ARTICLE IN PRESS

Available online at www.sciencedirect.com

ScienceDirect

Experimental and Toxicologic Pathology ▮ (▮▮▮▮) ▮▮▮–▮▮▮

EXPERIMENTAL AND TOXICOLOGIC PATHOLOGY

www.elsevier.de/etp

SHORT COMMUNICATION

Gender-selective toxicity of thimerosal ☆

Donald R. Branch[a,b,c,*]

[a]*Departments of Medicine and Laboratory Medicine and Pathobiology, University of Toronto, 67 College St., Toronto, Ontario, Canada M5G 2M1*
[b]*Division of Cell and Molecular Biology, Toronto General Research Institute, Toronto, Ontario, Canada*
[c]*Research and Development, Canadian Blood Services, Immunology Hub, Toronto Centre, Toronto, Ontario, Canada*

Received 20 March 2008; accepted 22 July 2008

Abstract

A recent report shows a correlation of the historical use of thimerosal in therapeutic immunizations with the subsequent development of autism; however, this association remains controversial. Autism occurs approximately four times more frequently in males compared to females; thus, studies of thimerosal toxicity should take into consideration gender-selective effects. The present study was originally undertaken to determine the maximum tolerated dose (MTD) of thimersosal in male and female CD1 mice. However, during the limited MTD studies, it became apparent that thimerosal has a differential MTD that depends on whether the mouse is male or female. At doses of 38.4–76.8 mg/kg using 10% DMSO as diluent, seven of seven male mice compared to zero of seven female mice tested succumbed to thimerosal. Although the thimerosal levels used were very high, as we were originally only trying to determine MTD, it was completely unexpected to observe a difference of the MTD between male and female mice. Thus, our studies, although not directly addressing the controversy surrounding thimerosal and autism, and still preliminary due to small numbers of mice examined, provide, nevertheless, the first report of gender-selective toxicity of thimerosal and indicate that any future studies of thimerosal toxicity should take into consideration gender-specific differences.

Keywords: Thimerosal; Thimerosal toxicity; Gender-selective toxicity; Maximum tolerated dose; Autism

Introduction

Thimerosal is an organic compound that contains mercury and has been used historically as a preservative in vaccines and pharmaceutical products. The breakdown product, ethylmercury, in thimerosal-preserved childhood vaccines has been suggested to be neurotoxic and to contribute to the etiology of neurodevelopmental disorders, including autism; however, this supposition is highly controversial (Mutter et al., 2005; Geier et al., 2007; Ng et al., 2007; Zareba et al., 2007; Thompson et al., 2007, Schechter and Grether, 2008). It has, however, been shown that mercury and thimerosal administration results in the decreased production of

☆ *Ethical Statement*: All animal studies were performed under an approved animal use protocol (AUP) for the care and use of animals (mice) by Nucro-Technics, 2000 Ellesmere Road, Scarborough, Ontario, Canada. Nucro-Technics is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and the Canadian Council on Animal Care. The study was conducted under the direction of Dr. Albert Licollari, DVM, Ph.D.

*Corresponding author at: Departments of Medicine and Laboratory Medicine and Pathobiology, University of Toronto,67 College St., Toronto, Ontario, Canada M5G 2M1. Tel.: +1 416 313 4458; fax: +1 416 974 9757.
E-mail address: don.branch@utoronto.ca.

0940-2993/$ - see front matter © 2008 Elsevier GmbH. All rights reserved.
doi:10.1016/j.etp.2008.07.002

the proinflammatory cytokines, TNFα, IL-6 and IL-12p70 from LPS-stimulated human monocyte-derived dendritic cells (Agrawal et al., 2007). These investigators also showed that thimerosal could differentially affect cytokine production from T lymphocytes through depletion of intracellular glutathione. However, a relationship to neurodevelopmental disorders has not been shown (Berman et al., 2008).

In humans, a relationship between thimerosal administration and autism has recently been reported where it was found that the incidence of autism was significantly higher (2.35-fold) in children born of Rh-negative compared to Rh-positive women, where the administration of immunoglobulins containing thimerosal had been common practice to prevent a disease known as hemolytic disease of the newborn (Geier and Geier, 2007). However, this conclusion is disputed by another recent study (Miles and Takahashi, 2007). Nevertheless, whether or not pre- or postnatal exposure to mercury-containing thimerosal had a role in some children who subsequently developed autism remains unresolved.

Our studies were not trying to determine whether thimerosal has any neurotoxicity or whether there is any connection of thimerosal to autism. Instead, we were doing an unrelated study that was testing only for the maximum tolerated dose (MTD) levels for thimerosal in a mouse model to be subsequently used in a study of reversal of immune-mediated platelet destruction (Rampersad et al., 2005). However, during our MTD studies, we serendipitously discovered that thimerosal shows a differential toxicity that depends upon whether the test animals are male or female.

Materials and methods

Animals

Female or male CD1 mice (*Mus musculus*) approximately 5 weeks old and 18–20 g were purchased from Charles River Canada Inc., Montreal, PQ. All MTD testing was performed by Nucro-Technics, Scarborough, Ontario, after an approved animal protocol. Nucro-Technics is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and the Canadian Council on Animal Care. The study was conducted under the direction of Dr. Albert Licollari, DVM, PhD.

Protocol

Mice were individually weighed and marked prior to treatment and a single dose of thimerosal was administered intravenously via the tail vein. A dose escalation study was initiated in accordance with standard testing to determine the MTD. Two or three male and female mice were used for each dose level and animals were observed for 7–14 days with necropsies performed at the end of the study. The escalating doses were not applied in parallel but were done subsequently with 2–5 days between each dose level. Stock solutions of thimerosal (BioShop Canada Inc., Burlington, Ontario) were prepared in 0.5-mL dimethyl sulfoxide (DMSO) and were stored at 2–8 °C for not more than 1 week. Dosing solutions were freshly prepared in phosphate buffered saline (PBS) on the day of dosing. 5% DMSO was sufficient to solubilize thimerosal up to a dose of 19.2 mg/kg. When doses were being prepared at 38.4 mg/kg, it was noticed that not all the thimerosal was being solubilized and a concentration of 10% DMSO was used for this and higher dosing. We had previously determined that CD1 mice can tolerate 10% DMSO.

Results

Initially, using a dose escalating approach, all mice tolerated thimerosal in 5% DMSO well up to doses of 38.4 mg/kg (Table 1). Although there was some initial weight loss during 2–3 days after dosing, animals appeared normal and regained pre-treatment body weights. When using 38.4-mg/kg thimerosal, however, it was noticed that the thimerosal was not going completely into solution; hence subsequent dosing with thimerosal was in 10% DMSO, starting with the next escalated dose of 115.2-mg/kg thimerosal. However, at this dose, two male and two female mice died (Tables 1 and 2). Thus, a 'step-down' approach was initiated as indicated in Table 1. During this step-down series of experiments, gender-related differential toxic effects were seen starting at 76.8 mg/kg. Even at 38.4 mg/kg when using 10% DMSO, all male mice died but no female mice. Indeed, within this dose range of 38.4–76.8-mg/kg thimerosal, seven of seven male mice died or had to be sacrificed in moribund condition (substantial weight loss, lethargy, piloerection) (Tables 1 and 2). In contrast, within the same dose range, no females showed any significant toxic effects of thimerosal, although there was some transient weight loss; however, there were no gross findings at necropsy. A summary of the gender-selective toxicity of thimerosal is provided in Table 2.

Discussion

The organomercurial preservative thimerosal has been used in various vaccine and immunoglobulin preparations for use in humans since the 1930s to help prevent potentially life-threatening contamination with

Please cite this article as: Branch DR. Gender-selective toxicity of thimerosal. Exp Toxicol Pathol (2008), doi:10.1016/j.etp.2008.07.002

Table 1. Step-up/step-down dosing results using thimerosal in CD1 male mice[a]

Dose (mg/kg)	DMSO (%)	Date dosed	Outcome
4.8	5	03/02/05	Animals appeared normal
9.6	5	03/04/05	Animals appeared normal
19.2	5	03/08/05	Animals appeared normal
38.4	5[b]	03/09/05	Animals appeared normal
115.2	10[c]	03/14/05	Animals died or were sacrificed
76.8	10[c]	03/22/05	Animals died or were sacrificed
51.2	10[c]	03/28/05	Animals died or were sacrificed
38.4	10[b]	03/30/05	Animals died or were sacrificed
38.4	5[b]	04/21/05	Animals appeared normal

[a]No female mice showed significant toxicities except when using 115.2 mg/kg where two of two female mice died or were sacrificed.
[b]Two mice of each sex dosed at 38.4 mg/kg with 5% DMSO survived and appeared healthy after being dosed. This was the maximum tolerated dose level and in order to have at least 5 mice/sex an additional 3 mice/sex were added (DMSO was 10%) and then mortalities were observed, thus a third group of 3 mice/sex was added (38.4 mg/kg at 5% DMSO).
[c]Due to solubility issues the amount of DMSO was increased from 5% to 10%.

Table 2. Summary of thimerosal toxicity in male and female CD1 mice

Group	Dose level (mg/kg)	DMSO(%)[a]	Number of males	Number of females	Number of deaths/moribund	
					Males	Females
1	4.8	5	2	2	0	0
2	9.6	5	2	2	0	0
3	19.2	5	2	2	0	0
4	38.4	5	5	5	0	0
5	38.4[b]	10	3	3	3	0
5	51.2[b]	10	2	2	2	0
6	76.8[b]	10	2	2	2	0
7	115.2[b]	10	2	2	2	2

[a]Due to decreased solubility, the concentration of DMSO was increased from 5% to 10%.
[b]There was no toxicity due to DMSO alone and this was so even in female mice administered thimerosal in 10% DMSO except when given a dose of 115.2 mg/kg.

harmful microbes. Recent attention due to the mercury contained within the thimerosal molecule has focused on the potential impact of thimerosal use on the development of autism (Berman et al., 2008; Geier and Geier, 2007; Geier et al., 2007; Miles and Takahashi, 2007; MMWR, 2007; Ng et al., 2007).

An important consideration when trying to evaluate the possible role of thimerosal toxicity is that autism occurs approximately four times more frequently in males compared to females (MMWR, 2007). Thus, if males have an increased sensitivity to thimerosal, this would have to be taken into account with analyses of any outcomes. Our results that demonstrate a significant difference in the MTD of thimerosal depending upon whether the test animal is male or female is, thus, an important finding. Indeed, in mice, we found when using 10% DMSO to completely dissolve the thimerosal prior to injection that the MTD of thimerosal in males was only 25.6 mg/kg; whereas, in females, the MTD was 76.8 mg/kg. Thus, thimerosal has a 3-fold increased toxicity in males compared to females. Use of 10% DMSO was necessary to completely solubilize the thimerosal. Thus, results using 5% DMSO may be somewhat misleading as tolerated results obtained at the higher doses, particularly when using 38.4 mg/kg in 5% DMSO, may be due to incomplete solubilization of the thimerosal. Regardless of the concentration of DMSO, only male mice were susceptible to severe thimerosal toxicity between the dose range of 38.4–76.8 mg/kg. This is the first report of gender-selective toxicity for thimerosal.

Although the *in vivo* levels of thimerosal used in our studies are far higher than what would be expected in humans, this may not preclude the impact of our findings as we were using mortality as our outcome. Although our study is not extensive as the group sizes are small, nevertheless, we clearly found that male mice were more susceptible to the toxic effects of thimerosal than were female mice. Indeed, this is the first report of gender-selective toxicity for thimerosal and it is intriguing as to why this compound would show this differential effect.

Please cite this article as: Branch DR. Gender-selective toxicity of thimerosal. Exp Toxicol Pathol (2008), doi:10.1016/j.etp.2008.07.002

It is believed that autism has a genetic basis (Zhao et al., 2007), although the specific genes involved have not, as yet, been clearly delineated. Recently, it has been argued that prenatal and neonatal testosterone exposures may be risk factors for autism (Knickmeyer and Baron-Cohen, 2006). Furthermore, it has been reported that estrogens are protective against neurotoxic effects (Wang et al., 2006) and may protect against certain neurodegenerative diseases, such as Parkinson's disease (Benedetti et al., 2001; Dean and McCarthy, 2007). Thus, our findings that thimerosal has increased toxicity in males compared to female mice suggest that estrogens produced in female mice may have some protective effect on the toxicity of thimerosal, while testosterone may have no effect or, perhaps, accelerate these toxic effects. This hypothesis requires future studies when using animal models to distinguish between male and female outcomes.

In summary, this is the first report of gender-selective toxicity of thimerosal. As autism occurs much more frequently in males than in females (MMWR, 2007), our findings may relate to a potential selectivity of thimerosal for toxic effects in some male children, particularly *in utero*. Thus, our results suggest that any future studies of thimerosal toxicity, as it may relate to childhood autism, need to take into account a potential for gender-selectivity of the effects of thimerosal.

References

Agrawal A, Kaushl P, Agrawal S, Gollapudi S, Gupta S. Thimerosal induces TH2 responses via influencing cytokine secretion by human dendritic cells. J Leuk Biol 2007;81: 474–82.

Benedetti MD, Maraganore DM, Bower JH, McDonnell SK, Peterson BJ, Ahlskog JE, et al. Hysterectomy, menopause, and estrogen use preceding Parkinson's disease: an exploratory case–control study. Mov Disord 2001;16:830–7.

Berman RF, Pessah IN, Mouton PR, Mav D, Harry J. Low level neonatal thimerosal exposure: further evaluation of altered neurotoxic potential in SJL mice. Toxicol Sci 2008;101:294.

Dean SL, McCarthy MM. Steroids, sex and the cerebellar cortex: implications for human disease. Cerebellum 2008; 7:38–47.

Geier MR, Geier DA. A prospective study of thimerosal-containing Rho(D)-immune globulin administration as a risk factor for autistic disorders. J Matern Fetal Neonatal Med 2007;20:385–90.

Geier DA, Sykes LK, Geier MR. A review of thimerosal (merthiolate) and its ethylmercury breakdown product: specific historical considerations regarding safety and effectiveness. J Toxicol Environ Health B Crit Rev 2007; 10:575–96.

Knickmeyer RC, Baron-Cohen S. Fetal testosterone and sex differences in typical social development and in autism. J Child Neurol 2006;21:825–45.

Miles JH, Takahashi TN. Lack of association between Rh status, Rh immune globulin in pregnancy and autism. Am J Med Genet A 2007;143:1397–407.

MMWR, Morbidity and Mortality Weekly Report, Department of Health and Human Services, Centers for Disease Control and Prevention, 2007;56:No.SS-1.

Mutter J, Naumann J, Schneider R, Walach H, Haley B. Mercury and autism: accelerating evidence? Neuro Endocrinol Lett 2005;26:439–46.

Ng DK, Chan CH, Soo MT, Lee RS. Low-level chronic mercury exposure in children and adolescents: meta-analysis. Pediatr Int 2007;49:80–7.

Rampersad GC, Suck G, Sakac D, Fahim S, Foo A, Denomme GA, Langler RF, Branch DR. Chemical compounds that target thiol-disulfide groups on mononuclear phagocytes inhibit immune-mediated phagocytosis of red blood cells. Transfusion 2005;45:384–93.

Schechter R, Grether JK. Continuing increases in autism reported to California's developmental services system: mercury in retrograde. Arch Gen Psychiatry 2008;65:19–24.

Thompson WW, Price C, Goodson B, Shay DK, Benson P, Hinrichsen VL, et al. Early thimerosal exposure and neuropsychological outcomes at 7–10 year. N Engl J Med 2007;357:1281–92.

Wang X, Dykens JA, Perez E, Liu R, Yang S, Covey DF, et al. Neuroprotective effects of 17beta-estradiaol and nonfeminizing estrogens against H_2O_2 toxicity in human neuroblastoma SK-N-SH cells. Mol Pharmacol 2006: 395–404.

Zareba G, Cernichiari E, Hojo R, Nitt SM, Weiss B, Mumtaz MM, et al. Thimerosal distribution and metabolism in neonatal mice: comparison with methyl mercury. J Appl Toxicol 2007;27:511–8.

Zhao X, Leotta A, Kustanovich V, Lajonchere C, Geschwind DH, Law K, et al. A unified genetic theory for sporadic and inherited autism. Proc Natl Acad Sci 2007;104: 12832–6.

Please cite this article as: Branch DR. Gender-selective toxicity of thimerosal. Exp Toxicol Pathol (2008), doi:10.1016/j.etp.2008.07.002