N-acetylcysteine protects against neurodevelopmental injuries induced by methylmercury exposure during pregnancy and lactation

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Abstract

As an extremely dangerous environmental contaminant, methylmercury (MeHg) results in detrimental health effects in human brain nervous system, one of its main targets. However, as a developmental toxicant, the brain of offspring is vulnerable to MeHg during pregnancy and lactation exposure. Unfortunately, mechanisms of neurodevelopmental injuries induced by MeHg have not been fully elucidated. *N-acetylcysteine* (NAC) has been used for several decades as an antioxidant to antagonize oxidative stress. However, the molecular mechanisms of NAC alleviating MeHg-induced neurodevelopmental toxicity are not clear. Here, for evaluation of the dose-dependent effects of MeHg exposure on neurodevelopmental injuries of offspring, and the possible protective effects of NAC, the pregnant female mice were exposed to MeHg and NAC from gestational day 1 (GD1) to postnatal day 21 (PND21). Our results indicated that administering MeHg caused behavioral impairment and neuronal injuries in the cerebral cortex of newborn mice. MeHg dose-dependently caused reactive oxygen species (ROS) overproduction and oxidative stress aggravation, together with expression of Nrf2, HO-1, Notch1, and p21 up-regulation, and CDK2 inhibition. NAC treatment dose-dependently antagonized MeHg-induced oxidative stress that may contribute to alleviate the neurobehavioral and neurodevelopmental impairments. These results give insight into that NAC can protect against MeHg-induced neurodevelopmental toxicity by its antioxidation capacity.

Introduction

Methylmercury (MeHg), a developmental toxic substance, is known to affect the central nervous system of children exposed to the metal during pregnancy. Microorganisms can convert inorganic mercury into MeHg, which accumulates in seafood and causes harm to human body. Intake of methylmercury contaminated seafood is considered to have adverse effects on fetal neurodevelopment (Al Osman et al. 2019; Branco et al. 2017). There is no difficulty for MeHg to penetrate blood brain by forming MeHg-cysteine complex under the action of neutral amino acid transport system (Takahashi and Shimohata 2019). Furthermore, MeHg can also effortlessly be transported across placental barrier (Straka et al. 2016). Severe neurological symptoms and mental retardation are described as a consequence of a mother ingesting food contaminated with MeHg during pregnancy, even for children born to asymptomatic mothers (Julvez et al. 2019; Rothenberg et al. 2011). Recently, a cohort study in Seychelles showed that prenatal exposure to MeHg can lead to neurodevelopmental impairment in 7-year-old children (Cediel Ulloa et al. 2021). Previous study in Shanxi province in northern China have concluded that prenatal Hg exposure is associated with increased risk for neural defects (Tong et al. 2021). In addition, the change of motor coordination ability and the impairment of learning and memory ability are the neurobehavioral effects caused by MeHg exposure (Falluel-Morel et al. 2012; Jacob and Sumathi 2019). Nevertheless, the explicit mechanism of MeHg neurodevelopmental toxicity during pregnancy and lactation exposure is not discovered completely.

MeHg is an inhibitor of enzyme catalysed reactions and protein synthesis. In addition, excessive production of reactive oxygen species (ROS) may be the key factor of MeHg-induced cranial nerve...
dysfunction by destroying redox reaction or reducing antioxidant defense (Ferrer et al. 2022). Therefore, MeHg-induced oxidative stress may have a wide range of effects on the homeostasis of the intracellular environment. Nuclear factor erythroid 2 related factor (Nrf2) is an important member of cellular defense pathway and plays an important role in the nervous system against MeHg-induced oxidative stress (Wei et al. 2021). Previous studies have shown that MeHg-induced oxidative stress plays an important role mediating the activation of apoptosis pathway in rat cortical neurons, while Nrf2 and its downstream gene Heme oxygenase-1 (HO-1) are up-regulated in these processes. (Liu et al. 2016; Liu et al. 2019). Furthermore, Notch family members are essential signaling molecules that control a diverse array of cellular responses during neural cells development. In the central nervous system, activated Notch1 signaling can regulate the growth and development of neurons (Peng et al. 2019). The proliferation and differentiation of neural stem cells are mediated by Nrf2 and Notch1 signaling pathways. The downstream effector of Notch1 signaling pathway plays an important role in the growth and development of progeny. There are one or more functional antioxidant response elements (ARE) sequences in Notch1 promoter. Therefore, Nrf2 can directly regulates Notch1 gene expression (Wakabayashi et al. 2010). Previous studies have found that MeHg induced the activation of Notch signaling through two downstream transcription factors of proteolytic Notch signaling pathway (Raciti et al. 2019; Tamm et al. 2008). However, the exact regulatory mechanisms have not clearly understood, thus, further studies are necessary to elucidate the regulatory role of Nrf2 and Notch1 pathway and its downstream factors in MeHg-induced neurodevelopmental toxicity.

N-acetylcysteine (NAC) is an effective antidote to metal toxicity in central nervous system. NAC can alleviate the oxidative stress of intracellular environment because it can increase the concentration of glutathione (GSH), and GSH is the most important bio-mercaptan that leads to the imbalance of redox in cells (Coles et al. 2018). NAC can enhance the resistance of cells to ROS-mediated oxidative damage because it contains a sulfhydryl group, which has antioxidant properties. Thus, we herein speculate that NAC might exert a neuroprotective effect by scavenging ROS and antioxidation, which contribute to inhibit or reverse MeHg-induced neurodevelopmental toxicity. Although the capacity of NAC to restrain neurotoxicity has been broadly explored (Faria et al. 2019; He et al. 2021; Mahmoud et al. 2019; Tenório et al. 2021), evidence supporting the protective role of NAC in MeHg-induced neurodevelopmental toxicity during pregnancy and lactation needs further investigation. Therefore, to verify our hypothesis, NAC were given in the mice model to explore the effects of MeHg exposure on neonatal mice, which can provide theoretical and experimental basis for the treatment of MeHg-induced developmental neurotoxicity.

**Materials And Methods**

**Chemicals and Reagents**

Methylmercury chloride (purity ≥ 95.0%) was provided by Laboratory of Dr. Ehrenstorfer-Schafers (Augsburg, Germany). NAC (purity ≥ 99.0%) was provided by Beyotime Biotechnology (Nantong, China). Reactive Oxygen Species Assay Kit was purchased from Beyotime Biotechnology (Nantong, China). GSH and malonyldialdehyde (MDA) kits were purchased from Nanjing Jiancheng Bioengineering Institute.
(Nanjing, China). RNAiso Plus, Prime Script® RT reagent Kit with gDNA Eraser, and SYBR® Premix Ex Taq™ II analysis kits were provided by TaKaRa Biotechnology Company (Dalian, China). SP and DAB Color Development kits were obtained from ZSGB-BIO (Beijing, China). Hematoxylin-Eosin Staining Kit was obtained from Solarbio LIFE SCIENCE (Beijing, China). Nissl Staining Solution was purchased from Beyotime Biotechnology (Nantong, China). SDS-PAGE gel preparation kit was obtained from Beyotime Biotechnology (Nantong, China). Rabbit monoclonal primary antibodies include Nrf2 (#A1244), Notch1 (#A16673), HO-1 (#A1346), β-actin (#AC038), β-tubulin (#AC008) were obtained from ABclonal Technology (Woburn, MA, USA). Rabbit monoclonal primary antibodies against p21 (#27296-1-AP), cyclin-dependent kinase 2 (CDK2) (#10122-1-AP) were obtained from Proteintech Group Inc (Chicago, IL, USA). Other analytically pure chemicals were purchased from local chemical providers.

Establishment of the animal models

Healthy adult C57BL/6 mice (8-week old) were provided by the Animal Laboratory of China Medical University (SPF grade, certificate number SCXK, 2018–0008), 20 males (initial body weight 32 ± 2g) and 40 females (30 ± 2g). Male and female mice were housed in opaque plastic cages (two females to one male in each cage). The animals were housed in cages, in which temperature always is 25 ± 2 °C, humidity always is 50 ± 5%, and natural daylighting. Commercial food and tap water were provided by China Medical University. After pregnancy (appearance of vaginal plug was considered as gestational one (GD 1) of pregnancy), the males were removed from the cages and the females were subjected to experimental treatments.

All pregnant mice were divided into two sections (Fig. 1). Section one: The pregnant mice were divided into 4 groups (n = 3), control group received distilled water only, other groups were exposed to 4mg/L, 8mg/L, or 12mg/L MeHg, dissolved in distilled water, through drinking the water freely. Section two: The pregnant mice were divided into 5 groups (n = 3), the first group served as the control group and received distilled water only, the mice in MeHg-treated group were exposed to 12mg/L MeHg, other three groups were subcutaneously injected with 50mg/kg, 100mg/kg, or 150mg/kg NAC in the back of neck, dissolved in the saline, respectively, together with 12mg/L MeHg exposure. The volume of injection was 10 ml/kg. NAC were administered on an alternate-day from GD1 to postnatal day 21 (PND21). The dosages of NAC were selected based on previous studies (Muniroh et al. 2020). All treatments were given from GD1 to PND21, behavioral tests were performed 8 days after PND21. All neonatal mice were killed 2 days after the behavioral tests. Briefly, the brain was carefully removed, then cerebral cortex was separated on ice bath. According to experimental needs, tissues were processed immediately or frozen at -80°C until assay. All the experimental procedures were processed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23, 1996) and supported by the Laboratory Animal Ethics Committee of China Medical University.

Determination of body weight, body length, and brain viscera index of offspring
The body weight and length of offspring mice were measured on PND5, PND10, PND15, PND21, the brain viscera index was measured on PND21, respectively (Miao et al. 2017).

**Open field test**

The spontaneous and exploratory abilities of mice were evaluated by open-field experiments in a black box of $50 \times 50 \times 40$ cm. The bottom of the box is divided into 16 squares, and a camera is placed 2 meters directly above the box. Each experiment lasted for 5 min. Ethvision XT 11 software was used to automatically record the total distance traveled (cm), average speed (cm/s), time spent in the central zone (s), and frequency of each mouse.

**Morris water maze test (MWM)**

The MWM test was performed as described in pervious experiment (Dinel et al. 2020). The water maze is a black plastic circular pool with a diameter of 100cm and filled with $23 \pm 0.5^\circ C$ water. Right above it is a camera that records the mouse's swim through the water. Mice were collected for 5 days. Each mouse had to swim in four different quadrants each day (the water maze was divided into four quadrants on average), and the time it took each mouse to find the hidden platform was recorded. The platform had a diameter of 10 cm and was hidden 1.5 cm below the surface. If the mouse did not find the platform within 60 seconds, it was gently placed on the platform for 20 seconds. 48 hours after the end of the acquisition test, the probe test was performed, at which point the platform was removed. The swimming time of the probe test was 60 seconds. The first search latency in the target quadrant, the dwell time in the target quadrant (%) and The Times of crossing the original position of the platform (crossing times) were recorded by software (Noldus Information Technology Inc Noldus EthoVision XT).

**Hematoxylin and Eosin (H&E) Staining**

The brain tissue of each group was first placed in 4% Paraformaldehyde overnight, dehydrated and embedded in paraffin blocks. The paraffin-embedded brain tissue was cleaned, dehydrated, cleaned, waxed, embedded, sectioned, coated, baked, stained with he in sagittal plane about 5µm thick, and finally observed under a light microscope (from Hitachi, Japan).

**Nissl staining**

The brains of the mice were fixed in 4% formaldehyde for four days. Immobilized brain tissue was transferred to ethanol and xylene to dehydrate. 5µm thick sections were successively dewaxed, rehydrated, stained with toluidine blue, and soaked at $38^\circ C$ for 30 min. Sections were dehydrated in 100% alcohol and washed in xylene for transparency (twice for 2 min each). The neurons in the cerebral cortex were visualized, the number of surviving nissl bodies and morphological change were quantified using ImageJ 1.8.0_112.

**Quantification of ROS formation**

ROS levels in cortical neuronal cells were quantified by flow cytometry using the oxidation-sensitive fluorescent dye DCFH-DA as described previously (Wang et al. 2020). In brief, cells were incubated with
10 mM of DCFH-DA at 37°C for 30 mins. Then the fluorescence was measured and monitored at 488nm (excitation)/525nm (emission) by flow cytometry. Four separate experiments for each treatment group were determined.

**GSH and MDA assays**

The GSH and malondialdehyde (MDA) contents in cell lysates were assessed using GSH and MDA assay kits (Nanjing Jiancheng Bioengineering Institute), respectively, according to the manufacturer’s instructions. Absorbance was determined using a microplate reader with 412 nm for GSH, and 532 nm for MDA, respectively.

**Western blotting**

The extraction and quantification of cerebral cortex proteins were performed according to the modified method described previously (Bass et al. 2017). Total protein was extracted in an ice bath supplemented with RIPA buffer and protease inhibitors. Protein concentrations were quantified with the bicinchoninic acid (BCA) reagent. For Western blotting experiments, equal amounts of proteins (30 µg per lane) were separated by electrophoresis on 8 or 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membrane was then blocked for 1.5 h at room temperature in Tween 20 Tris-buffered saline (TBST) containing 5% bovine serum albumin (BSA). Then the PVDF membranes were incubated with Nrf2 (1: 1000), HO-1 (1 1000), CDK2 (1 1000), p21 (1 1000), Notch1 (1 1000), β-tubulin (1 5000), or β-actin (1 1000) antibodies diluted with TBST overnight at 4 °C. The PVDF membranes were washed three times for 10 min in TBST, and then combined with HRP-conjugated antibodies (1:5000) for 1 h. The intensity of bands was evaluated semi quantitatively by densitometry using image analyzing software (ImageJ 1.8.0_112). β-actin or β-tubulin were used as the internal control.

**Immunohistochemistry staining**

The immunohistochemistry (IHC) staining of the cerebral cortex was performed as described below. Briefly, the paraffin brain slices of cerebral cortex were subjected to dehydration in alcohol and transparency was achieved with washes in dimethylbenzene (twice for 2 min each), and then antigen recovery, and incubated with 10% goat serum (30 min) following by incubation with the appropriate dilution of primary antibody [Nrf2 (1:200), and Notch1 (1:200)], at 4 °C overnight. The next day after washing, the sections were serially incubated with reaction enhancer (20 min) and goat anti-rabbit IgG polymer (20 min) at 37 °C. Then, a suitable amount of DAB chromogenic solution was used for color development. Lastly, the sections were rinsed with tap water and then subjected to normal dehydration and transparency. For each sample, three representative interested fields were analyzed by Image Pro Plus 6.0, and the mean integrated optical density value of positive cells were obtained for statistical analysis.

**The reverse transcription quantitative polymerase chain reaction (RT-qPCR)**
In RNAiso Plus, the cortex was completely ruptured. Chloroform was added and centrifuged to form supernatant layer, intermediate layer and organic layer. The supernatant layer containing RNA was collected. Total RNA was recovered by precipitation with isopropanol and finally dissolved in RNase-free water. The nucleic acid concentration is determined by the nucleic acid concentration analyzer. Genomic DNA is removed through Prime Script RT reagent Kit with gDNA Eraser. The gene primer sequences were designed by TaKaRa company as shown in Table 1. Subsequently, the reverse transcription reaction was carried out using two-step PCR reaction program using by the 7500 Real-time PCR system, and the comparative CT method \(2^{-\Delta\Delta CT}\) was used to assess the relative expression analysis.

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>Nrf2-F</td>
<td>5'-TCTTGGAGTAAGTGCAGAAGTGT-3'</td>
</tr>
<tr>
<td>Nrf2-R</td>
<td>5'-GTGGAAACTGAGCAGAAAAGGC-3'</td>
</tr>
<tr>
<td>Notch1-F</td>
<td>5'-CCGTGTAAGAATGCTGGAACG-3'</td>
</tr>
<tr>
<td>Notch1-R</td>
<td>5'-AGCGACAGATGTATGAAGACTCA-3'</td>
</tr>
<tr>
<td>HO-1-F</td>
<td>5'-AAGCCGAGAATGCTGAGTACA-3'</td>
</tr>
<tr>
<td>HO-1-R</td>
<td>5'-GCCGTGTAGATATGGTACAAGGA-3'</td>
</tr>
<tr>
<td>β-actin-F</td>
<td>5'-GGCTGTTATTCCTCCATCG-3'</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>5'-CCAGTTTGGTAAATGCCATGT-3'</td>
</tr>
<tr>
<td>Gapdh-F</td>
<td>5'-AGGTCGGTGTGAACGGATTG-3'</td>
</tr>
<tr>
<td>Gapdh-R</td>
<td>5'-TGTAACCATGTAGTTGAGGTCG-3'</td>
</tr>
</tbody>
</table>

### Statistical analysis

All the data were expressed as mean ± S.D., and analyzed by SPSS 25.0 software. Differences were determined by one-way analysis of variance, and then LSD-t test was used to detect the difference between two groups with special significance. \(P\)-value < 0.05 was considered to be statistically significant.

### Results

1. **MeHg exposure inhibited the neonatal mice developmental growth**

After being administrated from GD1 to PND21, we recorded the postnatal body weight, body length and brain viscera index data of offspring at 5, 10, 15 and 21 days. Based on the findings, there was a s dose-
dependently and statistically significant decrease in body weight, body length and brain viscera index of MeHg-treated neonatal mice compared to that of control mice (Fig. 2A, B and C, \( P < 0.01 \) or \( P < 0.05 \)).

2. MeHg induced neurobehavioral impairments in neonatal mice

The effects of MeHg on neurobehavioral ability were assessed by behavioral tests after pregnancy and lactation continuous exposure to MeHg. To analyze the effect of MeHg on locomotion ability, we performed an open-field test. In the open-field test, we found that the total distance decreased at 4mg/L and 8mg/L MeHg-treated groups compared with the control (Fig. 3B, \( P < 0.05 \)). Moreover, time spent in the center at MeHg-treated groups decreased significantly (Fig. 3B, \( P < 0.05 \)). However, no significant changes in speed parameters were observed among the control group and MeHg-treated groups. Taken together, these results suggested that MeHg induced neurobehavioral disorders in neonatal mice.

The MWM test was used to examine learning and memory abilities. MeHg-treated groups prolonged the latency period on day 5 (Fig. 3D, \( P < 0.01 \)). The platform was removed after an interval of 48 h, and the test data showed no difference in the moving speed and frequency of crossing the platform area among the groups, however the proportion of time remaining in the target quadrant and the first time latency by MeHg-treated mice decreased significantly (Fig. 3E, \( P < 0.01 \) or \( P < 0.05 \)). Our data showed that MeHg induced neurobehavioral impairments in neonatal mice.

3. MeHg induced pathological changes in neonatal mice cerebral cortex

The pathological changes were detected by inverted microscope. Routine HE histological examination in cerebral cortex revealed that the degree of damage was gradually aggravated with the increase of MeHg concentration (4, 8 or 12 mg/L), including the number of normal cells decreased significantly, and some cells showed nuclear condensation with vacuolar degeneration and necrosis (Fig. 4A). In addition, the result of Nissl staining indicated that the amount of Nissl bodies decreased dose-dependently in the MeHg-exposed neonatal mice cerebral cortex when compared with control group (Fig. 4B, C, \( P < 0.01 \)).

4. MeHg induced oxidative stress and Nrf2/Notch1 pathway activation in neonatal mice cerebral cortex

Aberrant oxidative injury is a driving forces in MeHg-induced neurotoxic pathology. To determine whether MeHg induced oxidative stress in neonatal mice cerebral cortex, we examined the effects of MeHg on the formation of ROS by flow cytometry, immunohistochemistry staining, and the mRNA, protein levels of Nrf2 and HO-1. The level of the ROS significantly increased after 12 mg/L MeHg treatment (Fig. 5A, \( P < 0.01 \)). In addition, we also examined the levels of MDA and GSH after the MeHg treatment. As results, compared to the control group, administering MeHg significantly reduced GSH content in the 12 mg/L MeHg-treated group (Fig. 5B, \( P < 0.01 \)). Compared with the control, an increase in MDA content was
observed in the 12 mg/L MeHg-treated group (Fig. 5B, P < 0.05). Similarly, compared with the control, the expression levels of the Nrf2 protein increased by 2.59-fold (P < 0.01) in 8 mg/L MeHg group, 3.42-fold (P < 0.01) in 12 mg/L MeHg group. Moreover, level of HO-1 was significantly increased by 1.40 times (P < 0.05) in 12 mg/L MeHg group (Fig. 5C, D). As is shown in Fig. 5F, G, we found that increasing the dosage of MeHg caused a clear increase in the level of Notch1 and p21 (P < 0.05, P < 0.01) and decrease in the level of CDK2 (P < 0.01). The mRNA level of Nrf2, HO-1 and Notch1 in 8 and 12 mg/L MeHg-treated groups were significantly increased, relative to control group, respectively (Fig. 5E, H, P < 0.05, P < 0.01).

Collectively, these data suggested that oxidative stress might be involved in MeHg-induced neurodevelopmental injuries in neonatal mice cerebral cortex.

5. NAC treatment antagonized MeHg-induced neonatal mice neurobehavioral impairments

The effects of NAC treated on neurobehavioral ability after MeHg exposure were assessed by behavioral tests after continuous exposure to MeHg during pregnancy and lactation. The MWM test was used to examine learning and memory abilities. 100 and 150 mg/kg NAC-treated neonatal mice were relieved of MeHg-induced memory impairments, which was manifested as a significant decrease in the latency of 12 mg/L MeHg-treated mice (Fig. 6B, P < 0.01). The platform was removed after an interval of 48 h, and the test data showed no difference in the moving speed and frequency of crossing the platform area among the groups, NAC-treated mice were obviously protected from this effect in frequency of crossing the platform area, the proportion of time remaining in the target quadrant and the first time latency among the 100, 150 mg/kg NAC-treated groups, relative to 12 mg/L MeHg-treated group, respectively (Fig. 6C, P < 0.01 or P < 0.05). In brief, our data showed that NAC alleviated MeHg-induced learning and memory impairments.

6. NAC treatment alleviated MeHg-induced pathological changes in neonatal mice cerebral cortex

As is shown in Fig. 7A, HE staining demonstrated that NAC treatment alleviated the MeHg-induced number of normal cells decreased, nuclear condensation with vacuolar degeneration and necrosis. Furthermore, we observed that a dose-dependent increase in the number of Nissl bodies was estimated the cerebral cortex in NAC-treated neonatal mice when compared with 12 mg/L MeHg-treated group (Fig. 7B, C, P < 0.01).

7. NAC treatment alleviated MeHg-induced oxidative stress and Nrf2/Notch1 pathway activation in neonatal mice cerebral cortex

To examine whether NAC alleviates MeHg-induced oxidative stress in neonatal mice cerebral cortex, we examined the effects of MeHg and NAC on the formation of ROS by flow cytometry, IHC staining, and the
mRNA, protein levels of Nrf2 and HO-1. The formation of ROS decreased significantly in the 100mg/kg and 150mg/kg NAC-treated groups in comparison with the 12 mg/L MeHg-treated group (Fig. 8A, P < 0.01). As is demonstrated in Fig. 8B, MeHg-administered neonatal mice had obvious decreases in GSH content among the NAC-treated mice groups (P< 0.01), moreover, there was a significant change in MDA content in the 150mg/kg NAC-treated mice group relative to the 12 mg/L MeHg-treated group, respectively. The oxidative stress effect was counteracted by conditioning with NAC, compared with the 12 mg/L MeHg group, the expression levels of the Nrf2 and HO-1 proteins were significantly decreased, Nrf2 level decreased by 17.29%(P 0.05) in 100mg/kg NAC-treated group, 33.08% P 0.01 in 150mg/kg NAC-treated group and HO-1 level decreased by 56% in 150mg/kg NAC-treated group (Fig. 8C D). The mRNA level of Nrf2 and HO-1 decreased in NAC-treated groups versus 12 mg/L MeHg group Fig. 8E, P 0.01. We observed that NAC downregulated the Notch1 and p21 protein expression, and upregulated the CDK2 protein expression compared with the 12 mg/L MeHg group (Fig. 8F and G, P < 0.05, P < 0.01). In addition, compared with the 12 mg/L MeHg group, the Notch1 mRNA expression in NAC-treated groups were downregulated in 100 and 150mg/kg NAC-treated groups (Fig. 8H, P < 0.05). Similarly, Results of immunohistochemistry staining showed that NAC administration markedly decreased the Nrf2 (Fig. 9A, C, P < 0.01) and Notch1 expression (Fig. 9B and D, P < 0.01) in 100 and 150mg/kg NAC-treated groups, relative to the 12 mg/L MeHg-treated group, respectively. Briefly, these results suggested that NAC protected MeHg-induced neurodevelopmental toxicity by its antioxidation capacity.

Discussion

Methylmercury is a heavy metal existing in the environment, which can penetrate the placental barrier, blood-brain barrier and damage the central nervous system during neurological development (Li et al. 2021). We provided in vivo experimental evidence that NAC effectively attenuated MeHg-induced neurodevelopmental toxicity in the cerebral cortex of neonatal mice by reducing neuronal damage and balancing oxidative stress. However, the clear mechanisms of neurodevelopmental toxicity of MeHg in pregnant and lactating offspring are not fully understood. Therefore, further investigation of the toxicity targets of MeHg in developing brain is needed.

Preliminary research found that MeHg exposure led to reactive oxygen species (ROS) overproduction, Ca$^{2+}$ dyshomeostasis, antioxidant enzyme activity decreased, lipid peroxidation (Liu et al. 2014; Liu et al. 2017). However, the toxicity of exposure during pregnancy and lactation is often overlooked, and numerous studies have reported developmental neurotoxicity induced by MeHg during pregnancy and lactation. The development of the blood-brain barrier and placental barrier transport systems is known to protect the fetal brain from drug exposure from the fetal central nervous system (Goasdoué et al. 2017; Tetro et al. 2018). The neurotoxicity of MeHg is initially manifested by deficits in behavior and memory function. Our tests showed that in the open-field test, these MeHg-treated mice responded emotionally to an unfamiliar environment, which was reflected by their abnormal locomotor activity, as well as a decline in learning and memory in the MWM test, which is in line with a previous study (Weiss et al. 2005). In addition, there is evidence that MeHg can be distributed to all brain regions across the blood-brain barrier
and placental barrier by neutral amino acid transport system (Granitzer et al. 2021; Ke et al. 2019), low dose MeHg exposure during pregnancy also caused developmental defects of nerve cells, including abnormal migration, differentiation and growth (Cambier et al. 2018; Ou et al. 2018). Ghrelin is the only recognized peripheral hormone that stimulates food intake, chronic MeHg exposure disrupted this normal hormone transduction process and led to weight loss in mice (Ferrer et al. 2021), which is in line with our study. The results showed that 8mg/L and 12mg/L MeHg-treated groups body weight decreased significantly, which suggested that MeHg crossed through the placenta of pregnant mice and exerted its toxic effects, which caused neurobehavioral disorders. MeHg is a widely recognized environmental toxicant, which is highly toxic to the central nervous system. Our previous study found that the degree of pathological injury gradually worsened with the increase of MeHg concentration (Ni et al. 2021). Therefore, HE and NissL staining were arranged to further explore the cerebral cortical damage caused by MeHg during pregnancy and lactation. The staining results showed that compared to the control group, nuclear deformation, shrinkage, cells were loosely arranged and began to form cavities, and Nissl bodies were few and incomplete in the MeHg-treated groups. These observations reflected that MeHg could cross the placental barrier and blood brain barrier in the offspring and caused nerve damage in the cerebral cortex of newborn mice.

The mature CNS of adults is fully functional, while the developing CNS is fragile, so it is more susceptible to MeHg (Franco et al. 2006). MeHg is a soft electrophilic agent, which preferentially reacts with proteins and nucleophilic groups of low molecular weight molecules (thiol and selenol) with high affinity. Strong evidence suggests that mitochondria are the major site of oxidative stress induced by methyl high light and that methyl high light induced damage to the mitochondrial electron transport chain leads to an imbalance between ROS production and antioxidant defense systems, thereby triggering oxidative damage and increasing GSH consumption (Liu et al. 2019). Interestingly, there is increasing evidence that ROS play well-known roles in regulating cell proliferation and survival processes (Liang and Ghaffari 2014; Zhang et al. 2016). The results of our study showed that MeHg significantly increased ROS and GSH levels, which is consistent with the results of Straka, Nogara et al (Nogara et al. 2019; Straka et al. 2016). ROS can be oxidized together with biofilms, leading to changes in biofilm fluidity and permeability, resulting in biofilms susceptible to lipid peroxidation, which may be an important aspect of MeHg neurotoxicity (Pinho et al. 2017; Su et al. 2019). we noted that MeHg significantly increased MDA level in 12mg/L MeHg-treated group. These results suggest that MeHg-induced ROS accumulation results in oxidative damage in the cerebral cortex during pregnancy and lactation.

Nrf2 is an essential anti-oxidative stress transcription factor that is also involved in cell development (Hahn et al. 2015). Previous studies have shown that Nrf2 mainly exists in the cytoplasm in the physiological environment, and when the intracellular ROS concentration level is above a threshold, activated Nrf2 regulates the transcriptional promoter of anti-oxidative stress proteins such as HO-1, thereby scavenging ROS (Suzuki and Yamamoto 2015). Notch family members have neuroprotective effects and are important signaling molecules that control a variety of cellular responses during embryonic development (Kopan and Ilagan 2009). At the same time, a functional ARE was found upstream of the major transcription start site of Notch1, thus Nrf2 is a regulator of Notch1 gene
activation and expression (Wakabayashi et al. 2010; Wakabayashi et al. 2015). A series of studies has testified that p21 is one of several transcriptional genes activated by Notch1 (Cialfi et al. 2014; Dabral et al. 2016), moreover, p21 can regulate the expression of downstream cyclins (such as CDK2, etc.) as an inhibitor of cyclin kinase (Wu et al. 2020). Consistent with the previously reported interaction between Nrf2 and Notch1, we observed that the protein levels of Nrf2, HO-1, Notch1 and p21 were significantly increased, while the expression of CDK2 was decreased with the increase of MeHg dose (Ye et al. 2020; Zimta et al. 2019). These findings verified that MeHg can induce oxidative stress via relate antioxidant stress pathways and activate transcription of downstream molecules in newborn mice during pregnancy and lactation, MeHg at high doses can hinder the normal development of developing mice.

NAC is a compound containing reactive sulfhydryl groups that enhance antioxidant and anti-free radical damage through direct antioxidant effects, and NAC is a precursor of GSH (Kumar et al. 2007). It has been shown that NAC treatment reduces the neurotoxicity of Hg in the hippocampus of developing rats (Falluel-Morel et al. 2012). Based on the experimental results in the previous section, we hypothesized that NAC could act as a protective agent against MeHg-induced developmental neurotoxicity in a mouse cortical model. As we predicted it, NAC incorporation protects against MeHg-induced developmental neurotoxicity in the mouse cerebral cortex. As a favourable metal-chelating agent, NAC obviously decreased transcription levels of metal-induced Nrf2 (Wolfram et al. 2020). Evidence indicates that NAC reduces ROS levels and the expression of the downstream molecule Notch1 (Wei et al. 2017; Yao et al. 2017). In our study, we observed the powerful neuroprotective effects of NAC on reducing MeHg-induced behavioral impairments and nerve cell injury. Consistently, our data showed NAC mitigate dose-dependent MeHg-induced cellular damage due to their ability to activate antioxidant defenses, inhibit signaling pathways related to the activation of oxidative stress, such as Nrf2, HO-1, and activate antioxidant enzymes such as GSH. In addition, NAC alleviated the developmental dysfunction in offspring mice through downregulating the expression of Notch1, p21 and CDK2. Therefore, these results confirmed that NAC alleviated the MeHg-induced oxidative stress and neurodevelopmental injury in newborn mice during pregnancy and lactation.

**Conclusions**

In conclusion, as has been discussed above, NAC is a potential therapeutic drug to treat MeHg-induced neurodevelopmental injury because it inhibited the oxidative damage and alleviated oxidative stress in the cerebral cortex cells during pregnancy and lactation. This work provides new insights for exploring the pathogenesis of nerve injury after MeHg explore during pregnancy and lactation, emphasizing that NAC may be a candidate for prevention of nerve injury or early treatment in pregnant woman with MeHg exposure. In addition, as the abnormal oxidative stress in newborn provide clues to the potential novel mechanisms of neurological diseases, the property of NAC to maintain the balance of oxidative stress homeostasis brings another way to employ its widespread neuroprotective effects.

**Declarations**
CRediT authorship contribution statement

Xiaoyang Li: Designed the study and supervised the data, Carried out the experiments, Data analyses and prepared Figures, Conceived and drafted the manuscript. Jingjing Pan: Carried out the experiments. Haihui Liu: Carried out the experiments. Chen Wang: Carried out the experiments. Si Xu: Carried out the experiments. Tianyao Yang: Carried out the experiments. Wei Liu and Jingyi Sun: Designed the study and supervised the data, Carried out the experiments, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References


Figures
Figure 1

Establishment of the animal models and the timeline.
Figure 2

MeHg exposure inhibited the neonatal mice developmental growth. (A) Body weight of neonatal mice from PND5 to PND21. (B) The body length of neonatal mice from PND5 to PND21. (C) The brain viscera index of neonatal mice in PND21. The results are presented as mean ± SD (n=10). **P<0.01 compared with control group.
**Figure 3**

**MeHg induced neurobehavioral impairments in neonatal mice.** (A) A representative search trace of the open field test. (B) Evaluation of the spontaneous movements of mice on the open field test. (C) A representative search trace on day 6 of the MWM test. (D) Mean latency time to reach the platform each day during spatial training for the MWM test. (E) The MWM test after removing the platform (48 h). The results are presented as mean ± SD (n=8). *P<0.05, **P<0.01 compared with control group.
Figure 4

MeHg induced pathological changes in neonatal mice cerebral cortex. (A) Representative images of cerebral cortex HE staining. (B) Representative images of cerebral cortex Nissl staining. The magnification of images is 20X. (C) The quantitative analysis of Nissl bodies (n=3). The scale is 100 μm. The black arrows refer to nuclear condensation. **P<0.01 compared with control group.
Figure 5

MeHg induced oxidative stress and Nrf2/Notch1 pathway activation in neonatal mice cerebral cortex. (A) The intracellular ROS level in the cerebral cortex was measured by flow cytometry (n=4). (B) Analysis of the GSH level and the MDA level (n=6). (C) (D) After Western blotting experiments, the band products of Nrf2, HO-1 and β-tubulin, as well as the semi-quantitative analyses of the Nrf2 and HO-1 protein expressions were shown (n=4). (E) The alteration of Nrf2 and HO-1 mRNA expressions were quantified with the method 2^ΔΔCT (n=4). (F) (G) After Western blotting experiments, the band products of Notch1, CDK2, p21 and β-tubulin, as well as the semi-quantitative analyses of the Notch1, CDK2 and p21 protein expression were shown (n=4). (H) The alteration of Notch1 mRNA expression was quantified with the method 2^ΔΔCT (n=4). *P<0.05, **P<0.01 compared with control group.
NAC treatment antagonized MeHg-induced neonatal mice neurobehavioral impairments (A) A representative search trace on day 6 of the MWM test. (B) Mean latency time to reach the platform each day during spatial training for the MWM test. (C) The MWM test after removing the platform (48 h). The results are presented as mean ± SD (n=8). **P<0.01 compared with control group. #P<0.05, ##P<0.01 compared with 12 mg/L MeHg-treated group.
Figure 7

NAC treatment alleviated MeHg-induced pathological changes in neonatal mice cerebral cortex. (A) Representative images of cerebral cortex HE staining. (B) Representative images of cerebral cortex Nissl staining. The magnification of images is 20X. (C) The quantitative analysis of Nissl bodies (n=3). The scale is 100 μm. The black arrows refer to nuclear condensation. ##P<0.01 compared with 12 mg/L MeHg-treated group.
**Figure 8**

NAC treatment alleviated MeHg-induced oxidative stress and Nrf2/Notch1 pathway activation in neonatal mice cerebral cortex. (A) The intracellular ROS level in the cerebral cortex was measured by flow cytometry (n=4). (B) Analysis of the GSH level and the MDA level (n=6). (C) (D) After Western blotting experiments, the band products of Nrf2, HO-1 and β-tubulin, as well as the semi-quantitative analyses of the Nrf2 and HO-1 protein expressions were shown (n=4). (E) The alteration of Nrf2 and HO-1 mRNA expressions were quantified with the method $2^{-\Delta\Delta CT}$ (n=4). (F) (G) After Western blotting experiments, the band products of Notch1, CDK2, p21 and β-tubulin, as well as the semi-quantitative analyses of the Notch1, CDK2 and p21 protein expressions were shown (n=4). (H) The alteration of Notch1 mRNA expression was quantified with the method $2^{-\Delta\Delta CT}$ (n=4). *$P<0.05$, **$P<0.01$ compared with control group. #$P<0.05$, ##$P<0.01$ compared with 12 mg/L MeHg-treated group.
Figure 9

NAC treatment alleviated MeHg-induced Nrf2/Notch1 pathway activation in neonatal mice cerebral cortex. (A) and (B) Representative images of Nrf2 and Notch1 IHC. (C) and (D) The mean optical density of Nrf2 or Notch1 evaluated using ImageJ software (n=4). **P<0.01 compared with control group. ##P<0.01 compared with 12 mg/L MeHg-treated group.