academicJournals

Vol. 7(7), pp. 77-84, July 2013
DOI: 10.5897/JCAB2013.0368
ISSN 1996-0867 ©2013 Academic Journals
http://www.academicjournals.org/JCAB

Journal of Cell and Animal Biology

Full Length Research Paper

The protective effects of vitamin C and folic acid against methylmercury teratogenicity in chick embryo

Gamal M. Bekhet[1,2]*, Mohamed A. Al-Kahtani[1] and Ashraf M. Abdel-Moneim[1,2]

[1]Department of Biological Sciences, Faculty of Science, King Faisal University, Al Hassa 31982, Saudi Arabia.
[2]Department of Zoology, Faculty of Science, Alexandria University, Alexandria 21511, Egypt.

Accepted 13 June, 2013

This study was undertaken with the aim of investigating the teratogenic effects of methylmercury (MeHg) on the chick embryos and evaluating the protective role of vitamin C and folic acid. Fertilized eggs received MeHg dose (2.5 mg/egg) alone or with addition of vitamin C (100 mM) and folic acid (100 mM) or with vitamin C and folic acid alone. Control eggs received saline. The eggs were injected on day zero of incubation. On day five, embryos were examined for viability, gross retardation and gross malformation and the hearts were processed for light and electron microscopy. Results showed that MeHg decreased the survival rate of embryos and caused gross malformations such as hypomorphic hearts, abnormal position of atria, exencephaly, hydrocephaly, anencephaly, everted viscera, microphthalmia, twisted body, limb malformation and hemorrhage, compared with control embryos. Histological examination of the hearts clearly revealed that the heart tissues have failed to develop correctly in the MeHg treated embryos. The corresponding pathological alterations were mainly characterized as cell death at the ultrastructural level. Embryos injected with MeHg plus vitamin C and folic acid were almost comparable to the control. Data obtained in this study suggest that supplementation with vitamin C and folic acid during pregnancy may prevent defects in heart development brought about by MeHg and other environmental xenobiotics.

Key words: Chick embryo, methylmercury, vitamin c, folic acid, teratogenicity.

INTRODUCTION

Methylmercury (MeHg), a highly toxic form of mercury in the environment, is known to induce neurodevelopmental toxicity in both animals and humans (Johansson et al., 2007). Number of synchronous mechanisms are likely associated with MeHg-induced neurotoxicity, including impairment of intracellular calcium homeostasis (Atchison, 2005), alteration of glutamate homeostasis (Farina et al., 2003a) and oxidative stress (Franco et al., 2007). Particularly, the glutathione system, an antioxidant tool for protecting cells against oxidative damage, represents a molecular target for MeHg (Stringari et al., 2008). MeHg can bioaccumulate in aquatic food webs, leading to elevated concentrations in birds (Scheuhammer et al., 2007). In highly contaminated areas, when MeHg is transferred from a mother bird to her eggs. Studies by Heinz et al. (2006) demonstrated the toxicity of MeHg to embryos of several bird species in various experimental conditions, using mainly different

*Corresponding author. E-mail: g.bekhet@yahoo.com. Tel: (+966) 569323209.

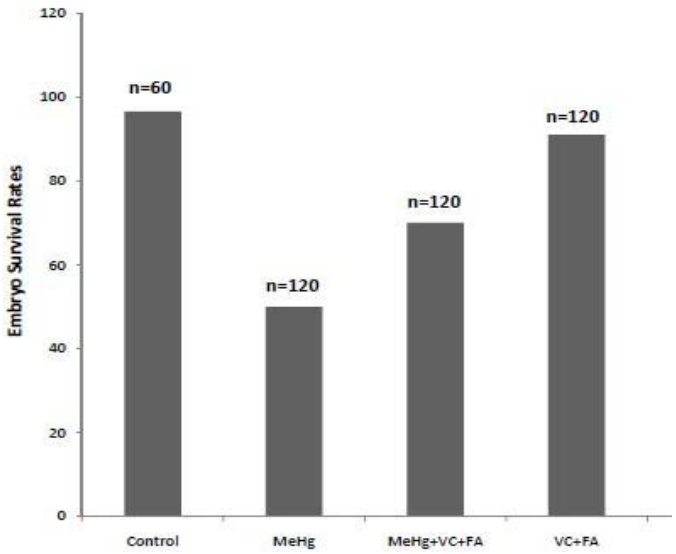


Figure 1. Overall embryo survival rates at harvesting on ID8 following the administration of MeHg and/or VC+FA into the egg yolk. Control embryo survival rates also shown x10.

solvents, injection sites, and embryo ages ranked the sensitivities of the embryos of many species of birds to MeHg. Bertossi et al. (2004) showed that MeHg induces neurotoxicity in chicks, motor impairment cerebellar damage (Carvalho et al., 2007), and alterations in glutathione homeostasis (Stringari et al., 2008) have been described as important molecular mechanisms involved with MeHg-induced neurotoxicity in rodents and primates, such indications of toxicity have not been examined in birds.

Vitamin C or ascorbic acid is a water-soluble compound synthesized by liver cells in many animals but not in humans, so humans rely totally on their diet to get enough vitamin C. The current recommended dos during pregnancy in the western world is 60 to 80 mg/day; because of its antioxidant properties, vitamin C may protect the embryo from oxygen free radical damage (Lee et al., 2004). The term folate represents all forms of the B vitamin series with the same structure and function, including many derivatives found in biological systems (Tamura and Picciano, 2006). An adequate supply of dietary folate in pregnancy is essential for normal embryonic development, including that of the cardiovascular system, and its supplementation lowers the incidence of congenital defects (Botto et al., 2003; Torrens et al., 2006). Several epidemiological studies showed an association between 'folic acid' deficiency and the occurrence of congenital heart disease and a significant reduction of conotruncal heart defects with the daily intake of 'folic acid' fortified cereals (Memon and Pratten, 2009).

This study was conducted to detect the teratogenicity of MeHg in avian embryos. Also, the protective effects of vitamin C and folic acid against methylemercury induced developmental abnormalities were examined.

MATERIALS AND METHODS

Fertile chicken eggs (obtained from local hatchery) were incubated at 38°C with a relative humidity of 70%. The first day of incubation was termed day zero. MeHg, vitamin C or folic acid were directly injected (single dose for each) into the air cell of the egg using an established protocol (Drake et al., 2006). Briefly, eggs were removed from the incubator and wiped with 70% ethanol, followed by introduction of a 1.5-mm-diameter hole in the blunt end using a metal probe. Using a 20-gauge needle, injections were slowly introduced directly into the air cell of the egg, after which the injection aperture was sealed with paraffin wax, and the egg was rotated 180° and returned to the incubator. For evaluating the toxic effects of MeHg, 120 eggs received a standard injection volume of 2.5 mg/egg in sterile 0.9% NaCl. For detecting the protections of antioxidants, 120 eggs were injected with a combination of 2.5 mg/egg MeHg with 100 mM vitamin C and 1 mM folic acid and 120 eggs were injected with combination of antioxidants 100 mM vitamin C and 1 mM folic acid. For control, 60 eggs received equivalent volumes of vehicle, sterile physiologic saline. After the fifth day of incubation, all eggs were opened and surviving and dead embryos were carefully separated from the yolk sac, cleaned and examined for gross abnormalities and classified according to Hamburger and Hamilton (1951), whereas infertile eggs were discarded.

Embryonic death was determined by the absence of a beating heart; this assessment was made in ovo after carefully removing shell from a 1 cm^2 area directly over the embryo. For assessing the potential embryotoxic effect of MeHg, the following parameters were determined: 1) mortality rate of embryos in which development was arrested; 2) incidence of gross malformations; 3) heart tissues were examined using light and electron microscopy. For light microscopy, representative samples of the cardiac tissues were fixed by immersion in Bouin's fluid for 8 h, dehydrated in a graded series of ethanol, paraffin embedded and then cut taking 5 mm thick sections which were subsequently stained using hematoxylin/eosin. For electron microscopy, small pieces of the appropriate heart tissues were fixed at 4°C for 2 h in 2% glutaraldehyde solution buffered with 0.1M sodium cacodylate pH 7.2, then post-fixed in 1% osmium tetroxide in the same buffer for 2 h, dehydrated in a graded series of ethanol, transferred to propylene oxide and then embedded in an Epon-Araldite mixture. Ultra-thin sections were contrasted in a 50% alcohol-uranyl acetate solution and lead citrate before examination on electron microscope (Jeol-1011, Japan) operated at 80 kV.

RESULTS

Data for the overall survival of embryos are given in Figure 1. Of the 60 eggs treated with 0.1 ml of 9.0% sodium chloride as controls only 58 (96.6%) survived until ID 5 (HH stages 29 to 31). In the survivors of the control group (n = 58), abnormalities were not recorded but there were only two cases showed exclusively reduced body size. Of the 120 eggs injected with MeHg, only 60 (50%) survived until ID 8 (HH stages 29 to 31). Among the

survivors of the group of MeHg–treated embryos (n = 60), abnormalities were recorded in every case (100%) (Figure 2). Defects included gross external ones such as 30 cases out of 60 (50%) showed heart defects, represented by 5 cases (8.3%) hypomorphic heart; 7 cases (11.7%) showed abnormal position of the heart shifting to the left, 5 cases (8.3%) showed shifting of the heart to the right, 9 cases (15.0%) with exposed heart and 4 cases (6.7%) showed haemorrhage all over the heart. Otherwise, the remaining 30 cases out of 60 characterized by congenital abnormalities represented by 3 cases (5%) with hydrocephaly, 4 cases (6.7%) with exencephaly, 4 cases (6.7%) with anencephaly, 5 cases (8.3%) with everted viscera, 3 cases (5%) with microphthalmia, 2 cases (3.3%) with shortened and twisted body, 5 cases (8.3%) with haemorrhage all over the body, one case (1.7%) with reduced body size, 1 case (1.7%) with limb reduction malformation and 3 cases (5%) showing misshapen body. Heart and brain abnormalities were very common. Moreover, of the 120 eggs injected with MeHg combined with VC and FA, only 84 (70%) survived until ID 8 (29 to 31).

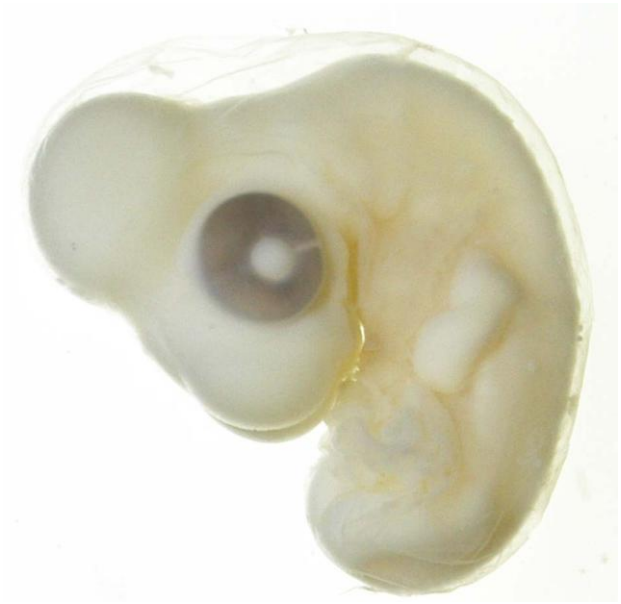
The gross external malformation exhibited in about 20 cases 84 (23.8%), 8 cases out of 20 (9.5%) showed heart defects represented by 2 cases (2.4%) hypomorphic, one case (1.2%) showed shifting of the heart to the left, one case (1.2%) showed shifting of the heart to the right, 3 cases (3.6%) with exposed heart and one case (1.2%) showed haemorrhage all over the heart. Otherwise, the remaining 12 cases out of 20 characterized by other malformations represented by 2 cases (2.4%) with hydrocephaly, 2 cases (2.4%) with exencephly, one case (1.2%) with anencephaly, 1 (1.2%) with everted viscera, one case (1.2%) with microphthalmia, 2 cases (2.4%) with haemorrhage all over the body, one case (1.2%) with reduced body size and 2 cases (2.4%) showing misshapen body. In the case of embryos injected with the protective vitamin C and folic acid, it was showed about 110 cases survived. Only 6 cases (5.5%) with malformations, 2 of them (1.8%) showed heart defects included one case (0.9%) with hypomorphic heart and the other one (0.9%) with haemorrhage in the heart. Meanwhile, the remaining 4 cases (3.7%) exhibited other types of external malformations such as anencephaly, microphthalmia, shortened and twisted body and reduced body size; one case (0.9%) was recorded for each type.

The heart was quite well differentiated in control chicks (Figure 3A). It had well developed atria and ventricles with complete interventricular septum. The ventricular walls had well developed myocardial layer. When these heart structures were studied in MeHg treated chick embryo, heart tissue was found to be affected adversely, for example development of tubular heart, thinning and occasional loss of myocardium and ventricular septal defect (Figure 3B). Treatment with additional VC and FA restores normal development (Figure 3C). The ultra-structural characteristics of control cardiac myoblasts included intact nuclei with delicate heterochromatin against a pale background, dense and well-developed mitochondria, and an intact sarcolemma. A certain degree of disorganization of myofibrils was evident in the myoblasts. Most of the myofilaments were grouped together to form short small bundles which were occasionally crossed by opaque material of the Z-band pattern (Figure 4A). Z-material might be found in the cytoplasm isolated from the myofilaments. In-contrast, myoblasts of embryos exposed to MeHg (Figure 4B) exhibited severe cardiotoxic features. The extent of the damage was rather heterogeneous among the myoblasts, but the presence of cellular injury was characteristic for all myoblast population. The nuclear chromatin was clumped with condensed heterochromatin and convoluted perinuclear membrane that often was split and edematous areas were evident. Many nuclei were reduced in sizes. The diameters of these shrunken nuclei decreased to half or one-third of the normal ones. The damage induced by MeHg was characterized by clumping of nuclear chromatin, sarcolemmal rupture, intermyofibrillar edema and increased vacuolation. No signs of apoptotic degeneration of cardioblasts were found.

The mitochondria became pale and swollen, accompanied by rupture of the internal septa and membranes. The arrangements of sarcomeres, myofibrils and myofilaments were lost and the integrity of the actin and myosin filaments was not visualized. In high magnification, holes were noted in the cell membranes (arrows). Loss of adherence between cardiac myocytes was prominent. These typical oncotic and necrotic structural damage in the myocytes were significantly attenuated by vitamin C and folic acid (Figure 4C) as indicated by preservation of mitochondria, conservation of myofilaments structure and maintenance of cellular integrity.

DISCUSSION

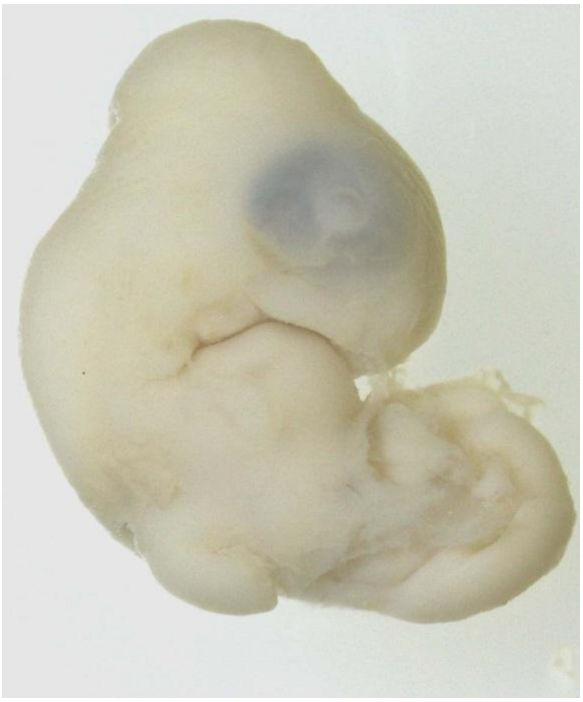
MeHg is a known human teratogen and therefore has been extensively investigated. This study was conducted to study MeHg induced developmental toxicity and also to investigate any protective effects of vitamin C and folic acid in preventing teratogenic effects brought about by MeHg in ovo using chick embryos. Avian embryos were chosen as a model system for these studies as these are cost effective, easy to handle, less time consuming, involve no maternal sacrifice and provide reliable results. The main finding of our in ovo study was that MeHg when injected caused a destruction of many organ systems of chick embryos. About half of the embryos were dead and



A

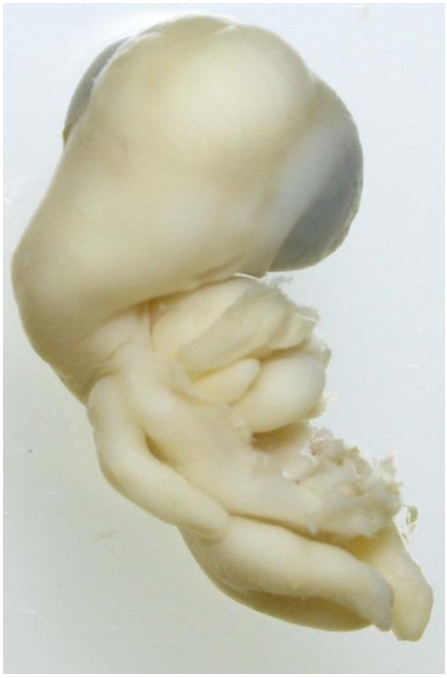
B

C

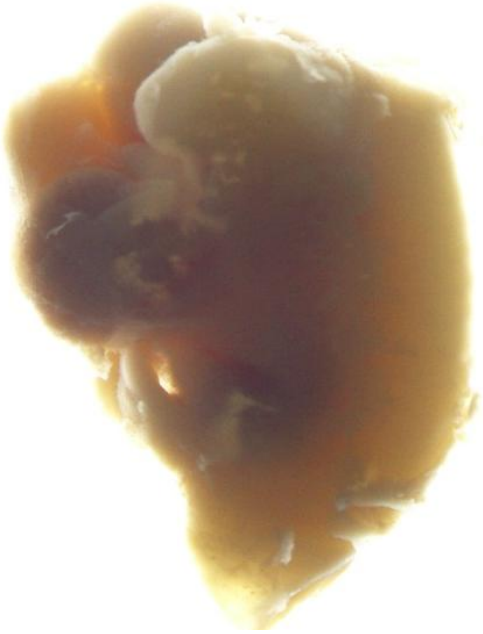


D

Figure 2. (A) Control. Hamburger and Hamilton stage 29 to 31 chick embryo, after 72 h of preincubation in ovo. (B-I) Teratogenic features of 3 day-chick embryos treated in ovo with MeHg. Note: heart defects including hypomorphic heart (in B), heart shifted to the right (in C), or to the left (in D), exposed heart (in E), and haemorrhage (in F). Note also exencephaly (in B), hydrocephaly (in G), anencephaly (in H), microphthalmia (in H), everted viscera (in E), reduced body size (in B), twisted body (in D, E), limb reduction and malformation (in B, H), misshapen body (in I) x10.

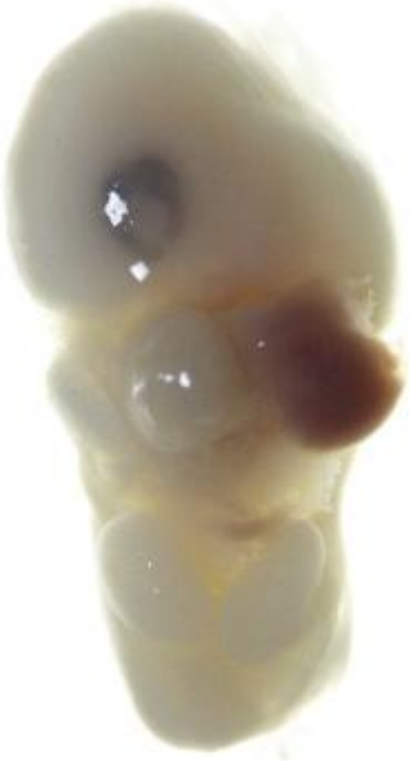


E



F

G



H

Figure 2. Contd.

Figure 2. Contd.

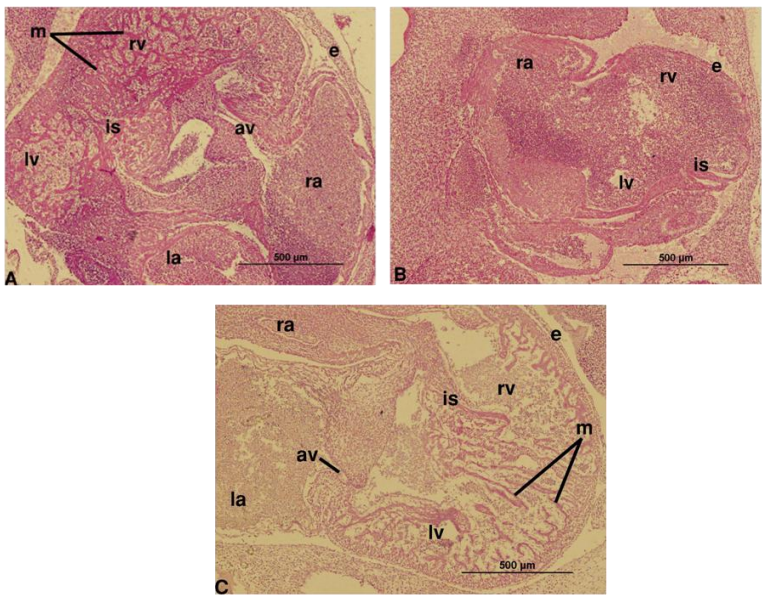


Figure 3. Sections of the heart from 3-day control embryo (A), embryos treated with MeHg (B), and with combination of VC and FA (C). All sections were stained with hematoxylin and eosin. The MeHg treated embryo clearly shows developmental ventricular septal defects. av: atrioventricular valve, e: epicardium, is: interventricular septum, la: left atrium, lv: left ventricle, m: myocardium, ra: right atrium, rv: right ventricle.

almost all remaining embryos were underdeveloped and showed teratogenic features including CNS malformations and optic defects. These findings are consistent with previous studies performed on many species of birds, which showed similar types of abnormalities when treated with MeHg (Heinz et al., 2011). It is not possible

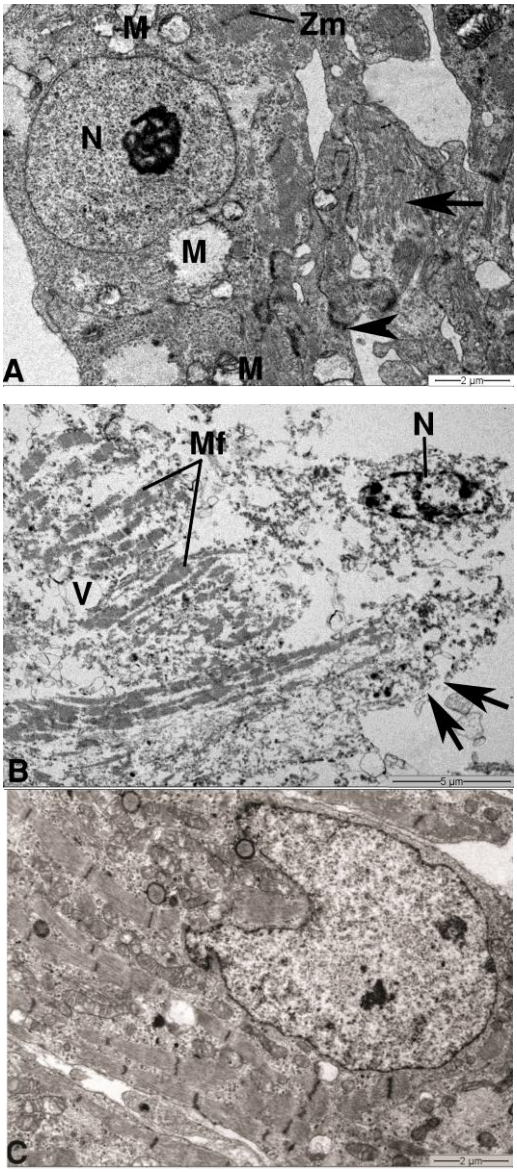


Figure 4. Cardiac myoblasts from 3-day chick embryo heart. (A) Control. Roundish shape myoblast can be seen. The myofilaments within the sarcoplasm are well developed and appear arranged in bundles (arrows). Other organelles such as mitochondria (M) are present and cell membranes are clearly defined. Arrowhead indicates a point of close apposition of plasma membranes of neighbouring cells suggesting the occurrence of focal tight junction. (B) MeHg group. Edematous myoblast displays degenerative process. Irreversible changes consist of degradation of myofilaments (Mf) and broken mitochondria. Margination of chromatin in the nucleus (N) and gaps in the cell membrane (arrows) are also evident. V: intracytoplasmic vacuole, a non- specific response of cell injury. (C) MeHg+VC+FA group showing less myocardial necrosis than MeHg-treated embryos.

to relate our findings of developmentally toxic levels of MeHg precisely with humans; however, epidemiological studies showed a direct relationship of MeHg exposure in excess of the recommended dose and potential malformations in the newborns. Furthermore, recent findings in adults suggest that MeHg exposure is associated with increased cardiovascular mortality (Grandjean et al., 2004). The present study also demonstrates a protective effect of VC and FA on MeHg treated cardiac myocytes in ovo. The effective concentration shown to have protective effect is 100 µM which is consistent with the amount of VC used by Peng et al. (2005) to treat ethanol induced growth retardation and microcephaly in *Xenopus laevis* embryos. The concentration of FA with protective effect is 1 µM which is similar to the amount of folinic acid used to treat the malformations of branchial arch derived structures in rats in whole embryo culture by Zhang et al. (2006). In a case control study, conducted in Atlanta, a direct relationship of prevention of cardiac defects, especially outflow tract and ventricular septal defects, with periconceptional use of multivitamins containing FA and VC was suggested (Willcox et al., 2008). Similar results were also found in a randomized control trial in Hungary which showed a reduction of 50% in cardiac defects with supplemental FA during pregnancy (Gardinar, 2006).

The observations that supplemental VC and FA offered a remarkable protection against the effects of MeHg might be due to their antioxidant properties. In an investigation of the protective effect of glutathione against MeHg toxicity in cultured mouse neuroblastoma cells, Kromidas et al. (1990) proposed that MeHg causes damage to microtubules by oxidation of tubulin sulfhydryls and peroxidative injury. Under these conditions, reactive oxygen species may be continuously formed as a by-product of metabolic reactions. Furthermore, such reactive oxygen species have also been linked with embryonic heart defects (Hobbs et al., 2005) caused by a variety of environmental toxins and maternal diseases. However, the extent of reactive oxygen species production and the subsequent likelihood of a developmentally toxic effect depend on the time and dose of exposure. Therefore, in order for normal development of embryos to be achieved under conditions where free radicals or reactive oxygen species are generated at abnormally high levels, there must be a balance between generation and degradation of free radicals and VC, may be useful in protecting embryos against damage.

ACKNOWLEDGMENT

The financial support for this project from the Deanship of Scientific Research, King Faisal University, Saudi Arabia

is gratefully acknowledged.

REFERENCES

Atchison WD (2005). Is chemical neurotransmission altered specifically during methylmercury-induced cerebellar dysfunction? Trends Pharmacol. Sci. 26:549–557.

Botto LD, J Mulinare, JD Erickson, 2003. Do multivitamin or folic acid supplements reduce the risk for congenital heart defects? Am. J. Med. Genet. 121A: 95-101.

Carvalho MC, Franco JL, Ghizoni H, Kobus K, Nazari EM, Rocha JBT, Nogueira CW, Dafre AL, Muller YMR, Farina M (2007). Effects of 2,3-dimercapto-1-propanesulfonic acid(DMPS) on methylmercury-induced locomotor deficits and cerebellartoxicity in mice. Toxicology 239:195-203.

Drake VJ, SL Koprowski, JW Lough, SM Smith (2006). Gastrulating chick embryo as a model for evaluating teratogenicity: Acomparison of three approaches. Birth Defects Res. Clin. Mol. Teratol. 76:66-71.

Farina M, Dahm KCS, Schwalm FD, Brusque AM, Frizzo MES, Zeni G, Souza DO, Rocha JBT (2003a). Methylmercury increases glutamate release from brain synaptosomes and glutamate uptake by cortical slices from suckling rat pups: Modulatory effect of ebselen. Toxicol. Sci. 73:135-140.

Franco JL, Braga HC, Nunes AKC, Ribas CM, Stringari J, Silva AP, Pomblum SG, Mora AM, Bohrer D, Santos ARS (2007). Lactational exposure to inorganic mercury: Evidence of neurotoxic effects. Neurotoxicol. Teratol. 29:360-367.

Gardinar HM (2006). Keeping abreast of advances in fetal cardiology. Early. Hum. Dev. 82:415-419.

Grandjean P, Murata K, Budtz-Jørgensen E, Weihe P (2004). Cardiac autonomic activity in methylmercury neurotoxicity: 14-year follow-up of a Faroese birth cohort. J. Pediat. 144:169-176.

Hamburger V, Hamilton HL (1951). A series of normal stages in the development of the chick embryo. J. Morphol. 88:49-92.

Heinz GH, Hoffman DJ, Kondrad SL, CA Erwin (2006). Factors affecting the toxicity of methylmercury injected into eggs. Arch. Environ. Contam. Toxicol. 50:264-279.

Heinz GH, Hoffman DV, Klimstra JD, Stebbins KP, Kondrad SL, Erwin CA (2011). Teratogenic effects of injected methylmercury on avian embryos. Environ. Toxicol. Chem. 30:1593-1598.

Hobbs CA, MACleves W, Zhao KS, Melny S, James SJ (2005). Congenital heart defects and maternal biomarkers of oxidative stress. Am. J. Clin. Nutr. 82:598-604.

Johansson C, Castoldi AF, Onishchenko N, Manzo L, Vahter M, Ceccatelli S (2007). Neurobehavioural and molecular changes induced by methylmercury exposure during development. Neurotox. Res.11:241-260.

Kromidas L, Trombetta LD, Jamall IS (1990). The protective effects of glutathione against methylmercury cytotoxicity. Toxicol. Lett. 51:67-80.

Lee BE, Hong YC, Lee KH, Kim YJ, Kim WK, Chang NS, Park EA, Park HS, Hann HJ (2004). Influence of maternal serum levels of vitamins C and E during the second trimester on birth weight and length. Eur. J. Clin. Nutr. 58: 1365-1371.

Memon S, Pratten M (2009). Developmental toxicity of ethanol in chick heart in ovo and in micromass culture can be prevented by addition of vitamin c and folic acid. Rep. Toxicol. 28:262-269.

Peng Y, Kwokb KHH, Pai-Hao Y, Samuel SMN, Jie L, Wong OG, Ming-Liang HE, Hsiang-Fu Kung MCM, Lin MC (2005). Ascorbic acid inhibits ROS production, NF-[kappa]B activation and prevents ethanol-induced growth retardation and microencephaly. Neuropharmacology 48:426-434.

Scheuhammer AM, Meyer MW, Sandheinrich MB, Murray ME (2007). Effects of environmental methylmercury on the health of wild birds, mammals, and fish. Ambio 36:12-18.

Stringari J, Nunes AKC, Franco JL, Bohrer D, Pomblum SG, Dafre AL, Milatovic D, Souza DOG, Rocha JBT, Aschner M (2008). Prenatal methylmercury exposure hampers glutathione antioxidant system ontogenesis and causes long-lasting oxidative stress in the mouse brain. Toxicol. Appl. Pharmacol. 227:147-154.

Tamura T, Picciano M (2006). Folate and human reproduction. Am. J. Clin. Nutr. 83: 993-1016.

Torrens C, Brawley L , Anthony FW , Dance CS , DunnRD , AA Jackson AA ,L Poston L , Hanson MA (2006). Folate supplementation during pregnancy improves offspring cardiovascular dysfunction induced by protein restriction. Hypertension 47:982-987.

Willcox BJ, Curb JD, Rodriguez BL (2008). Antioxidants in cardiovascular health and disease: key lessons from epidemiologic studies. Am. J. Cardiol. 101:S75-86.

Zhang Z, Xu Y, Li L, Han J, Zheng L, Liu P, Li Y (2006). Prevention of retinoic acid induced early craniofacial abnormalities by folinic acid and expression of endothelin-1/dHAND in the branchial arches in mouse. Br. J. Nutr. 96:418-425.