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Mercuric resistant bacteria *Aeromonas* exhibits neurologic toxic effects on the developmental motor reflexes, and brain oxidative stress in mice offspring

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ABSTRACT

Mercury and its derivatives even in small concentration may cause a major human health problem. Though not reported in detail, there are various aquatic bacterial species that produce small quantities of methyl mercury (MM) growing under aerobic conditions. Consumption of food derived from sources contaminated with such bacteria within therapeutic doses and exposure to different forms of MM compounds through such sources may induce substantial toxic effects. In the present study, the perinatal oral exposure of pregnant mice to two strains of mercury resistant bacteria (MRB), *Aeromonas* KSU5 MRB and KSU6 MRB resulted in a significant reduction in postnatal body weight gain, delays in the opening of the eyes and appearance in the body hair fuzz, and deficits in the developing sensory motor reflexes in the mice pups during their weaning period on post-natal day (PD)7, PD14 and PD21. A significant and MM producing concentration-dependent disturbance in the levels of neurotransmitters like dopamine (DA) and serotonin (5-HT); non-enzymatic oxidative stress (OS) indices like thiobarbituric acid-reactive substances (TBARS) and total reduced glutathione (GSH); and enzymatic OS indices like glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) were observed in the forebrain region of the offspring at weaning period (PD7, PD14, and PD21), at adolescent age (PD30), and at adult age (PD36). Thus, perinatal exposure to MRB can affect developing fetus, raising the concerns for it's potential neurotoxic hazards. A reduced exposure to mercury during pregnancy is of crucial importance in preventing mercury-induced neurotoxicity in the offspring.

1. Introduction

There is enough evidence that small concentration of mercury and its derivatives such as phenyl mercury are although nontoxic, the transformation of these compounds into methyl or dimethyl mercury (MM) in the aquatic environment cause a major human health problem [1,2]. Though not reported in details, the major mercury discharge as divalent mercury ($Hg2+$); metallic mercuric or phenyl derivatives are possible sources of Minamata disease. There are also reports of various bacterial species producing small quantities of MM, growing under aerobic conditions, usually considered as mercuric resistant bacteria (MRB) [3–5]. These bacteria can get into the human food chain through seafoods or drinking water. Prolonged exposure of such sources of MM through MRB, within therapeutic doses and exposure to its different forms of MM compounds through various sources may also induce substantial toxic effects. Microbes resistant to mercury convert inorganic mercury to MM, which has higher toxicity level [6,7]. Thus a small environmental concentration of mercury and subsequent presence of MRB at a site increases the chances of accumulation of mercury at higher levels in the food chain through bio magnifications. Studies show that many Asian coastal areas are polluted by mercury compounds and the amount ranges from 2 to 15 ng of dissolved mercury [8].

Mercuric ion resistance involves a diverse set of genes, which are widespread in both Gram-positive and Gram-negative bacteria. The bacterial *mer* (mercury resistance gene) operons encode a cluster of genes involved in the detection, mobilization and enzymatic detoxification of mercury [9]. Long-term developmental effects of MRB have not been studied adequately in human populations, although continuous exposure of sea food throughout the gestation period might be associated with perinatal complications like transient neurodevelopmental deficits, depressed neurological functions in the newborns, teratogenic risk to the developing fetus and toxic effects in the neonatal offspring [10,11]. However, the possibility of realistic long term effects on the developing fetal brain through such bacteria due to mercury during pregnancy cannot be ruled out completely [12–14]. It is well

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documented that the brain develops mostly during the first trimester of the pregnancy making it highly vulnerable to the neurological impact of various drugs used during pregnancy [15,16].

The aim of this study was to identify and isolate the MRB and explore the effects of perinatal oral administration of isolated MRB to pregnant mice on their offspring at various postnatal developing ages for neurobehavioral and biochemical (brain neurotransmitters and oxidative stress indices) effects to assess for a possibility of longer lasting effect of mercury toxicity in the offspring. Data on maternal effects, however; has not been included herein and shall form a part in a separate communication.

2. Materials and methods

2.1. Experimental animals

Three females to one male Swiss–Webster strain mice (10–12 weeks old) were maintained in each opaque plastic cages measuring 30 × 12 × 11 cm, under reversed lighting conditions (with white lights on from 22.30 to 10.30 h local time) and at an ambient temperature (regulated between 18 and 22 °C). On the day one of pregnancy (appearance of the vaginal plug was considered as day one of pregnancy), the males were removed from the cages and the females were subjected to experimental treatments. Food (Pilsbury's Diet) and water were available ad libitum unless otherwise indicated. All study protocol and animal handling procedure were approved by the Research and Ethics Committee of King Saud University, Riyadh, Saudi Arabia, and all precautions were taken to minimize animal stress and pain in the animals.

2.2. Mercury resistant bacterial strain

MRB were isolated from the sediments collected from nearby sea shore and plated by primary enrichment method, directly plated on media with amended mercury. In brief, the isolation of mercury resistant bacteria was done using 1 g of sea sediment in water nutrient broth composed of: peptone 5.0 g, beef extract 3.0 g, aged seawater 750 mL and deionised water 250 mL; final pH 7.5). To induce MRB growth, about 10 ppm HgCl2 salt was added into broth prior to inoculation. Tubes were incubated at 24 °C–30 °C in the different set for 24 h in a shaker. Growth was determined visibly by turbidity and streaking of a loopful of liquid culture on Luria Bertani (LB) agar plates supplemented with HgCl2. Single colonies were obtained following spreading techniques on agar plates supplemented with HgCl2. Phenotypic identification of MRB was done by simple biochemical tests according to standard protocol. [17].

2.3. Antibiotic susceptibility profile

The antibiotic resistance profile of isolated MRB was determined by antibiotic disc diffusion method using different antibiotics such as Streptomycin (10 μg/ml), Ampicillin (30 μg/ml), Chloramphenecol (30 μg/ml), Gentamicin (10 μg/ml), and Rifampicin (10 μg/ml), Carbapenems (30 μg/ml), nalidixic acid (10 μg/ml) and tetracyclin (10 μg/ml). The antibiotic discs were placed on nutrient agar plates previously seeded with 18 h broth culture of the test organisms. The plates were incubated at 37 °C for overnight, after which diameter of zones of inhibition and minimum inhibitory concentration (MIC) was examined. *Escherichia coli* ATCC 25922, Pseudomonas *aeruginosa* ATCC 25923, *Staphylococcus aureus* ATCC 27853 and *A. hydrophila* ATCC 7966 were used as reference strains.

To determine the minimum inhibitory concentration of these isolates to mercury (MIC), agar plates assay was performed. Cultures were grown in trypticase soy broth (TSB) and then 2 μL of each culture was spotted onto respective brain heart infusion (BHI) (Oxoid Hampshire, England) agar plates containing 0–600 μg mL/HgCl2. Plates were incubated at 37 °C. The lowest concentration inhibited the growth was considered the MIC for that particular isolates.

2.4. Molecular tests

All isolated MRB strains were tested in a mercury volatilization assay by the X-ray film method as described [18]. The presence of *mer*A and *mer*R genes which are meant for mercuric resistant ability was assessed by PCR amplification with primers by the method described earlier [5]. Isolation of total DNA from mercuric-resistant strains was done according to standard protocols. PCR reactions were done with PCR Master Mix (2X) (Fermentas) and amplifications involved standard cycles of PCR.

2.5. MRB treatment and experimental design

The pregnant mice were divided into 3 groups of 10 pregnant mice in each. The first group (Group I) serving as the control group received plain tap water only. Whereas the second and third groups (Group II and III respectively) were treated with MRB bacterial suspension containing 1×10^8 cfu/mL/kg body weight per day with KSU5 MRB and KSU6 MRB strains respectively, dissolved in plain tap water, through oral administration. For the treatment purpose, we selected KSU5 MRB and KSU6 MRB strains because they could resist the highest concentration (510 and 600 μg of mercury/ml respectively). Our pilot studies have shown that the normal and/or pregnant mice on an average can tolerate 1×10^8/mL of MM/kg per day without lethal effect. These bacterial doses of KSU5 MRB and KSU6 MRB formed the sole drinking fluid source for the experimental group of pregnant mice during the required period of the experiment and fresh bacterial suspension were replaced in the drinking bottle every day. All pregnant mice were housed individually. Treatment started from day one of pregnancy and was continued until post-natal day 15 (PD15) and thereafter the mothers were switched to plain tap water. The pups of each experimental group were culled to only eight per dam on the postnatal day 1 (PD1) after birth and were left with their mothers until PD22. During this weaning period, three male pups of each litter were color marked from the others, and were subjected to various behavioral tests (described below) under dim lighting (ca 8 lux). In all, 21 pups belonging to seven litters from each treatment category were considered. All observations were recorded on PD1 and repeated every other day until PD21 in the same cohort of three color marked male pups of each litter. These observations were used to measure the early development of sensory motor coordination reflexes together with morphological development in the pups. For statistical analysis, the mean of all three cohorts (color marked pups) per litter was considered as a single score. Thus, seven replicates from each treatment category were considered for the following observations.

2.6. Physical assessment during weaning period

Physical developmental landmarks like body weight, opening of the eyes and appearance of body hair fuzz, were evaluated in the developing offspring starting from day 1 after birth (PD1) through the entire weaning period until PD21.

2.6.1. Body weight

Weight is a useful indicator of development. Thus, the pups were weighed every alternate day from PD1 until PD21.

2.6.2. Eye opening and hair appearance

The day at which the body hair fuzz appeared, and the eyes opened was also recorded. These two parameters are also useful morphological indicators of development.

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