cell with irregular damaged nuclear membrane, rupture of myelin fiber. Mitral nerve cell with a destructed nucleus, many cytoplasmic vacuoles, swollen RER, vacuolated Mitochondria, Neuropil vacuolation and irregular nuclear membrane (Fig 5b and 6b1, b2).

**Manganese Chloride + propolis treated group.** Normal granular cells arranged in a cluster. Mitral cells appeared with abundant cytoplasm containing Nissl substance in the cell body and deeply stained nucleus. Some cells showed vacuolated cytoplasm and vacuolation of neuropil is still present. (Fig 2c and 3c)

Immunohistochemically stained sections showed GFAP +ve reaction. (Fig 4c, table I)

Electron microscopic examination showed a normal cluster of granular cells, vacuolation in neuropil was still present in some areas. Mitral nerve cells appeared with euchromatic nuclei, prominent nucleoli, cytoplasm containing rough endoplasmic reticulum, some swollen mitochondria with loss of its cristae were still noticed. (Fig 5c and 6c1, c2).

**DISCUSSION**

Manganese is a vital nutritional component which plays an appropriate function of numerous biological processes such as blood clotting, bone growth, metabolism, free-radical defense, and production of brain neurotransmitter. Mn is one of the most worldwide used metal [41]. Pure Manganese is used in the manufacture of dry batteries, glass, steel industry [20]. Manganese deficiencies infrequently occur in humans; otherwise, its high levels are very destructive to human, independent from exposure route [45].
The current study observed that mitral cells were the most affected cells after exposure to Manganese Chloride, they showed dark pyknotic nuclei, pericellular vacuolated destructed nucleus, many cytoplasmic vacuoles. Also swollen RER, vacuolated Mitochondria, irregular nuclear membrane, congested blood vessels and dark apoptotic cells in the granular cell layer.

Many studies revealed that Mn toxicity is manifesting in central nervous system [11]. High levels of Manganese in the brain lead to a neurotoxic disease (manganism), which resembles idiopathic Parkinson Disease (iPD) [23]. Olfaction has been given a lot of value, clinically. Researchers have indicated that olfactory dysfunction in Alzheimer's disease (AD) may precede the clinical emergence of cognitive impairment, and it may be an early sign of brain alteration. Those with olfactory impairment were more likely to develop AD than others [12].

Mn compound with transferrin will attach to receptors of transferrin in the capillaries of cerebral hemispheres for endocytosis to capillary endothelial cells [18]. Then it is released from systemic transferrin to complex with brain synthesized transferrin to reach the brain tissue. A few amounts of Mn is bound to citrate, then cross the blood-brain barrier through monocarboxylate transporter [4]. Mn carriage to brain cells was confirmed to pass through neurons of the olfactory system; its Solubility seems to play a major role in transport, with soluble MnCl2 and MnSO4 concentration, inhalation of Mn concentrates mainly in the olfactory bulb [13]. Re-localization mechanism of Mn after it reached the brain had not been fully understood [26]. Mitochondria are the primary target organelles for Mn accumulation, when its concentrations are elevated, Mn2+ is oxidized to Mn3+, which leads to the production of reactive oxygen species ROS [9]. However, Mn3+ is a potent inhibitor of complex I in the electron transfer chain of mitochondria, that decrease ATP release and increased the outflow of electrons and oxygen radical formation [46]. Mn toxicity may be due to accumulation in mitochondria, causing oxidative stress and dopaminergic neurotoxicity [3]. This finding is agreed with the current study, which showed destruction and vacuolation of mitochondria with absent of its crista.

The beginning of Mn toxicity was characterized by anxiety, schizophrenia, movement disorders and impaired memory [8]. These findings agreed with the present results as rat in Mn group failed in Buried Food Test in comparing to other groups.

The current study showed many pyknotic astrocytes in Mn group which agreed with other studies that revealed increase accumulation of Mn level in astrocytes. Disorder occurred when extra Mn load on glial cells and astrocytes interrupts their capability of controlling the environment of a neuron. This leads to an increase neurons susceptibility to (ROS), excitotoxicity, and toxic product
handled by astrocytes [5]. Astrocytes offer neurons with glycine, precursors, and cysteine essential in neuronal GSH production [14]. Dysfunction of astrocyte delays normal function of the neuron; so, it is reasonable to consider astrocytes as a target of Manganese neurotoxicity. Neuronal death is eventually caused by Manganese toxicity, which is initiated directly, or via neuronal/astrocyte disorder. Many hypotheses demonstrate how Mn toxicity leads to neurodegeneration. ROS generation and change in energy metabolism are usually supposed to be reputed mechanisms [21].

Antioxidants are elements that prevent and interrupt oxidation of substrate whereas finding in tiny quantity. Nutritional antioxidants act through different mechanisms as free radical scavengers, decrease peroxide concentrations, repair oxide membranes and reduce ROS release [19]. Antioxidants have controlled the expression of many genes, signal regulatory pathways, and avoid the occurrence of cell death [44].

The present work showed histological improvement in Manganese Chloride + propolis treated group.

Propolis is the most antioxidant studied in the research, it is qualified in cardioprotective, neuroprotective effects and suggested as a protective element against Alzheimer’s [31] and Parkinson’s diseases [25]. The basic molecular mechanisms of propolis act as a treatment for cognitive brain function. It significantly inhibits the H2O2 lead to mitochondria ROS generation and nuclear DNA damage, which induce dysfunction of synaptic efficiency in neuronal cells [31].

Pinocembrin, a flavonoid abundant in propolis, has powerful neuroprotective effects by improving mitochondrial function, inhibiting inflammatory responses, reducing oxidative damage and neuronal apoptosis [28]. Many studies described that, there was a link between oxidative damage, impaired mitochondrial function and increases activities of mitochondrial respiratory complexes II and IV without affecting mitochondrial membrane potential [30]. Recently found that propolis protects against the hypoxia-induced microglia-mediated neuroinflammation [29]. The concomitant administration of propolis with paclitaxel reduced the neurodegenerative changes induced by paclitaxel supported by previous reports where propolis stimulates the neural stem cells differentiation into neurons and enhances the transcription of the pro-neural genes thus it has an anti-apoptotic effect [2]. Besides, propolis attenuates caspase-3 activities, strengthening the anti-apoptotic effect [40]. Propolis attenuates the diazinon induced nephrotoxicity which confirms its effectiveness as antioxidant and anti-inflammatory [34]. Propolis showed protective effects in spinal cord injury in rabbits exposed to spinal ischemia-reperfusion injury [22]. Propolis protective actions are through a reduction in expression of several inflammatory cytokines in the nucleus and inhibition
of oxidative damage to proteins, DNA/ RNA, lipids, carbohydrates, and influencing immune responses. So, it is considered a potent neuroprotective agent due to its biological activities based on its flavonoids and infrequent side effects [17]. The possible toxicological effects of Propolis extracts were investigated. The results showed that it was quiet safe, no mortalities or signs of toxicity in mice when administered orally at doses up to 5000 mg/kg b.wt [32]. Currently, herbal remedies such as propolis becoming popular because of their beneficial effects with fewer side effects compared to synthetic and semi-synthetic drugs.

**CONCLUSIONS**

Manganese chloride (MnCl2) lead to toxic effects on rat olfactory bulb, especially of mitral cells which showed destructed nucleus, many cytoplasmic vacuoles, swollen RER and vacuolated Mitochondria. Manganese Chloride + propolis treated group showed improvement compared to Mn Cl2 group. So, it was concluded that Propolis can ameliorate the toxic changes of manganese Chloride on rat olfactory bulb.

**Ethical consideration:** All procedures performed in this study involving animals were done accordance the ethical standards of the Institutional Animals Ethics Committee of Suez Canal University.

**Conflict of interest:** There are no conflicts of interest.

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Table I. The mean values of area percentage of GFAP immunoreaction (mean ± SD) in different groups

<table>
<thead>
<tr>
<th></th>
<th>Control group Mean ± SD</th>
<th>Mncl2 group Mean ± SD</th>
<th>Mncl2+ propolis group Mean ±SD</th>
<th>Propolis group Mean ± SD</th>
</tr>
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<tbody>
<tr>
<td>Area percentage of GFAP immunoreaction</td>
<td>5.949 ± 0.66</td>
<td>11.297 ± 0.71* #</td>
<td>6.243 ± 0.87</td>
<td>5.899 ± 0.64</td>
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</tbody>
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P > 0.05

*Significant compare to control and propolis group
#Significant compare to Mncl2+propolis group

Figure 1. Mean ± SD duration in seconds of Buried Food Test. P >0.05

Figure 2. (a1) A photomicrograph of rat olfactory bulb section control group showing olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL) and granular cell layer (GCL) (a2) mitral cells (arrow) with abundant cytoplasm, euchromatic nuclei, prominent nucleoli and granular cells. (b) MnCl2 group showing darkly stained mitral cells (arrow), dark pyknotic nuclei, congested blood vessel (Bv) and dark apoptotic cells in granular cells (GC) (c) MnCl2+ propolis group showing granular cells and mitral cells (arrow) with abundant cytoplasm.

Figure 3. (a) A photomicrograph of rat olfactory bulb section control group showing Mitral cells (MC) reside in a single row, with abundant cytoplasm contain Nissl substance, euchromatic nuclei and prominent nucleoli, granular nerve cells (GC) with scanty cytoplasm and normal nuclei. Golgi type II cells (Go) (b) MnCl2 group showing darkly stained mitral cells (arrows) with pyknotic nuclei, pericellular spaces and vacuolation. Pyknotic astrocyte (arrowhead), granular cell (Gc) and Golgi type II cell (Go) (c) MnCl2 + propolis group showing normal granular cells and mitral cells with abundant cytoplasm, deeply stained nucleus and normal neuropil.
**Figure 4.** (a) A photomicrograph of rat olfactory bulb section control group showing a few positive immunoreaction cells. (b) MnCl2 group showing GFAP positive reaction. (c) MnCl2+ propolis group showing GFAP positive immuno-reaction.

**Figure 5.** (a) An electron micrograph of rat olfactory bulb control group showing normal granular nerve cells with clumped heterochromatin and scanty cytoplasm. (b) MnCl2 group showing abnormal granular cell with irregular damaged nuclear membrane (arrowhead), rupture of myelin fiber (arrow) and neuropil vacuolation (c) MnCl2+ propolis group showing cluster of granular cells and vacuolation in neuropil.

**Figure 6.** (a1) An electron micrograph of rat olfactory bulb control group showing Mitral nerve cell with euchromatic nuclei (N) and prominent nucleoli (arrow). Abundant cytoplasm, rough endoplasmic reticulum (RER) and Mitochondria. Normal granule cells (GC). (a2) Mitochondria (M) with normal crista and dense matrix.(b1) MnCl2 group showing Mitral cell with destructed nucleus, irregular and damaged nuclear membrane, many cytoplasmic vacuoles, swollen RER and vacuolated Mitochondria (b2) Irregular nuclear membrane and Neuropil vacuolation with destructed Mitochondria (c1) MnCl2+ propolis group showing Mitral cell with euchromatic nuclei, prominent nucleoli and cytoplasm containing RER, some swollen mitochondria and normal neuropil. (c2) Cytoplasm with some slight swollen mitochondria and loss of its cristae.