Effect of thimerosal on the neurodevelopment of premature rats

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Original article

Background: **This study was undertaken to determine the effect of thimerosal on the neurodevelopment of premature rats.**

Methods: **Thimerosal was injected into premature SD rats at a dose of 32.8, 65.6, 98.4 or 131.2 μg/kg on postnatal day 1. Expression of dopamine D4 receptor (DRD4) and serotonin 2A receptor (5-HT2AR), apoptosis in the prefrontal cortex on post-injection day 49, and learning and memory function were studied and compared with those in a control group injected with saline.**

Results: **Expression of DRD4 and 5-HT2AR and learning function decreased, and apoptosis increased significantly in the 131.2 μg/kg group (P<0.001). Memory function was significantly impaired by 65.6 (P<0.05), 98.4 and 131.2 μg/kg (P<0.001).**

Conclusions: **The negative adverse consequences on neurodevelopment observed in the present study are consistent with previous studies; this study raised serious concerns about adverse neurodevelopmental disorder such as autism in humans following the ongoing worldwide routine administration of thimerosal-containing vaccines to infants.**

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Key words: **dopamine D4 receptor; neurodevelopment; serotonin 2A receptor; thimerosal**

Introduction

Neurological alterations that may result from thimerosal exposure have recently become a hot topic. Thimerosal exposure via vaccination is thought to cause brain disorder.[1] Since there is no appropriate agent to replace, thimerosal is used as a preservative in vaccines. Therefore, it is necessary to determine the appropriate levels of thimerosal for neurodevelopment. Studies[2,3] have been focused on neurological alterations after exposure to thimerosal in rats, but further study is required to demonstrate the acceptable levels of exposure for neurodevelopment.

Rat model is considered feasible for research in intoxication following metal exposure. Learning and memory are important brain functions. And the prefrontal cortex is a critical region receiving stimulation for the development of learning and memory function,[4] which is mainly executed by neurotransmitters. The variants of dopamine D4 receptor (DRD4) are reported to be associated with memory function of rats,[5] whereas serotonin 2A receptor (5-HT2AR) is correlated with impaired episodic memory performance.[6] It was reported that in the human neuroblastoma cell line, thimerosal induced mitochondria-mediated apoptosis.[7]

In the present study, we investigated whether thimerosal could induce alterations in expression of DRD4 and 5-HT2AR, apoptosis of the prefrontal cortex, and learning and memory functions in the premature rats.

Methods

The protocol of this study was approved by the Institutional Ethics Committee of Xi'an Jiaotong University Health Science Center, Xi'an, China. Thirty premature Sprague-Dawley rats (Laboratory Animal Center of Xi'an Jiaotong University Health Science Center) were delivered on day 20 of gestation (term=day 22) by hysterotomy, and they were randomly divided into five groups, with six rats in each group. Thimerosal (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline and injected into the gluteus maximus of four

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groups on postnatal day 1 at a dose of 32.8, 65.6, 98.4, or 131.2 μg/kg, respectively. In a control group, the rats received saline injection at the same time. There was no significant difference in the weight and sex between the five groups. The rats in each group were killed on post-injection day 49.

The Morris water maze (MWM) test was conducted on post-injection days 44-48 to evaluate spatial learning and memory function. The latency to escape (learning function) and the percentage of time spent in the target quadrant (memory function) were calculated. The rats were anesthetized and perfused on post-injection day 49. The brains of the rats were sectioned according to a rat brain atlas.[8] The specimens of the prefrontal cortex were fixed and sectioned at 5 μm. Immunohistochemical staining was used to detect the expression of DRD4 and 5-HTR2A, and the integrated optical density (IOD) was normalized to the corresponding values from the control samples. The terminal deoxynucleotidyl transference-mediated biotinylated deoxyuridine triphosphate nick end labeling technique was used to detect nerve cell apoptosis. Significance differences between the five groups in the expression of DRD4 and 5-HT2AR, apoptosis, and memory function in the MWM test were analyzed with one-way ANOVA. Group differences in the learning data of the MWM test were analyzed by two-way ANOVA with repeated measures. Differences between pairs of the groups were analyzed by a post hoc test. Correlation analyses were used to measure the relationship between apoptosis and expression of DRD4 and 5-HT2AR. The influencing factors in the MWM test were analyzed by multivariate regression analysis. The statistically significant level was set to 0.05. Statistical analyses were performed with SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA).

Results

The MWM test showed that learning function was significantly different between training days (P<0.001) and groups (P<0.001). The 131.2 μg/kg thimerosal group showed a significant increase in learning function compared with the control group (P<0.001). The memory function was significantly different among the five groups (F=59.85, P<0.001). In the 65.6 μg/kg (P<0.05), 98.4 μg/kg (P<0.001) and 131.2 μg/kg (P<0.001) thimerosal groups, the memory function was significantly decreased compared with the control group.

The expression of DRD4 and 5-HT2AR differed significantly among the five groups (both P<0.001). The IOD values for the expression of DRD4 (P<0.001, Fig. 1) and 5-HT2AR (P<0.001, Fig. 2) in the 131.2 μg/kg thimerosal group were significantly decreased compared with those in the control group. The apoptosis rate was also significantly different among the five groups (P<0.001), and the 131.2 μg/kg thimerosal group showed a significant increase compared with the control group (P<0.001, Fig. 3).

Correlation analyses of apoptosis relating with the expression of DRD4 and 5-HT2AR revealed an inverse relationship (r=-0.912, -0.928, respectively; P<0.001). Multivariate regression analysis indicated that the expression of DRD4 and 5-HT2AR as well as apoptosis influenced learning (b=-0.32, -0.43, 0.54, P<0.05) and memory function (b=0.57, 0.53, -0.59, P<0.05). DRD4 and 5-HT2AR expression levels affected apoptosis, and

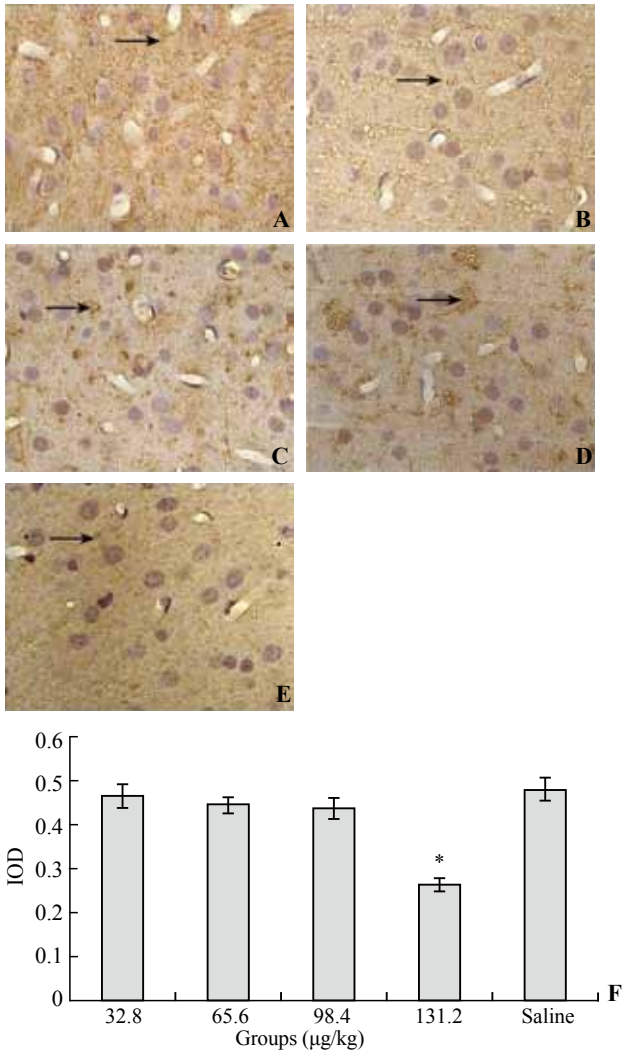

Fig. 1. The expression of DRD4 (arrows) in the saline **(A)**, 32.8 μg/kg **(B)**, 65.6 μg/kg **(C)**, 98.4 μg/kg **(D)**, and 131.2 μg/kg **(E)** groups visualized by immunohistochemical staining (original magnification × 400); **F**: comparison of the prefrontal cortex immunohistochemical staining integrated optical density (IOD) of DRD4 between the 5 groups. *: P<0.001. DRD4: dopamine D4 receptor.

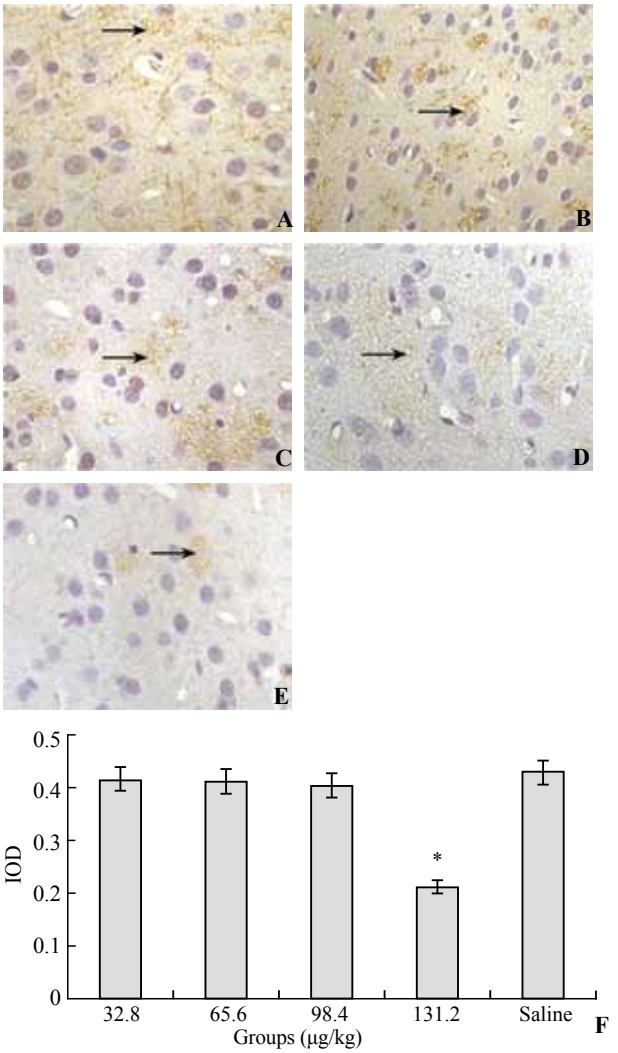

Fig. 2. The expression of 5-HT2AR (arrows) in the saline **(A)**, 32.8 μg/kg **(B)**, 65.6 μg/kg **(C)**, 98.4 μg/kg **(D)**, and 131.2 μg/kg **(E)** groups shown by immunohistochemical staining (original magnification × 400); **F**: comparison of the prefrontal cortex immunohistochemical staining integrated optical density (IOD) of 5-HT2AR between the 5 groups. *: P<0.001. 5-HT2AR: serotonin 2A receptor.

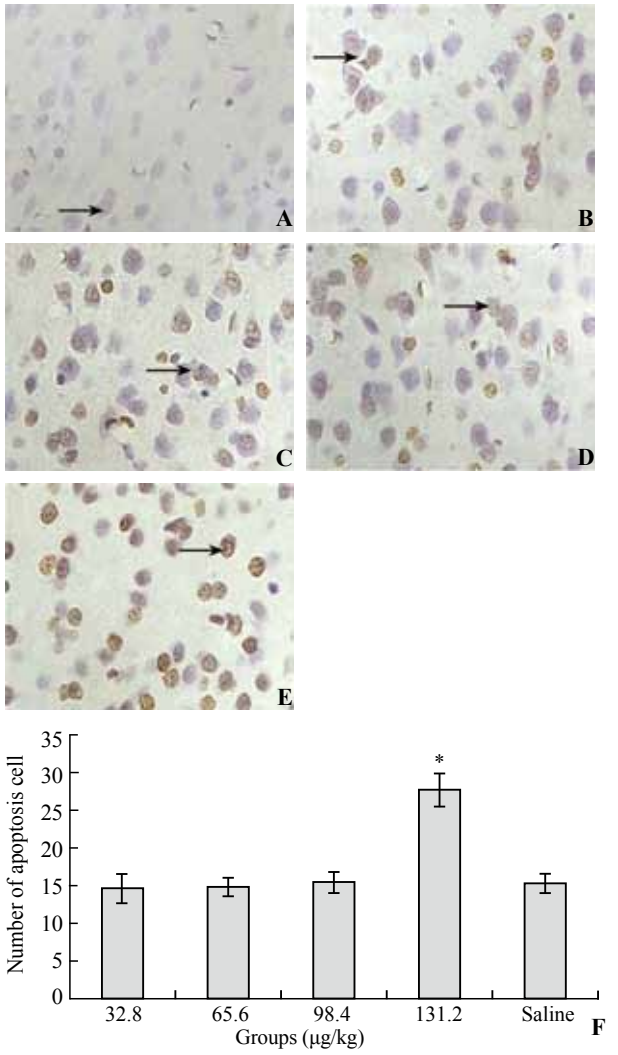

Fig. 3. Apoptotic cells (arrows) from the saline **(A)**, 32.8 μg/kg **(B)**, 65.6 μg/kg **(C)**, 98.4 μg/kg **(D)**, and 131.2 μg/kg **(E)** groups shown using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL); **F**: comparison between the 5 groups of apoptosis in the prefrontal cortex using TUNEL (original magnification × 400). *: P<0.001.

DRD4, 5-HT2AR and apoptosis were involved in the spatial cognitive function of the premature rats.

Discussion

Thimerosal contains 49.5% ethylmercury. It can be converted to ethylmercury and accumulate in the brain.[9] Under normal conditions, intracellular glutathione has a role in detoxifying ethylmercury, and high levels of ethylmercury can deplete the available glutathione. Thus, cytotoxicity can result from excess ethylmercury. Therefore we hypothesized that a higher dose of thimerosal would induce neurological alterations in the premature rats, and the results of our study were consistent with this hypothesis.

Our results suggested that premature rats receiving 65.6, 98.4 and 131.2 μg/kg of thimerosal exhibited abnormal functions of spatial learning and memory. DRD4 and 5-HT2AR are important neurotransmitters for learning and memory function, and are highly expressed in the prefrontal cortex in humans. 5-HT2AR regulates Ca^{2+} influx via phosphatidylinositol, which is important for learning and memory.[10,11] DRD4 triggers the inhibition of cAMP synthesis, thereby modulating G-protein-regulated Ca^{2+} channels, which are also important for learning and memory.[12] Previous studies[2,3] on rats have shown that thimerosal exposure in the early life produced cerebral perturbation of the serotonergic and dopamine systems. This finding is

similar to the present study. Analysis of the factors affecting the function of learning and memory revealed that decreased expression of DRD4 and 5-HT2AR could be one of the mechanisms that diminish the functions of learning and memory. However, the change in memory function did not show the same tendency as the alteration of DRD4 and 5-HT2AR expression. A possible explanation is that the lesion in the other brain regions for instance the hippocampus could affect memory function as well.

In our study, apoptosis was increased in rats treated with 131.2 μg/kg of thimerosal. A previous study[13] also showed that thimerosal enhanced human neuroblastoma cell apoptosis. Because of the correlation between apoptosis and DRD4 and 5-HT2AR expression in our studies, we postulated that the hypofunction of DRD4 and 5-HT2AR might be partly due to the mechanisms of thimerosal-mediated apoptosis. When cultured hippocampal neurons were treated with thimerosal, the electrophysiological responses to N-methyl-D-aspartate (NMDA) were impaired.[14] NMDA may mediate the function of 5-HT2AR through cell signaling and maintenance of the cell membrane function. DRD4 is also able to modulate NMDA receptors in the prefrontal cortex by inhibition of active Ca^{2+}-calmodulin-dependent kinase II. Considering NMDA-induced toxicity including apoptosis, we postulated that the function of DRD4 and 5-HT2AR in thimerosal-mediated apoptosis is mediated by NMDA.

Rats exposed to thimerosal in their early postnatal life showed lasting neuropathological changes.[15,16] Research on hamsters, rhesus macaques, and mice[17-19] showed adverse neurodevelopment outcomes after neonatal cumulative exposure to thimerosal-containing vaccine. Neonatal exposure to thimerosal-containing vaccines is associated with disorders of neurodevelopment.[1,20]

In conclusion, our results are consistent with previous studies in mice, rats, rhesus macaques, and humans, demonstrate that exposure to mercury from thimerosal-containing vaccines in susceptible populations, such as premature infants, may be associated with neurodevelopmental disorders like autism.

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