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Evaluation of blood nitrotyrosine and nitric oxide levels in acute mercury intoxication in children

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Abstract

Aim: Multiple processes have been demonstrated to elucidate the biological toxicity of mercuric chloride, among which oxidative stress has been identified as a contributing factor. The superoxide radical has the potential to induce peroxidation of lipid membranes, alter the activities of proteins and antioxidant enzymes, and modulate gene transcription. Additionally, it has the ability to swiftly deactivate nitric Oxide, resulting in impairment of endothelial function and causing harm to macromolecules, membranes, and DNA by generating more harmful radicals including peroxynitrite and hydroxyl radicals. The formation of nitrotyrosine occurs through the interaction between peroxynitrite and tyrosine residues found in proteins. Nitrotyrosine serves as a useful marker for assessing the possible cytotoxic impacts of nitric oxide. While there have been previous animal tests undertaken, the existing literature we have reviewed does not provide evidence regarding the impact of direct mercury exposure and mercury toxicity on nitrotyrosine and nitric oxide. In order to achieve our research objectives, we have devised a plan to investigate the presence of nitrotyrosine and nitric oxide in the blood serum of children who have been exposed to mercury in our study.

Materials and Methods: Our study included 65 patients, 42 girls and 23 boys, who had accidentally come into contact with mercury in the laboratories of some schools in Kahramanmaraş, and whose blood mercury level was over 10 µg/l and/or whose urine mercury level was over 15 µg/l. The control group of the study included a total of 23 children, 17 girls and 6 boys, who applied to the pediatric clinic with various complaints, without intoxication or neurological findings, and from whom blood samples would be taken for different diagnoses.

Results: Nitric oxide and nitrotyrosine levels were found to be higher in children exposed to mercury than in the control group ($p<0.01$). Nitric oxide, nitrotyrosine and mercury levels in the patients were high in both genders, and no gender-related difference was detected ($p>0.05$). The mean duration of mercury exposure was 45 minutes. Of the 65 patients, 20 were asymptomatic and 40 were symptomatic. The most common symptoms were headache and nausea.

Conclusion: The existing body of research predominantly focuses on investigating the association between mercury poisoning and oxidative stress biomarkers through animal studies, with a limited number of studies conducted on human subjects. Our study has made a valuable contribution to the existing literature by successfully detecting elevated levels of nitric oxide and nitrotyrosine in children who have been diagnosed with mercury poisoning.

Introduction

Mercury is a chemical element that can be found in the air, water, and soil. It exists naturally as inorganic salts and organic compounds, and all forms of mercury can be dangerous [1]. Oxygen radicals, which are chemicals generated within organisms, are continuously produced and frequently removed by the antioxidant defence system. Oxidative stress arises when the equilibrium between oxidation and reduction tilts in favour of free radicals, either

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due to a deficient antioxidant defence mechanism or an augmented production of free radicals [2,3].

Nitric oxide (NO); It is a reactive molecule that functions as an oxidative signaling molecule in various physiological processes such as neurotransmission and blood pressure. Excessive production of reactive nitrogen species (RNT), called nitrosative stress, causes cytotoxic and mutagenic effects. While increased NO levels increase apoptosis in some tumor cells, when it is at low levels, it can increase vascularity and protect cells from apoptosis. NO creates nitrosative stress by producing 3-nitrotyrosine (3-NT) [4]. 3-NT; It is a product of tyrosine nitration mediated by RNTs. 3-NT is a biomarker that indicates cell injury, inflammation, and the generation of NO [5].

Mercury (Hg) is a very dangerous metal that has no biological role in the organism and can have toxic effects even at very low levels. For this reason, it is considered a global pollutant that has very harmful effects on all living things [6]. Multiple studies have demonstrated that metals including mercury, lead, and cadmium can induce cellular harm by generating free radicals and triggering oxidative stress in numerous organs [7,8].

There is a scarcity of research examining the impact of sudden mercury poisoning on indicators of oxidative stress, such as NO and Nitrotyrosine. Furthermore, the precise consequences of this poisoning on human beings remain incompletely understood. Therefore, this study was conducted to investigate the influence of mercury on indices of oxidative stress in human volunteers.

Materials and Methods

A total of 65 patients, 42 girls and 23 boys, who were admitted to Kahramanmaras Sutcu Imam University Faculty of Medicine Child Neurology Polyclinic and Gaziantep Children's Hospital Pediatrics Clinic with acute mercury poisoning between February 20, 2012 and March 3, 2012, were included in this study. The control group consisted of patients who presented at the Pediatric Polyclinic with various ailments, but did not exhibit any signs of poisoning or neurological abnormalities. A total of 23 healthy volunteers, comprising 17 girls and 6 boys, were recruited in the study to obtain blood samples for different diagnostic purposes. The sample size was calculated as a minimum of 17 with a 5% deviation and 95% confidence level using the information that the prevalence of heavy metal intoxication is 1.1%.

It was learned that the cases considered as the patient group were involved in an accident that occurred in the school laboratory, when a jar filled with elemental mercury broke, and they played with mercury in the classroom. All of the children inhaled mercury, 4 patients handled mercury directly without any protective equipment, and 3 patients tasted mercury. Blood and urine samples were collected from individuals who exhibited symptoms such as nausea, headache, and skin rash, and were confirmed to have had exposure to mercury. The study included patients whose blood mercury levels exceeded 10 µg/l and/or urine mercury levels exceeded 15 µg/l [9]. All patients were hospitalized for treatment on the same day.

Patients with mercury poisoning were treated with oral chelator therapy to ensure excretion of mercury in the urine. [The recommended dosage for D-penicillamine is 50 mg/kg/day, divided into four equal doses. Alternatively, 2,3-Dimercaptopropanesulfonic acid (DMPS) should be taken at a dosage of 20 mg/kg/day, also divided into four equal doses]. DMPS was preferred for patients with neurological symptoms. N-acetylcysteine (15 mg/kg/day) was added to the patients' treatment regimen. 24-hour urine samples were collected from patients daily, and mercury levels above 15 µg/L were considered toxic, and asymptomatic patients with urine mercury levels below 15 µg/L were discharged.

Blood and urine mercury levels were studied using the ICP-MS method in the Ankara Hygiene Center Presidency Poison Research Directorate Laboratory. The determination of NO was determined by the diazotization of nitrite sulfanilamide and its associated N-naphthylethylenediamine (NNDA), produced by the Griess reaction and modified cadmium reaction, and by spectrophotometric measurement of the color resulting from the reaction at 545 nm. Results were expressed in µmol/L. The concentration of 3-NT in serum samples was quantified using the double sandwich enzyme-linked immunosorbent assay (ELISA) technique. Our study was approved by the Kahramanmaraş Sütçü İmam University Clinical Research Ethics Committee on 14.04.2014 with decision number 2014/01-07.

Statistical analysis

The statistical analyses were conducted using the SPSS20.0 programme. The descriptive statistics are reported as the mean plus or minus the standard deviation (SD). The chi-square test, Wilcoxon test, and Student's t-test or Mann-Whitney U-test were employed to compare categorical and continuous data between groups, respectively. The confidence interval was established with a level of confidence of 95%, and the threshold for statistical significance was chosen at $p<0.05$. The Independent Samples t-test was employed to assess disparities between the groups.

Results

Among the cohort of patients diagnosed with mercury poisoning, 42 individuals (64.6%) were female, while 23 individuals (35.4%) were male. Within the control group, there were 17 cases (73.9%) of females and 6 cases (26.1%) of boys. There was no statistically significant disparity in gender between the two groups ($p = 0.415$). While the average age of the mercury poisoning group was 12.3 ± 2.2 years, the average age of the control group was 7.95 ± 3.8 years. A statistically significant difference was detected between the two groups in terms of age ($p<0.001$).

The average duration of mercury exposure was 45 minutes (range, 25 – 120 minutes). Of the 65 patients, 20 (38.5%) were asymptomatic and 40 (61.5%) were symptomatic. The most common symptoms were headache (n:21, 32.3%) and nausea (n:11, 16%). Physical and neurological examination findings of 39 (60%) of the children were normal (Table 1). When average blood NO levels were compared in the patient and control groups; The NO level was 12.84 ± 3.59 (µmol/liter) in the patient group

Table 1. Sociodemographic characteristics of the patients, sign and symptom distribution.

Features of Children	n = 65 (100%)
Gender	
Male	23 (35.4%)
Female	42 (64.6%)
Average age	12.3 ± 2.2
Mode of exposure	
Inhalation	65 (100%)
Skin contact	4 (6.15%)
Oral	3 (4.6%)
Exposure time	
Median (min-max)	45 minute (25-120)
Symptoms of Patients	
Asymptomatic	20 (38.5%)
Headache	21 (32.3%)
Nausea	11 (16%)
Vomiting	4 (6.15%)
Skin rash	2 (3.07%)
Cough	3 (4.6%)
Diarrhea	1 (1.5%)
Vertigo	2 (3.07%)
Myalgia	4 (6.15%)
Blurred vision	1 (1.5%)
Laboratory	
Proteinuria	2 (3.07%)
Liver enzyme elevation	4 (6.15%)

Table 2. Nitric oxide and Nitrotyrosine levels in patient and control groups.

	NO (µmol/lt)		
Groups	Mean ± SD	Min- max	P
Control group (n=23)	5.64 ± 5.64	1.68-10.32	p<0.001
Patients (n=65)	12.84 ± 3.59	7.20-20.40	
	Nitrotyrosine (nmol/lt)		
Control group (n=23)	707.13 ± 167.2	438.90-996.40	p<0.001
Patients (n=65)	1004.96 ± 129.78	641.30-1436.30	

Independent samples t-test, statistically significant value: (p <0.05), SD: standard deviation, min: minimum, max: maximum.

and 5.64 ± 5.64 (µmol/liter) in the control group. The disparity in NO levels between the two groups exhibited a statistically significant distinction (p<0.001) (Table 2). In the patient group, the mean blood NO level in girls was 13.06 ± 3.66 (µmol/liter) and in boys it was 12.42 ± 3.51 (µmol/liter). There was no statistically significant difference in NO levels between genders (p = 0.49).

When average blood Nitrotyrosine levels were compared in the patient and control groups; Nitrotyrosine level was 1004.9 ± 129.7 nmol/l in the patient group and 707.1 ± 167.2 nmol/l in the control group. The disparity between the two groups exhibited statistical significance. (p<0.001) (Table 2). While the average blood Nitrotyrosine level in the girls in the patient group was 1000.31 ± 119.96 (nmol/liter), it was 1013.45 ± 148.53 (nmol/liter) in the boys. There was no statistically significant difference in NO levels between genders (p = 0.70).

The median blood mercury level in the patient group was 41 µg/l (5.6-2968) (Table 2). The average mercury level in female patients (n:42) was 225,02 ± 543.1 µg/l, and in male patients (n:23) it was 122,13 ± 310.13 µg/l. Nevertheless, there was no substantial disparity observed between the genders (p = 0.22). Within the same group, the mean blood mercury concentration was 186.13 ± 414.06 µg/l in children aged 7-12 (n:32) and 191.03 ± 531.97 µg/l in children aged 13-19 (n:33). There was no significant difference between the two age groups (p = 0.96). In the 7-12 age group, the average NO level was 12.36 ± 3.20 (µmol/lt), while in the 13-19 age group, it was 13.30 ± 3.93 (µmol/lt). There was no statistically significant difference observed between the two groups (p = 0.29). The mean Nitrotyrosine concentration was 976.65 ± 119.11 (nmol/lt) in the 7 - 12 age group and 1032.42 ± 135.50 (nmol/lt) in the 13 - 19 age group. The investigation revealed that there was no statistically significant disparity between the two groups, as indicated by a p-value of 0.83. While there was a statistically significant disparity in average age between the control group and the sick group, we believe that this discrepancy did not impact the outcomes, as there was no significant distinction observed in age and NO and Nitrotyrosine levels.

The Spearman correlation test was utilised due to the non-normal distribution of the data. A substantial statistical association was observed between the levels of NO and Nitrotyrosine (p=<0.01, r=0.605). There was no observed link between NO and Mercury (p=0.153), as well as between Nitrotyrosine and Mercury (p=0.819) (Table 3).

Discussion

Toxic metals, such as mercury, aluminium, and lead, induce oxidative stress by generating reactive oxygen species (ROS). Multiple investigations conducted in adult individuals have demonstrated that mercury has the potential to facilitate the generation of free radicals and induce cellular harm by beginning oxidative stress in many tissues [10]. Mercury has been proposed to interfere with cellular antioxidant defence mechanisms, primarily by blocking crucial antioxidant enzymes or depleting the intracellular antioxidant glutathione. Additionally, it has been reported that mercury achieves this by promoting the generation of ROS. Mercury is a significant heavy metal because of its pro-oxidant properties [11,12].

NO is a ubiquitous biological mediator that is present throughout the body and is involved in a wide range of physiological and pathological processes [13]. Although other free radicals are detrimental at any level of concentration, NO serves a crucial function in significant physiological processes when present in low quantities. Nitric oxide has a dual impact, as it induces peroxynitrite-mediated lipid oxidation events within cells. Additionally, NO acts as both a pro-oxidant and an antioxidant, capable of inhibiting the production of lipid radical chains [14].

Table 3. Correlation between blood mercury, NO and nitrotyrosine levels.

			NO level	Nitrotyrosine level	Mercury level
Spearman's rho	NO level	Correlation Coefficient	1.000	.605**	.179
		Sig. (2-tailed)	.	.000	.153
		N	88	88	65
	Nitrotyrosine level	Correlation Coefficient	.605**	1.000	.029
		Sig. (2-tailed)	.000	.	.819
		N	88	88	65
	Mercury level	Correlation Coefficient	.179	.029	1.000
		Sig. (2-tailed)	.153	.819	.
		N	65	65	65

**. Correlation is significant at the 0.01 level (2-tailed). Spearman correlation test.

Nevertheless, an overabundance of unregulated NO synthesis might be detrimental to cells. Nonetheless, due to these characteristics, NO emerges as an optimal physiological messenger molecule [15]. Excessive production of NO leads to its reaction with superoxide radical, resulting in the formation of peroxynitrite. This process significantly contributes to cellular damage [16]. Peroxynitrite forms 3-NT by introducing a nitro group to the phenolic ring of tyrosine, either within proteins or in its unbound state. The reaction can either occur spontaneously or be facilitated by transition metals, superoxide dismutase (SOD), CO_2, and myeloperoxidase [17-19]. The measurement of nitrotyrosine is considered a valuable indicator for diagnosing in vivo damage caused by NO, as nitrotyrosine is the stable final product of peroxynitrite oxidation [20]. Although 3-NT is not detectable in the plasma and tissues of healthy persons, its levels considerably rise under situations characterised by elevated production of NO and oxidative stress, such as inflammatory and degenerative processes. Nitrotyrosine and NO are present in several cells, but their utility as indicators of brain injury has been increasingly demonstrated in recent times. Mercury exposure was administered to pregnant mice throughout the period of foetal brain development, and subsequently, the level of nitrergic activity in the molecular layer of the dentate gyrus was assessed. It was shown that there was an increase in nitrogenous activity in this specific brain region [21]. Elevated levels of 3-NT have been seen in patients with Alzheimer's disease and Amyotrophic Lateral Sclerosis (ALS) compared to control groups [22,23].

In their study, Sumathi et al. [24] orally administered methyl mercury to male Vistar rats for a duration of 21 days. They also administered methyl mercury to a control group of rats, along with oral doses of Bacopa Maniere extracts, a plant commonly used in alternative medicine in India for its neuroprotective properties. Subsequently, erythrocytes were detected only in the group that received methyl mercury alone. The activities of SOD, Catalase (CAT), and Glutathione peroxidase (GPx) were observed to decrease significantly. Additionally, levels of NO_2^- and NO_3^- were found to increase. These metabolites were considered as indicators of free radical damage, and it was hypothesised that NO production could contribute to oxidative damage. Furthermore, Bacopa Maniere extracts were administered concurrently with methylmercury. Within the group, it was discovered that the presence of methylmercury in the brain led to a decrease in oxidative damage.

In a study conducted by Moneim [25], he investigated whether the berberine plant was protective against neurotoxicity and oxidative damage caused by mercury. In this study, adult male albino rats were injected with Mercury (II) chloride ($HgCl_2$) for 7 days. It was shown that NO production increased after the injection, antioxidant enzymes decreased, but glutathione increased and NO and lipid peroxidation decreased in those given beriberin herb before exposure.

Karapehlivan and his colleagues [26] conducted a study to examine the defensive impact of omega-3 fatty acids against $HgCl_2$ poisoning in mice. This study investigated the levels of Malondialdehyde (MDA), glutathione, NO, and total sialic acid (TSA). Additionally, histological alterations in specific organs were analysed. A total of 28 mice were divided into 4 groups. In Group 1, the mice were injected with saline solution into their peritoneal cavity. In Group 2, the mice were injected with 0.4 mg/kg/day of mercuric chloride into their peritoneal cavity. In Group 3, the mice were injected with 0.4 mg/kg/day of mercuric chloride into their peritoneal cavity and simultaneously received a subcutaneous dose of 0.5 g/kg/day of omega-3 fatty acid. In Group 4, the mice were only given a subcutaneous dose of 0.5 g/kg/day of omega-3 fatty acid. Throughout the duration of this investigation, all applications were maintained for a period of 7 days. In comparison to group 1, it was seen that group 2 exhibited elevated levels of MDA, NO, and TSA, while groups 3 and 4 displayed lower amounts of these substances. Group 4 demonstrated the highest degree of diminished glutathione (GSH) levels. Group II animals exhibited severe deterioration in the liver and kidney, as observed through histopathological analysis. Consequently, Karapehlivan et al. This study shown that omega 3 fatty acid can mitigate the toxicity generated by $HgCl_2$ by modulating the antioxidant system and acute phase response in mice. Mercury possesses toxicity when it is inhaled, ingested, or absorbed via the skin. Existing literature primarily demonstrates the correlation between mercury poisoning and oxidative stress indicators through animal studies, with limited research conducted on people.

Our study revealed elevated levels of NO and Nitrotyrosine

in children affected by mercury poisoning. We hypothesised that administering antioxidant treatment, in addition to chelator treatment, during the acute phase could potentially mitigate oxidative stress. However, further research is required to validate this hypothesis.

Ethical approval

Approval was received from the Kahramanmaraş Sütçü İmam University Clinical Research Ethics Committee dated 14.04.2014 and with the decision number 2014/01-07.

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