Published in final edited form as:

Viral Immunol. 2015 November ; 28(9): 532–537. doi:10.1089/vim.2015.0037.

Increased Dietary Salt Intake Does Not Influence Influenza A Virus–Induced Disease Severity in Mice

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Abstract

Influenza viruses are pathogens of significant public health importance. The influence of nutritional status on severity of disease has become increasingly recognized. In particular, high dietary salt intake has been linked to cardiovascular disease, but the effects on infectious diseases have not been studied. This study investigated the impact on influenza-induced morbidity and mortality in mice fed isocaloric diets containing 10-fold increments of sodium by altering the salt levels. Following infection, despite higher levels of IFN-gamma cytokine in the lung as well as virus-neutralizing antibody in the serum of mice fed the lowest salt level, the amounts of dietary salt intake had no substantial impact on the disease severity or the ability to respond immunologically to the infection.

Introduction

With an estimated three to five million cases of severe disease worldwide and approximately 250,000 to 500,000 deaths annually (22), influenza viruses remain a significant public health concern. Hospitalizations and deaths attributed to seasonal influenza occur more frequently among persons at the extremes of age and/or with underlying medical conditions (10,20). In 2009, the pandemic H1N1 influenza virus (pH1N1) caused more than 80 million infections within a year (6); pregnancy, diabetes, and obesity were identified as risk factors for severe disease (18). In light of this, it is important to investigate the risk factors that contribute to disease severity, so that health status–specific intervention strategies can be identified for all age groups.

Knowledge of the contribution of nutrition to infectious disease remains limited. Recent studies demonstrate an important relationship between the host's nutritional status and

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Author Disclosure Statement

No competing financial interests exist.

influenza virus–induced disease severity (3,19). One such nutrient is sodium, a cation and micronutrient important for maintenance of extracellular fluid volume, and is mostly obtained by the body from dietary salt. The causal link between sodium intake and high blood pressure, a critical health issue, has been well documented (15), although some recent studies suggest an inverse relationship between sodium consumption and death from cardiovascular disease (8). Considering that both children and adult populations consume more salt than the recommended amounts (5,7), as well as the association of hypertension with increased risk of 2009 pdm H1N1 infection (2,11,16) and the newly emerged H7N9 outbreak in China (12), understanding the consequences of high dietary salt intake on infectious disease outcomes is warranted. Moreover, although there are no published reports on the effects of salt intake on susceptibility to respiratory infections, epidemiological studies on markers of hydration and salt intake have demonstrated conflicting results with broncho-pulmonary disorders (1,14). In this study, using an approach described by Wu *et al.* (23), adult mice were fed with isocaloric diets containing varying percentages of salt. Different levels of salt had no effect on influenza virus–induced disease severity. In addition, varying the levels of dietary salt had no negative impact on the primary immune responses to the virus.

Materials and Methods

Mice and virus infections

Four-week-old female C57BL/6 mice (Jackson Laboratory) were placed on isocaloric diets (Harlan Laboratories; see Supplementary Table S1) containing 8% NaCl (high salt diet; HSD), 0.49% NaCl (normal salt diet; NSD, recommended for laboratory mice), or 0.05% NaCl (low salt diet; LSD), on *ad libitum* access, for a period of 4 weeks. Preliminary "pair feeding" studies showed comparable levels of feed consumption among all three groups of mice. After 4 weeks, mice were anesthetized by injection of 300 μL of avertin intraperitoneally, and infected intranasally, as described elsewhere (19), with either a low dose (50 mouse infectious doses; 50 MID_{50}) or a high dose (1 lethal dose; $1LD_{50}$) of A/Puerto Rico/8/34 (PR8) in 50 μL of phosphate-buffered saline (PBS). All animal research was approved by the CDC-IACUC and was conducted in an AAALAC-accredited facility.

Virus titration and analysis of inflammatory proteins

Mice were sacrificed on days 3, 6, or 9 after $50MID_{50}$ infection, and lungs were collected and homogenized. Virus titrations were done as described previously (19). Inflammatory proteins in the lung tissue supernatants were analyzed by bioplex assay following the manufacturer's kit instructions (Bio-Rad Laboratories). IFN-β was measured using the Mouse IFN-β enzyme-linked immunosorbent assay kit (PBL Assay Science).

Hemagglutination inhibition, serum chemistry, and flow cytometry

For hemagglutination inhibition (HI) assay (19), a volume of 50 μL of receptor-destroying enzyme-treated serum was added to the 96-well plate and twofold serial dilutions performed with PBS. Four hemagglutination units of virus were added and incubated at room temperature for 30 min. Following incubation, 50 μL of 0.5% suspension of turkey red blood cells was added and incubated at room temperature for 30 min. The dilution of serum

showing complete HI was recorded as the HI titer. Serum clinical chemistry was performed by Comparative Clinical Pathology Services, LLC. For flow cytometry, single cell suspensions were prepared from lungs after collagenase (263 IU/mL) treatment at 37°C for 30 min. Spleens were processed by gently dissociating tissue over a 70 μm sieve followed by staining with anti-GL7, anti-MHCII, anti-CD138, anti-CD38, anti-B220, anti-CD8, anti-CD44 antibody (Biolegend), and influenza-NP-Pentamer (Proimmune) for CD8 T cell and B cell analysis. For intracellular cytokine detection, spleen cells were infected with 1 MOI followed by addition of GolgiPlug the next day for 5 h. Cells were washed twice with PBS followed by surface staining, with anti-CD4, anti-CD8, and anti-CD44. Cells were permeabilized using the fix/perm kit (BD Biosciences) followed by intracellular staining with anti-TNF-α, anti-IFN-γ, and anti-IL2 antibody. Samples were acquired on an LSRII (BD Biosciences) and data analyzed using the flowJo software (Treestar).

Histopathology

On day 6 post-infection, mice were euthanatized and tissues were collected in 10% neutral-buffered formalin. After 72 h, the samples were transferred to 70% ethanol, sectioned, and stained with hematoxylin and eosin.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software). A two-way analysis of variance (ANOVA) was used, in conjunction with the Bonferroni post-test, on water consumption, morbidity, virus titer, cytokine levels, serum chemistry, and B cell and T cell analyses. The Mann–Whitney test was used to determine significance of the HI titer. A one-way ANOVA was used for feed consumption data analysis. The log-rank (Mantel–Cox) test was used to compare percent survival among groups of mice. All differences were considered significant when the p-value was ≤ 0.05.

Results

Increased salt intake does not impact morbidity or mortality following influenza virus infection

To examine the effects of dietary salt on influenza virus infection, 4-week-old mice were put on isocaloric diets containing different levels of sodium containing salt (NSD, HSD, and LSD; Supplementary Table S1) for a period of 4 weeks. During this period, both feed and water intake were measured. While mice on the HSD consumed more water than NSD or LSD groups, there was no difference in the amount of feed consumption (Supplementary Fig. S1A and B; Supplementary Data are available online at www.liebertpub.com/vim). To determine the effects of dietary salt on growth, change in body weight was charted as a percentage of the original body weight. Irrespective of diet, mice showed comparable weight gain over the period; although the HSD group gained less weight, this was not statistically significant (Fig. 1A). In addition, analysis of serum for physiological parameters indicative of liver and kidney functions (Supplementary Table T2) and histological analysis of liver and kidney (Supplementary Fig. S1C) did not show any significant differences among the three groups.

After 4 weeks on the different diets, mice were infected with two different virus doses (low/50MID$_{50}$ and high/1LD$_{50}$) to determine the effect of increased salt intake on disease severity. Irrespective of the virus dose, there were no differences in the morbidity or mortality of mice on different salt diets (Fig. 1B). Next, the viral burden in the lungs of these mice was determined at three different time points post-infection. Similar levels of virus were observed in three groups of mice on days 3 and 6 post-infection. However, on day 9 post-infection, the HSD group of mice, when compared with NSD or LSD, had four- to sixfold higher ($p > 0.05$) virus titers (Fig. 1C). Furthermore, histological evaluation of lung tissue on day 6 post-infection did not show any striking differences in the extent of inflammatory cell influx into the bronchial epithelium or interstitial tissue (Fig. 1D).

Increased salt intake does not impact primary immune response to influenza

Next, the effect of dietary salt on immune responses following infection was examined. Mice that had been on the HSD, NSD, or LSD for 4 weeks were infected with 50MID$_{50}$ of influenza. On days 3, 6, and 9 post-infection, the levels of chemokines and cytokines in the lung tissue were measured using a bioplex assay. There were no significant differences in the levels of the various inflammatory mediators measured except for IFN-γ, which, when compared with HSD or LSD, was present at significantly higher levels in mice on the LSD, although only at day 6 post-infection (Fig. 2A). The levels of serum HI antibodies (Fig. 2B, upper panel) were determined, and no significant difference was found among the three groups at day 10 post-infection. At day 21 post-infection, geometric mean HI titers were comparable between the LSD and NSD groups, but the mean HI titers in the HSD group were modestly but significantly lower when compared with titers of the LSD group. However, analysis of germinal center B cells in the spleen, on day 21 post-infection, did not show any significant differences among the three groups (Fig. 2B, lower panel). Finally, assessment of splenic T cells on day 21 post-infection showed no difference in percent influenza-NP+CD8+ T cells (Fig. 2C; left panel) and percent T cells producing IFN-γ, TNF-α, or IL-2 (Fig. 2C; middle and right panels). Although there was a relative increase in the frequency of T cells producing IFN-γ in LSD group, the difference when compared with other two groups was not statistically significant. Thus, increasing the level of dietary salt does not substantially impact influenza-induced primary immune response and disease severity in mice.

Discussion

Influenza viruses can cause highly contagious, acute respiratory illness leading to a significant global health concern (22). With diet being increasingly recognized as an underlying and important risk factor for disease (3, 19), and studies showing reduction in salt intake can help with prevention of noncommunicable chronic respiratory diseases (9), this study set out to understand the relationship between increased intake of the sodium, a key micronutrient, and its effects on influenza-induced disease severity. The study shows that increasing the level of salt in the diet of young mice that had been fed the specific salt diet for 50% of their young lives (4–8 weeks) does not have a significant impact on the susceptibility of young mice to influenza virus infection. Previously, using a similar

approach, a significant effect in disease outcome was observed with diets supplementing distinct levels of protein energy (19).

Recent studies have examined the role of a HSD in autoimmunity. In models of experimental autoimmune encephalitis, autoreactive T cells have been postulated to play a role, with mice on a HSD displaying increased disease severity, mediated by a T_H17 polarization of the CD4 + T response (23). Using a similar approach, and custom diets, described by Wu *et al.*, a model was set up where mice were given diets that had either 10-fold less (LSD) or more sodium (HSD) compared with the levels normally used in the laboratory mouse diet (NSD) (23). Despite varying the levels of salt in the diet, the mice remained healthy, as suggested by their normal weight gain and serum clinical chemistry analysis (Fig. 1 and Supplementary Table S2). The three salt diets, despite having 10-fold increments of sodium, did not lead to any changes in serum sodium levels. An earlier study (21) using similar salt diets (0.3% NaCl and 4% NaCl) in mice also found that serum Na^+, Cl^-, and K^+ levels were unchanged in the two groups of mice. In the same study, Wang *et al.* found that serum electrolyte balance was maintained by increased urinary sodium excretion. In addition, as also demonstrated by Wang *et al.*, in the present studies, mice on the HSD consumed more water than mice on the other diets. In assessing the effects of salt on influenza infection, no difference was found in the morbidity or mortality of mice, irrespective of the levels of dietary salt. Consistent with this finding, no difference was observed in the histopathological analysis of the lung tissue among the groups.

The effect of high salt consumption on the innate and adaptive immune responses was also determined. As shown previously (13), influenza infection triggered the induction of several important antiviral and proinflammatory cytokines. In the HSD group, although the levels of some inflammatory mediators at day 9 (MIP-1β, IFN-β, and MCP-1) showed relatively higher levels, the differences were not statistically significant. Despite an increase in IFN-γ at day 6 post-infection in the LSD group, the virus titer at all three time points was comparable among all groups of mice. Moreover, no difference in extent of immune infiltrates in the lungs was observed among the three groups by flow cytometry (data not shown). With respect to adaptive immune responses, when compared with the NSD group, there were no significant differences in percent germinal center B cells, serum HI antibody titer, or T cell responses (percent T cells and percent cytokine producing T cells) with the HSD group, suggesting that a HSD may not substantially impact the response to influenza vaccination. Taken together, these data, although derived from experiments that did not involve any assessments of hypertension, demonstrate that increased salt consumption did not result in enhanced disease or diminished primary immune responses to infection in young, otherwise healthy mice.

Interestingly, two recent studies investigating the association between underlying medical conditions and susceptibility to influenza infection in humans found that hypertension was a major risk factor for both pdmH1N1 and H7N9 associated mortality (2,12). Therefore, studies in mice involving long-term use of custom salt diets and other mouse models of hypertension (4,17) may help in further exploring any likely association between salt intake/hypertension and susceptibility to influenza infection. Although the present data show that increasing the level of salt in the diet may not impact influenza disease severity, considering

the recommendation for reducing the salt intake in the aged population (5), it remains to be seen if a HSD has any effect on the degree of disease severity in aged animals, particularly in an experimental setting that mimics hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Immunology and Pathogenesis Branch in the Influenza Division, Centers for Disease Control and Prevention, for providing constructive comments on this research. This study was supported by CDC intramural funding. SA was supported by Battelle Memorial Institute, 2987 Clairmont Rd, Suite 450, Atlanta, GA 30329.

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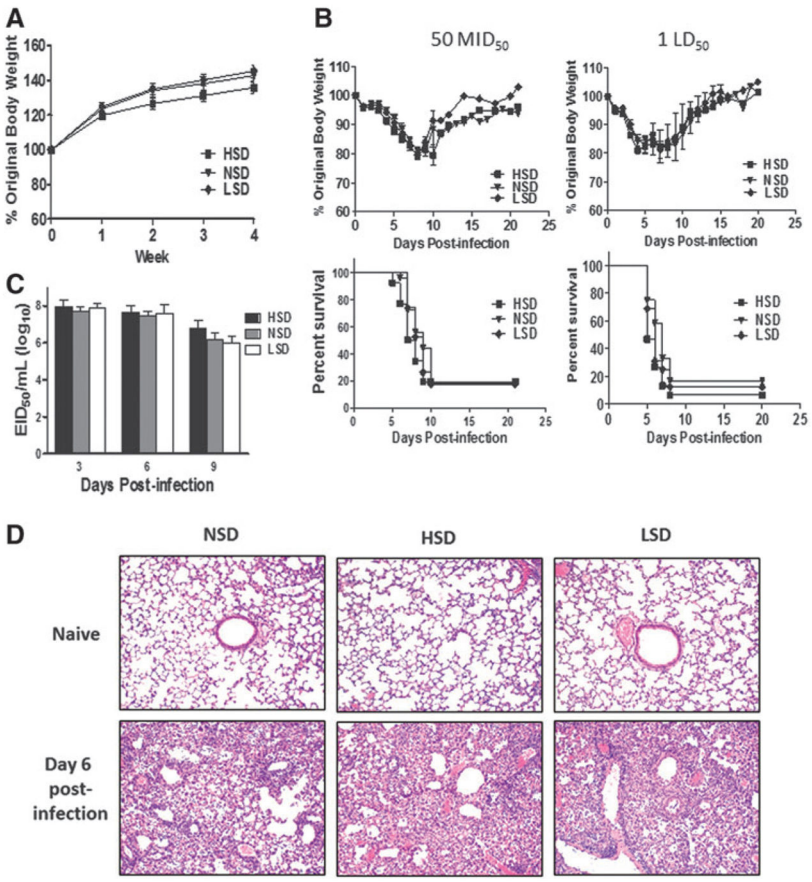

FIG. 1.

Increase in salt intake does not influence influenza A virus–induced disease severity. Groups of mice maintained on three different isocaloric diets (high salt diet [HSD], normal salt diet [NSD], and low salt diet [LSD]) for a period of 4 weeks were weighed weekly and changes in body weights were plotted **(A)**. They were subsequently infected with $50MID_{50}$ or $1LD_{50}$ and the morbidity (*upper panels*) and mortality (*lower panels*) were assessed **(B)**. Next, they were infected with $50MID_{50}$ and lung viral titers determined on days 3, 6, or 9 post-infection **(C)**, or had the lung tissue sections processed for histopathology **(D)**, as described in Materials and Methods. Data shown in **(A)** represent the mean ± standard error of the mean (SEM) from two independent experiments with 20 mice per group. Data shown in **(B)** represent mean ± SEM from four independent experiments with at least 23 mice per group for the $50MID_{50}$ and two independent experiments with at least 16 mice per group for the $1LD_{50}$. Data shown in **(C)** represent mean ± standard deviation (*SD*) from at least two independent experiments with a minimum five mice per group/experiment, and **(D)** represents images (magnification, 10×) from an experiment consisting of three mice per group.

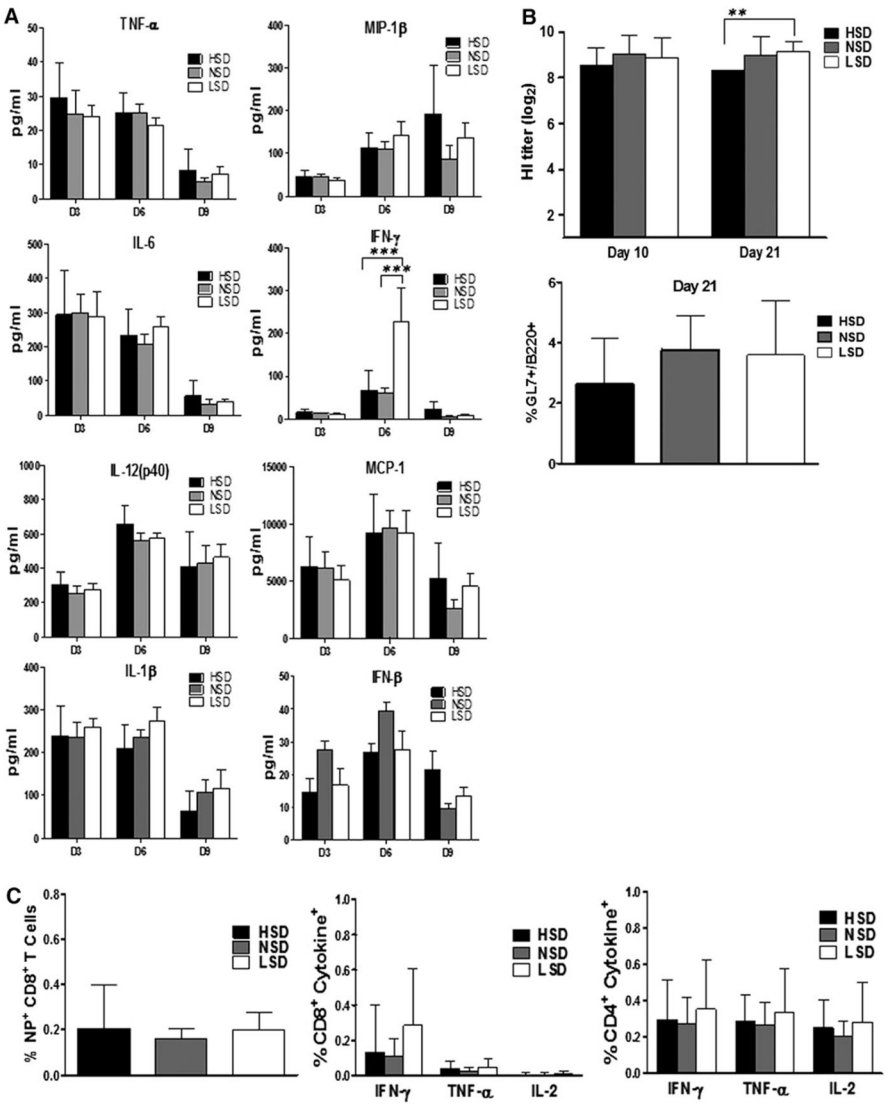

FIG. 2.

Increase in salt intake does not impact inflammatory mediators in the lung tissue and adaptive immune responses following influenza virus infection. Groups of mice maintained on three different isocaloric diets (HSD, NSD, and LSD) for a period of 4 weeks and infected with $50MID_{50}$ **(A)** were sacrificed on days listed and lung homogenates were analyzed for cytokines and chemokines using a bioplex or ELISA (IFN-*β*), **(B)** had serum collected on days 10 and 21 post-infection for HI titer (*upper panel*) and spleen tissue assessed for the percent germinal center B cells (*lower panel*), or **(C)** had spleens harvested on day 21 for analysis of percent NP+CD8+ T cells (*left panel*), CD8+ cytokine+ T cells (*middle panel*), and CD4+ cytokine+ T cells (*right panel*). Data shown in **(A)**, **(B)**, and **(C)** represent values (mean ± *SD*) from two independent experiments with at least five mice per group. ***$p \leq 0.001$; **$p \leq 0.01$.