World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Evaluation of the potential interaction between methylmercuric chloride and naringenin on pre- and postnatal rat development

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Received: 27-10-2015 / Revised: 18-11-2015 / Accepted: 28-11-2015

ABSTRACT

The present work aims to examine the potential role of methylmercury (MMC) in inducing developmental toxicity as well as to evaluate the role of naringenin in preventing or alleviating the induced defects. In the present work 80 pregnant rats were divided into four groups (each comprising 20 rats); control group, naringenin (Ng) treated group (50 mg /kg b.wt./day), MMC treated group with 1 mg/ kg bwt and co-treated group with both MMC and Ng at the same dosages. Half of the animals were treated daily from gestational day 7 to 19th day by gastric intubation, and the other half were treated daily from the 7th day of gestation to the postnatal day (PN) 29 by gastric intubation. All animals were killed in the day next to the last given dose. The results indicated that oral administration of naringenin to rats treated with MMC improved the fetal and pups growth rate and markedly reduced the fetal morphological and skeletal abnormalities. Naringenin also improved the percentage of fragmented DNA in fetal and neonatal brain as well as the histpathological lesions observed in cortex of both fetuses and pups maternally treated with MMC.

Key words: Fetotoxicity; Methylmercury; Naringenin; Neurotoxicity.

INTRODUCTION

Despite the ecological awareness that rose over the last few years, mercury is still an important environmental pollutant [1]. Naturally occurring inorganic mercurial compounds or effluents from industrial pollution can be converted to organic methyl mercury (MMC) by microorganisms which is more toxic and penetrates the blood–brain barrier (BBB) more easily than inorganic mercurial compounds. The major route of human exposure to mercurial compounds is through dietary intake of mercury-contaminated fish, seafood, grain, or processed food [2]. MMC is easily absorbed from the diet into the bloodstream and is distributed to all tissues. Fetuses are known to be a high-risk group for MMC exposure because of the high susceptibility of the developing brain itself [3]. In early studies, methyl mercury was shown to be embryogenic and teratogenic in golden hamsters, rats and mice [4, 5]**.** Various experimental studies demonstrated that methyl mercury crosses the placenta and accumulates in fetal tissues [3]. Several mechanisms have been proposed to explain the toxicity of Hg and/or its compounds. However the molecular mechanisms underlying mercury toxicity are not fully understood, although several studies now indicate that ROS production plays a central role [6] and frequently cause oxidative DNA damage [7]**.**

Recently, there is extensive interest in the progress of strategies to protect embryonic development from insults during organogenesis. Maternal dietary antioxidant supplementation has clearly been successful in improving fetal outcomes in animal models, such as experimental diabetes [8] or after exposure to specific teratogens [9,10]**.** Naringenin, a flavonoid found in grape fruit juice was related to a large number of beneficial effects such as antioxidant [11], cardioprotective [12], nephroprotective [13], hepatoprotective [14] and anti-viral effect [15]**.** It has the ability to improve the viability, growth and differentiation in mice embryos co-treated with hydroxyurea and naringenin [9]**.** However, there is no study concerning the effect of naringenin against MMC - induced developmental toxicity. Therefore, the current study was undertaken to evaluate the potential interaction between methylmercury and naringenin on fetal and neonatal development.

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MATERIALS AND METHODS

Chemicals: Methyl mercuric (II) chloride (MMC) and Naringenin (Ng) were obtained from sigma chemical Co., St. Louis Mo.,USA.

Animals: Eighty female adult albino rats of the strain *Rattus norvegicus* (weighting about 120–140 g) were used in this study. They were obtained from animal house of El-Salam farm Giza, Egypt. During the study, the female rats were kept individually in stainless-steel cages in a well-ventilated room. All animals had ad libitum access to a standard rodent chow diet and water. After one week of acclimatization, each two females were housed with one male, in stainless-steel cages, for mating. In the next morning, fertilization was investigated by the presence of sperm in the vaginal smears. Females with positive sperm were considered pregnant and the day of sperm detection was defined as the first day of gestation.

Animals grouping: In the present work 80 positively mated females were divided into four groups (each comprising 20 rats); control group, naringenin (Ng) treated group (50 mg /kg b.wt./day) [16], MMC treated group with 1 mg/ kg bwt [17] and co-treated group with MMC and Ng at the same dosages. Half of the animals of each group were treated daily from gestational day 7 to 19th day by gastric intubation then pregnant rats were sacrificed on the 20th day of gestation. The other half of dams were treated daily from the 7th day of gestation to the postnatal day (PN) 29 by gastric intubation and their pups were killed on PN 30. The following parameters were evaluated:

Clinical toxicity: All dams and the neonates were observed daily for determination of the mortality rate, the general appearance and their behavior during the experimental period. Also the body weight of control and treated dams as well as the neonates was recorded once weekly.

Teratological examination: The uteri of pregnant rats that sacrificed on the 20th day of gestation were removed and weighed, and the contents were examined for the implantation sites, the post implantation loss index [(number of implants – number of viable fetuses) / (number of implants)] x100, dead and live fetuses. Live fetuses were weighed and examined for any external abnormalities including those of the oral cavity. One third of the live fetuses were used for skeletal examination using alcian blue and alizarin red S according to the method described by **McLeod** [18].

DNA fragmentation by quantitative analysis using diphenylamine assay in fetal and neonates brain: Small pieces of fetal and neonatal brain of control and maternally treated groups were used to evaluate the DNA fragmentation by quantitative analysis using diphenylamine assay. This method is based on quantification of the fragmented DNA in fetal and neonatal cerebral tissues according to the method described by **Hickey et al.** [19]. The amount of fragmented DNA was expressed as the percentage of total DNA appearing in the supernatant.

Histological examinations: The whole brains of fetuses and neonates of different groups were carefully separated from the skull, fixed with buffered neutral formalin (10%), dehydrated with ethanol series, paraffin embedded, then the brains were coronal sectioned at 5 micrometer thickness through the cerebral cortex. The sections were stained with hematoxylin and eosin (H & E), and then checked for histological alterations using a light microscope.

Statistical analysis: Data were reported as mean ± standard error (S.E). One way analysis of variance (ANOVA) followed by post HOC least significant differences analysis (LSD) was performed using statistical package for social science (SPSS) version 20 to compare all the treated groups. The value of $p<0.05$ was considered statistically significant.

RESULTS

Clinical toxicity: MMC exposure induced some clinical signs of toxicity in dams, including decreased activity, loss of appetite, weakness and a significant reduction of body weight in comparison with the control. In addition, three dams from MMC group as well as two from MMC+Ng group were died during the investigation period. Pups treated maternally with MMC at 1 mg/kg b.wt. exhibited some clinical signs of toxicity, including weakness, alopecia, and a significant reduction of body weight gain. On the other hand, the usage of naringenin against MMC toxicity improved the loss in body weight gain decreased by the action of MMC (table 1).

Teratological examinations: Pregnancy outcome in different groups was summarized in table 1. Pregnant rats treated with MMC alone or in combination with naringenin showed a significant decrease ($P<0.001$) in uterine weight when compared to the control group. Also, there was a high frequency of abortion rate and postimplantation loss / litter in MMC treated rats versus control. On the other hand, no significant change was observed in the number of implantation sites and the number of live fetuses in MMC or any other treated group when compared to the control ($P>0.05$).

MMC exposure at 1 mg/kg caused a significant decrease in fetal body weight and length as well as litter weight (P<0.001) when compared to the control group. Co- treatment with MMC and naringenin caused a very highly significant improvement when compared to MMC treated group. MMC treated group showed more males (62.06±1.88 %) than the control (46.75±3.98 %), and the use of naringenin improved this percentage to 50.63±1.70%.

The percentage of external abnormalities/ litter in fetuses maternally treated with MMC increased to 72.36±1.61 % in relation to control 17.5±0.56% while in the group simultaneously treated with MMC and naringenin the rate of external abnormalities / litter markedly improved to 33.04±1.17 % . The most frequent anomalies observed in fetuses maternally treated with MMC were congestion, hemorrhage at the tail and head, red patches on several parts of the body (fig. 1) and paralysis at the right forelimb.

The fetuses taken from mothers treated with MMC showed growth retardation in general skeleton and exhibited several skeletal alterations like delayed ossification of skull bones and enlargement of fontanels (fig.2). Also, showed symmetrical cleavage of the thoracic centra (fig.3), appearance of extra ribs (fig.4), fused ribs (fig.5), partial loss of ossification of some sternbrae (fig.6), completely absence of the ossification of the sacral and caudal vertebrae (fig.7), and shortness or limitation of the growth of long bones and lack of ossification of metacarpals, metatarsals and phalanges of fore and hind limb (fig.8). On the other hand, the use of naringenin against toxicity of MMC modulates these alterations.

Proportion of DNA fragmentation of fetal and neonatal brain: Fetuses maternally treated with MMC showed a significant (P<0.001) increase in the percent of DNA fragmentation in their brain when compared with the control group. However, simultaneous administration of MMC and naringenin displayed a very highly significant (p <0.001) reduction in the percentage of DNA fragmentation caused by MMC. On the other hand, both sex of neonates maternally treated MMC exhibited a significant increase (p<0.001) in the percentage of fragmented DNA when compared to the control group. Co-treatment with naringenin and MMC induced a very highly significant decrease in the high percentage of fragmented DNA caused by MMC (table 2).

Histological studies on fetal and neonatal cerebral cortex: The microscopic examination of cerebral cortex of control fetuses showed the normal organization and distribution of the neurons in different zones of cerebral cortex; the cortical zone, the intermediate or migratory zone and the ventricular zone (fig.9 a1, b1 & c1). Fetuses maternally treated with MMC revealed reduction in cell population and vascular congestion, some neurons were hyperchromatic and shrunken, and some others showing pyknotic nuclei, other sections showed abnormal cellular distribution and hemorrhage (fig. 9 a2, b2 & c2). On the other hand co- treatment with naringenin improved more or less the abnormalities induced by MMC, where the cell population was almost normal in the cortical and ventricular regions, the cellular distribution was normal in all zones. The number of hyperchromatic cells or pyknotic nuclei was reduced (fig.9 a3, b3 &c3).

On the other hand, the cortical region of control neonate showed the cell bodies of neurons; the pyramidal cells and granular cells that usually have conspicuous cytoplasm as well as normal nuclei. The background neuropil, consists of many axons and dendrites of all nerve cells in the region. The glial cells which are non-neuronal cells that maintain homeostasis, form myelin, and provide support and protection for neurons characterized by their small, rounded and lymphocyte like nuclei (fig.10a). The cortical region of male or female pups in MMC group showed marked deformation or shrinkage of some nerve cells with pyknotic basophilic nuclei and interstitial deterioration of the neuropil (fig.10 b1&b2). The cortical degeneration was more pronounced in male pups than females. In MMC group co-treated with naringenin, the brain tissue damages were variably reduced. Compared to MMC group, the quantity and morphology of neurons as well as the neuropil were markedly improved (fig. 10 c1&c2).

DISCUSSION

The present study showed that MMC exposure at 1 mg/kg b.wt. induced some clinical signs of maternal toxicity, including decreased activity, loss of appetite, weakness and decrease in body weight gain. These observations are in accordance with **Fossato da Silva *et al.*** [20] and **Xu *et al.*** [21].Several investigations attributed the reduction of body weight due to mercury exposure to the anorexigenic effect of mercury [3] as well as to the interference of this metal in the absorption of nutrients such as the amino acids and glucose absorption [22]. In the present study, the decrease in maternal body weight may be attributed also, in part to the increase in incidence of abortion and fetal growth retardation. This notion is supported in this study by the finding of significant reduction in uterine weight and fetal body weight and length.

In the current investigation, there was a high frequency of abortion, postimplantation loss / litter, fetal growth retardation and external and skeletal abnormalities of fetuses maternally treated with 1 mg/kg bwt MMC through gastric intubation. These observations are in accordance with previous studies [17, 23]**.**The loss of fetal body weight was probably due to lower mitotic growth rate in the affected fetuses [24]**,** and/or due to the loss of skeletal ossification [25]**.** Also, ROS was found to play important roles in fetal development; being involved during implantation, growth and differentiation [26]**.** The embryonic and fetal development periods are believed to be extremely sensitive to high levels of ROS in part because effective free radical scavenging systems are not yet fully developed [27]**.** It was found that MMC can easily cross the placenta and then accumulate in the fetus, where it can deleteriously affect the offspring [28]. Also, **Lee *et al*. [29]** suggested that the causes related to low fetal and litter weight after MMC exposure may be due to the disability of fetuses to use nutrients from mothers during pregnancy period. Elevated ROS may underlie fetal developmental skeletal delay and altered embryonic organogenesis. The embryonic and fetal development periods are believed to be extremely sensitive to high levels of ROS in part because effective free radical scavenging systems are not yet fully developed [27]**.** Changes in prenatal bone health and fetal osteogenesis have been positively correlated with excessively elevated ROS that may lead to improper skeletal formation [30]**.** Oxidative stress leads to enhanced production of ROS, which can modify DNA, proteins, lipids and carbohydrates [31].Therefore, MMC may induce DNA damage by produce ROS [32]. Supporting this view, the present study showed that both fetuses and pups maternally treated with MMC exhibited a very highly significant increase in the percentage of fragmented DNA when compared to the control. These results are in accordance with many authors [33, 34]. Therefore, the high incidence of external and skeletal abnormalities rate in this investigation may be associated with DNA damage induced by MMC.

Methylmercuric chloride is known to induce DNA single strand breaks [35]**,** inhibit DNA repair system [36] and cause oxidative DNA base damage in mammalian cells [37]**.** Furthermore, the study of **Liu *et al*.,** [38] showed that MMC exposure increased the levels of 8-hydroxydeoxyguanosine (8-OHdG) and this could lead to severe DNA oxidative damage. The 8-OHdG is widely used as a biomarker of DNA oxidative damage. Therefore, the observed increase in DNA damage in the rats exposed to MMC could be caused either by the enhancement of DNA oxidation or by the inhibition of the DNA repair system as suggested by **Crespo-Lo´peza *et al.*** [39]. Oxidizing and reducing equivalent imbalance in turn leads to macromolecule damage namely protein modification, lipid peroxidation, and DNA oxidation, and if unchecked, oxidative damage can lead to cell death. Supporting this view, our histopathological examination of cerebral cortex of both fetuses and neonates exhibited reduction in cell population, vascular congestion, interstitial necrosis and some neurons were hyperchromatic with pyknotic nuclei. In previous studies, neonatal rats administered MMC for 10 days from PN2 showed minimal damage in the hippocampus and brainstem nuclei, while neonates treated for 10 days from PN15 showed widespread neuronal degeneration in the cerebral cortex, striatum and red nucleus [40]**.** Also, **Sirois and Atchison** [41] reported that MMC exposure caused a marked degeneration of cerebellar architecture, especially in the cerebellar granule cell layer.

Free radicals are known to be involved in toxicant triggered processes leading to induction of apoptosis as well as necrosis [42]**.** Mercury compounds induce a general collapse of the antioxidant mechanisms in the cell by binding to the sulfhydryl groups of glutathione peroxidase. Such a collapse results in cell degeneration inhibits lipid peroxidation and thereby induces loss of membrane integrity and finally cell necrosis [43, 44]. In the present study, treatment with naringenin against MMC showed an improvement in the embryotoxic effect caused by MMC, this data is in accordance with some authors who studied the protective effect of flavonoids on embryo toxicity as; **Pérez-Pastén et al.** [9] **and Ren et al.** [10]. In the present work naringenin significantly reduced the high percentage of fragmented DNA caused by MMC in fetal and neonatal brain and diminished the histopathlogical changes observed in the cerebral cortex of both fetuses and neonates induced by MMC alone. These observations are in accordance with **Mercer *et al*.** [45] who revealed that naringenin decreased the biomarkers of DNA oxidative damage in primary rat mesencephalic cultures after receiving N-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium hydrochloride. Also, in a study by **Cavia-Saiz *et al*.** [46] naringenin at different concentrations showed a partial protection to the electrophoretic pattern of DNA incubated with hydroxyl radicals. Furthermore, naringenin alone or in combination with other flavonoids decreased DNA damage in lymphocytes in diabetic mice [47] and in simvastatin treated rats [48]. It has also, the ability to prevent DNA fragmentation in rats treated with Fe [49]. **Al-Rejaie et al.** [50] found that naringenin treatment significantly corrected the impaired levels of nucleic acids and

total protein in colon tissue suggesting the cytoprotective properties of the naturally occurring flavonoid.

Several studies showed that the flavonoids had placental-barrier-crossing capacity such as the synthetic flavonoid 3-methyl-48,6- dihydroxy-38, 58-diiodoflavone [9] **,** a-naphthoflavone, [9, 51] , catechin [9] and epigallactocatechin gallate and neohesperidin dihydrochalcone [9] .In the same concern, naringenin may also cross placental blood barrier and improve developmental toxicity caused by MMC. Also, naringenin showed an antioxidant and free radical scavenging activity [52], so the protective effect may be due to this action. Supporting this view **Renugadevi and Prabu** [13] suggested that the lipophilic nature of naringenin can facilitate its adherence to lipid bilayer which might reduce the formation of free radicals and protects the cell membrane. Naringenin treatment may protect cell membranes against lipid peroxidation and protein oxidation by scavenging the intracellular ROS produced [11]**.** Furthermore, **Renugadevi and Prabu** [13] mentioned that the chelating property of naringenin enhances the elimination of cadmium from the renal tissue, which might reduce the Cd burden. On similar lines, the chelating property of naringenin may enhance the elimination of mercury in fetal and neonatal tissue. In addition, several investigators reported that naringenin was mutagenic inhibitor [53] and genotoxicity inhibitor [54]. Therefore, it can be concluded that MMC exposure during gestation and lactation had impact on fetal and neonatal development. It can induce growth retardation as well as external and skeletal abnormalities. MMC had particular effect on immature brain; it can increase the percentage of fragmented DNA and induce pathological effects on cerebral cortex of both fetuses and neonates. Co- treatment with naringenin can diminish the developmental toxicity caused by MMC.

Table (1): **Pregnancy outcome of pregnant rats on the 20th day of gestation of control (Cont), naringenin (Ng), methyl mercuric chloride (MMC) and both methyl mercuric chloride and naringenin group (MMC +Ng) as well as the body weight (g) of neonates at birth and at postnatal day 30 (PN30).**

	Cont	**Ng**	**MMC**	**MMC+ Ng**
Uterine weight (g)	32.65±0.92	34.77±1.02	26.36±0.91^{a3b3}	27.32±0.90^{a3b3}
% of partial abortion	0	0	10	0
No. of implantation sites/ litter	7.00±0.39	6.80±0.20	6.70±0.34	6.80±0.20
Postimplantation loss index	1.38±0.084	1.50±0.13	1.89±0.2 ^{a1}	1.57±0.16
No. Live fetuses/ litter	7.30±0.15	6.70±0.26	7.20±0.29	7.20±0.20
Fetal body weight (g)	2.87±0.11	3.19±0.1^{a1}	1.93±0.08^{a3b3}	2.24±0.1^{a3b3c3}
Litter weight (g)	20.25±0.62	23.70±0.873^{a1}	15.77±1.55^{a2b3}	16.18±0.70^{a2b3}
%of males	46.75±3.98	50.10±1.00	62.06±1.88^{a3b3}	50.63±1.70^{c3}
%of females	53.30±3.98	49.90±1.00	37.94±1.88^{a3b3}	49.38±1.70^{c3}
% of external abnormalities / litter	17.5±0.56	20.45±0.33	72.36±1.61 ^{a3b3}	33.04±1.17^{a3b3c3}
Pups body weight (g) at birth	7.00±0.21	6.00±0.21^{a2}	4.9±0.18^{a3b2}	5.40±0.2
Pups body weight (g) at PN 30	43.10±1.13	43.10±1.11	31.40±0.92^{a3b3}	38.20±1.16^{a2b2c3}

Data are represented as mean ±SE. **a**:significant value compared to control, **b**: significant value compared to naringenin & **c**: significant value compared to MMC group **1**:at level p< 0.05, **2**: at level p< 0.01 & **3**: at level p< 0.001

Table (2): **The percentage of DNA fragmentation (%) in the brain of fetuses and both male and female pups maternally treated with methyl mercuric chloride (MMC) without or with naringenin (Ng).**

	Cont	**Ng**	**MMC**	**MMC+ Ng**
Fetuses	15.09±1.13	17.19±0.31	52.66±2.28 ^{ab}	34.54±1.89 ^{abc}
Male pups	20.07±1.09	20.53±0.93	45.73±1.52^{ab}	32.56±0.89
Female pups	18.26±0.96	19.00±0.74	47.04±1.32^{ab}	35.02±1.57 ^{abc}

Data are represented as mean ±SE. **a**:significant value compared to control, **b**: significant value compared to naringenin & **c**: significant value compared to MMC group at level p< 0.001.

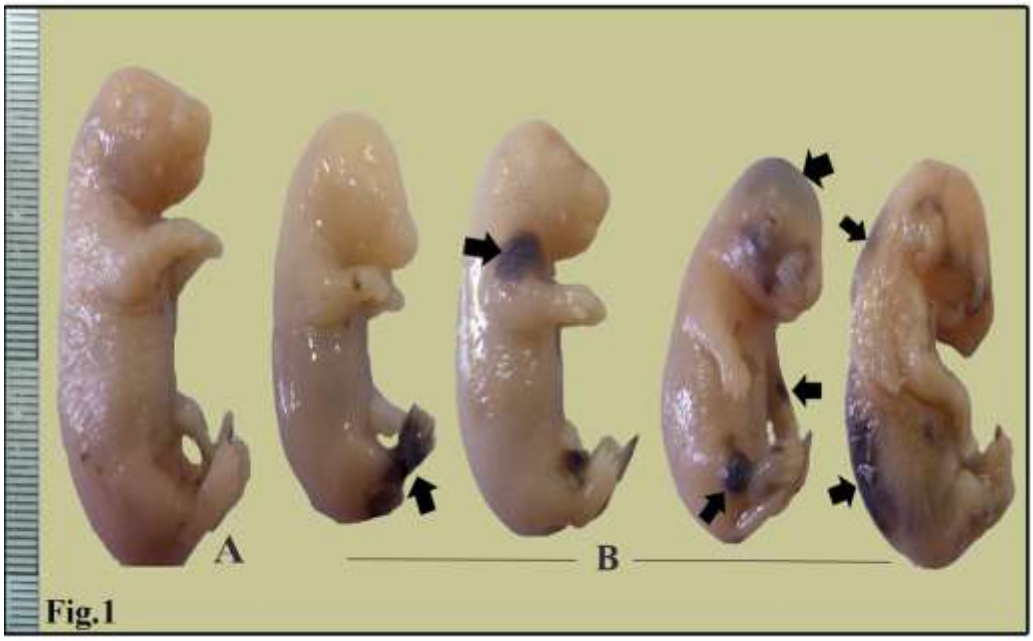

Fig. (1): photograph of fetuses on the 20th day of gestation of control (A) and fetuses obtained from rats treated with MMC showing subcutaneous hemorrhage in different parts of the body (arrows) and growth retardation (B).

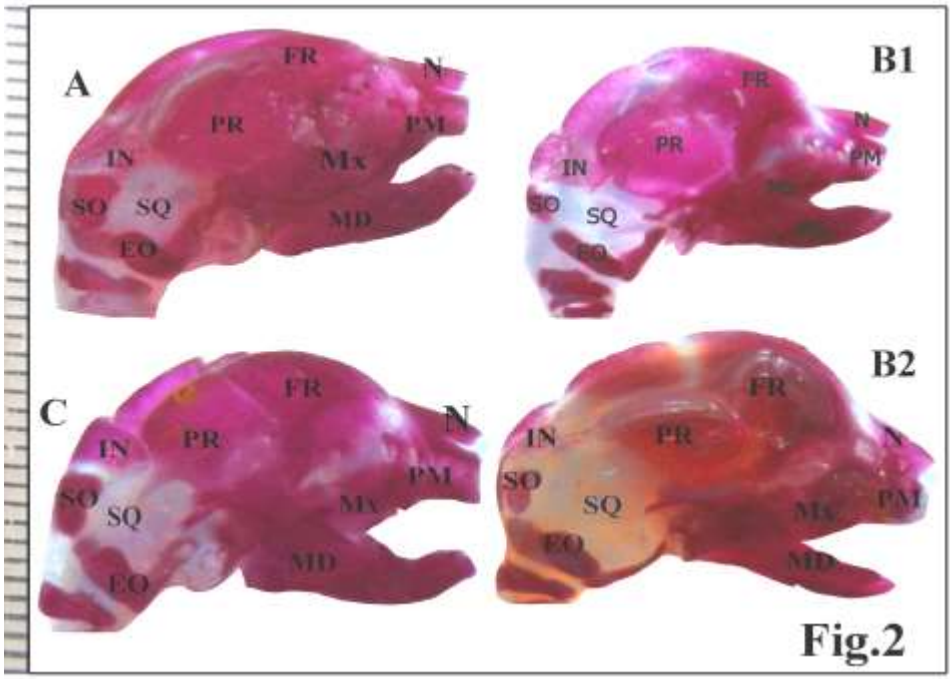

Fig.(2): A photograph of lateral view of the skulls of fetuses on the 20th day of gestation showing skull of control (A), skulls of fetuses maternally treated with MMC (B1&B2) showing delayed ossification of skull bones [parietal, (PR) interparaital (IN) and supraoccipital (SO)], dilation of the cranial sutures and reduction of lower jaw. A skull of fetus maternally treated with MMC and naringenin (C) showing an improvement of skull bone elements. (S: alizarin red and alcian blue).

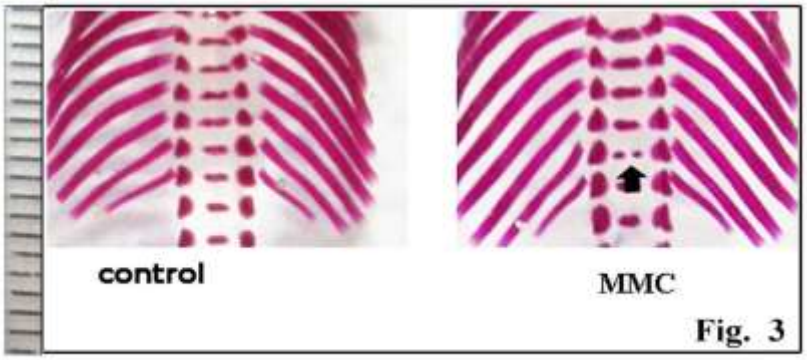

Fig. 3

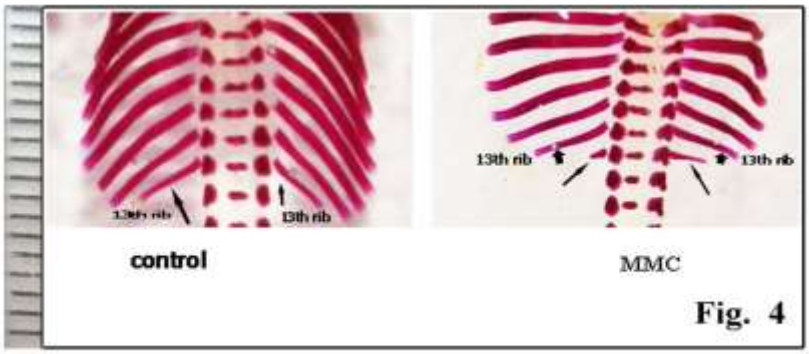

Fig. 4

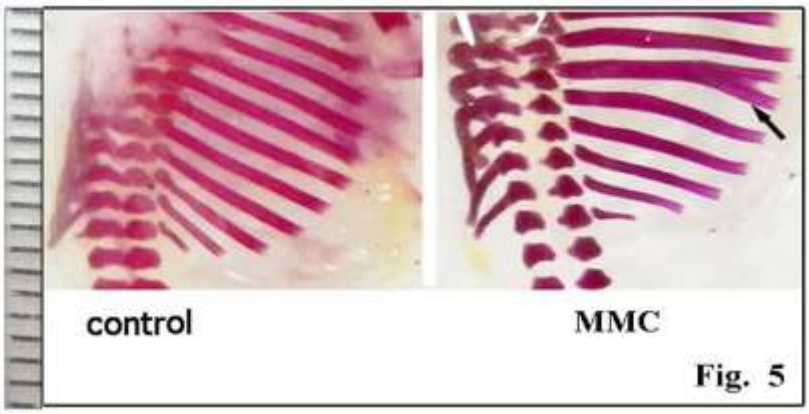

Fig. 5

Figs. (3-5): Photographs of ribs region of fetuses' skeleton on the 20th day of gestation of control and fetuses maternally treated with MMC showing symmetrical cleavage of the thoracic centra (arrow) [fig.3], appearance of extra ribs (arrows) [fig.4] and fused ribs (arrow)[fig.5]. (S: alizarin red and alcian blue).

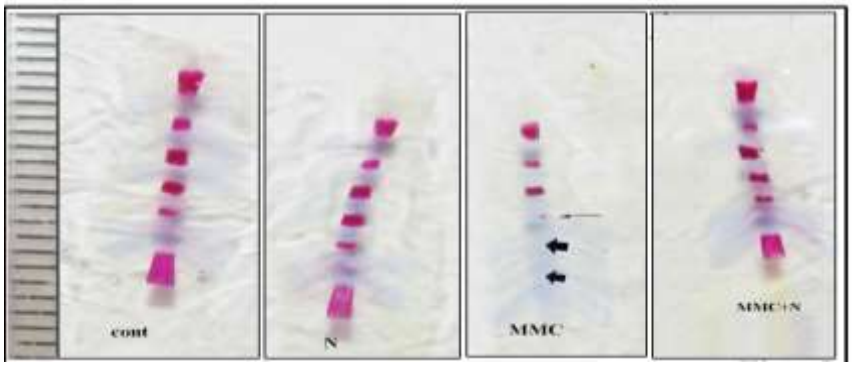

Fig. 6: A photograph of sternbrae of fetuses of control and naringenin (N) treated groups showing well ossified sternbrae. MMC showing complete absence of the ossification (thick arrow) and less degree of ossification (thin arrow), MMC+N showing an improvement. (S: alizarin red and alcian blue).

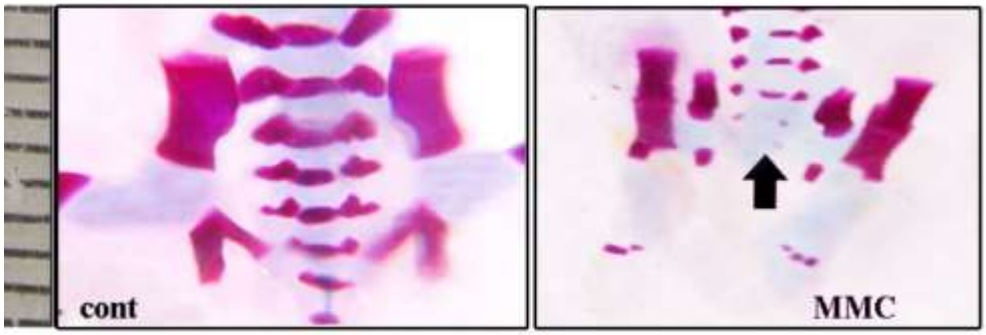

Fig.7: A photograph of the posterior region of fetuses of control showing well ossified sacral and caudal vertebrae. MMC showing poor ossification of sacral vertebrae and complete lack of calcified ossification centers in the caudal vertebrae (arrow). (S: alizarin red and alcian blue)

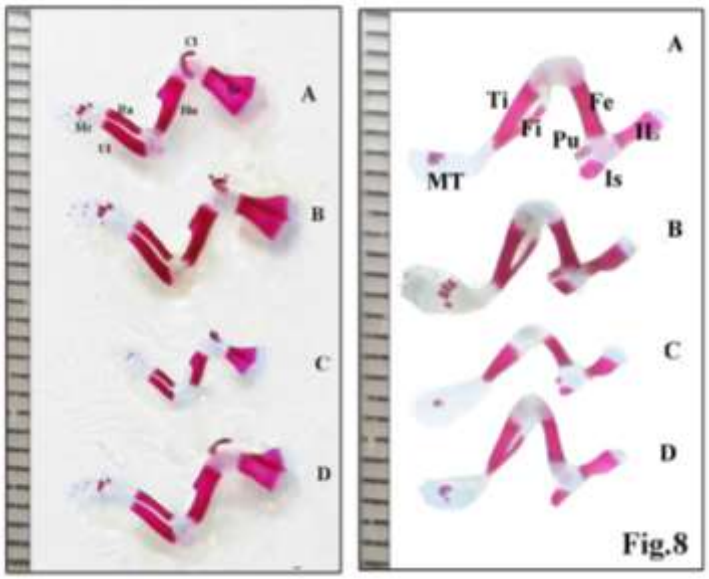

Fig.8: A photograph of the pectoral and pelvic girdles as well as the fore and hind limbs of fetuses of different groups: control (A), naringenin (B), MMC (C) showing shortening of long bones and MMC+N (D) showing an improvement. (S: alizarin red and alcian blue).

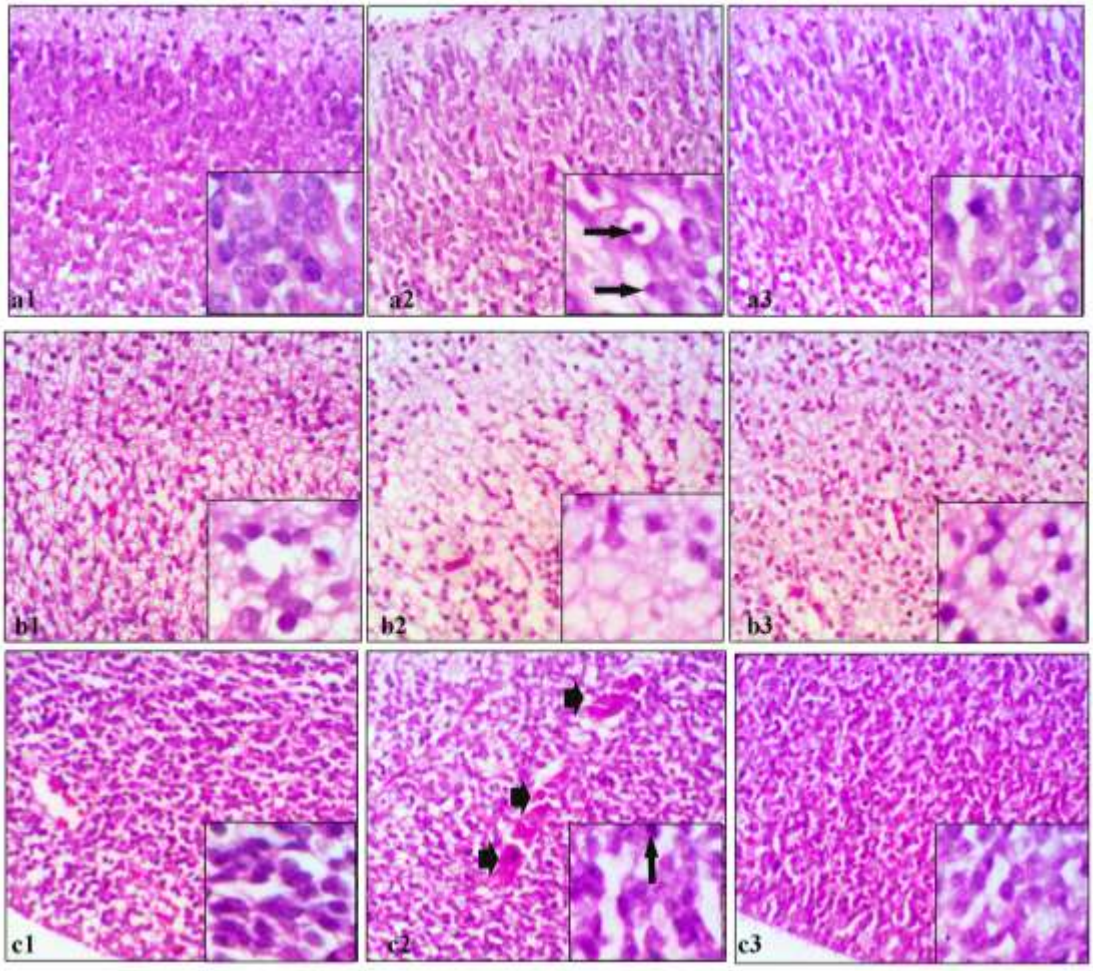

Fig.9

Fig.9: Photomicrographs of cerebral cortex of fetuses through the cortical zone (a), the intermediate zone (b) and the ventricular zone (c) of control (1), MMC treated mothers (2) and MMC plus naringenin treated dams (3). The cerebral cortex of MMC group showing reduction in cell population, shrunken neurons with basophilic nuclei (arrows) (a2&c2), abnormal cellular distribution (b2) and hemorrhage (arrows head) (c2). Fetuses maternally treated with both MMC and naringenin (a3, b3 &c3) showing almost normal cellular population and normal distribution in all cortical zones. The number of hyperchromatic cells or pyknotic nuclei was reduced.
S: H&E (X-400 and X-1000 for insertion).

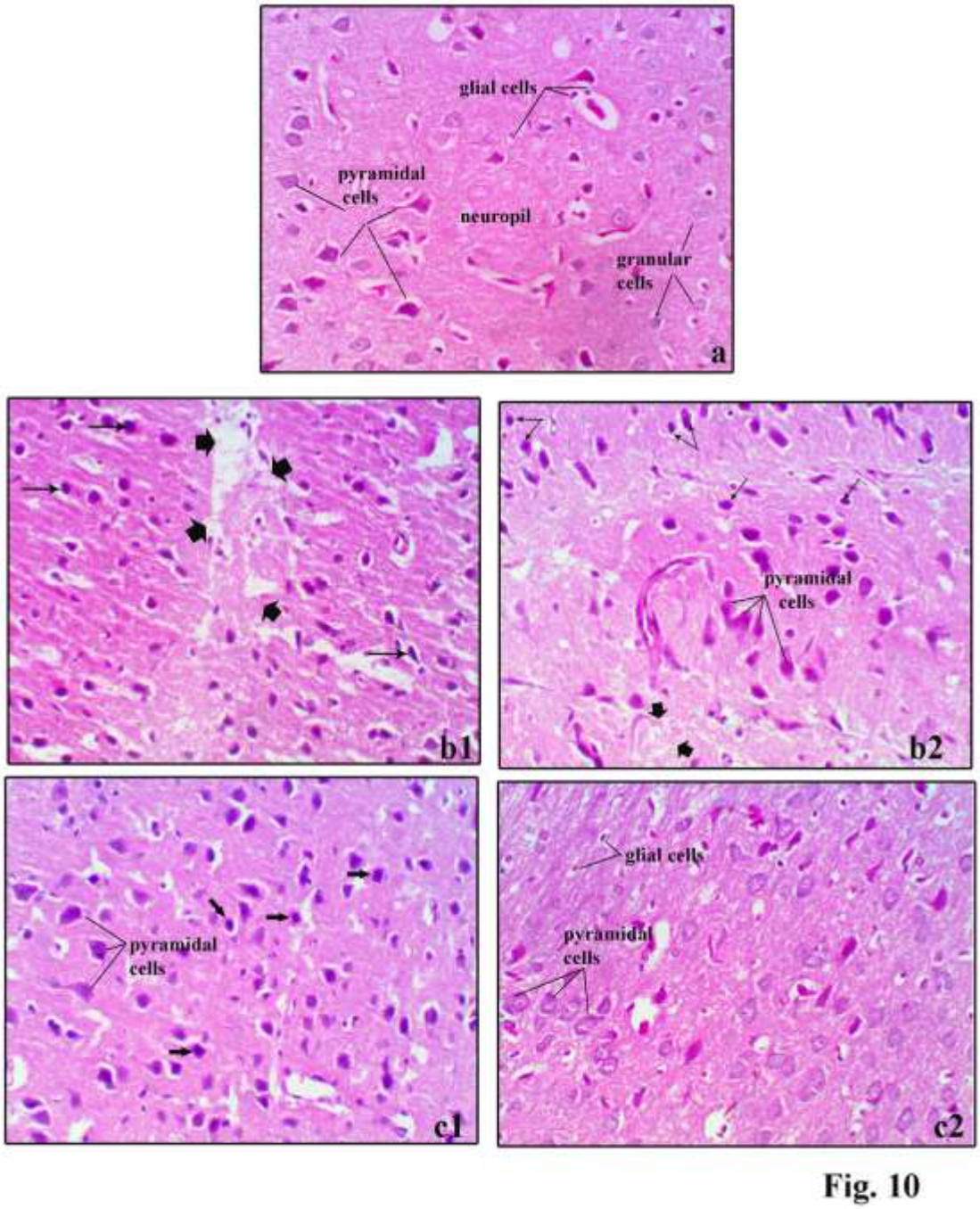

Fig. 10

Fig. 10: Photomicrographs of cerebral cortex of neonates of control (a) and neonates maternally treated with MMC (b1 male & b2 female) showing shrunken neurons with eosinophilic cytoplasm and pyknotic basophilic nuclei (arrows) and deterioration of the neuropil in the cortex (arrows head). Neonates maternally co-treated with MMC and naringenin (c1 male & c2 female) showing almost normal neurons and neuropil with few pyknotic nuclei (arrows). Note the degenerative changes were more pronounced in male pups than females. S: H&E (X-400)

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