

Original Research Article

In-vitro assay of effect of NaMSA on mercuric chloride induced oxidative stress in erythrocytes

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A B S T R A C T

Mercury induces very serious toxicological and biochemical dysfunctions which leads to dangerous health hazards. In the recent years much attempt has been made to find out an active ingredient present in the plant useful to nullify the toxic effect created by Hg. Recent therapeutic approaches against mercury toxicity is effective less. Flavonoids has proved its action against allergy and against metal chelation. But, till now no scientific evidence is available for using morin-5'-sulfonic acid sodium salt as therapeutic agent against mercuric chloride induced toxicity. Hence the present investigation was designed to study *In vitro* effect of Morin-5'-Sulfonic Acid Sodium Salt against the mercury induced biochemical changes in goat erythrocytes. In order to nullify the oxidative stress against 3 different concentrations of $HgCl_2$ NAMSA with $50\mu\text{m}$ used for the present experiment. The mercuric chloride decreased CAT, SOD and GPx activities and increased MDA levels and when the same $HgCl_2$ administered with NaMSA, the values were maintained within the normal compared with control rats. This was due to cytoprotective activity of NaMSA on the erythrocyte against the $HgCl_2$ triggered toxicity. Obtained results were supported by the earlier studies. Mercury induces oxidative stress in erythrocytes in a dose-dependent manner through the generation of free radicals and alterations in the antioxidant defense system of cells and Morin hydrate is an effective protector for human erythrocytes against lysis by peroxyl radical.

Keywords

Mercury;
Flavonoids;
induced
toxicity;
NaMSA;
CAT,
SOD
and
GPx activities.

Introduction

Mercury is a chemical element with the symbol Hg and atomic number 80. Hg is a

divalent metal without any biological functions. It is also known as quicksilver,

It primarily affect the CNS, liver and renal system. Mercuric poisoning symptoms are systemic. This means that it doesn't just affect one part of the body, but it affects every system in the body. In addition to this, mercuric poisoning inhibits the immune system and therefore the patient will have other diseases as well (Senese, 2007).

Mercury risk in India

The concentration of mercury in fish in other sea food consumed in certain costal areas reported in range of 0.03-10.82 mg/g compared to the permissible limit of 0.5 g/g. There is a potential risk to human health and environment due to the entry of mercury in food chain in and around chloralkali plant. The basket fruits and vegetables contain several folds higher concentration mercury in certain industrial area against prescribed Indian standards. The overall total mercury concentration ranged from 62.5 to 548ng/gm (189 ng/gm). However, organic mercury compounds are more readily absorbed via ingestion than inorganic mercury compounds (US Environmental protection agency, 2010).

Mechanism

Free radicals are defined as atoms or molecules that contain one or more unpaired electrons. (Halliwell and Gutteridge, 1999).

Multiple mechanisms have been proposed to explain the biological toxicity of HgCl₂ by investigating the biochemical fate of various Hg forms (Gutierrez *et al.*, 2006). Indeed, the Hg²⁺ form has shown a great affinity for endogenous biomolecules-associated with thiol (-SH) group (Clarkson, 1997) and it is invariable found

attached to SH-containing proteins, small-molecular weight peptides (such as glutathione) and amino acids (Such as cysteine) (Perottoni *et al.*, 2004b), leading to a profound deterioration of vital metabolic processes (Sener *et al.*, 2003; Wiggers *et al.*, 2008). Consequently, the oxidative stress was strongly suggested as one of the crucial mechanisms in Hg-induced pathological aspects (Lund *et al.*, 1993; Clarkson, 1997; Perottoni *et al.*, 2004a). However, biochemical parameters are still more indicative of early physiological changes following sub chronic and chronic Hg exposure (Wadaan, 2009).

An antioxidant is defined as any substance present at low concentration compared to those of an oxidizable substrate significantly delays or prevents oxidation of those substrates. (Diplock, 1991; Halliwell and Gutteridge, 1999). The intracellular antioxidants include low molecular weight scavengers of oxidizing species, and enzymes, which degrade various radicals especially O₂•- and H₂O₂ (Srivastava, UNEP)

Antioxidant enzymes such as superoxide dismutase, catalase, thio redoxin reductase, glutathione peroxidase, glutathione-S-transferase, aldo-keto reductase and aldehyde dehydrogenase exist in the cells convert ROS into less noxious compounds. These enzymes collectively provide a defense against various radicals and oxidants (Chaudiere and Ferrari-Iliou, 1999; Halliwell and Gutteridge, 1999; Arner and Holmgren, 2000; Kuhn and Borchert, 2002).

Superoxide dismutase (SOD) is a primary antioxidant enzyme, which catalyzes the dismutation of O₂•- to the less-reactive species H₂O₂ and O₂. The cellular SOD is

represented by a group of metalloenzymes with various prosthetic groups. The three forms are cytosolic Cu-Zn-SOD, mitochondrial Mn-SOD and extracellular Cu-SOD.

Antioxidant enzymes

Catalase (CAT) is a tetrameric hemeprotein, which is located in peroxisome and very efficiently promotes the conversion of H₂O₂ to water and molecular oxygen. (Halliwell and Gutteridge, 1999; Valko *et al.*, 2007).

Glutathione peroxidase (GPx) is a H₂O₂ degrading enzyme, which is widely distributed in animal tissues. The phospholipid hydroperoxide glutathione peroxidase (PHGPx) is a member of GPx family, which can act upon peroxidised fatty acid residues within membranes and lipoproteins and reduce them to alcohols. Glutathione-S-Transferase (GST) is a detoxification enzyme, which catalyses the conjugation of GSH to an electrophilic site of toxic compounds thereby protecting cells against xenobiotics. (Halliwell and Gutteridge, 1999).

Thus, GSH appears to play a central role in intracellular antioxidant defenses as it is involved in all the lines of protection against ROS (Sies, 1999). During the course of the reaction catalyzed by GPx and GST, the GSH is rapidly converted into oxidized glutathione (GSSG). The ratio of GSH/GSSG plays an important role in regulating the cellular redox status. The decrease in ratio of GSH/GSSG has a dramatic impact on cellular function (Schafer and Buettner, 2001).

Due to the fundamental role of GSH in scavenging and removal of deleterious ROS, GR also plays a crucial part in the

antioxidant defense mechanisms of the cell (Williams, 1992; Argyrou and Blanchard, 2004).

Non enzymatic antioxidants

The non-enzymatic antioxidants are low molecular weight substances, which includes vitamin C, vitamin E, carotenoids, thiol antioxidants (glutathione, total sulphhydryl groups (TSH), thioredoxin (TRX) and lipoic acid (Valko *et al.*, 2007).

Metal chelators

Inorganic ingestion such as mercuric chloride should be approached as the ingestion of any other serious caustic. Immediate chelation therapy is the standard of care for a patient showing symptoms of severe mercuric poisoning or the laboratory evidence of a large total mercuric load. (Risher, Amler 2005).

In the past 50 years there has been substantial progress in understanding, developing and clinical application of chelating agents used to treat acute and chronic mercury poisonings in humans. Particularly, tissues after exposure to inorganic mercury salts and mercury vapor (Baum, 1999).

Identifying and removing the source of the mercuric is crucial. Decontamination requires removal of clothes, washing skin with soap and water, and flushing the eyes with saline solution as needed. In most cases the final major toxic form of mercury found in the affected tissues, blood (Magos, Halbach, Clarkson, 1978) and other cells.

Chelating agents are primarily sulfhydryl-containing compounds such as mono- or

dithiol molecules. At the molecular level, the chelation process appears as an inevitable tug of war between the chelating agents and the competing biological ligands (Andersen and Molecular, 2002).

Drugs such as DMP not effectively cross the cell membrane nor the blood brain barrier (Aposhian., 1983), Use 20% temporarily (Risher and Amier, 2005). The affinity of EDTA for mercury is low, there is no evidence that calcium disodium EDTA is able to remove mercury from human tissues (Aposhian *et al.*, 2003) AAND in addition to that many chelating therapeutic agents available in practice for acute inorganic mercuric poisoning with the following efficiency DMPS> DMSA> penicillamine>ALA> EDTA removed 20 percent.

Flavonoids

Flavonoids have recently attracted a great interest as potential therapeutic agents against a large variety of diseases, such as anti-viral, anti-allergic, anti-platelet and anti-inflammatory, and possibly protective effects against chronic diseases (Chantal *et al.*, 1996: Hollman *et al.*, 1999). NaMSA is easily soluble in water and keep properties of the parent compounds. The aqueous solubility of NaMSA under the same conditions was 2.7.10⁻² mol/dm³. Sulfonic morin derivative can be considered to be multiprotic acids, which dissociate in aqueous solutions yielding respective anions and NaMSA was used as antioxidant. NaMSA is characterized by low toxicity to laboratory animals (mice and rats) (Kopacz, 2002 and Szelag, 2003). Flavonoids are mutagenic only under aerobic conditions (Nagao *et al.*, 1981) strongly suggests a role for active oxygen. It may be useful in the

prevention of human disease attributed to free radical damage

Materials and Methods

Chemicals used

The fine chemicals Alanine, used for the present study purchased from Mercuric chloride, morin-5-sulfonic acid sodium salt, Dimethyl sulfoxide from Sigma chemicals, USA procured from its Bagalore supplier. Nitroblue tetrazolium salt (NBT), reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), phenazine methosulphate (PMS) and thiobarbituric acid (TBA) were purchased from Merck Company (A.R.Grade) dealer, chennai.

Methods

Standard methods were used for the estimation of factors. The level of reduced glutathione (GSH) by the method of Moran *et al.*, (1979), SOD by the method of Kakkar *et al.*, (1984), catalase by the method of Sinha (1972). The activity GPx by the method of Rotruck *et al.*, (1973),

Preparation of Morin-5'-Sulfonic Acid Sodium Salt

Morin was purchased from sigma chemicals, USA .is not soluble in water but soluble in alcohol 50 mg/ml. so, it is necessary to convert insoluble form of morin to water soluble Morin-5'-Sulfonic Acid Sodium Salt .hence, sulphonation reaction was carried out and morin was converted as Morin-5'-Sulfonic acid Sodium Salt (Kopacz, 2003) and used for the presented experiment.

Erthrocyte preparation

150 millilitres of fresh goat blood was collected in dry tubes from a healthy goat through cervical decapitation and using heparin anticoagulant. Erythrocytes were separated from blood plasma by centrifugation (1600 rpm at 40C for 5 min), then washed three times with a cold isotonic saline solution (0.9% NaCl). The supernatant and the buffy coat were carefully removed after each wash. After separation, packed erythrocytes were suspended in phosphate buffer (170 ml of Na₂HPO₄ (1.41 g/l) solution+77 ml of NaH₂PO₄ (1.19 g/l) solution + NaCl (8.8 g/l)], at pH 7.40 to obtain a 50% cellular suspension.

The mixtures were thawed, the erythrocytes were destroyed by osmotic pressure and then subjected to centrifugation, Supernatants were isolated and MDA levels and the activities of SOD, CAT and GPx were measured by spectrophotometer (Shimadzu UV-1700, Japan).

Experimental design

Group A: (Control) Erythrocytes incubated for 1 hour at 37°C in 0.9% saline.

Group B: Erythrocytes incubated for 1 hour at 37°C with 1.052 μM HgCl₂ (Rao et al., 2001).

Group C: Erythrocytes incubated for 1 hour at 37°C with 5.262 μM HgCl₂ (Rao et al., 2001).

Group D: Erythrocytes incubated for 1 hour at 37°C with 10.524 μM HgCl₂ (Rao et al., 2001).

Group E: Erythrocytes incubated for 1 hour at 37°C with 1.052 μM HgCl₂ (Rao et al., 2001) and morin-5'-sulfonic acid

sodium salt 50 μM (Venkatesan and Mohamed Sadiq, 2013).

Group F: Erythrocytes incubated for 1 hour at 37°C with 5.262 μM HgCl₂ (Rao et al., 2001) and morin-5'-sulfonic acid sodium salt 50 μM.

Group G: Erythrocytes incubated for 1 hour at 37°C with 10.524 μM HgCl₂ (Rao et al., 2001) and morin-5'-sulfonic acid sodium salt 50 μM.

Statistical Analysis

The values were expressed as mean value (n=6) of + S.E.M, The *in vitro* experimental data were analysed using one way analysis of variance by the Duncan's Multiple Comparison Test to determine the level of significance and p<0.05 was considered as statistically significant.

Results

Mercury induces oxidative stress in erythrocytes in a dose-dependent manner through the generation of free radicals and alterations in the antioxidant defense system of cells (Zabinsky et al., 2000). Chronic mercury exposure in stomach and intestine showed has significant effects on the functions of both red and white the blood cells and reduction from exposure results in the improvement of this condition (Huggins, 1999; and Dietrich Klinghardt, 2008) whereas mercury vapor is lipid soluble and has affinity for RBC (Goyer et al., 1993). Binding of mercury to glutathione and the subsequent elimination of intracellular glutathione levels are lowered in several specific types of cells on exposure to all forms of mercury, Glial cells (Lee, 2001), human erythrocytes (Queiroz, 1998) and mammalian renal tissue (Zalups, 2000).

In the presented work, Mercuric chloride alone administered RBC showed significant ($P<0.05$) decrease in the activity of CAT, SOD and GPX but MDA level was found to be high ($p<0.05$) compared with control group in which the Erythrocytes were not treated with neither $HgCl_2$ nor NaMSA. Whereas, administration of NaMSA along with $HgCl_2$ causes significant protection from the decline of CAT, SOD, GPX level and MDA from increase. this was due to the chelating effect of NaMSA on $HgCl_2$ activity (Figure.1 and Figure.2)

Erythrocyte SOD, CAT and GPx activities were decreased upon $HgCl_2$ -administration which was due to induced-oxidative stress in vitro, most likely due to its nucleophilic property (Faix *et al.*, 2003), has been shown to induce oxidative stress in erythrocytes through the generation of free radicals and alteration of the cellular antioxidant defense system on the levels of malonaldehyde (MDA) and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Dilek Duak., 2010).

Effect of NaMSA on $HgCl_2$ influenced MDA level in erythrocytes MDA level

The obtained results explains that $HgCl_2$ increased the MDA level significantly ($p<$ compared to control group where the same administered with NaMSA did not affect the MDA normal (Figure.1).

Normal erythrocyte function is wholly dependent on an intact erythrocyte membrane. The toxic effect of many environmental chemicals and pesticides (also includes Hg) is largely due in large part to their effect on erythrocyte membranes (Schara *et al.*, 2001; Brandao *et al.*, 2005) and mercury exposure has

been demonstrated to induce membrane lipid peroxidation detected by increased MDA content in many tissues (Emanuelli *et al.*, 1996; Kim and Sharma, 2003).

MDA is one of the major oxidation products of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of LPO. It has been shown previously that $HgCl_2$ increase MDA level in tissues (Mahboob *et al.*, 2001; Augusti *et al.*, 2008). In this study, MDA levels were increased in $HgCl_2$ -treated erythrocytes, which suggests that MDA levels could be used as a marker of $HgCl_2$ injury.

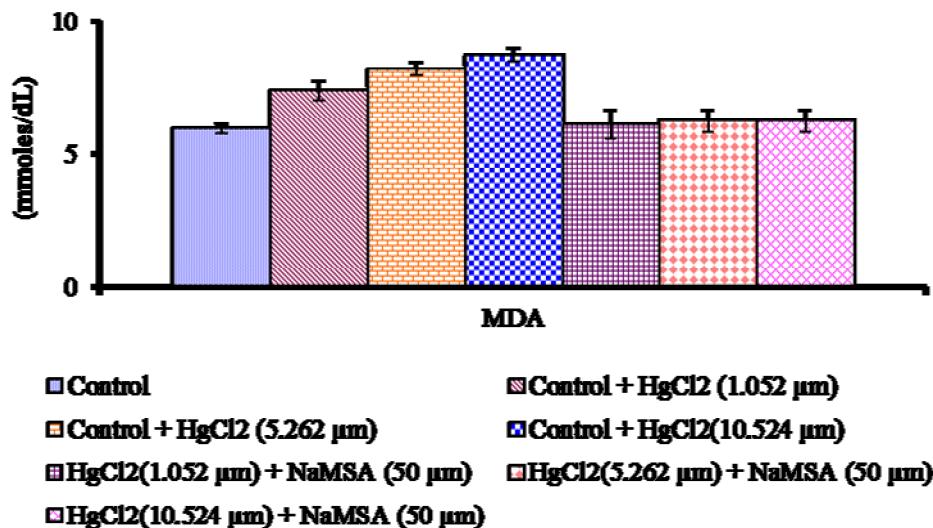
Influence of NaMSA on $HgCl_2$ induced changes in the activities of SOD, CAT and GPx in erythrocytes

As per the results recorded in the experiment, The CAT,SOD and GPx values declined from normal significantly ($p<0.05$) upon mercuric chloride addition, but the result was reversed to normal when NaMSA added with $HgCl_2$.

Bansal *et al.*, (1992) reported the in vitro toxicity of mercuric chloride on human erythrocytes in relation to their effect on lipid peroxidation and some enzymes. MDA is one of the major oxidation product of per oxidized polyunsaturated fatty acid and increased MDA content is an important indicator of LPO, $HgCl_2$ exposure in human erythrocytes increased the levels of MDA and SOD, CAT and GPx activities were decreased (Mahboob *et al.*, 2001; Augusti *et al.*, 1998).

Erythrocyte SOD, CAT and GPx activities were decreased in $HgCl_2$ -treated erythrocytes and this effect was prevented by pretreatment with combination of VC and VE.

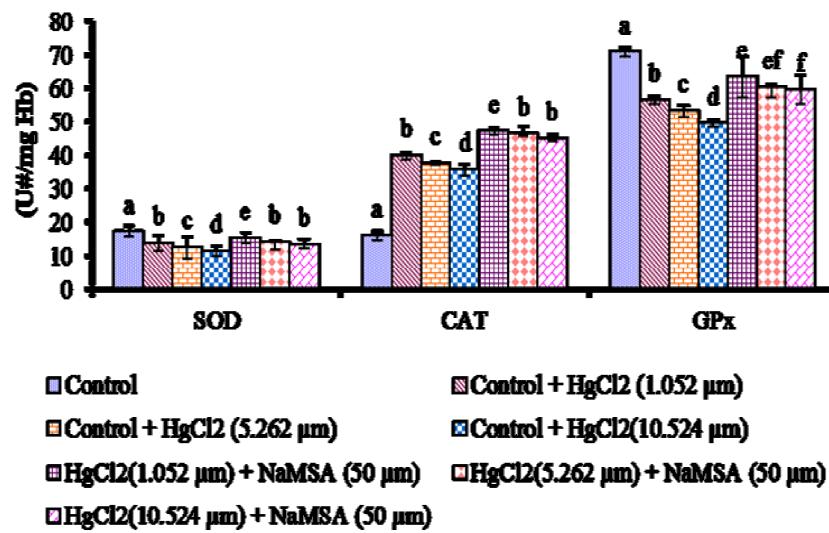
Figure.1 *In vitro* assay of activity of NaMSA on HgCl₂ induced changes in erythrocytes MDA level



HgCl₂ - Mercuric chloride; NaMSA – Morin sulfonic acid -5'-sodium salt

Values are means \pm S.D for six rats. Values not sharing a common superscript differ significantly at P< 0.05 (DMRT).

Figure.2 *In vitro* assay of activity of NaMSA effect on HgCl₂ induced changes in erythrocytes SOD, CAT and GPx level



HgCl₂ - Mercuric chloride; NaMSA – Morin sulfonic acid -5'-sodium salt

Values are means \pm S.D for six rats.

Values not sharing a common superscript differ significantly at P< 0.05 (DMRT).

Molecular damage of the cells in mercury toxicity is by the formation of peroxy radicals which can also be formed in lipid and non-lipid systems such as proteins (Dean *et al.*, 1993). The scavenger role of antioxygenic enzymes in removing toxic electrophiles helps the cell to maintain its internal environment to a limited extent at lower concentration of mercury. An increase in the oxidative stress may be due to a decrease in the antioxidant defenses or due to an increase in the processes that produce oxidants (Hussain *et al.*, 1999; Lukaszewicz-Hussain and Moniuszko-Jakoniuk, 2003).

GPx is well known to defense against oxidative stress in the cellular environment. So, the enhanced activity reported in the present investigation occurred probably as an adaptive cellular response against hydroperoxides generated by HgCl₂, as described by Santos *et al.* (1997) and Augusti *et al.* (2008).

Morin is a constituent of many fruits and used as a food additive because of its antioxidant activity (Hanasaki *et al.*, 1994; Ramanathan *et al.*, 1994). In addition, it possesses anti-inflammatory potential (Baumann *et al.*, 1980; Galvez *et al.*, 2001; Nakadate *et al.*, 1984).

The sulfonic derivative of morin is potent in its cytostatic and cytotoxic activities. However, its solubility in water was greater than that of the original agents and higher culture medium concentrations of NaMSA was obtained (Krol *et al.*, 2002).

However NaMSA and HgCl₂ simultaneous administration protected the erythrocytes from oxidative stress. This may be the antidote effect of morin-5-sulfonic acid sodium salt against the mercuric chloride

induced oxidative stress. Morin hydrate is an effective protector for human erythrocytes against lysis by peroxy radical (Wu, 1994).

In vivo studies, morin hydrate has also been found to prevent necrosis of liver (Wu., 1993.; Wu., 1994). Morin (2', 3, 4', 5, 7-pentahydroxyflavone), a member of the flavonoid family, demonstrated cyto protective properties against oxidative stress via antioxidant effects. Although previous papers reported that morin exhibits antioxidant effects on free radical, in addition to protecting cells such as myocytes, hepatocytes, and neuron cells against oxidative stress (Wu.,1995; Kok, 2000 and Gottlieb, 2006) and the same result is supported by our findings. From the obtained it concluded that HgCl₂ generates free radicals that leads to disrupt the architecture of RBC due to the decrease in CAT, SOD, Gpx and Increase in MDA. Whereas the NaMSA protects the RBC by chelating mercury and forming complex with the same. Hence the antioxidants retained in the normal level.

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