Studies on H1N1 vaccine-induced monoamines alternations and oxidative stress on brain of adult mice

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ARTICLE INFO

ABSTRACT

Over the past decade illness outbreaks have posed a serious threat to human life and well-being. The 2009 outbreak H1N1/A influenza virus also was expected to disproportionately affect healthy young persons under the age of 25 years. A small amount of the preservative thimerosal is routinely added to many vaccine preparations, including H1N1 vaccine. Thimerosal is an organic mercurial containing an ethylmercury moiety attached to the sulfur atom of thiosalicylate. Since the 1930s, thimerosal has been used as an antiseptic and a preservative in a wide variety of products, to investigate the monoamines alternation and oxidative stress induced after H1N1 vaccine injection, adult male Swiss mice were injected with thiomerosal, adjuvant, H1N1 antigen and H1N1 vaccine. Results obtain on the present study showed that thiomerosal, H1N1 antigen and H1N1 vaccine were caused significant decrease in norepinephrine (NE) and dopamine (DA) contents of hypothalamus, striatum and cerebral cortex. The alternation in NE and DA was associated with significant increase in oxidative markers namely lipid peroxidation and nitric oxide, oxidation induction was extent to cause significant decrease in glutathione level. In conclusion, the present study demonstrated that H1N1 vaccine as a whole and/or its ingredient caused oxidative stress and monoamines alternations in brain of mice. The present observation could be due to the presence of thiomerosal.

INTRODUCTION

New communicable disease influenza A (H1N1) affected geographically diverse areas around the world in 2009. Person to person transmission has led to increase the numbers of patients. The current H1N1 virus, which was previously referred as Swine Flu is totally a new virus subtype. This new virus subtype is efficiently able to be transmitted from human to human which may cause Pandemic Influenza (Gangurde *et al.,* 2011). Influenza virus infection, one of the most common infectious diseases, is a highly contagious airborne disease that causes an acute febrile illness and results in variable degrees of systemic symptoms, ranging from mild fatigue to respiratory failure and death. These symptoms contribute to significant loss of workdays, human suffering, mortality, and significant morbidity (Islam and Rahman, 2010). Strategies to shorten the time between emergence of a human influenza pandemic virus and the availability of safe and effective

pandemic influenza vaccines are of the highest priority in global health security. There are limited immunogenicity and safety data, and no efficacy data would be available when human pandemic influenza vaccines are first administered after a pandemic is declared.

The risks and benefits of pandemic influenza vaccine will need to be studies post marketing (Bouvier and Palese, 2008). Vaccines contain live viruses, killed viruses, purified viral proteins, inactivated bacterial toxins, or bacterial polysaccharides. In addition to these immunogens, vaccines often contain other substances. For example, vaccines may contain preservatives that prevent bacterial or fungal contamination (eg, thimerosal); adjuvants that enhance antigen-specific immune responses (eg, aluminum salts); or additives that stabilize live, attenuated viruses (eg, gelatin, human serum albumin).

Furthermore, vaccines may contain residual quantities of substances used during the manufacturing process (eg, formaldehyde, antibiotics, egg proteins, yeast proteins) (Offit and Jew, 2003).

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Thimerosal (sodium ethylmercurithiosalicylate) was developed in the 1930s as an effective bacteriostatic and fungistatic preservative and has been widely used in multidose vials of vaccines and in ophthalmic, otic, nasal, and topical products. Prior to 2001, a child may have received a cumulative dose of over 200 mg/kg in the first 18 months of life (Blanusa *et al.,* 2012). Although the neurotoxicity of methyl mercury has been relatively well studied, limited information is available on the relative neurodevelopmental toxicity of ethylmercury, the mercury metabolite of thimerosal. Following recommendations, thimerosal was subsequently removed as a preservative from most children's vaccines in the US. However, influenza vaccines and Rho D immunoglobulin shots containing thimerosal are still recommended to pregnant women, and many vaccines given to children in developing countries still contain thimerosal (James *et al.,* 2005).

Previous mechanistic studies of methylmercury toxicity in neurons have implicated reactive oxygen species (ROS) and depletion of intracellular glutathione as major contributors to mercury-induced cytotoxicity. Organic mercury has a high affinity for the thiol (–SH) group on glutathione, a tripeptide composed of cysteine, glutamate, and glycine (Sanfeliu *et al.,* 2001). The cysteine moiety of glutathione carries the active thiol group that binds and detoxifies a variety of heavy metals, including organic and inorganic mercury. Normally, the intracellular concentration of glutathione is extremely high, in the mM range; however, with depletion of this essential antioxidant, excess free mercury is available to bind to cysteine thiol groups present in essential cellular proteins, leading to functional inactivation and cytotoxicity (James *et al.,* 2005). To the authors' knowledge, no similar studies have been done to elucidate the oxidative stress and alternation in monoamines of brain after exposure to H1N1 vaccine. So, the purpose of the present study was to determine the monoamines alternation and oxidative stress induction in brain of mice after H1N1 vaccine injection.

Materials and methods
Experimental animals

Adult male Swiss albino mice weighing 25–30g were obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). After an acclimatization period of one week, the animals were divided into five groups (28 mice /group) and housed in wire bottomed cages in a room under standard conditions of illumination with a 12-hours light-dark cycle at 25±1℃. They were provided with water and a balanced diet *ad libitum*. All animals received care in compliance with the Egyptian rules for animal protection.

Experimental protocol

Animals within different treatment groups were maintained on their respective diets for 12 to 24 days as follows: the first group (Con), received a subcutaneous injection of saline at the zero day, and the 14th day and serve as control at dose of 100 µl. /mice, the second group received a subcutaneous injection of thiomersal (Thio) at the zero day, and the 14th day at dose of 5 µl/kg dissolved in 100 µl saline, the third group received a subcutaneous injection of Adjuvant (Adj; AS03) at the zero day, and the 14th day at dose of 5 µl/mice dissolved in 100 µl saline, the forth group received a subcutaneous injection of antigen (Ag) at the zero day, and the 14th day at dose of 10 µl/mice dissolved in 100 µl saline and the fifth group received a subcutaneous injection of mixture of antigen and adjuvant (Mix) at the zero day, and the 14th day at dose of 5 µl/mice dissolved in 100 µl saline.

Animals of all groups; Con, Thio, Adj, Ag and Mix were decapitated after 7, 14, 21 and 28 days post-treatment (n= 7). The brains of the mice were carefully removed and dissection of the brains was performed on an ice-cold glass plate for the separation of hypothalamus, striatum and cerebral cortex regions according to the method described by Glowinski et al. (1966). The three regions of the first portion of each brain were blotted and frozen for further determination of norepinephrine (NE) and dopamine (DA). The 2nd portion of each brain was weighed and homogenized immediately to give 50% (w/v) homogenate in ice-cold medium containing 50 mM Tris-HCl, pH, 7.4. The homogenate was centrifuged at 3000 rpm for 10 min in cooling centrifuge at 4°C. The supernatant (10%) was used for the various biochemical determinations.

Determination of monoamines levels in brain regions

Norepinephrine (NE) and dopamine (DA) were extracted and measured in the three regions of each brain using fluorometric technique according to the modified method of Ciarlone (1978). The fluorescence was measured in Jenway 6200 fluorometer.

Determination of lipid peroxidation in brain regions

Lipid peroxidation (LPO) in brain homogenate were determined according to the method of Ohkawa et al. (1979) by using 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67%, followed by heating in a boiling water bath for 30 min. Thiobarbituric acid reactive substances were determined by the absorbance at 535 nm and expressed as malondialdehyde (MDA) equivalents formed.

Determination of nitric oxide

The assay of nitric oxide content in brain homogenates was done according to the method of Green et al. (1982). In an acid medium and in the presence of nitrite the formed nitrous acid diazotise sulphanilamide was coupled with N-(1–naphthyl) ethylenediamine. The resulting azo-dye had a bright reddish–purple colour which could be measured through spectrophotometry at 540 nm.

Estimation of glutathione

Glutathione (GSH) was determined chemically in brain homogenate using Elman's reagent (1959). The method based on the reduction of Elman's reagent (5, 5` dithiobis (2-nitrobenzoic acid) "DTNB") with GSH to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

Histological study

Brains of two rats was washed in saline and fixed in 10% neutral formalin, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin for light microscopic observations.

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Duncan's test was used as a *post hoc* test according to the statistical package program (SPSS version 17.0).

RESULT

Monoamines disturbances results

Multiple doses of thiomersal, adjuvant, antigen and mixture of vaccine ingredients on given for 28 days were produced disturbances in norepinephrine (NE) contents on different brain regions (Figure 1). In view of the present results, thiomersal injection was induced a significant ($p < 0.05$) decrease in NE contents of hypothalamus, striatum and cerebral cortex at all time intervals compared to control mice. In addition, in mice treated with antigen and mixture of H1N1 vaccine for 28 days, a significant decrease in NE contents of hypothalamus and striatum was observed at all time intervals as compared to control animals. The decreased in NE content on hypothalamus and striatum after mixture of H1N1 vaccine injection was also presented in cerebral cortex at all time intervals. The toxic effects of H1N1 vaccine in NE contents was also extended to dopamine (DA) contents as it shown in Figure 2. Injection of thiomersal, antigen and H1N1 whole vaccine were induced a significant ($p < 0.05$) reduction in DA contents of all investigated brain regions at all time intervals as compared to control group.
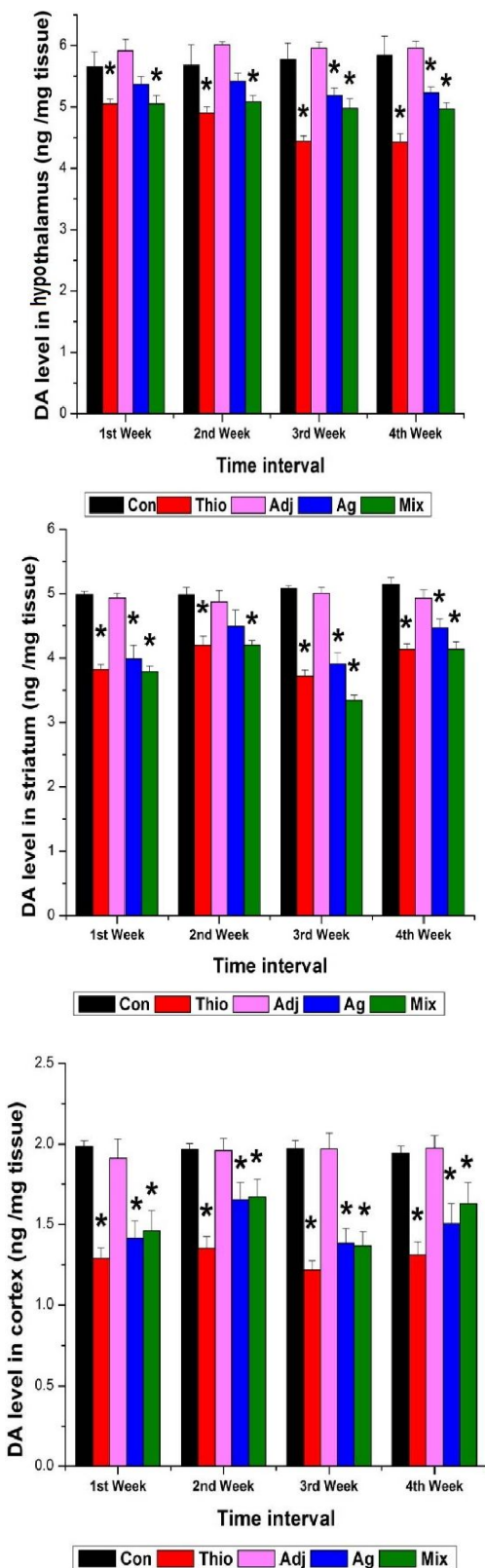
Oxidative stress results

Figure 3 summarizes the effect of H1N1 vaccine on lipid peroxidation (LPO) in neural tissue homogenates. Thiomersal, adjuvant, antigen and mixture of vaccine ingredients were caused a significant ($p < 0.05$) increase in LPO levels on brain tissues at all time intervals compared to control group. The maximum elevation in LPO level was observed at the 4th week post-injection of Ag and Mix (17.57% and 18.21%, respectively). In addition, Thio and Adj injection on mice were caused maximum elevation in LPO levels at the 4th week with a percentage difference of 15.00% and 15.88%, respectively.
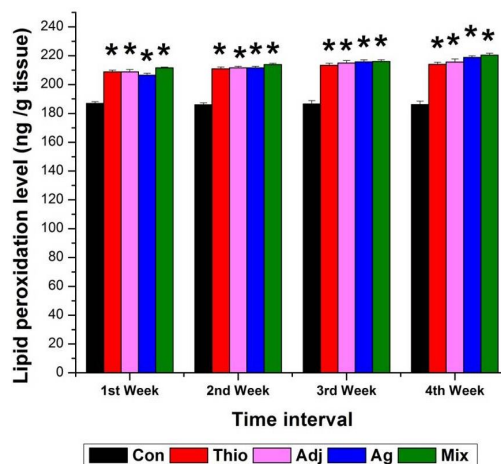
As shown in Figure (4), the subcutaneous injection of Thio, Adj, Ag and Mix was caused a significant ($p < 0.05$) elevation in nitric oxide (NO) level on brain homogenates of mice at all time intervals as compared to control group. Moreover, NO level in mice injected with thiomersal showed maximum elevation at the 2nd week. In addition, the mixture injection in mice caused maximal elevation in NO level at the 3rd week with percentage difference of 23.63%.
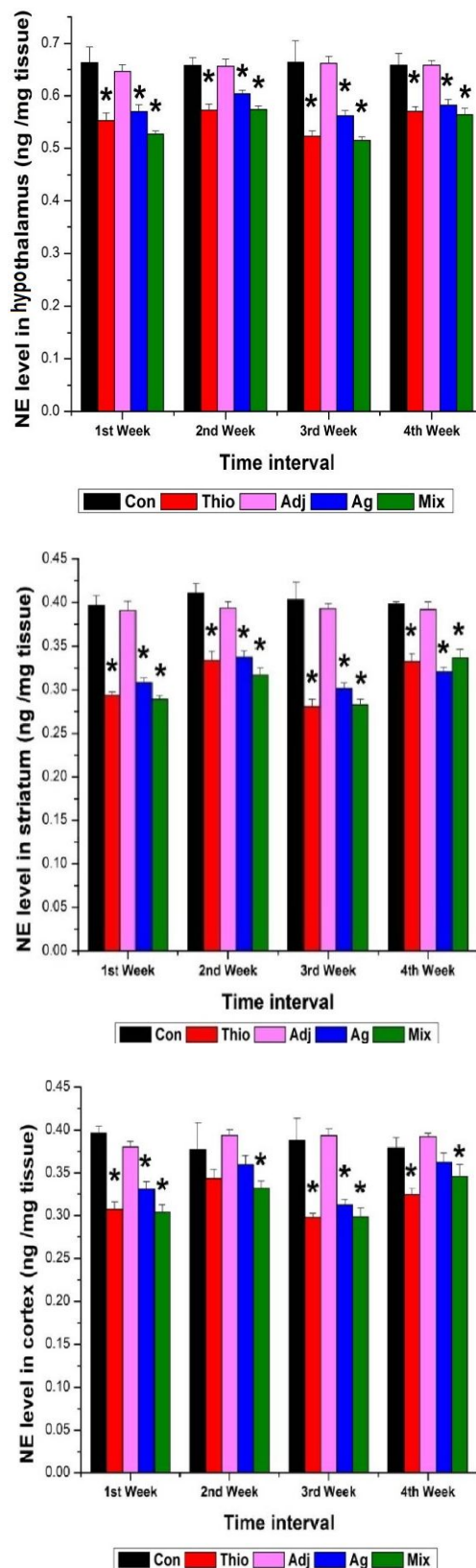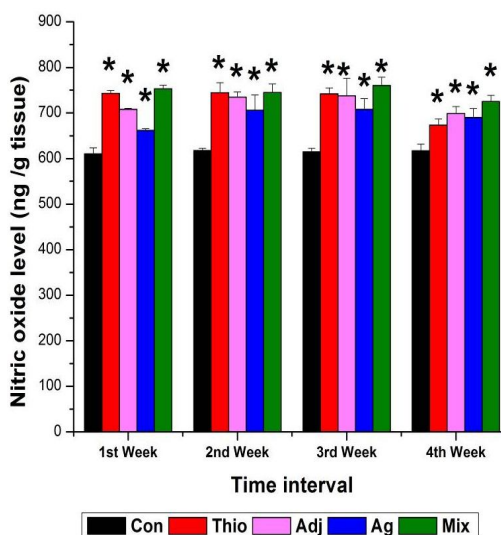

Fig. 1: Changes in the norepinephrine contents in the hypothalamus, striatum and cerebral cortex of male mice treated with thiomersal, adjuvant, antigen and mixture of H1N1 vaccine.*, significant change at *p* <0.05 with respect to **Con** group.

Fig. 3: Lipid peroxidation levels in brain homogenates of male mice treated with thiomersal, adjuvant, antigen and the mixture of H1N1 vaccine.*, significant change at $p < 0.05$ with respect to **Con** group.

Fig. 4: Nitric oxide levels in brain homogenates of male mice treated with thiomersal, adjuvant, antigen and the mixture of H1N1 vaccine.*, significant change at $p < 0.05$ with respect to **Con** group.
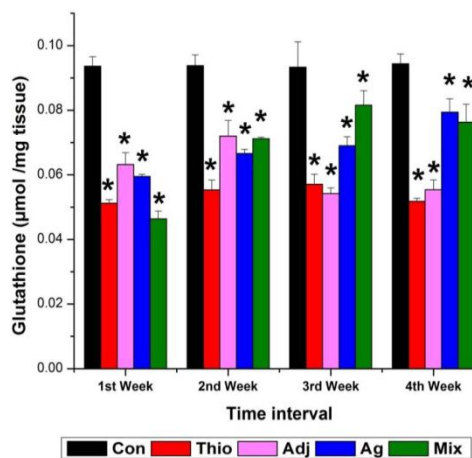

Fig. 2: Changes in the dopamine contents in the hypothalamus, striatum and cerebral cortex of male mice treated with thiomersal, adjuvant, antigen and mixture of H1N1 vaccine.*, significant change at $p < 0.05$ with respect to **Con** group.

Fig. 5: Glutathione content in brain homogenates of male mice treated with thiomersal, adjuvant, antigen and the mixture of H1N1 vaccine.*, significant change at $p < 0.05$ with respect to **Con** group.

The effects of thiomersal, adjuvant, antigen and mixture of vaccine ingredients on glutathione (GSH) contents in homogenates are presented in Figure 5.

Thio, Adj, Ag and mixture injection for 28 days caused a significant ($p < 0.05$) reduction in GSH content in brain tissues at all time intervals compared to control mice. Moreover, Thio and Adj injection were caused maximum dimension in GSH content at the 1st (-45.29%) and 3rd (-41.91%) weeks post-injection. However, the maximal reduction in GSH content was observed at the 1st week post-injection of the antigen and mixture with a percentage difference of -36.43% and -50.43%, respectively.

Histopathological observation

Figure 6 showed regular histological features of brain of control group. Sections of thiomersal and H1N1 vaccine exposed brains showed vacuolation and degenerated neurons as well as necrotic and apoptotic neurons in hypothalamus, striatum and cerebral cortex. Brains of mice treated with adjuvant and antigen showed vacuolated neurons and many apoptotic and necrotic neurons in striatum and cerebral cortex with less injury in hypothalamus.
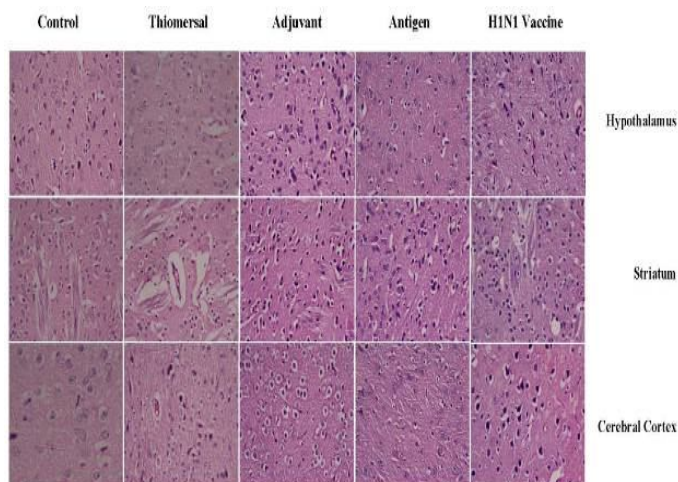
Fig. 6: (A) Normal histological features of different brain regions of control group with well formed neurons. (B) Sections of thiomersal exposed brain with damaged hypothalamus, striatum and cerebral cortex. (C) Sections of adjuvant treated brain regions with damaged striatum and cerebral cortex. (D) Sections of brain regions of brain after antigen. (E) Sections of H1N1 vaccine exposed brain with damaged hypothalamus, striatum and cerebral cortex (400X).

DISCUSSION

Considerable concern has been expressed recently over the cumulative dose of mercury given to children through routine immunizations given in the 1990s. The source of mercury in vaccines is the antimicrobial preservative, thimerosal, containing 49.9% ethyl mercury by weight. All forms of mercury are well known to be neurotoxic, especially during early brain development (Costa *et al.,* 2004). The high affinity binding of mercuric compounds to the thiol (–SH) group of cysteines in essential proteins is thought to be the basis for mercury-induced cytotoxicity. *In vivo* studies in rodents have shown that ethyl mercury is able to cross the cell membrane and then is converted intracellularly to inorganic mercury which accumulates preferentially in the brain and kidney (James *et al.,* 2005). Intracellular accumulation of inorganic mercury was shown to be higher for ethyl compared to methylmercury with equimolar exposure, although the clearance rate of ethylmercury was faster than methylmercury (James *et al.,* 2005). A recent in vitro study of thimerosal exposure to fibroblasts and human cerebral cortical neuron cell lines demonstrated that short term exposure in concentrations similar to those used in the present study induced DNA strand breaks, membrane damage, caspase-3 activation, and cell death (Baskin *et al.,* 2003). Mercury can cause biochemical damage to tissues and genes through diverse mechanisms, such as interrupting intracellular calcium homeostasis, disrupting membrane potential, altering protein synthesis, and interrupting excitatory amino acid pathways in the central nervous system. Mitochondrial damage, lipid peroxidation, microtubule destruction, and the neurotoxic accumulation of serotonin, aspartate, and glutamate are all mechanisms of methylmercury neurotoxicity (Yee and Choi, 1996). Increased reactive oxygen species (ROS) were reported in previous studies during thimersal injection, which was attributed to electron leakage, enhanced mitochondrial activity and increased electron chain activity. ROS subsequently attack almost all cell components including membrane lipids and producing lipid peroxidation (Chauhan and Chauhan, 2006). Therefore, it can be hypothesized that oxidative stress may be one of the contributing factors for mercury induced toxicity. LPO is one of the main manifestations of oxidative damage and it has been found to play an important role in the toxicity of many xenobiotics. The present study suggests a significant increase in LPO in brain which implicates free radicals-induced oxidative cell injury in mediating the toxicity of thiomersal (Chauhan *et al.,* 2004). Nitric oxide has protective or regulatory functions in the cells at a low concentration and toxic effects at higher concentration (Abdel Moneim, 2012). The present results show elevated NO level in the brain due to thiomersal injection, indicating that thiomersal induced toxicity may involve changes in NO production. Mercury in both organic and inorganic forms is neurotoxic. Methylmercury accumulates in the brain and becomes associated with mitochondria, endoplasmic reticulum, golgi complex, nuclear envelopes, and lysosomes. In nerve fibers methylmercury is localized primarily in myelin sheaths, where it leads to demyelination, and in mitochondria. (Chang, 1977). The adverse affect of mercury on GSH has secondary effects on the levels of Na^+, K^+ and Mg^{++}ATPases, all of which are dependent on sulfhydryl compounds. These enzymes, all critical for proper functioning of nervous and other tissues, are all inhibited by various mercurial compounds. (Magour *et al.,* 1987). Mercury also affects the release of neurotransmitters from presynaptic nerve terminals. This may be due to its ability to change the intracellular concentration of Ca^{2+} by disrupting regulation of Ca^{2+} from intracellular pools and increasing the permeability of plasma

membranes to Ca^{2+}(Atchison and Hare, 1994). While there is undoubtedly much more learn about the specific mechanisms of mercury-induced neurotoxicity. Mercury is also known to inhibit synaptic uptake of dopamine, (Rajanna *et al.,* 1990) serotonin, (Oudar *et al.,* 1989) and norepinephrine. (Rajanna and Hobson, 1985). Mental health symptoms are also quite common with mercury toxicity. Evidence linking mercury exposure to psychological disorders has been accumulating for 60 years. The recognized psychological symptoms of mercury toxicity include irritability, excitability, temper outburst, quarreling, fearfulness, restlessness, depression, and in some cases insomnia. In a study of individuals with amalgam fillings who had them removed, the majority noted psychological improvement. The greatest improvements were found in anger outbursts, depression, irritability, and fatigue. (Siblerud, 1989). In conclusion, the present study demonstrated that H1N1 vaccine as a whole and/or its ingredient is a neuronal toxin in mice. The present study demonstrated that this vaccine cause oxidative stress and monoamines alternations in brain of mice. The present observation could be due to the presence of thiomerosal.

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