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PROTECTIVE EFFECT OF ASCORBIC ACID AND TIRON (4-5 DIHYDROXY-1, 3 BENZENDISULFONIC ACID) AGAINST MERCURY CHLORIDE-INDUCED OXIDATIVE STRESS AND NEUROTOXICITY IN RABBIT

(With 3 Tables and 5 Figures)

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الأثر الوقائي لفيتامين ج والتيرون (أحماض ٤-٥ هيدروكسى ١-٣ بنزين داى سلفونيك) ضد التأثير المثبط لكلوريد الزئبق على إنزيمات الأكسدة والمسبب للسمية العصبية في الأرانب

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يعد التلوث الناشىء من المخلفات الصناعية هو المصدر الأساسي للتلوث البيئي. ويعتبر الزئبق والعديد من مركباتة مجموعة من اخطر هذة المخلفات الملوثة للبيئة , ولذلك فقد أجريت هذة الدراســـة للتعرف علي إمكانية الوقاية من التأثير السمي لهذة المركبات وذلك بإستخدام أحد العناصر التمخلبية وهو عنصر التيرون وأيضا تم إستخدام أحد العناصر المضادة للأكسدة وهو فيتامين ج. وقد تمت الدراسة على عدد ٤٠ أرنبة بيضاء نيوزلندي وقد قسم هذا العدد إلى أربعة مجموعات كل منها يتكون من عدد ١٠ أرانب كالتالي : المجموعة (أ) قد أعطيت مركب كلوريد الزئبق بجرعة قدرها ملليجرام واحد/ كيلو جرام من وزن الجسم عن طريق الفم بإستخدام أنبوب اللي المعدي يوميا ولمدة ثلاث شهور. المجموعة (ب) قد أعطيت نفس الجرعة من مركب كلوريد الزئبق مضافا إليها جرعات من فيتامين ج بواقع ١ جرام / لتر ماء فى ماء الشرب يوميا ولمدة ثلاث شهور, المجموعة (ج) وقد أعطيت نفس الجرعة من مركب كلوريد الزئبق يوميا ولمدة ثلاث شهور,ثم تم حقنها بريتونيا بالعنصر التمخلبى التيرون بجرعة قدرها٤٧١ ملليجرام / كيلو جرام من وزن الجسم فى عدد ٦ جرعات متتالية ولمدة أسبوعين بواقع عدد ٣ جرعات أسبوعيا . أما المجموعة (د) فقد أستخدمت كمجموعة ضابطة للتجربة. وقد تم عمل تحليل كيميائي حيوي لقياس نشاط كل من الإنزيمات الخاصة بالأكسدة فى خلايا المخ مثل إنزيم جلوتاثيون بيرأوكسيداز وإنزيم الصوديوم ديسميوتاز وأيضا تم قياس مستوى الإ نزيمات الخاصة بتأكسد الدهون الموجودة بالجدار الخلوي لخلايا المخ. وقد تم أيضا قياس مستوى نشاط إنزيم الكولين أستيراز كناقل عصبي داخل أنسجة وخلايا المخ. علاوة على الفحص الباثولوجى للعديد من قطاعات المخ المختلفة والتى شملت الفصوص الأمامية للمخ وجذع المخ والمخيخ والهيبوكامبس وذلك بصباغتها بنوعين مختلفين من الصبغات المتخصصة. وقد تم الفحص المجهرى على مستوى الخلايا العصبية ذات الأنواع المتعددة والتي توجد فى الأجزاء المختلفة من المخ والتي سبق ذكرها. وقد أسفر ت النتائج التحليلية للمجموعة (أ) والمتعرضة لمركب كلوريد الزئبق فقط عن نقص معنوي فى مستوى نشاط كل من إنزيم الجلوتاثيون بير اوكسيداز وإنزيم الصوديوم ديسميوتاز بينما كانت هناك زيادة معنوية كبيرة فى مستوى الإ نزيمات الخاصة بتأكسد الدهون الموجودة بالجدار الخلوي لخلا يا المخ. كما أظهرت النتائج نقصا معنويا فى مستوى نشاط إنزيم الكولين إ ستيرا ز فى خلا يا المخ مقارنة بالمجموعة الضابطة .أما نتائج الفحص الباثولوجى لخلايا وأنسجة المخ فى الأجزاء المختلفة فقد أظهرت تحلل وفقد , كما وجد تنكرز لبعض الخلايا العصبية مقارنة بالمجموعة الضابطة. وقد تم عمل إحصاء عددي لبعض أنواع الخلايا المعروفة بلبركينجى وهى نوع من الخلايا الهرمية الموجودة فى المخيخ. وأيضا تم فحصها باثولوجيا فأظهرت النتائج نقصا معنويا فى عدد الخلايا فى الحيوانات المتعرضة مقارنة بالمجموعة الضابطة,كما أظهر الفحص المجهرى تحلل وفقد وتغير فى شكل الخلايا. وبتحليل النتائج السابقة تبين وجود نقص فى إنزيم الجلوتاثيون بير اوكسيداز وإنزيم الصوديوم ديسميوتازو هي

الإنزيمات المضـادة للأكسـدة داخل الخلايا وزيادة فى مسـتوى الإنزيمات الخاصـة بتأكسـد الدهون الموجودة بالجدار الخلوي وهى المسؤلة عن تنفس الخلايا مع نقص ملحوظ فى مستوى نشاط إنزيم الكولين إ ستيرا ز فى خلا يا المخ وهو إنزيم يعمل كناقل عصبي بين الخلايا, مما يؤدى إلى خلل فى تنفس الخلايا العصبية مؤديا الى فقد وتحلل وأيضا موت لبعض هذه الخلايا وهو ما تأكد حدوثة بالفحص الباثولجي للخلايا العصـــبية داخل أجزاء المخ المختلفة والذي يؤكد بدورة على حدوث درجة من درجات التسـمم العصـبى نتيجة للتعرض لمركب كلوريد الزئبق غير العضـوي. وقد كان هناك تحسـنا معنويا ملحوظا فى مسـتوى جميع هذه القياسـات وأيضـا للتغيرات الباثولوجية في كلا من مجموعتي (ب) التي تم علاجها بفيتـامين ج والمجموعـة (ج) التي تم علاجهـا بـالتيرون ذلـك عند مقـارنتهمـا احصـائيا بالمجموعة (أ). وبالنظر لهذة النتائج يمكن أن نسـتنتج وجود تأثيرا واقيا أو علاجيا لكل من فيتامين ج كعنصر مضاد للأكسدة أو للتيرون كعنصر تمخلبى وان هذا التأثير العلاجي قد ساعد فى الحد من الآثار والأعراض العصبية الناتجة عن التعرض لمركب كلوريد الزئبق غير العضوي في إناث الأرانب البيضاء. وقد كانت من النتائج الهامة لهذة الدراسة ملاحظة أن التأثير العلاجي للعنصر التمخلبى التيرون كان أكثر وضوحا (إحصائيا) من العنصر المضـاد للأكسدة فيتامين ج على مستوى كل القياسـات السابقة,مما يجعل هذة الدراسة توصى باستخدام هذا العنصر فـى الـوقـايـة او عـلاج حـالات الـتسـمـم أو الـتـعـرض لـمـركـبـات الـزئـبـق غـيـر العضوية.

SUMMARY

The current study was performed to assess the potential of 4-5 dihydroxy-1, 3 benzendisulfonic acid (Tiron) and L-ascorbic acid (vitamin C) against inorganic mercury (mercuric chloride-HgCl2) induced oxidative stress and neurotoxicity in female New Zealand white rabbits. 10 rabbits per group were assigned to one of four treated groups: 0 mg HgCl2, 0 mg Ascorbic acid and 0 mg Tiron (control); 1mg Hg Cl2/kg BW orally; 1mg HgCl2/kg BW orally plus 1gm ascorbic acid /liter in drinking water; HgCl2/kg BW orally plus 471mg Tiron/kg BW I/P. Rabbits were administered HgCl2 and ascorbic acid for three months while Tiron administered in 6 concessive doses for 15 days at the level of three doses per week. Biochemical analyses on oxidative stress-related parameters and acetylcholine esterase activity as neurotransmitter were carried out. Histopathological analyses for detecting the cellular damage in brain tissues of exposed rabbits were also performed. Results obtained showed that HgCl2 significantly ($p<0.05$) increased malondialdehyde and 4-hydroxyalkenals (MDA&4-HAE the marker of lipid oxidation) in brain tissues, while the activities of superoxide dismutase (SOD), Glutathione peroxidase (GSHPx) and acetylcholine esterase (AChE) activities were significantly ($p<0.05$) decreased. Histopathological analysis of the brain revealed that neuronal degeneration with apoptotic features in cerebral cortex, hippocampus and cerebellum. Loss and significant ($p<0.05$) decrease of purkinje cells number in cerebellum was detected. Also the purkinje cells lost the normal shape and became distorted. Most of the above parameters responded positively with either Tiron or vitamin C therapy, but more pronounced beneficial effects on the previous described parameters were observed in Tiron treated group. It is concluded that the protective effect of vitamin C as antioxidant and Tiron as a chelating agent against mercury chloride - induced neurotoxicity. Tiron was more effective than vitamin C in restoration of the most investigated parameters.

Key words: *Tiron, mercury chloride, purkinje cells, glutathione peroxidase*

INTRODUCTION

Mercury is important environmental toxicant that causes neurological and developmental impairment in both humans and animals. Within the environment, the mercury exists in three different chemical forms (elemental mercury vapor, inorganic mercury salts, and organic mercury) that are all important for human exposure (Clarkson, 1997). Gold mining emits elemental mercury vapor that is inhaled and absorbed into the blood stream (Grandjean *et al.,* 1999). Methylmercury (MeHg), an organic mercury compound, is found in fish and seafood, and fishing communities are highly exposed to MeHg due to the high consumption of MeHg contaminated fishes (Clarkson *et al.,* 2003). Human exposure to inorganic mercury is mainly occupational, which is often related to mining and industrial activities (Berzas *et al.,* 2003). In addition, inorganic mercury is believed to be the toxic species produced in tissues after inhalation of mercury vapor, which produced as industrial waste (WHO, 1991). The distribution, metabolism, and toxicity of mercury are largely dependent upon its chemical form. Inorganic mercury is toxic to the renal, reproductive and nervous systems (Frumkin *et al.*, 2001), and human exposure to inorganic mercury is often related to specific working conditions e.g. mining, spillage of mercury compounds on work clothes or in the working environment, handling of mercury salts in the chemical industry and laboratories (Berlin *et al.,* 1986; Wide, 1986; Bluhm *et al.,* 1992), accidental (Shamley and Sack, 1989) and intentional (Winship, 1985) events can also contribute to human exposure to inorganic mercury. Through consumption of mercury in food, the populations of many areas, particularly in the developing world, have been confronted with catastrophic outbreaks of mercury induced diseases and mortality (WHO, 1991).

Even though mercury neurotoxicity is not well understood, it has been shown that alterations in calcium and glutamate homeostasis (Aschner *et al.,* 1994; Sirois and Atchison, 2000; Aschner *et al.,* 2000; Farina *et al.,* 2003), oxidative stress (Ou *et al.,* 1999; Franco *et al.,* 2006) and oxidation of protein thiols (Hansen *et al.,* 2006) represent the important molecular mechanisms by which both organic and inorganic mercury may cause neurotoxicity. Several studies have pointed to the antioxidant glutathione system as a potential target for the deleterious effects of organic and inorganic mercurials. Particularly important: the cerebellar antioxidant glutathione system represents an important molecular target for such effects (Manfroi *et al.,* 2004; Stringari *et al.*, 2006). There is evidence that cerebral cells are selectively targeted by mercurials in vivo (Sanfeliu *et al.*, 2003). Lakshmana *et al.* (1993) reported that mercury chloride may be induced neurotoxicity through alteration of levels of some neurotransmitters as noradrenalin, dopamine, serotonin and acetylcholine esterase activity in different regions in rat brain.

In mercurial poisoning, supportive care is given when necessary to maintain vital functions and the use of chelating agents assists the body's ability to eliminate mercury from the tissues (Aposhian, 1983; Tchounwou *et al.*, 2003). Numerous studies have documented the efficacy of antioxidant vitamins and chelating therapy in promoting the elimination and restoration of the deleterious effects induced by mercury and other heavy metals in animals and human subjects (Sharma *et al.,* 2002; EL-Demerdash, 2004; Mathur *et al.*, 2004; Zaidi *et al.*, 2005; Sharma *et al.*, 2007; Yousef *et al.*, 2007). Antioxidant vitamins can protect against oxidative damage through its ability to re-oxidation the reduced form of glutathione, is the natural antioxidant present within the cells (Zaidi *et al.*, 2005). Ascorbic acid is one of the important antioxidant vitamin known. Rao *et al.* (2001) reported that the protective role of vitamin C on mercury chloride-induced genotoxicity in human blood cultures. It was used in the present study to investigate its potentiality to modulate neurotoxicity induced by mercury chloride in vivo.

Chelating therapy has been reported as a useful approach for counteracting mercurial toxicity. Chelating agents compete with the in vivo binding site for the metal ion through the process of ligand exchange (Jones, 1994) and metal-chelator complex is mainly excreted from the body through urine or feces. Among chelating agents currently available the sodium salts of 4-5 dihydroxy-1, 3benzendisulfonic acid (Tiron) has been found to be highly effective against several metallic toxicities (Mathur *et al.*, 2004; Sharma *et al.*, 2002 and 2007). Tiron is a diphenolic-chelating compound, which forms water-soluble complexes with a large number of metal ions (Sharma *et al.*, 2007). AS already mentioned, although Tiron is able to modulate or decrease several metallic toxicities, there are no available studies in our knowledge showing beneficial effects of Tiron against HgCl2 induced neurotoxicity in rabbit. Taking this fact in consideration, our study was aimed to investigate the possible neuroprotective effects of both vitamin C as antioxidant and Tiron as a chelating agent against HgCl2- induced neurological damage and oxidative stress in rabbit. The potential neuroprotective effects of either Vitamin C or Tiron were evaluated in this study using biochemical and histological approach.

MATERIALS and METHODS

Chemicals

Mercury chloride (HgCl2), L- ascorbic acid (vitamin C) and 4-5 dihydroxy 1, 3 benzendisulfonic acid (Tiron) purchased from Sigma (St.Louis,Mo.,USA). Colorimetric assay kits for determination of glutathione peroxidase (GSHPx), superoxide dismutase (SOD), malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) lipid peroxidation and acetylcholine esterase (AChE) were obtained from Oxford Biomedical Research (USA). All other chemicals were of the highest grade available commercially.

Animals and treatment

Forty female New Zealand white rabbits (body weight: 1000-1200 g), which were supplied by medical experimental animal house Assiut University, Egypt, were used in accordance with guidelines on the care and use of laboratory animals. The ethical committee of Vet. Med. Assiut University approved the study.

All animals were housed at 23±2Ċ and 55±5% humidity under natural photoperiod for one week before the start of experiment. A commercial balanced diet and tap water ad *libitum* were provided. Animals were randomly divided into four groups (A-D) of 10 animals each. Group A (HgCl2) given (1mg/kg/daily) HgCl2 orally by gavage for three months. Group B (HgCl2/Ascorbic acid) as in group A plus daily Vitamin C administered 1g/litre in drinking water, for three months. Group C (HgCl2/Tiron) given (1mg/kg/daily) HgCl2 orally by gavage for three months, then Tiron intraproteneal (I/P) administered at a dose of 471-mg/kg/daily for 15 days in 6 concessive doses, at the level of 3 doses per week day by day. Group D (control group) given the saline only and kept as a control.

These animals were sacrificed under anesthesia with chloroform. Brain hemisphere was taken, sagitally cut into two halves. One half was stored at 60C for enzymatic assay and biochemical analysis. The other half was processed for histopathological study.

Sample preparation for biochemical analysis The samples of brain tissues were washed by NaCl 0.9% containing 0.16 mg /ml heparin to remove the blood cells. The samples were homogenised in 4-8 volumes (per weight tissues) of cold buffer 50 mM TRIS-HCl, PH 7.5, containing 5 mM EDTA and 1mM2-mercaeptoethanol, using ultra-turrax T25b homogenizer. The supernatant was prepared by centrifugation at (5000-10,000 xg) for 10-20 minutes at 2-8Ċ.

Biochemical analysis

Determination of glutathione peroxidase (GSHPx)

The activities and concentrations of (GSHPx) were determined spectrophotometrically using a commercially available kit. This procedure based on the method described by Paglia and Valentine (1967) and modified by Chu *et al.,* (1993). The activities of GSHPx were measured as the production of NADP+ by the activation of glutathione reductase (GR) on oxidized glutathione (GSSG) in the presence of NADPH. The absorbance determined at 340 nm and the activity was given as units per gram protein in brain tissues.

Determination of superoxide dismutase (SOD)

The brain tissues level of SOD was determined using a colorimetric kit according to Nebt (1991). Spectrophotometric assay of SOD activity was based on the enzyme's ability to inhibit superoxide-driven NADH oxidation. The rate of reaction was measured by recording to the change in the absorbance at 550 nm. The activity was expressed as units per gram protein in brain tissues.

Determination of lipid peroxidation

Measurement of malondialdehyde (MDA) and 4-hydroxyalkenals (HAE)

have been used as an indicator of lipid peroxidation. The colorimetric kit was used to determine the levels of oxidized lipid according to Esterbauer *et al.* (1991). This assay is based on the reaction of chromogenic reagent with MDA and HAE at 45 Ċ to yield a stable chromophore with maximum absorbance at 586 nm. The rate of lipid peroxidation was expressed as nmol of reactive substance formed/min/mg protein.

Determination of acetylcholinesterase activity

Brain acetylcholinesterase activity (AChE) was estimated by the method of Ellman *et al.* (1961), using acetylthiocholine iodide as a substrate. The rat of hydrolysis of acetyl thiocholine iodide is measured at 412 nm through the release of the thiol compound which, when reacted with DTNB, produces the colour- forming compound TNB. The rate of enzyme activity was expressed as nmol of reactive substance formed/min/mg protein.

Determination of protein

Protein concentrations were measured by the method of Bradford (1976), using bovine serum albumin as a standard.

Protein concentration used in the concentration of SOD or GSHPx or MDA & HAE and AChE can be expressed as activity per mg of protein by dividing the units /ml of protein concentration.

Histopathological studies

For light microscope studies, the brain tissues were fixed in 10% in natural buffer formalin and processed for paraffin embedding, sectioned at 6µm and stained with H&E (Young *et al.*, 1992). Some sections were stained with 1% Cresyl-violet solution according to Müller and Naujoks (1975) and Maldonado *et al.* (2002). The purkinje cells analysis was performed by direct counting of these cells from 10 random visual fields for every slide processed (10 rabbit per group) according to Carvalho *et al.* (2007).

Statistical analysis

A one-way analysis of variance ANOVA followed by Tukeyes HSD test was used to test for significance difference among treated groups. Data are expressed as mean± S.E.M. Differences were considered significant when $P<0.05$.

RESULTS

Biochemical assay

Mercury chloride administration, in female rabbit for three months, produced severe alteration in various brain biochemical parameters. It induced significant increase ($P<0.05$) in the overall means of brain MDA&4-HAE concentrations and decrease in superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and acetylcholine esterase (AChE) activities compared to control group. On the other hand, treatment with ascorbic acid (Vitamin C) or 4-5 dihydroxy 1, 3-benzendisulfonic acid (Tiron) caused a significant ($P <0.05$) restoration in the values of all these parameters. It is noticed that the restoration was more clear in Tiron group than vitamin C group, where there is a statistically significance differences at ($P <0.05$) when compared between Tiron and vitamin C groups (Table 1&2).

Histopathological studies

HgCl2 treated rabbit (group A) revealed that the most neurons of the cerebral cortex and hippocampus were degenerated and became smaller in size, where their nuclei became smaller and showed chromatolysis with cytoplasm vaculation (degenerative features) (Fig. 1a). Some neurons showed a margination of the nucleus with perineuronal gliosis (Fig. 1b), as compared to control group (Fig. 1c). The perineuronal tissues showed microglia cells reaction (gliosis) (Fig. 1d&e). There were perivascular edema and degenerated endothelium of blood vessels in the cerebral cortex (Fig. 2a). There was perivascular cough with lymphocytic infiltration and gliosis in hippocampus (Fig. 2b), as compared to control group (Fig. 2c). Histological analysis of the cerebellum showed a reduced number of cerebellar purkinje cells. The number of purkinje cells decreased significantly 5.31 ± 0.25 cells per field at $P<0.05$, as compared to control group (Table 3). A diffuse loss of purkinje cells was detected (Fig. 3a), the shape of the purkinje cells was distorted (Fig. 3b) in comparison with control group (Fig. 3c). In HgCl2/VitC treated rabbit (group B) the evidence of shrinkage and neuronal degeneration in few neurons in the cerebral cortex and hippocampus were noticed (Fig. 4a). The blood vessels still showed perivascular edema (Fig. 4b). In HgCl2/Tiron treated rabbit (group C) the neurons in the cerebral cortex and hippocampus appeared more or less similar to the control in H&E or Cresyl violet stain (Fig. 5a). In the perineural as well as perivascular structures, the pathological changes could not be detected (Fig. 5b). The cerebellum in both HgCl2/Vit.C treated rabbit (B) and HgCl2/Tiron (C) groups, showed increase in the number of purkinje cells (Table 3). Also the morphological appearance of the purkinje cells was restored to some extent in Vit. C group and completely in Tiron group (Fig. 4c&5c).

Table 1: Oxidative stress parameters and lipid peroxidation metabolites in brain homogenates of exposed and treated female rabbits

Parameters	MDA&HAE	SOD	GSHPx

Groups	(nmol/mg protein)	(IU/mg protein)	(IU/mg protein)
HgCl2 (A)	6.96±0.22**	0.56±0.07**	0.523±0.136**
HgCl2/Vit.C(B)	4.35±0.16**a	0.88±0.08**a	0.758±0.237** a
HgCl2/Tiron(C)	3.83±0.23**ab	0.96±0.06**ab	0.804±0.317**ab
Contro l(D)	3.50±0.31	1.63±0.15	0.983±0.243

Values were expressed as means ±S.E.M. **indicate significant differences at ($P<0.05$) when compared to control group.

(a) indicate significant difference at ($P<0.05$) when compared group A and both B and C groups.

(b) indicate significant difference at ($P<0.05$) when compared B and C groups by one way ANOVA followed by Tukeyes HSD test.

Table 2: Acetylcholine esterase activity in brain homogenate of exposed and treated female rabbit.

Groups	Acetylcholine esterase activity (nmol/min/mg protein)
HgCl2 (A)	8.55 ±0.83**
HgCl2+ Vit.C (B)	15.50±±1.06**a
HgCl2+Tiron (C)	16.36±0.95±1.06**ab
Control (D)	18.05±0.89

Values were expressed as means ±S.E.M. **indicate significant differences at ($P<0.05$) when compared to control group.
(a) indicate significant difference at ($P<0.05$) when compared group A and both B and C groups.
(b) indicate significant difference at ($P<0.05$) when compared B and C groups by one way ANOVA followed by Tukeyes HSD test.

Table 3: Number of Purkinje cells in the cerebellum of HgCl2 and Vit.C or Tiron-exposed groups.

Groups	Purkinje cells
HgCl2 (A)	5.31 ±0.25**
HgCl2+ Vit.C (B)	6.90±0.23**a
HgCl2+Tiron (C)	7.60±0.28**ab
Control (D)	8.20±0.25

Data are presented as number of cells per visual field (40X) and expressed as mean ± S.E.
**indicate significant differences at ($P<0.05$) when compared to control group.
(a) indicate significant difference at ($P<0.05$) when compared group A and both B and C groups.

(b) indicate significant difference at ($P<0.05$) when compared B and C groups by one way ANOVA followed by Tukeyes HSD test.

LEGENDS

Fig. 1: Sections of the brain rabbits from group A ($HgCl_2$- treated group) and control group showing neurons and perineural tissues of the cerebral cortex.

- a- Central chromatolysis of the nucleus and cytoplasmic vaculation in the neurons of the cerebral cortex (arrows) [Group A. Cresyl violet (C.V). X50].
- b- Shrinkage and margination of the nucleus of the neurons in the cerebral cortex associated with perineural gliosis (arrows) [Group A. C.V. X50].
- c- Normal neurons of the cerebral cortex in the control [Group D. C.V .X50].
- d- Perineuronal microglia cells reaction (gliosis) (arrows) [Group A. H&E.X40].
- e- Perineuronal microglia cells reaction (gliosis) (arrows) [Group A. C.V .X40].

Fig. 2: Sections of the brain rabbits from group A ($HgCl_2$- treated group) and control group showing blood vessels of the cerebral cortex and hippocampus.

- a- Perivascular edema associated with congestion and damage in endothelial lining cells (arrows) of the blood vessels of the cerebral cortex [Group A. H&E.X40].
- b- Perivascular cuff have lymphocytic and microglia infiltration (arrows) in blood vessels of hippocampus [Group A. H&E.X40].
- c- Normal blood vessels of the cerebral cortex of control [Group D.H&E.X40].

Fig. 3: Sections of the brain rabbits from group A ($HgCl_2$- treated group) and control group showing different layers of the cerebellum.

- a- Decrease thickness of the granular layer (G) with diffuse loss of purkinje cells (arrows) [Group A. C.V. X50].
- b- The shape of purkinje cells was distorted and their arrangement was disrupted (arrows) [Group A. C.V. X50].
- c- In control group, the purkinje cells (arrows) are arranged side by side in a single row between the outer molecular layer (M) and the inner granular layer (G), they have normal flask- shape with extensively branched dendrites [C.V.X50].

Fig. 4: Sections of the brain rabbits from group B ($HgCl_2$/Vit.C- treated group) showing:

- a- Few neurons still have cytoplasmic vaculation (arrows) [Group B. C.V.X50].
- b- Neurons slightly normal [Group B. C.V. X50].
- c- Slight perivascular edema (arrows) [Group B. H&E.X40].
- d- Slight increase in the Purkinje cell number (arrows)[Group B. C.V.X20].

Fig. 5: Sections of the brain rabbits from group C ($HgCl_2$/Tiron- treated group) showing:

- a- Normal neurons and perineuronal tissues of the cerebral cortex [Group C.C.V. X50].
- b- Normal endothelial lining of blood vessels (arrows) [Group C. H&E. X20].
- c- Increase number of purkinje cells with normal arrangement [Group C. C.V. X20].
- d- Normal flasked shape of the purkinje cells (arrows). Group C. C.V. X50

DISCUSSION

Oral administration of $HgCl_2$ for 3 months to female rabbit resulted in a significant enhancement in the level of lipid peroxidation products (MDA& 4-HAE) in the brain while the antioxidant enzymes SOD and GSHPx were decreased significantly Table (1). The increase in MDA& 4-HAE levels in the exposed animals was pointing to an occurrence of oxidative damage to membrane lipid. In addition, the animals showed decrease in SOD and GSHPx activity where, glutathione GSH and GSH-related enzymes GSHPx are considered as major cell defences to counter act oxidative stress (Sies, 1999 and Dringen *et al.,* 2005). Most cells are equipped with enzymatic antioxidant systems such as SOD, GSHPx and catalase or non-enzymatic antioxidants system, such as uric acid, Vit. C, Vit. E and albumin: when these defences are over helmed, cell function is affected (Mostafa *et al.,* 2006). The oxidative damage is though to participate in the pathogenesis of neurodegenerative disorders and many chronic diseases by inducing oxidative changes to cellular lipid, proteins and DNA. Excessive reactive oxygen species (ROS) production, which related to oxidative stress, can occur during the normal aging process or following exposure to environmental toxicants (LEE and OPanashuk, 2004). Overproduction of reactive oxygen species and further oxidative stress is one of the most important consequences of toxicity of metals (Hansen *et al.,* 2006). Mercury induced neurotoxicity is related, at least in part, to its effect on the GSH antioxidant system (Manfroi *et al.,* 2004 and Farina *et al.,* 2005). Where, it can induce lipid peroxidation and this effect appears to be related to the ability of mercurial compounds to inhibit GSHPx activity (Farina et al., 2004 and 2005; Manfroi *et al.,* 2004). GSHPx belongs to class of enzymes that catalyzes the reduction of hydroperoxides by GSH and its main function is to protect against the damaging effects of endogenously formed hydroperoxides (Dringen *et al.,* 2005). HgCl2 may be included among ROS generating systems that are responsible for oxidative stress (Shanker and Aschner, 2003). The stimulating effect of mercurial compounds toward the reactive oxygen species ROS formation in biological systems has been proposed, indeed. Me-Hg induces ROS formation in vivo (rodent cerebellum) and in vitro (isolated rat brain synaptosomes) (Ali *et al.,* 1992). As well as in cerebellar neuronal cultures, hypothalamic neuronal cell line and in mixed reaggregating cell cultures (Sarafian *et al.,* 1994; park et al., 1996; Sorg *et al.,* .1998). Brain AChE activity was significantly inhibited in HgCl2 exposed group Table (2). It is well known that the inhibition of the brain AChE leads to the accumulation of acetylcholine in synapses that, in turn, induce hyperactivity of cholinergic pathways and interfered with neurotransmitter function leading to neurotoxicity (Silva *et al.,* 2006). Costa, (1988) reported that alterations in any parameter of neurotransmission could be resulted of neural death, due to the cytotoxic effect of neurotoxicants. These results indicate that HgCl2 can induce neurotoxicity through inhibition of AChE activity. Lakshman *et al.* (1993) reported that mercury chloride might be induced neurotoxicity through alteration of the levels of some neurotransmitters as noradrenalin, dopamine, serotonin and acetylcholine esterase activity in different regions in rat brain. Also Ji *et al.,* (2005) reported that mercury induce change in the neurotransmitters levels, where it induce decrease in AChE activity and increase in ACh content and this suggesting that these two indexes have the potential to biomarkers in assessment of health effects by mercury contamination.

The relationship between neurotransmitter and oxidative damage in the toxicity process was induced by mercurial compounds was studied by Cheng *et al.,* (2005). They concluded that methylmercury induced the change of neurotransmitter and free radical. They participated in the toxicity process of injury by methyl mercury. The damage of neurotransmitter may be because the chaos of free radical and the chaos of free radical may also do more damage to neurotransmitter vice versa. Also Jie *et al.,* (2007) reported that the long-term dietary consumption of mercury-contaminated rice induces the aggravation of free radicals and exerts oxidative stress.

The histopathological changes induced due to chronic mercury chloride exposure in rabbit for three months were involved the neurons, blood vessels and nerve fibers in examined cerebral cortex, hippocampus and cerebellum. The neurons undergo degenerative change. Some degenerative neurons became shrinkage and dark while others appeared swollen and disrupted with cytoplasmic vaculation Fig (1a). In agreement with our findings Nagashima *et al.* (1996) observed that mercurial compounds induced degeneration of cerebellar granule cells manifested by shrinkage and displayed marked nuclear pyknosis. The current study also demonstrated that the nuclei of these cells were chromatolysed, fragmented and marginated features, which are characteristic of degeneration in the neuronal cell body of cerebral cortex and hippocampus Fig (1b). Fallul-Morel *et al.* (2007) demonstrated that mercury induce apoptotic cell death in granular layer of hippocampus, hillus of dentate gyrus of exposed animals. Degenerative changes involving apoptotic processes were recognized in rabbit brain may be due to mercury – induced oxidative damage.

Cerebral edema either perineural or perivascular associated with angiopathic lesions was obtained in mercury treated group Fig (2a, b). Increase in the levels of lipid peroxidation products due to mercury exposure will enhance the angiopathic lesions and subsequently edema formation. It has reported that intoxication of mercuric chloride in the inner ears of guinea pigs may damage the blood vessels by causing swelling of the endothelial cells, mitochondrial disintegration, and sometimes protrusion of endothelial cell cytoplasm herniating into the blood vessel lumen. Chronic mercuric chloride intoxication resulted in distorted endothelial cells with an increase in cytoplasmic density (Anniko and Sarkady, 1977). Oxidative damage to the brain cell component may be an important mechanism mediating the neurotoxicity of mercury (Shanker *et al.,* 2004 and 2005). Significant increase in MDA & 4-HAE (lipid peroxidation marker) and significant decrease in the antioxidant enzymes SOD & GSHPx in mercury exposed group, in the present study, will confirm this suggestion.

Among various tissues, the cerebellar Purkinje cells were the most obvious staining targets for mercury accumulation (Warfvinge *et al.,* 1992). In this study, it was also demonstrated that distortion or obvious loss in the Purkinje cell layer and degeneration of Purkinje cells occurred following HgCl2 treatment Fig (3a, b). Sørensen et al., (2000) showed that a significant loss of Purkinje cells, granule cells and the volume of the granular cell layer were significantly reduced after mercurial compounds exposure. Møller-Madsen and Danscher, (1991) reported that after 20- days treatment of methyl mercuric chloride, mercury deposited in the cerebellar cortex were restricted to Purkinje cells and Golgi cells. A significant phenomena observed in this study was the significant reduced number of cerebellar Purkinje cells Table (3). In agreement with our findings, Carvalho *et al.* (2007) showed a reduced number of Purkinje cells in Me-Hg treated mice. It has been evidenced that cerebellar cells are selectively targeted by mercurial in vivo (Sanfeliu *et al.*, 2003).

Possible mechanisms of mercury neurotoxicity could be related to cell damage via excessive free radical formation (Shanker *et al.,* 2004), disruption of redox mediated toxicity and Ca2+ homeostasis (Gasso *et al.,* 2001). The studies invoke ROS as potent mediators in mercurial compounds cytotoxicity and support the hypothesis that excessive

ROS generation, at least in part, plays an important role in mercury –induced neurotoxicity (Shanker and Aschner, 2003).

Normally cells are equipped with endogenous defense comprising of both enzymatic and non-enzymatic antioxidants, tripeptides and others to safeguard the cells from probable oxidative injury. Still then, the cells suffer from oxidative assault when the antioxidant capabilities of the cells are inhibited by the heavy generation of ROS and its products resulting in the cells lost capacity to protect or to repair itself (Heffner and Repine, 1989). Vitamin C is a non – enzymatic antioxidant and, is therefore, potentially involved in protecting cells against oxidative stress (Anane and Creppy, 2001). Also, vitamin C is naturally occurring free radical scavenger; as such its presence assists various other mechanisms in decreasing numerous disruptive free radical processes from taking place, including lipid peroxidation (Knight *et al.,* 1993). The present study showed that ascorbic acid reduced MDA&4-HAE and increased the SOD & GSHPx in Hg Cl2+Vit. C exposed groups, the decrease in lipid peroxidation and the increase in antioxidant enzymatic activity after vitamin C supplementation, have already been reported (Anane and Creppy, 2001). Similarly aluminum and AFB1 induced cytotoxicity are reportedly minimized after vitamin C supplementation (Yousef *et al.,* 2003 and Yousef, 2004). These results in conclusively indicate the beneficial effects of vitamin C to overcome oxygen-dependant cytotoxicity in animals. Although, the detail mechanism of the action of vitamin C in scavenging oxygen radicals is not fully understood, it is believed that the vitamin C as an antioxidant might stimulate the 7-α hydroxylation of lipids and cholesterol nuclei thus enhancing their degradation to bile acids, which could be excreted from the body. Alternatively, vitamin C as apart of the redox buffer system can effectively scavenge harmful ROS (Yossef *et al.,* 2007). Vitamin C as a strong antioxidant having nucleophilic properties and binds to mercury ions Hg+2 to reduce mercury – induced DNA damage (Sato *et al.,* 1997). It further manifests its detoxification effect by removing or minimizing free radicals produced by mercury (Herbaczynska *et al.,* 1995). Rao *et al.* (2001) reported that the protective role of vitamin C on mercury chloride induced genotoxicity in human blood cultures. And they attributed this effect to strong antioxidant and nucleophilic nature of vitamin C. In the present study, supplementation of vitamin C to HgCl2-treated rabbits group has effectively increased the activity of the antioxidant enzymes (SOD& GSHPx) and the neurotransmitter AChE, while decreased MDA&4-HAE levels Table (1&2&3) and reduced the histopathological lesions in the brain tissues Fig (4 a, b and c), thereby minimizing the HgCl2- related oxidative stress.

Successful chelation therapy for metal poisoning lies in the mobilization of the metal and its excretion from the body by use of chelating agents. This would reduce the body burden of the metal and prevent its toxic effects (Sharma and Shuka, 2000). Results of the present study suggested that most of the above parameters responded positively with Tiron therapy, where, it was effective in returning the altered biochemical indices and histological changes largely to normal in HgCl2/Tiron treated rabbits Table (1& 2&3) and Fig (5a, b and c). The effectiveness of Tiron could be attributed to the chelating properties and available binding sites of Tiron, which leads to the decrease concentration of HgCl2 from the different organs. In previous studies, they confirmed the efficacy of Tiron against beryllium toxicity in experimental animals (Sharma and Shuka, 2000) and (Sharma *et al.,* 2002). The ortho-diphenolic chelator structure of Tiron forms stable water-soluble complexes and toxicity of these complexes is less than that of the metal ion they contain

(Sharma and Mishra, 2006). The no observable adverse effect level (NOAEL) for maternal and developmental toxicity of Tiron is 1500 mg/Kg/day (Bosque *et al.,* 1993). The therapeutic effectiveness (TEF) of Tiron was approximately equal to one. Tiron significantly increased urinary excretion of vanadium from the body. Tiron was also reported as an effective antidote for vanadyl sulphate intoxication in mice (Gomez *et al.,* 1991). The efficacy of Tiron to mobilize metal and restore the alterations in biochemical parameters may be due to the available binding sites and stability constant of the metal chelator complex (Shrivastava *et al.,* 2007). In the present study has clearly shown that Tiron was effective in the prevention of HgCl2 intoxication in rabbits, thereby decreasing the concentration of HgCl2 from the different organs. In conclusion, the chronic mercury chloride exposure in rabbits induced several biochemical alterations either at the level of oxidative damage or neurotransmitter activity, it also produce encephalopathy morphopathological lesions. The previous alteration and lesions were minimized in HgCl2/Vit.C treated group (B) and markedly improved in HgCl2/Tiron treated group (C). This will confirm the protective effect of vitamin C as antioxidant and Tiron as a chelating agent against mercury neurotoxicity. Tiron was found to be more effective than vitamin C in restoration of the most various parameters were investigated in this study. However, no previous information on the clinical use of Tiron in the therapy of toxicity by mercury compounds is available therefore, further investigations are required.

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