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Effects of maternal silver acetate exposure on immune biomarkers in a rodent model

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ABSTRACT

Male and female rats (26-day old) were exposed to 0.0, 0.4, 4 or 40 mg/kg body weight silver acetate (AgAc) in drinking water for 10 weeks prior to and during mating. Sperm positive females remained within their dose groups and were exposed to AgAc during gestation and lactation. Splenic and thymic lymphocyte subsets from F1 generation PD (postnatal day) 4 and 26 pups were assessed by flow cytometry for changes in phenotypic markers. Spleens from PD4 pups had lower percentages of $CD8^+$ lymphocytes in 4 and 40 mg/kg AgAc exposed groups and reduced Concanavalin A (Con A) response at all AgAc exposure groups. Splenic maturation increased in PD26 pups compared to PD4 pups. Con A and lipopolysaccharide (LPS) mediated splenic responses were lower in PD26 pups exposed to 40 mg/kg AgAc. Changes in PD 26 pup splenocyte phenotypic markers included lower TCR + cells at 4 and 40 mg/kg AgAc exposure and higher B cell population in the 40 mg/kg AgAc. PD26 pup splenic natural killer cell (NK) activity was higher in the 0.4 AgAc group and unchanged in 4 and 40 mg/kg AgAc groups. In conclusion, maternal exposure to AgAc had a significant impact on rat splenic development during the early lactation period.

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1. Introduction

Use of silver in its ionic form or as nanoparticles is promoted worldwide due to its antibacterial properties. Typically, uses of silver in biomedical applications include management of dental caries (Peng et al., 2012), wound dressing, prevention of catheter associated infections and during skin regeneration (Chaloupka et al., 2010; Murphy and Evans, 2012). In addition, Lansdown has reviewed various applications of different forms of silver, its absorption, metabolism and toxicity in detail (Lansdown, 2010). It is also applied as a disinfectant coating in water purification filters (Mpenyana-Monyatsi et al., 2012), and in food contact substances as in packaging materials (Martinez-Abad et al., 2012). Although food contact substances are not intended to be components of food there is a potential for them to migrate into the food matrix resulting in human exposure. Silver has been shown to be released from storage containers in both ionic and nanoparticle forms (von Goetz et al., 2013), thus warranting an in vivo safety assessment study.

Assessment of immunotoxicity is an important safety measure for foods, drugs and chemical compounds as an increasing number of food additives or contact substances have been shown to have biological effects beyond nutrition and the adverse effects include but are not limited to immunosuppression and unwanted immunostimulation. At present there are limited studies on the effects of subchronic oral exposure to silver in ionic and nanoparticle forms (Park et al., 2010; van der Zande et al., 2012.) and intravenous exposure to silver nanoparticles on immunological endpoints (De Jong et al., 2013). Results from these studies demonstrated that 28-day repeated oral exposure resulted in dose related tissue accumulation of silver without any effects on innate or adaptive immunity in rats (van der Zande et al., 2012) although there were increased inflammatory responses in mice (Park et al., 2010). In contrast, intravenous exposure to silver nanoparticles caused immunotoxicity as shown by an almost complete suppression of

Abbreviations: AgAc, silver acetate; ConA, concanavalin A; IL, interleukin; LPS, lipopolysaccharide; MNC, mononuclear cells; NK, natural killer; PD, post-natal day.

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splenic natural killer cell activity and altered cytokine and antibody production (De Jong et al., 2013). Conversely Haase et al. (2014) have reported that exposure to both silver nanoparticles and ionic silver cause elevated intracellular levels of reactive oxygen species and the second messenger Zn^{2+} in cells of the innate immune system. These effects were primarily observed on neutrophil granulocytes and macrophages in vitro and although these effects were correlated with both silver nanoparticle and ionic silver exposure it appeared that the reported effects were primarily due to direct exposure to ionic silver or silver ions released from the silver nanoparticles. Małaczewska (2014) reported a number of immunological effects as a consequence of colloidal silver exposure including an increase in CD4+/CD8+ T cell distribution, a decrease in NK and NKT cell distribution (doses of 0.25 and 2.5 ppm) and an increased CD4+:CD8+ ratio (25 ppm); a depression in lymphocyte proliferation (0.25 ppm) and the stimulation of phagocytosis and respiratory burst of granulocytes and monocytes (all doses). These findings suggest there is a potential risk of developing an inappropriate immune response in an organism exposed to prolonged administration of this substance. While a number of studies address the effect of nanosilver on various aspects of reproduction/development, recent studies have not specifically addressed the potential of ionic silver to induce an inappropriate immune response, including a possible effect on the thymus and spleen. The present study was conducted to assess the effects of long term low dose exposure to silver acetate on reproduction and development.

2. Materials and methods

2.1. Test material and analysis of the dosing solutions

Silver acetate (purity 99%; CAS 563-63-3; KSCN %Ag - 63.7–65.5%), was obtained from Sigma-Aldrich and stored according to the conditions detailed by the manufacturer. Dosing solutions were prepared in double distilled water (Hydro-Pico Systems, Inc., Research Triangle Park, NC, USA) and dosing concentrations were verified using an Orion Star pH/ISE meter (Thermo) and Silver/Sulfide electrode with corresponding double-junction reference electrode (thermos) using a direct calibration method following the manufacturer's recommendation for use. The specifics of the dosing solution analysis can be found in Sprando et al., 2016. Silver acetate concentrations utilized in the study were 0.40, 4.0 and 40.0 mg/kg bw and were selected in order to achieve the equivalent of approximately 0, 0.25, 2.5 and 25 Ag + mg/kg bw/day. Dosage by water bottle was selected to mimic the route of human exposure.

2.2. Test animals

Healthy P generation males and nulliparous females were randomized into 4 treatment groups through a standard (by weight) block randomization procedure. P generation male and nulliparous females were exposed to silver acetate via water bottle at the silver acetate concentrations mentioned above for 10 weeks prior to mating. Sperm/plug positive P generation females remained within their dose groups and were continuously exposed to silver acetate through gestation and lactation up through lactation day 21. Pup weight was monitored as soon as possible after birth and on lactation days 0, 4, 7, 14 and 21. At lactation day 4, litters were culled by random selection to 5 males and 5 females when possible. Randomly selected culled animals (PD4) were utilized for immunological evaluations (n = 10 pups/gender/dose group). At lactation day 21, randomly selected F generation male and female pups were individually housed but were not exposed to silver acetate. At postnatal day 26 randomly selected pups were utilized for immunological observations (n = 10 pups/gender/dose group).

The animal protocol was approved by the IACUC committee of the Center for Food Safety and Applied Nutrition of the U.S. Food and Drug Administration and the study was conducted according to the Animal Welfare Act guidelines. This study was designed and conducted according to the current FDA protocol for testing foods and food additives (FDA CFSAN Redbook, 2000) A detailed description of dose analysis, dose administration, mating procedures and information relating to feed and fluid consumption and body weight gain and animal husbandry etc. can be found in Sprando et al., 2016.

2.3. Immunological evaluation

PD 4 pups were euthanized by decapitation and PD 26 pups were euthanized with carbon dioxide. The thymus and spleens from Post-natal Day (PD) 4 and PD 26 pups were collected from 10 pups/gender/dose group. In this study, the effects of silver acetate exposure during pre-mating and pre and post-gestational periods on post natal (PD4 and PD26) splenic and thymic cell populations and functional adaptive and innate immunocompetence were determined using ex vivo models.

2.3.1. Isolation of mononuclear cells from spleen and thymus

The splenic and thymic mononuclear cells were isolated as described elsewhere (Babu et al., 2014). Briefly, spleens and thymuses were minced in Hanks' Balanced Salt Solution, filtered and the single cell suspensions were over-layered on Histopaque (density 1.083 g/ml). Mononuclear cells (MNC) were obtained from the interface after centrifugation at 400*g* for 30 min at room temperature. Cells were washed and the final pellet was adjusted to 4×10^6 cells/ml for proliferation assay (in vitro mitogen reactivity) or to 1×10^7 cells/ml for natural killer (NK) activity measurement and for flow cytometric analyses of phenotypic cell surface markers.

2.3.2. Mitogenic reactivity in vitro

Mitogenic reactivity to concanavalin A (Con A, Sigma, St Louis, MO) and E. coli lipopolysaccharide (LPS, Sigma) was assessed as described previously with minor modifications (Babu et al., 2014). Aliquots of splenic MNC (2×10^5 cells) in RPMI 1640 medium with 10% fetal bovine serum were incubated with Con A (0.4, 0.8 or 1.6 μg/well) or LPS (1.25, 2.5 or 5.0 μg/well) for 72 h at 37 °C and in 5% CO_2 atmosphere. At the end of 48 h, cells were incubated with 0.5 μCi ^{3}H-thymidine (PerkinElmer, Waltham, MA), for an additional 24 h. Cells were harvested using a 96 well automatic harvester and counted in a microbeta counter (PerkinElmer). Data are presented as stimulation indices, which is the ratio of the counts from stimulated cultures and those from non-stimulated cultures.

2.3.3. Immunophenotyping by flow cytometry

Immunophenotyping was carried out using freshly isolated MNC from spleen and thymus as described previously (Babu et al., 2014), and quantified using FACS Aria II flow cytometer (BD Biosciences, CA). Briefly, splenic and thymic MNCs were incubated with the monoclonal antibodies specific to rat cell surface antigens including CD3 labeled with Phycoerythrin (PE)-conjugated anti-rat CD3 (clone G4.18, total T), CD4 labeled with fluorescein isothiocyanate (FITC)-conjugated anti-rat CD4 (clone OX-35, T helper/inducer), CD8 labeled with FITC- conjugated anti-rat CD8a (clone OX-8), anti-rat CD8b (clone 341,T suppressor/cytotoxic), CD45 labeled with FITC-conjugated-anti rat CD45RA (clone OX-33, B cell marker) and αβ TCR labeled with FITC-conjugated-anti rat TCR (clone R73), for at least 45 min at 4 °C. Samples were then washed and fixed until further analysis. Antibody pairing was done for two-