EFFICACY OF PROBIOTIC (PROTOXINE) ON MERCURY-INDUCED NEPHROTOXICITY AND LIPID PEROXIDATION IN RATS

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ABSTRACT

Mercury is the third most dangerous heavy metal and its toxicity causes serious risks to health through unfavorable pathological and biochemical effects of oxidative stress. The aim of the present study was to elucidate the possible protective role of protoxine, a probiotic, on mercuric chloride-induced oxidative stress and histopathological changes in the kidneys of the experimented rats. Adult male Wistar albino rats were daily exposed to mercuric chloride (0.25 mg kg^{-1}) in the diet and water together for 20 days. Treatment with mercuric chloride led to oxidative stress by elevating malondialdehyde (MDA) level and also decreasing superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. Furthermore, mercuric chloride exposure induced histopathological changes characterized by tubulonecrosis in the renal tissue of intoxicated rats. MDA level, SOD, GSH-Px activities and histopathological changes were modulated in concomitantly daily supplementation of protoxine (12 mg rat^{-1}) to mercuric chloride-treated groups. Results from current study suggested mercuric chloride treatment resulted lipid peroxidation, alterations of enzymatic antioxidant defense system and histolopathological changes in the kidneys of male rats. The administration of protoxine proved to be beneficial in ameliorating the mercuric chloride-induced nephrotoxicity.

Key words: Mercuric chloride, Oxidative stress, Nephrotoxicity, Probiotic, Histopathology.

INTRODUCTION

Mercury present in the environment is a well-established toxicant to human and animal health. Exposure to mercury brought harmful effects to human health, but changes resulting from human exposure to mercury only called the attention of the scientific society after the accidents in Japan and Iraq (Bernhoft, 2012). In Iraq, an outbreak mercury poisoning occurred in 1971 when wheat grains were treated with fungicides containing organic mercury. Over 500 people who ate bread made

with contaminated wheat were killed (Bakir *et al.*, 1973). In Japan, in the (Minamata Bay) a tremendous poisonous cases occurred resulting from the deposition of industrial waste with large quantities of mercury. Mercury was then ingested by human through fish intake. Signs and symptoms such as ataxia, speech impairment, visual field constriction, sensory disturbance, deafness, blindness, tremors, involuntary movements, mental retardation, coma, and death were recorded. Infants whose mothers were infected developed mental retardation, peripheral neuropathy, cerebral palsy and blindness. These changes became known as Minamata disease or Russell-Hunter syndrome (Takeuchi *et al*., 1996 ; Yee and Choi, 1996 ; Gochfeld, 2003). Mercury in the inorganic form such as salts, for example, mercury (II) chloride, primarily affects gastrointestinal tract and kidneys. Since it cannot cross the blood brain barrier easily, mercury salts inflict little neurological damage without continuous or heavy exposure. Mercury (II) salts are usually more toxic than their mercury (I) counterparts because of their greater solubility in water and rapid absorption by the gastrointestinal tract (Lund *et al*., 1993).

Nephrotoxicity is a serious side effect of mercury and is believed to be related to reactive oxygen species in the kidney. It is well known that inorganic mercury causes severe kidney damage after acute and chronic exposure (Monisha *et al*., 2014). Pretreatment with $ZnCl_2$ abolished mercury-induced delta-ALA-D-inhibition in kidneys. In face of zinc effects to prevent Hg-delta-ALA-D inhibition and to alter Hg-deposition levels in kidney these results suggest that these effects may be partially due to the synthesis of metallothioneins (Peixoto *et al*., 2003). Recent investigations have proved the crucial role of nutritional antioxidants to prevent the damage caused by toxic compounds (Ahmed *et al*., 2010). Probiotics are defined as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host. These micro-organisms are normally isolated from intestinal microflora of the intended species and selected based on conditions such as resistance to stomach acids, bile salts, ability to colonize the intestinal harmful microorganisms (Chaucheyras and Durand, 2010 ; Cho *et al*., 2011 ; Zahra *et al.*, 2017). Some probiotics are also known to have antioxidative properties in human subjects, which may be another important characteristic for mercury toxicity protection. On the basis of these special functions, lactobacilli seem to have potency against mercury toxicity. Alla *et al*., (2017) found that probiotics can decrease cadmium pollution effectively. This hypothesis was also

proposed in a recent review (Monachese *et al*., 2012), but to our knowledge, very few studies on the protective effects of lactobacilli against mercury toxicity have been carried out so far and the mechanism of such protection has not been studied yet. Therefore, it is interesting to investigate whether some probiotic like protoxine might play a role in the alleviation and/ or amelioration of mercury toxicity. The current study was designed to determine the antioxidative stress impact of using probiotic (protoxine) on the biological effects of mercurial toxicity in rat model.

MATERIALS AND METHODS

Animals

Eighteen adult male Wister albino rats weighing 250-275 g were used for the present study. They were reared at the animal house of the college of pharmacy, University of Baghdad, Baghdad, Iraq with the approval of animal rights review committee. They were acclimatized for 1 week on normal diet of pelletized rat chow, with water given *ad libitum* at room temperature (25-28 ^{o}C) within a 12:12 hrs. light and dark cycle before the commencement of the experiment.

Experimental induction of nephrotoxicity

Mercuric chloride was chosen to induce nephrotoxicity in rats. Mercuric chloride was added to the food and water of the experimental rats at a non lethal dose of (0.25 mg $HgCl_2$ kg^{-1} food or water).

Probiotic Material Used

PROTOXINE (total viable count= $2.00x10^{12}$) a combination of beneficial probiotic microorganisms in dextrose monohydrate:

Lactobacillus plantarum
Lactobacillus delbrueckii spp
Bulgaricus PXN39
Lactobacillus acidophilus
Lactobacillus rhamnosus
Bifidobacterium bifidum
Streptococcus salivarius spp
Thermophilus
Enterococcus faecium

Experimental Setup

Rats were divided into three groups comprising of six animals each:

Group I: Served as control (Received standard diet and normal tap water).
Group II: Received contaminated diet and water with $HgCl_2$ continuously for 21 days.
Group III: Received contaminated diet and water with $HgCl_2$ continuously mixed with probiotic (protoxine) in a dose of 12 mg each rat^{-1} for 20 days.

Collection and Processing of Rat tissues

At the 0 and 21 days, Blood samples were collected by puncture from the heart in clean test tubes, serum separated by centrifuging the blood samples at 4000 rpm for 5 min to obtain the serum for the estimation of malondialdehyde (MDA) oxidative stress biomarker, GSHP-x (Glutathione peroxidase) and SOD (Superoxide dismutase). Furthermore, at the end of experimental period (21 days), the animals were humanly sacrificed by using 60 mg/kg body weight of sodium pentothal. Kidneys were removed and washed with ice-cold saline and immersed 5 days in 10% neutral buffered formalin, processed in automatic tissue processor and stained with hematoxiline and eosin for histopathologic study.

Histopathological Studies

Kidneys were fixed for 48 hr in 10% neutral buffered formalin saline. Tissues were embedded in paraffin and sectioned at 5-µm thickness using a rotary microtome. Sections were stained with hematoxylin-eosin (H and E) according to Luna (1968) for light microscopy examination.

Biochemical Analysis

Determination of MDA by Thiobarbituric acid reactive substances (TBARS): The proteins in 0.15 ml serum were first precipitated by using sulphuric and phosphotungstic acid and then the levels of MDA were measured in these samples. Precipitate obtained incubated with TBA in a water bath at 95 °C for 60 minutes in an environment with oxygen and pH=3.4. The colored complex that occurred was refrigerated to room temperature. Then the complex was taken into n-buthanole phase. At the end, the complex of MDA-(TBA) was measured by using Shimadzu UV-1201V spectrophotometry at 532 nm TEP (1, 1, 3, 3-tetraethoxypropane) was used as standard MDA.

Serum activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) of all groups were analyzed on Randox diagnostic's kits by automated chemistry analyzer.

Statistical Analysis

Statistical analysis concluded analysis of variance of mean±SD were performed using the SPSS version 18 statistical package, 2010, (SPSS Inc. Chicago, IL, USA). Data were expressed in as mean ± SEM. *P*- value of less than 0.05 was considered as statistically significant.

RESULTS

Oxidative-antioxidative stress biomarkers

At day 0 there were no significant statistical differences ($p<0.05$) between all groups regarding the level of serum malondialdehyde (MDA) and activites of both superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (Table 1).

Table 1. The protective role of protoxine against the toxic effects of mercuric chloride on serum malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in rats at day 0

Variable	Group 1 (Control) *N=6*	Group 2 ($HgCl_2$) *N=6*	Group 3 ($HgCl_2$+Protoxine) *N=6*
MDA (*M* mol l^{-1})	0.55 ± 0.19^{A}	0.57 ± 0.21^{A}	0.57 ± 0.11^{A}
SOD (µ mg^{-1} Hb)	11.01 ± 1.26^{A}	10.90 ± 1.88^{A}	10.13 ± 1.15^{A}
GSH-Px (µ mg^{-1} Hb)	25.35 ± 0.69^{A}	25.27 ± 0.39^{A}	24.95 ± 1.61^{A}

Values are given as mean ± SEM for 6 rats $group^{-1}$.
Each value not sharing a common letter superscript is significantly different ($p<0.05$).

Whereas, at day 21 there were a significant statistical differences ($p<0.05$) between both control (Group 1) and $HgCl_2$ +Protoxine (Group 3) comparing to the $HgCl_2$ (Group 2) regarding the estimations of MDA level and SOD activity. Frank and significant statistical differences ($p<0.05$) in the activity of GSH-Px were seen between all groups at this day (Table 2).

Table 2. The protective role of protoxine against the toxic effects of mercuric chloride on serum malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in rats at day 21

Variable	Group 1 (Control) *N=6*	Group 2 ($HgCl_2$) *N=6*	Group 3 ($HgCl_2$+Protoxine) *N=6*
MDA (*M* mol l^{-1})	0.57 ± 0.19^{A}	1.88 ± 0.16^{B}	0.59 ± 0.41^{A}
SOD (µ mg^{-1} Hb)	10.86 ± 1.14^{A}	6.50 ± 1.17^{B}	9.93 ± 1.15^{A}
GSH-Px (µ mg^{-1} Hb)	24.18 ± 0.79^{A}	12.17 ± 0.49^{B}	20.45 ± 1.01^{C}

Values are given as mean ±SEM for 6 rats $group^{-1}$.
Each value not sharing a common letter superscript is significantly different ($p<0.05$).

Histopathological findings

No histopathological lesions and normal tissue architecture had been seen in the kidneys of the control group (Fig. 1). In Group 2 the histopathological findings show that $HgCl_2$ cause diffuse coagulative necrosis of the distal part of the proximal convoluted tubules, mainly the pars recta and in the outer stripe of outer medulla, respectively. Furthermore, nephropathy was characterized by nephrosis, foci of hydropic tubular degeneration, thickened tubular basement membrane and scattered dilated tubules containing hyaline casts. Massive and diffuse cellular necrosis was observed in the proximal tubules of kidneys from rats treated with $HgCl_2$ alone. The dead cells were in an advanced stage of karyolysis and cytoplasmolysis, fragmentation and dissolution. In addition, the lumen of these tubules were filled with numerous dead cells, fragments of cells, sometimes membrane-bound and debris were shed into the lumen of the tubules with degenerated and desquamated epithelial cells, massive hyaline casts, and very few infiltrated inflammatory cells in the form of mononuclear cells (Fig. 2 and 3).

On the contrary, in the treated group with protoxine the damages exclusively, inducing vacuolization of some renal convoluted tubules and rarely early signs of necrosis in few proximal convoluted tubules where seen in limited areas of kidney. There was a significant reduction in lesions caused by epithelial and nuclear changes typically associated with tubular necrosis, observed only in the animals that were continuously treated with protoxine (Fig. 4). This was evidenced by preservation of tubular morphology compared to the group treated with $HgCl_2$ alone.

A statistically significant difference was observed in treated animals and controls comparing to the intoxicated group. The kidneys of all treated rats are summarized in (Table 3). Light microscopic examination of kidneys from control and probiotics treated rats showed almost normal architecture to mild structural alterations in renal tissues.

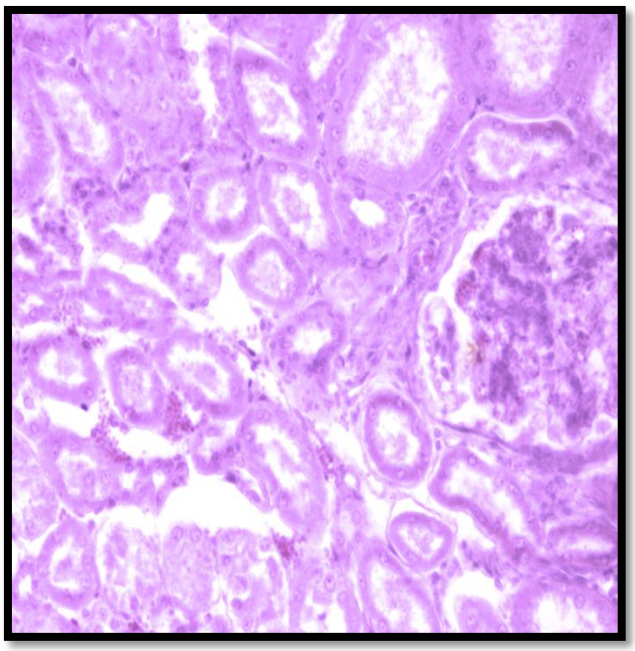

Figure 1. Kidney of rat from control group, normal tissue architecture

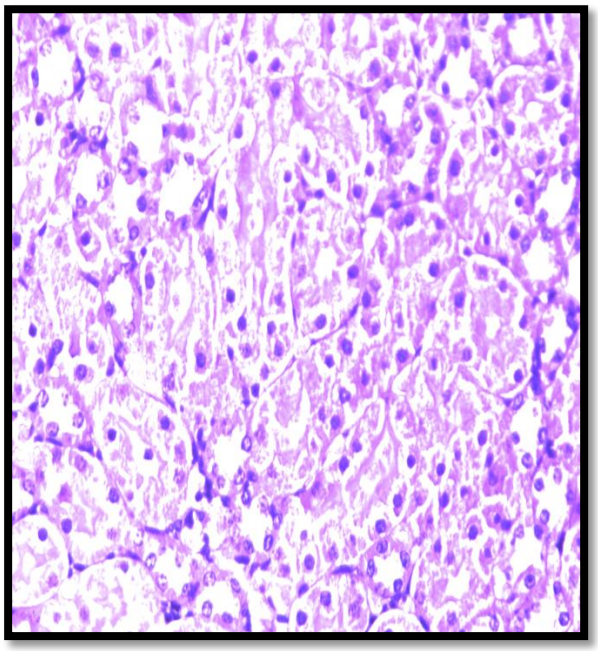

Figure 2. Kidney of rat from $HgCl_2$ treated group, nephropathy was characterized by nephrosis, foci of hydropic tubular degeneration, thickened tubular basement membrane and scattered dilated tubules containing hyaline casts. A few casts are present in the distal part of the pars recta between the vascular bundles

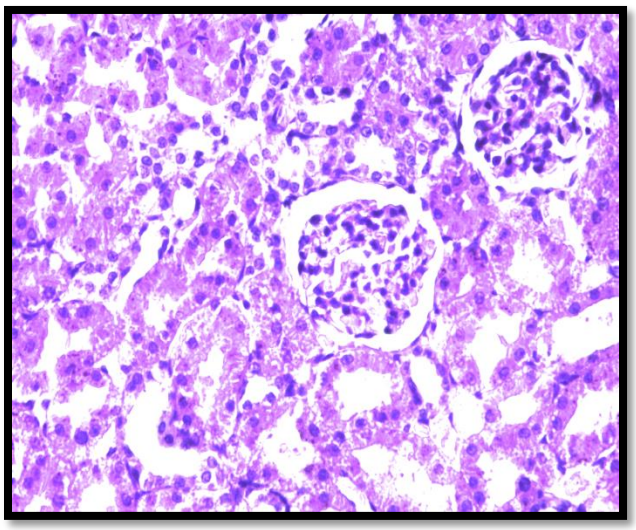

Figure 3. Kidney of rat from $HgCl_2$ treated group, first part of pars recta showing pyknosis, karyorrhexis, karyolysis, cytoplasmolysis and mild degree of mononuclear cells infiltration as advanced nuclear & cytoplasmic signs of coagulative necrosis

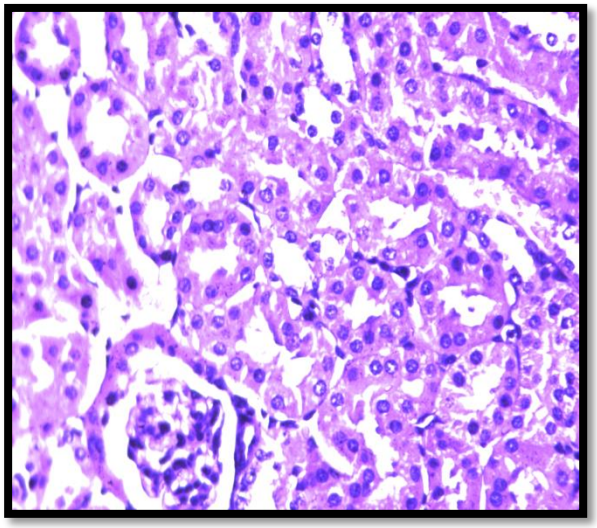

Figure 4. Kidney of rat from Group 3 ($HgCl_2$+Protoxine) treated group, a significant reduction in lesions observed only in the animals that were continuously treated with protoxine, there was pronounced preservation of tubular morphology compared to the group treated with $HgCl_2$ alone

Table 3. Histopathologic scoring of renal lesions in kidneys of Group 1 (Control), Group 2 ($HgCl_2$) treated group and Group 3 ($HgCl_2$+Protoxine) treated group at the end of the experimental period 21 days

Lesions	Group 1 (Control)	Group 2 (HgCl2)	Group 3 ($HgCl_2$+Protoxine)
Nephrosis	-	+ + + + + +	+ +
Pyknosis in the endothelium of the proximal convoluted tubules	-	+ + + + + + +	+ + +
Karyorrhexis	-	+ + + + + + +	+ + +
Karyolysis	-	+ + + + + +	+ + +
Cytoplasmolysis	-	+ + + + + + + +	+ +
Infiltration of inflammatory cells	-	+ +	+

DISCUSSION

Current study may be the first to evaluate some of the protective mechanisms of the oral administration of the probiotic (protoxine) as (beneficial microorganism) against mercurial nephrotoxicosis. This study provides biological evidence that protoxine can reduce lipid peroxidation, and reverse deficits in antioxidant defense systems to alleviate subsequent bad consequences of mercurial nephrotoxicosis.

Oxygen free radicals are considered to be important mediators of mercury-induced renal failure (Stuart *et al*., 2006). Characters of mercury nephrotoxicity are oxidative stress and inflammation. Generation of reactive oxygen metabolites may be the basis of a variety of insults, such as lipid peroxidation. The nephrotoxicity of mercury is associated with its accumulation in the renal cortex, which is leading cell damage and death. Treatment with several natural and synthetic antioxidant substances has been extensively studied and shown to be useful for either the prevention or amelioration of nephrotoxicity in experimental rats.

Taken together, the results showed that compared to the mice that received mercury only, probiotic treatment can effectively decrease and alleviate renal oxidative stress, and ameliorate renal histopathological changes. Our results suggested that probiotic treatment is more effective against mercuric toxicity than a simple antioxidant treatment due to its special physiological functions and that it can be considered a new dietary therapeutic strategy against nephrotoxicity. Previous studies confirmed that some species of lactic acid bacteria (LAB) including *Lactobacillus rhamnosus*, *L. plantarum*, and *Bifidobacterium longum* are

capable of binding heavy metals *in vitro* (Halttunen *et al*., 2007 ; Halttunen *et al.*, 2008). Furthermore, multiple recent reports confirmed that other probiotics may also be protective against heavy metal toxicity. A mixture of *L. rhamnosus* Rosell-11, *L. acidophilus* Rosell-52 and *B. longum* Rosell-175 significantly reduced Cd-induced genotoxicity both *in vitro* using liver tissue culture and in rats (Jama *et al*., 2012).

Frankly, the known histopathological lesions (Haagsma and Pound, 1980) and antioxidant enzyme levels associated with Hg-induced nephrotoxicity in rats were also observed. These findings were confirmed by observation of histopathological lesions such as epithelial desquamation, tubular necrosis, tubular casts, and infiltration of inflammatory cells, indicating that the kidney is very sensitive to Hg toxicity. A significant decrease in oxidative stress (MDA) biomarker and significant increase in antioxidative stress biomarker, however, were observed in the Hg+ probiotic group when compared with the Hg alone group. These data indicate that probiotic at selected dose has exerted a protective effect against Hg nephrotoxicity. The administration protoxine may effectively decreased intestinal Hg absorption and alleviated tissue oxidative stress, reversed renal damage and ameliorated the corresponding histopathological changes. These findings are in the the line of Hemaiswarya *et al.,* (2013) explanations about the mechanism of action of probiotics. The evidence of this study confirms the relationship between oxidative-anti oxidative parameters and histopathological findings, Furthermore, Fermentation breaks down the nutrients in foods by the action of beneficial microorganisms and creates natural chelators that are available to bind toxins and remove them from the body may added as another mode of action to minimize the potential toxic effects of mercurial toxicosis.

CONCLUSION

The selected combination of beneficial microorganisms (Protoxine) has some significant protective effect on experimental nephrotoxicity induced by $HgCl_{(2)}$ in rats and it is related to inhibition of lipid peroxidation (MDA production), which involves triggering of antioxidative stress master enzymes SOD and GSH-Px too. Based on current findings there is a need to try a more profound investigation into the antioxidant abilities of probiotics and prebiotics based on the available experimental and clinical data.

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تأثير إعطاء المسكن الحيوي (البروتوكسين) في تسمم وأكسدة الدهون في كلى الجرذان المعاملة بالزئبق

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المستخلص

يشكل الزئبق ثالث اخطر المعادن الثقيلة اهمية إذ تسبب سميته مخاطرا صحية جدية من خلال تاثيراته المرضية غير المرغوبة المتاتية من عملية الجهد التاكسدي التي يحدثها. الهدف من الدراسة التجريبية الحالية هو تخمين دور الحماية التي يوفرها اعطاء السابق الحيوي المسمى بروتوكسين على عملية الجهد التاكسدي والتغييرات النسيجية المرضية الناشئة في كلى الجرذان المستخدمة. تضمن البحث استخدام جرذاناً بالغة من

جنس وستر البابنو التي تم اعطائها كلوريد الزئبق عن طريق الماء والعلف بجرعة 25 ملغم لكل كغم ولمدة عشرين يوما. سبب كلوريد الزئبق لوحده جهدا تاكسديا تمت ملاحظته عبر ارتفاع مستوى المالون داي الديهايد المؤشر الحيوي لعملية اكسدة الدهون وانخفاضا لفعالية بعض الموءشرات الانزيمية الحيوية المضاده للجهد التاكسدي مثل انزيم الـ سوبراوكسياید دسميوتيز وانزيم الكلوتاثايون بيروكسيديز فضلاً عن إحداث تغيرات نسيجية مرضية تمثلت بالنخر النبيبي في كلى الجرذان المعامله به. احدثت اضافة السابق الحيوي البروتوكسين بجرعة يومية مقدارها 12 ملغم لكل حيوان والمستخدم سوية مع كلوريد الزئبق انخفاضا هاما في مؤشر الجهد التاكسدي وارتفاعا في اقيام الانزيمات المضادة لذلك الجهد مع احداث تعديلا ايجابيا في هيئة الافات النسيجية المرضية الكلوية. أكدت نتائج البحث الحالي التاثير السمي لكلوريد الزئبق وقدرته على احداث عملية اكسدة دهنية نشأ جرائها تغيرات نسيجية مرضية في كلى الجرذان المعاملة به وتغيرات سلبية في النظام الانزيمي المضاد. ان الاستخدام المرافق للسابق الحيوي البروتوكسين مع كلوريد الزئبق في هذه التجربة اثبتت قدرته على احداث تحسن احصائي هام في وضع السمية الكلوية الناشئة عن تناول الجرذان لمركب كلوريد الزئبق.

الكلمات المفتاحية: البروتوكسين، مضاد أكسدة الدهون، كلى الجرذان، التسمم بالزئبق.