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Susceptibility of Overweight Mice to Liver Injury as a Result of the ZnO Nanoparticle-Enhanced Liver Deposition of Pb^{2+}

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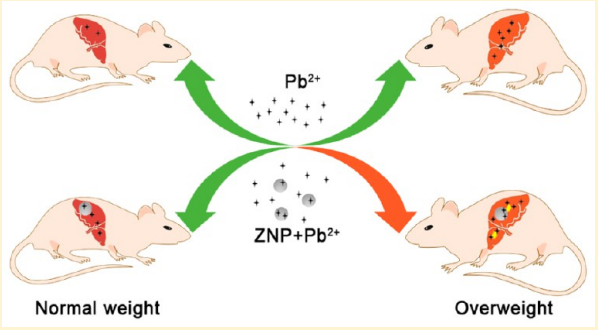
Supporting Information

ABSTRACT: The prevalence of the applications of nanomaterials in consumer products and water treatment facilities increases the chance that humans will be exposed to both nanoparticles and environmental pollutants such as heavy metals. Co-exposure to nanoparticles and heavy metals may adversely affect human health, especially in susceptible populations such as overweight subjects. To evaluate the impact of such co-exposures, we orally administered zinc oxide nanoparticles (ZNPs; 14 or 58 nm) and/or $Pb(Ac)_2$ at tolerable doses to both healthy overweight and healthy normal weight mice. The ZNPs enhanced the deposition of Pb in all major organs in the overweight mice compared with that in the normal mice. As a result, higher levels of hepatic reactive oxygen species, pro-inflammatory cytokines, and liver injury were observed in the overweight mice but not in the normal weight mice. Our findings underscore a potentially enhanced risk of nanoparticle/heavy metal co-exposure in the susceptible overweight population.



INTRODUCTION

Nanoparticles have large surface areas and high surface reactivity. These characteristics are associated with superior performance in pollutant absorption, catalysis, and sensing, which enhances their environmental applications. However, the increased production and use[1] of nanomaterials increase the likelihood of their release into the environment and their coexistence with various pollutants, which further increases the chance of human exposure to both nanoparticles and pollutants such as heavy metals. For instance, zinc oxide nanoparticles (ZNPs) and heavy metal ions such as Pb^{2+}, Cd^{2+}, Cu^{2+}, and Ni^{2+} are common pollutants found in groundwaters. ZNPs have a large surface area and a high surface energy, allowing them to strongly adsorb metal ions.[2] The potential risk of humans being orally exposed to these pollutant adducts is high. Nanoparticles can enter cells[3] and exhibit toxicity in plants,[4] human cells,[5] and animal models.[6] Moreover, they synergistically interact with heavy metals in both aquatic organisms[7,8] and healthy adult mammals,[9,10] leading to an aggravated toxicity. However, the toxicity that may result from co-exposure to nanoparticles and heavy metals in susceptible populations is still not well understood.

Being overweight and obesity has become a major health threat worldwide in the past several decades. The population of overweight and obese subjects has increased from 28.8% in 1980 to 36.9% in 2013 for men and from 29.8% in 1980 to 38.0% in 2013 for women.[11] Among the 1.9 billion overweight and obese adults in 2014, 1.3 billion of them were overweight (body mass index between 25 and 30 kg/m^2), as reported by the World Health Organization.[12] A high-fat diet (HFD) is a major contributing factor to being overweight and obesity. These conditions in turn lead to various health issues such as fatty liver disease, diabetes, cardiovascular disease, and cancers.[13,14] HFD-induced overweight subjects also face intestinal permeability dysfunction because of the alteration of bile acid metabolism and a decrease in the level of expression of tight junction proteins,[15,16] and being overweight impacts human health in a variety of ways. Therefore, the consequences of oral exposure to nanoparticle/heavy metals adducts in the overweight population need to be investigated.

In this study, we compared the responses of overweight and normal mice to two-week oral exposures to ZNPs (ZNP-14 and ZNP-58) and/or $Pb(Ac)_2$ at doses that are nontoxic to healthy mice. We found that the co-exposure enhanced the accumulation of Pb in all major organs, induced higher levels of hepatic reactive oxygen species (ROS) and pro-inflammatory cytokines, and aggravated liver injury only in HFD-fed mice.

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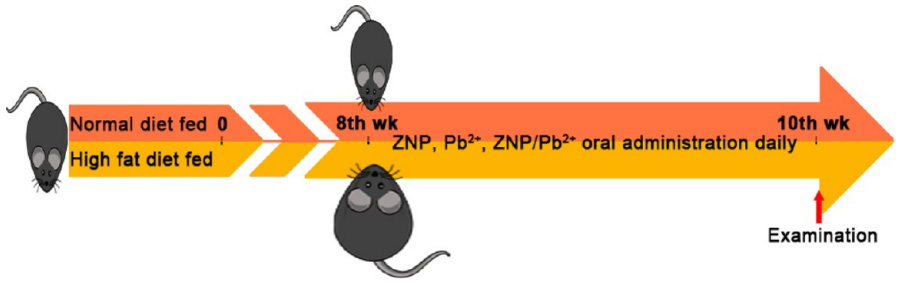

Figure 1. Overweight mouse model and ZNP and Pb^{2+} co-exposure scheme. C57BL/6J mice were orally administered ZNP-14 or ZNP-58 (200 mg/kg), $Pb(Ac)_2$ (150 mg/kg), or ZNP-14 and $Pb(Ac)_2$ or ZNP-58 and $Pb(Ac)_2$ (200 and 150 mg/kg, respectively) daily for 2 weeks after being fed the ND or HFD for 8 weeks.

MATERIALS AND METHODS

Preparation and Characterization of ZNPs and ZNP with Pb^{2+}. ZNPs of two sizes (zinc oxide nanopowder, <50 and <100 nm particle sizes) were purchased from Sigma-Aldrich Co. LLC (Shanghai, China), and lead(II) acetate trihydrate was purchased from Shanghai Jingchun Co., Ltd. (Shanghai, China). The morphological characteristics and sizes of ZNPs were analyzed using a transmission electron microscope (JEM-1011, Jeol). The hydrodynamic size and ζ potential of ZNPs in the presence and absence of Pb^{2+} were measured using a particle size analyzer (Nano ZS90, Malvern Instruments, Malvern, U.K.). The ZNPs were dispersed in deionized water and sonicated for 30 min before being administered.

Animals and Exposures. Male C57BL/6J mice (5 weeks old) were obtained from the Institute of Laboratory Animal Science of the Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC) (Beijing, China). All animal experiments were approved by the Animal Care and Use Committee of Shandong University and were in accordance with the National Institutes of Health guidelines in its Guide for the Care and Use of Laboratory Animals. After being acclimated for 1 week, 84 mice were randomly divided into two groups and fed a normal diet (ND) or a high-fat diet [HFD; acquired from the Shanghai Laboratory Animal Center (SLAC, Shanghai, China), with the composition shown in Table S1] for 8 weeks, during which time the body weights were recorded every week. By the end of the eighth week, mice in each group were then randomly divided into seven subgroups, and one of these seven subgroups was sacrificed to confirm the overweight mouse model. The other six groups of both ND- and HFD-fed mice were orally administered sterile water, $Pb(Ac)_2$ (150 mg/kg), ZNP-14 (200 mg/kg), ZNP-58 (200 mg/kg), ZNP-14 and $Pb(Ac)_2$ (200 and 150 mg/kg, respectively), or ZNP-58 and $Pb(Ac)_2$ (200 and 150 mg/kg, respectively) daily for an additional 2 weeks (Figure 1). The body weights were recorded every day during these various exposures.

Biodistribution of ZNPs and Pb. On the second day of the final administration, the mice were sacrificed after being anesthetized using sodium pentobarbital. The heart, liver, spleen, lung, kidney, and small intestine were collected from each animal and weighed. Samples of each tissue were added to 10 mL of 70% nitric acid and digested in microwave ovens. The Zn and Pb contents of the various organs were determined using an inductively coupled plasma mass spectrometer (model 7700, Agilent, Santa Clara, CA).

Histopathological Examination. Additional samples of the spleens, kidneys, and livers were fixed in 10% buffered formalin for 24 h and then embedded in paraffin. The paraffin-embedded sections were stained with hematoxylin and eosin (H&E staining). The organ injuries were scored by an experienced pathologist who was blinded to the experimental procedures.

Biochemical Analysis. Plasma samples were harvested by centrifugation at 600*g* for 10 min. The plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using an automatic biochemical analyzer (AU400, Olympus, Tokyo, Japan).

Oxidative Stress and Inflammation Levels in the Liver. The liver tissues were weighed and then homogenized in cold phosphate-buffered saline using a tissue homogenizer (PRO200 homogenizer, Bio-Gen, Oxford, CT). The supernatants were collected after centrifugation of the homogenates at 3000*g* for 15 min at 4 °C. The superoxide dismutase (SOD) activities and malondialdehyde (MDA) levels in the supernatants were analyzed using SOD and MDA assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The interleukin-1β (IL-1β) and IL-6 levels in the supernatants were determined using IL-1β and IL-6 enzyme-linked immunosorbent assay kits (Boster Biological Technology Co., Ltd., Wuhan, China).

Statistical Analysis. All statistical calculations were performed using Sigma Plot 12.0. The numerical data are presented as the means ± the standard deviation of no fewer than five independent determinations. The comparisons between the control and experimental groups were analyzed using one-way analysis of variance followed by post hoc least significant difference or Tukey's tests. A P value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Characterization of ZnO Nanoparticles. The physical and chemical properties of nanoparticles determine their biological effects in the body of an animal. We first thoroughly characterized the ZNPs before and after incubation with $Pb(Ac)_2$. The average sizes of ZNP-14 and ZNP-58 were 13.9 ± 2.4 and 57.7 ± 15.8 nm, respectively, as measured by transmission electron microscopy (TEM) (Table 1). During biological interactions, nanoparticles are always suspended in aqueous medium. The hydrodynamic diameters of ZNP-14 and ZNP-58 in water were 14.2 ± 0.1 and 60.8 ± 0.9 nm, respectively, as measured by *in situ* dynamic light scattering (DLS), which indicated that both ZNPs were suspended well in the aqueous solutions. Because of the adsorption of proteins, the hydrodynamic diameters of ZNP-14 and ZNP-58 increased to 15.8 ± 0.3 and 63.9 ± 0.3 nm, respectively, in 10% fetal bovine serum (FBS). Both ZNPs carried positive charges as shown by ζ potential values of 38.7 ± 0.1 and 26.2 ± 0.6 mV,

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Table 1. Characterization of the ZnO Nanoparticles

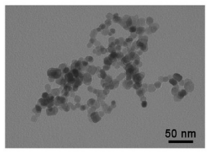
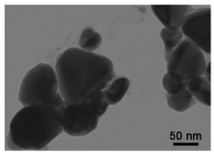
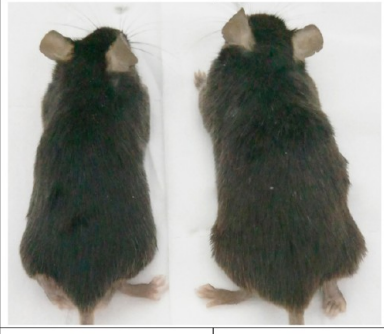
	ZNP-14	ZNP-58
TEM		
Size by TEM (nm)	13.9±2.4	57.7±15.8
Hydrodynamic diameter in water (nm)	14.2±0.1	60.8±0.9
Hydrodynamic diameter in 10% FBS (nm)	15.8±0.3	63.9±0.3
Zeta potential in water (without Pb^{2+}, mV)	38.7±0.1	26.2±0.6
Zeta potential in water (with Pb^{2+}, mV)	60.0±0.9	57.2±0.4
Zeta potential in 10% FBS (without Pb^{2+}, mV)	-20.8±0.6	-23.5±1.2
Zeta potential in 10% FBS (with Pb^{2+}, mV)	-11.3±0.9	-11.8±0.4

Table 2. Overweight Mouse Model

		Normal diet	High fat diet
Body weight (g)	Day 0	21.15±1.05	21.14±0.99
	8^{th} week	25.95±1.30	31.30±1.20**
Gross morphology			
Plasma cholesterol (U/L)		2.03±0.39	4.25±0.51**
Plasma high density lipoprotein (U/L)		1.08±0.05	1.95±0.08**
Plasma low density lipoprotein (U/L)		0.22±0.04	0.55±0.08**

**$P < 0.01$, compared with the normal diet group.

respectively, in water. Interactions with $Pb(Ac)_2$ shifted the ζ potential values of the ZNPs to more positive values (60.0 ± 0.9 and 57.2 ± 0.4 mV, respectively), indicating that both ZNPs strongly adsorbed Pb^{2+} ions under these conditions. In 10% FBS, the ζ potential values of the ZNPs were shifted to −20.8 ± 0.6 and −23.5 ± 1.2 mV, respectively. The adsorption of Pb^{2+} to the ZNPs in the presence of serum proteins shifted the ζ potentials to −11.3 ± 0.9 and −11.8 ± 0.4 mV for ZNP-14 and ZNP-58, respectively, which indicated that protein coatings were formed. Similar charges on ZNPs and protein coatings may further stabilize nanoparticles in biological media.

ZNPs can be partially dissolved and release Zn^{2+} ions in aqueous solution, especially at a low pH value, such as in gastric fluid.[17,18] Our data showed that ZNPs exhibited similar dissolution rates, with approximately 0.3 and 8.5% (17 mg/kg in this study) Zn^{2+} release in water and acidic gastric fluid (AGF), respectively, within 24 h (Figure S1). Previous studies showed that oral dosing of Zn^{2+} ions at a concentration of 268.4 mg/kg for 13 consecutive weeks induced no significant changes in the body weight of Sprague-Dawley rats,[17] demonstrating that the effect of Zn^{2+} (at 17 mg/kg) on rodents after oral administration is negligible.

Overweight Mouse Model. To compare the toxicity effects in the normal and overweight mice, we first established this mouse model. It is well-known that C57BL/6J mice are susceptible to diet-induced obesity. Sex differences also exist in the progression of diet-induced obesity, and male C57BL/6J mice are more vulnerable to HFD-induced body weight and fat mass gains and metabolic alterations than are females.[19,20] Therefore, male C57BL/6 mice are often used as the standard animals for diet-induced obesity research.[21−23] In this study, male C57BL/6J mice (6 weeks old) were fed with a HFD (Table S1) for 8 weeks. At the end of the eighth week, the body weight and plasma lipid levels were determined. The results showed that HFD feeding resulted in a 20.6% increase in body weight. The serum cholesterol level in HFD mice was doubled, and the levels of high-density lipoprotein and low-density lipoprotein in the HFD mice were 1.8- and 2.5-fold higher, respectively, than the levels in the ND mice (Table 2).

According to a previous report, a mouse with a body weight 20% heavier than that of the normal diet-fed mouse is classified as overweight.[24] In this study, HFD feeding for 8 weeks led to an increased body weight of 20.6%, accompanied by increased levels of plasma lipids. Although the data did not indicate a severely obese condition, these data showed the successful establishment of an overweight mouse model.

Dosage Selection. The concentrations of ZNP in the environment are highly variable and are not well-known. According to the Organization for Economic Cooperation and Development (OECD), the suggested doses for investigating the toxicity of new substances are 5, 50, 300, 2000, and 5000 mg/kg.[25] Because nanoparticles agglomerate in aqueous solution at high concentrations, an oral dose of 300 mg/kg has often been used in previous studies to evaluate subchronic toxicity of ZNPs in mice.[26,27] To establish a tolerable dose, both ND and HFD mice were orally administered ZNP-14 (200 or 300 mg/kg) and $Pb(Ac)_2$ (150 mg/kg) daily for 2 weeks. At the 300 mg/kg dose, death occurred in both the ND and HFD groups [three of six in the HFD group on the fourth day and one of six in the ND group on the third day (Figure 2)]. Daily exposures of the mice to a ZNP-14 dose of 200 mg/kg and a $Pb(Ac)_2$ dose of 150 mg/kg did not induce death throughout the 14-day period, which indicated that a daily dose of ZNP and $Pb(Ac)_2$ (200 and 150 mg/kg, respectively) was well tolerated by the mice.

Altered Distribution of Pb^{2+} in Major Organs. Oral co-administration of ZNPs and $Pb(Ac)_2$ may alter the absorption of these substances into the blood or their deposition in the various organs. To examine these dynamic absorption and tissue deposition processes, we used ICP-MS to examine the contents of Zn and Pb in the major organs after exposure for 14 days. The results showed that large amounts of the ZNPs were internalized and trapped in the intestines. This result indicated that ZNPs could cross the intestinal membranes and might be absorbed into the circulation. In the animals that were exposed to ZNP-14, the Zn levels in the intestines of the ND and HFD mice were 2.4 and 2.1 times the basal Zn level, respectively, and for ZNP-58, these values were 2.0 and 2.3 times the basal level, respectively (Figure 3A). Compared with the administration of

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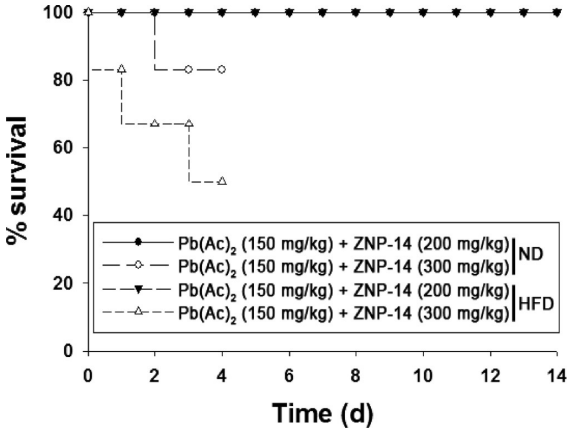

Figure 2. Percent survival of ND/HFD mice orally administered ZNP-14 (200 or 300 mg/kg) and $Pb(Ac)_2$ (150 mg/kg). Oral co-administration of ZNP-14 (200 mg/kg) and $Pb(Ac)_2$ (150 mg/kg) for 2 weeks was tolerated by the mice, whereas co-administration of ZNP-14 (300 mg/kg) and $Pb(Ac)_2$ (150 mg/kg) was lethal to mice.

the ZNPs alone, co-administration of $Pb(Ac)_2$ further increased the intestinal levels of Zn in the ND and HFD mice (1.6 and 1.9 times the levels observed after administration of ZNP-14 alone or 3.7 and 4.1 times, respectively, higher than the basal Zn level). This result suggested that the positive charge (adsorption of Pb^{2+}) on the ZNPs facilitated their absorption through the intestinal membranes. The same effect was also observed for ZNP-58 [1.7 and 1.6 times higher than the levels after ZNP-58 administration in the ND and HFD mice, respectively (Figure 3A)]. After the administration of ZNP-14 or ZNP-58, the absorbed ZNPs were mainly deposited into the kidneys and the liver (Figure 3B,D), which was consistent with previous studies.[27,28] Co-exposure to ZNP and $Pb(Ac)_2$ induced only a slight alteration in the ZNP deposition in the kidneys (Figure 3B). The amounts of Zn that were found in the other organs after administration of ZNP or ZNP and $Pb(Ac)_2$ were similar in the ND and HFD mice.

On the other hand, Pb^{2+} was well absorbed and widely deposited in various organs, including the kidneys, spleen, liver, lungs, and heart after the administration of $Pb(Ac)_2$. Co-exposure to ZNP and $Pb(Ac)_2$ further increased the rate of deposition of Pb in the various organs. Compared to the levels resulting from the administration of Pb^{2+} alone, the co-administration of ZNP-14 or ZNP-58 increased the levels of Pb deposited in the kidneys (11.8- or 11.1-fold, respectively), in the spleen (4.0- or 3.4-fold, respectively), in the liver (1.8- or 2.0-fold, respectively), in the lungs (1.7- or 1.6-fold, respectively), and in the heart (3.3- or 2.3-fold, respectively) in the ND mice (Figure 4). A similar effect was also observed in the HFD mice when ZNP-14 or ZNP-58 was co-administered with Pb^{2+}. Specifically, in these mice, the co-administration of ZNP-14 or ZNP-58 with Pb^{2+} resulted in increases in the rate of Pb deposition compared to those reached after administration of Pb^{2+} alone that were 2.4- or 2.5-fold higher in the kidneys (23.3- or 24.3-fold higher than those in the ND mice after administration of Pb^{2+} alone, respectively), 2.7- or 3.8-fold higher in the spleen (7.3- or 10.3-fold higher than those in the ND mice after administration of Pb^{2+} alone, respectively), 1.2- or 1.1-fold higher in the liver (2.8- or 2.5-fold higher than those in the ND mice after administration of Pb^{2+} alone, respectively), 1.6- or 1.7-fold higher in the lungs (3.0- or 3.2-fold higher than those in the ND mice after administration of Pb^{2+} alone, respectively), and 2.7- or 2.6-fold higher in the heart (4.9- or 4.7-fold higher than those in the ND mice after administration of Pb^{2+} alone, respectively), respectively.

The organs of HFD mice are more susceptible to uptake of foreign ions or molecules. This is because HFD feeding increases the intestinal permeability as characterized by the decreased level of expression of various tight junction proteins, including claudin-1, claudin-3, occludin, and junctional adhesion molecule-1,[16] alterations in the bile acid metabolism,[15] and depletion of the intestinal eosinophils.[29] In this

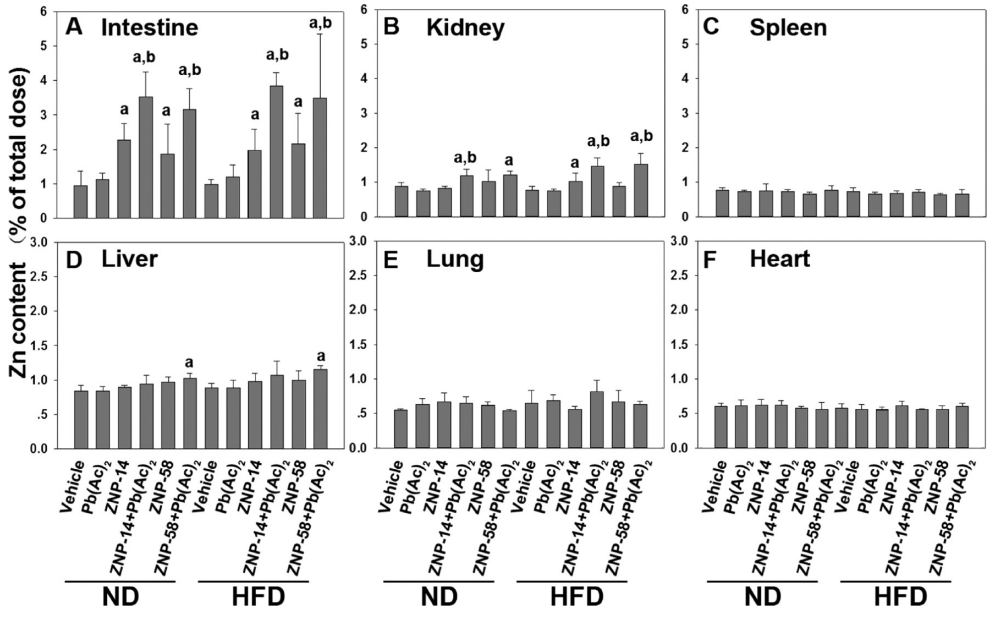

Figure 3. Distribution of Zn in the major organs (means ± standard deviation; $n = 5$). The data are presented as the Zn concentrations determined in the major organs as a percentage of the total exposure dose. [a]$P < 0.05$, compared with the vehicle control. [b]$P < 0.05$, compared with the ZNP control.

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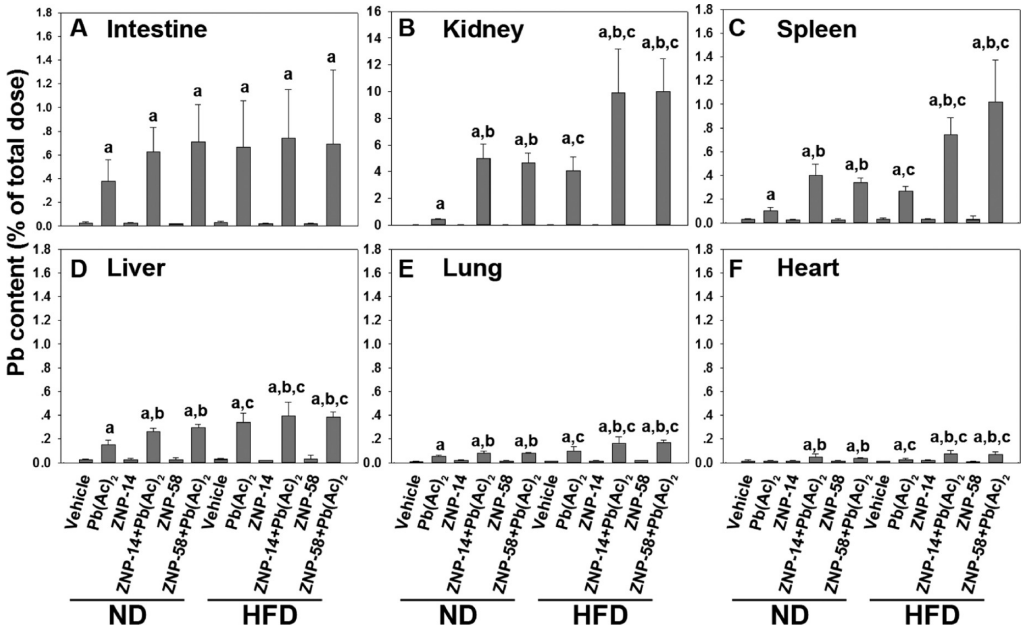

Figure 4. Distribution of Pb in the major organs (mean ± standard deviation; $n = 5$). The data are presented as the Pb concentration determined in the major organs as a percentage of the total exposure dose. [a]$P < 0.05$, compared with the vehicle control. [b]$P < 0.05$, compared with the $Pb(Ac)_2$ control. [c]$P < 0.05$, compared with the ND-fed mice with the same treatment.

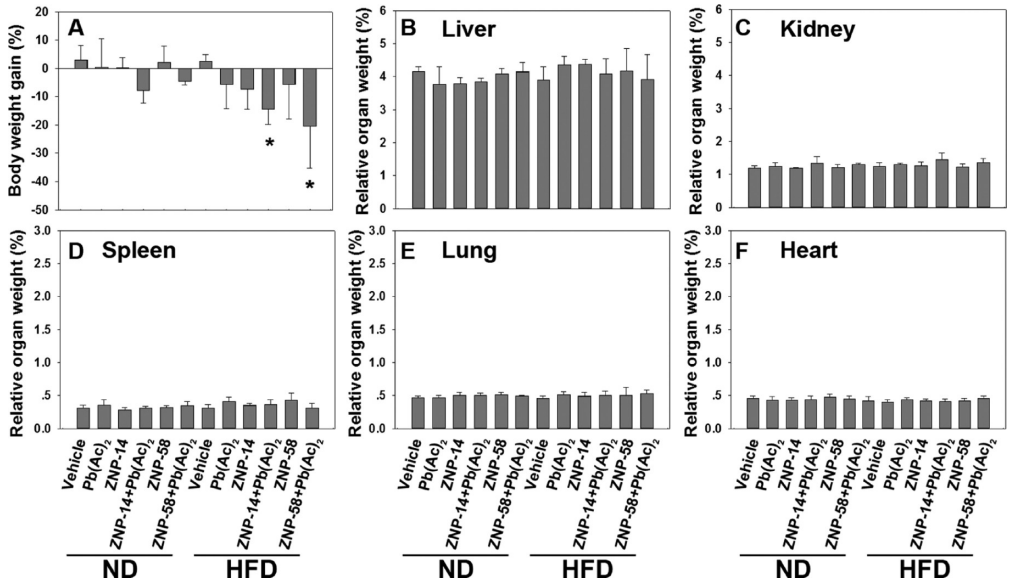

Figure 5. (A) Body weight gain and (B–F) relative organ weights of the ND and HFD mice after various treatments (means ± standard deviation; $n = 6$). The body weight gains in both ND and HFD mice after administration of ZNP, Pb^{2+}, and ZNP with Pb^{2+} were calculated as (final body weight after daily treatment for 2 weeks − initial bodyweight)/(initial body weight). After the various treatments, the major organs (liver, kidney, spleen, heart, and lung) of the ND and HFD mice were collected and weighed. The relative organ weights (organ weight/body weight) were calculated. *$P < 0.05$, compared with the vehicle control.

study, we observed that the rate of deposition of Pb in HFD mice exposed to Pb^{2+} alone was increased 9.7-, 2.7-, 2.3-, 1.9-, and 1.8-fold in the kidney, spleen, liver, lungs, and heart, respectively, compared with the levels in the ND mice under the same conditions (Figure 4).

Although the nanoparticle-assisted accumulation of heavy metal ions in the organs of healthy adult mice[9,10] and pregnant mice[30] has been reported, the underlying mechanism is still not clear. In our case, adsorption of Pb^{2+} increased the ζ potential values of both ZNP-14 and ZNP-58 (Table 1). On one hand, ZNPs might act as carriers to assist Pb^{2+} deposition in organs. On the other, the ZNP/Pb^{2+} nanoadducts deposited in the various organs should have a clearance rate lower than that of free Pb^{2+} ions. These may be responsible for the enhanced Pb^{2+} deposition and accumulation in organs under the co-administration conditions. Another possibility is that co-administration might enhance Pb^{2+} deposition in the various organs by causing nanoparticle-induced damage to various organ barriers. TiO_2 NPs were shown to damage the blood–milk barriers, which permitted the transfer of nanoparticles to pups.[31] These nanoparticles also damaged the cell membranes and increased the rate of cellular uptake of Cd^{2+} metal ions.[32]

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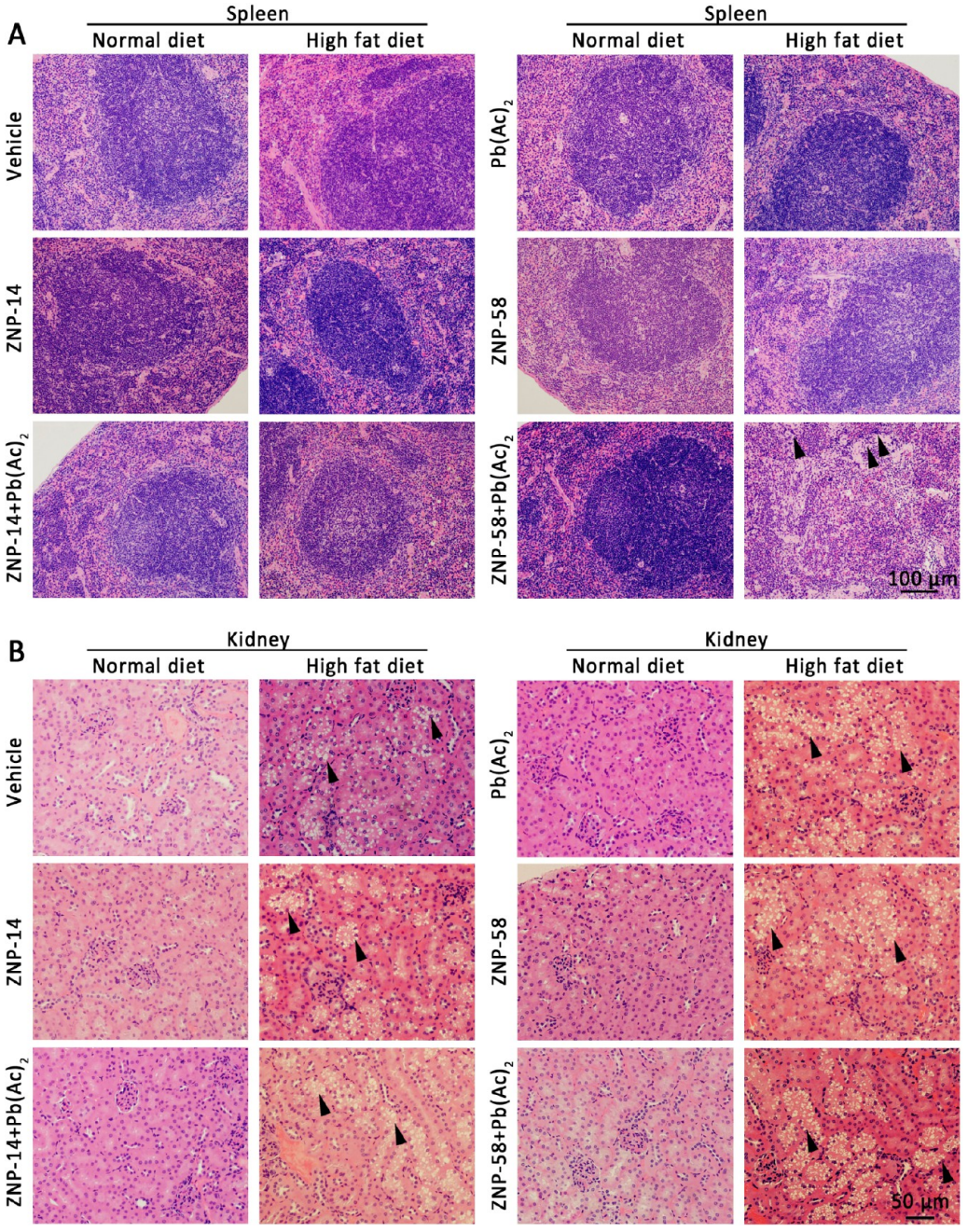

Figure 6. Histological examination of the spleen and kidney in ND and HFD mice after treatment with the ZNPs and $Pb(Ac)_2$. (A) Histopathology of the spleen tissue in ND and HFD mice after exposure to ZNPs and $Pb(Ac)_2$ by daily oral administration for 14 days. The triangles in panel A indicate a slight increase in the number of fibroblasts that was observed only in the spleens of the HFD mice co-exposed to ZNP-58 and $Pb(Ac)_2$. (B) Histopathology of the kidney tissues in the ND and HFD mice after exposure to ZNPs and $Pb(Ac)_2$ by daily oral administration for 14 days. The arrows in panel B indicate the vacuolar degeneration in the epithelia of the renal tubules.

An increased rate of deposition of Pb^{2+} in the major organs of the ND and HFD mice under co-administration conditions could result in enhanced organ injuries. These were further examined experimentally.

Susceptibility of Overweight Mice to ZNP and $Pb(Ac)_2$. To evaluate the toxicity caused by the oral exposures to the ZNPs and ZNP/Pb^{2+} adducts, we first examined the body weight gains and relative organ weights of the mice after various treatments. Noticeable body weight losses of 15 and 20% were detected in HFD mice after treatment with ZNP-14 with Pb^{2+} and ZNP-58 with Pb^{2+}, respectively (Figure 5A). In contrast, there was no loss of body weight in the ND mice after the same treatments or in HFD mice after the ZNP-only or Pb^{2+}-only treatments (Figure 5A). These data demonstrated that the overweight mice were more susceptible to the toxic effects caused by the ZNP/Pb^{2+} complex than were the normal adult mice. Although we did not observe any alterations in the relative weights of the major organs (Figure 5B–F), we performed more investigations of the integrity of the various organs.

Weak Toxic Effects on the Spleen or the Kidneys. Because higher levels of Pb accumulated in the spleen and the kidneys than in the other organs (Figure 4), we first analyzed the pathologic changes in the spleen and the kidneys in the ND

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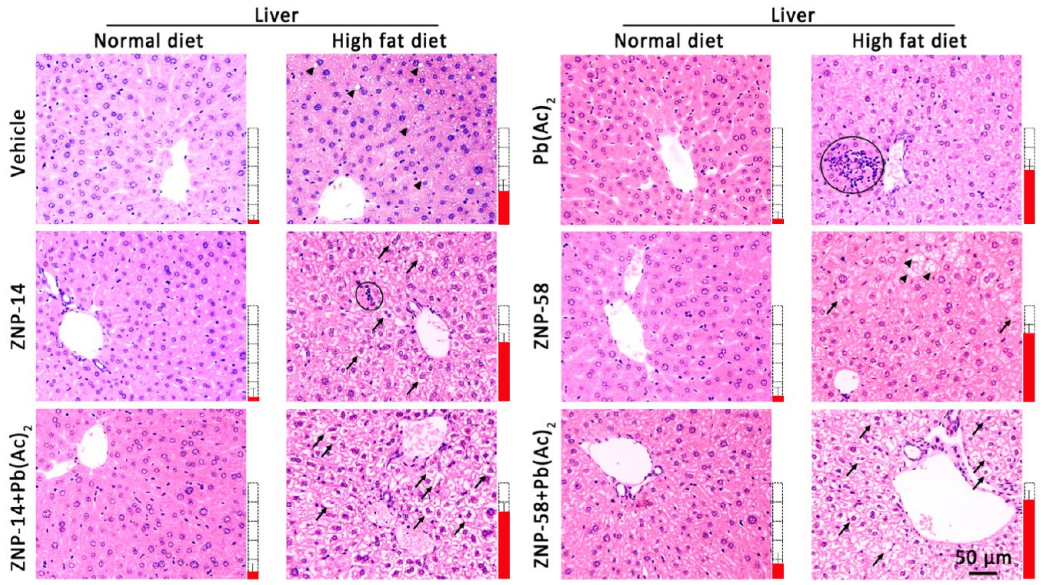

Figure 7. Pathologic alterations in the ND and HFD mice after various treatments. Histological examination of the liver after the various treatments indicated liver injury. Steatosis, vacuolar degeneration, and spotty necrosis are marked with triangles, arrows, and circles, respectively. The scores for the degree of liver injury on the basis of the images in the H&E-stained pathological sections of the liver tissues are shown on the right of each panel.

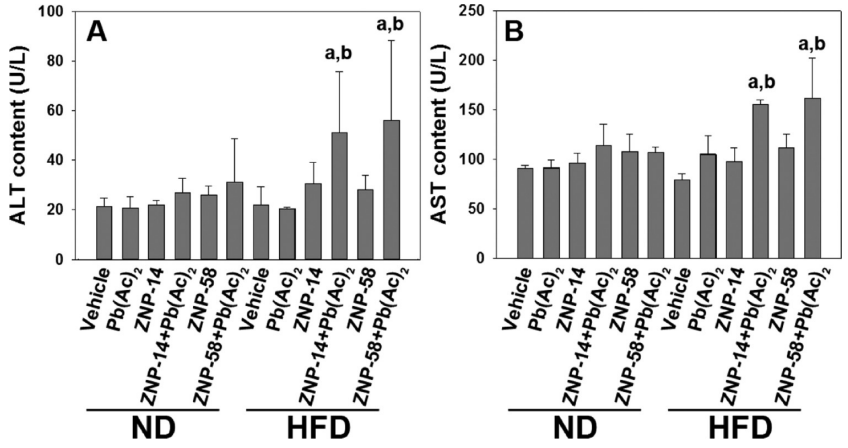

Figure 8. ZNP and $Pb(Ac)_2$ co-administration affected the liver function of the HFD mice. Liver function tests were performed by measuring the serum (A) ALT and (B) AST levels in both the ND and HFD mice after the various treatments. Noticeably increased levels of ALT and AST were observed only in the HFD mice co-exposed to ZNPs and Pb^{2+}. $^{a}P < 0.05$, compared with exposure to only Pb^{2+}. $^{b}P < 0.05$, compared with exposure to only ZNP.

and HFD mice after the various treatments. A slight increase in the number of fibroblasts was found in the spleens of the HFD mice after treatment with ZNP-58 and Pb^{2+} (Figure 6A). In the kidneys, HFD feeding alone caused vacuolar degeneration in epithelia of renal tubules (Figure 6B). However, no exacerbation of the renal injury was observed in the kidneys of the HFD mice after exposure to ZNP and Pb^{2+}. These data suggested that only a minor toxic effect of ZNP and Pb^{2+} in the spleen and the kidneys occurred in both the ND and HFD mice under our experimental conditions, despite the accumulation of Pb.

The kidney is a target organ for Pb^{2+}-induced toxicity.[33−35] Pathologic changes that were characterized by tubular atrophy and interstitial fibrosis in the kidneys first appeared at 6 months when rats were given 0.5% $Pb(Ac)_2$ in their drinking water (~55 mg/kg/day).[33] More severe damage was found after exposing rats to $Pb(Ac)_2$ (2% in drinking water or 220 mg/kg/day) for 2 months.[35] In the study presented here, both normal and overweight mice were exposed to $Pb(Ac)_2$ at a dose of 150 mg/kg/day for 14 days. Although the HFD feeding and the existence of ZNPs enhanced the deposition of Pb in the kidneys, the relatively short exposure period might be responsible for the less noticeable pathologic changes in the kidneys.

Enhanced Liver Injury in HFD Mice under Co-Administration Conditions. The liver is a major organ for clearing xenobiotic chemicals and a target organ for nanoparticle accumulation.[36] Oral administration of ZNPs at a dose higher than 300 mg/kg induced liver injury in healthy adult mice, as indicated by increased levels of plasma ALT and AST.[26,27] The overweight mice tested in this study all suffered from an average degree of steatosis or fatty liver disease. The fatty livers of HFD mice become more sensitive to environmental pollutants.[23,37] Therefore, we next analyzed the hepatic injury in the ND and HFD mice after the treatments with ZNP and $Pb(Ac)_2$. Exposure of the ND mice to ZNP-14 and ZNP-

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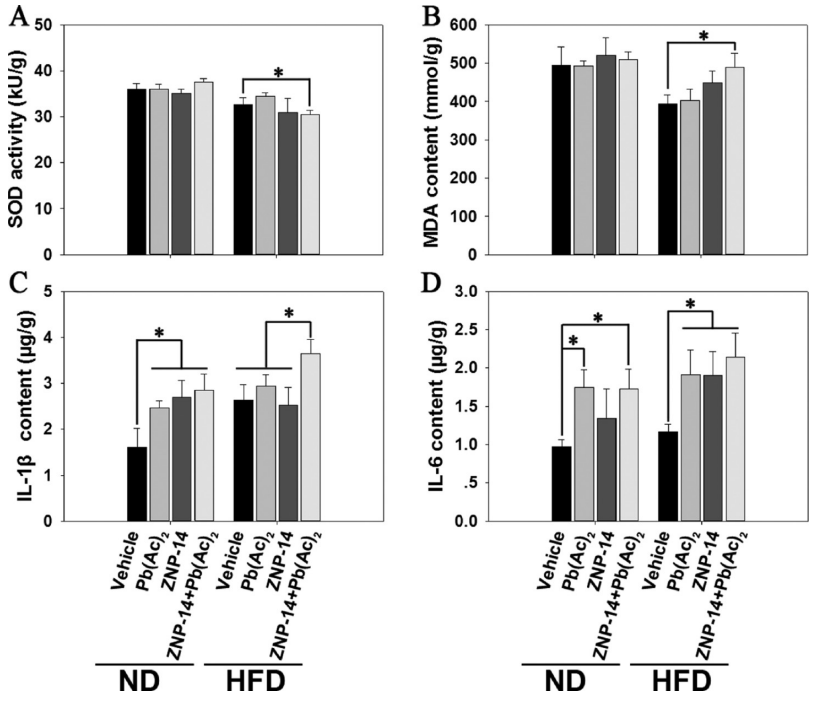

Figure 9. Hepatic ROS and pro-inflammatory cytokine production in mice treated with ZNP-14 (200 mg/kg) and/or $Pb(Ac)_2$ (150 mg/kg). The hepatic (A) SOD activity and (B) MDA content were determined after administration of the various combinations of agents. The inflammation in the liver was confirmed by measuring the levels of hepatic (C) IL-1β and (D) IL-6. *$P < 0.05$, analyzed using Sigmaplot 12.0.

58 (200 mg/kg) and/or $Pb(Ac)_2$ (150 mg/kg) did not induce liver injury, with the exception of a slight vacuolar degeneration in the liver after the co-administration of ZNP-58 and Pb^{2+} (Figure 7). The same exposures in the HFD mice induced significant liver injury. In addition to the HFD-induced hepatic steatosis in HFD mice, $Pb(Ac)_2$ exposure at a dose of 150 mg/kg induced spotty cell necrosis and a mild vacuolar degeneration in the liver (Figure 7). Exposure to both ZNP-14 and ZNP-58 at a dose of 200 mg/kg also induced moderate vacuolar degeneration (Figure 7). These results showed that individual $Pb(Ac)_2$ or ZNP administration enhanced the hepatic injury in the HFD mice but caused no harm in ND mice. Furthermore, co-exposure of ZNP-14 or ZNP-58 with $Pb(Ac)_2$ caused severe vacuolar degeneration in the livers of the HFD mice (Figure 7). The degree of liver injury was scored on the basis of pathologic examinations of 50 microscopic fields (10 liver slices) from five different mice, and the results are shown in Figure 7. To substantiate these findings, we also analyzed the serum ALT and AST levels in the mice. The serum ALT and AST levels in HFD mice after treatment with ZNP-14 and Pb^{2+} were 2.3- and 2.0-fold higher, respectively, than those of the vehicle-treated control mice. For the animals treated with ZNP-58 and Pb^{2+}, the levels were 2.6- and 2.1-fold higher, respectively (Figure 8A,B), whereas no alterations were observed in the ND mice subjected to the same treatments or in the HFD mice after treatment with either ZNP or Pb^{2+} alone. Together, these data demonstrated that exposure of HFD mice to ZNP-14 and Pb^{2+} or ZNP-58 and Pb^{2+} caused more severe liver damage than in the ND mice subjected to the same treatments.

Increased Hepatic ROS and Pro-Inflammatory Cytokine Production in HFD Mice. An increase in oxidative stress may be a key determinant of both Pb^{2+}- and ZNP-induced toxicity.[27,38] Because heavy accumulations of both ZNPs and Pb^{2+} were observed in the livers, we performed a series of experiments to determine the ROS levels in the livers of both ND and HFD mice. We treated mice with ZNP-14 and/or $Pb(Ac)_2$ and determined the changes in reactive oxygen species (ROS) production in the liver. A reduction in the SOD activity and an increase in the MDA content were observed only in the HFD mice after treatment with ZNP-14 and $Pb(Ac)_2$, which indicated that the treatment with ZNP-14 and $Pb(Ac)_2$ induced oxidative stress in the livers (Figure 9A,B). The same treatment did not induce an increase in the ROS of the livers of the ND mice.

Inflammation is a protective response of the body against harmful stimuli. Previous studies have shown that Pb^{2+} exposure caused an increase in the level of production of pro-inflammatory cytokines such as IL-1β and IL-6 both *in vitro* and *in vivo*.[39−41] In addition, inflammatory responses accompany nanoparticle-mediated toxicity.[42,43] Therefore, we further measured the hepatic pro-inflammatory cytokine production in mice after treatment with ZNP-14 and Pb^{2+}. In the ND mice, the hepatic IL-1β and IL-6 contents were both 1.8-fold higher than those of the vehicle control after administration of ZNP-14 and $Pb(Ac)_2$ (Figure 9C,D) in the absence of significant inflammatory injury in the liver (Figure 7). In contrast, in the HFD mice, the hepatic IL-1β and IL-6 levels were 1.4- and 1.8-fold higher, respectively, than the control levels after the treatment with ZNP-14 and $Pb(Ac)_2$. The hepatic IL-1β and IL-6 levels in the HFD mice were 1.3- and 1.2-fold higher, respectively, than those in the ND mice after the treatment with ZNP-14 and $Pb(Ac)_2$, which suggested a higher level of inflammation in the livers of the HFD mice.

Pb^{2+} causes oxidative damage in liver tissues by enhancing lipid peroxidation in the cell membranes[44,45] and inhibiting the activity of antioxidants,[46] which result in the derangement of hepatic biochemical pathways and energy metabolism.[47] These toxic effects depend on the dosage and exposure time of the Pb^{2+} treatment. Furthermore, Pb^{2+} enhances LPS-induced liver

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injury, which suggests that it has a pro-inflammatory effect.[48] Here, our findings show that co-exposure to ZNP and Pb^{2+} enhanced the liver deposition of Pb^{2+}, the liver ROS levels, and the pro-inflammatory cytokine production, which eventually resulted in liver injuries.

The toxicity of environment pollution is due to the combined effects from all pollutant components to which we are exposed. The main drinking water pollutants include nanoparticles with their payloads of pollutants, such as heavy metal ions. To evaluate the toxicity of such nanoadducts in susceptible overweight populations, we here investigated the effects of co-exposure to ZNPs and $Pb(Ac)_2$ in overweight mice. Such co-exposures enhanced the absorption and organ deposition of Pb^{2+}, especially in the overweight mice in which the HFD had already increased the intestinal permeability to Pb^{2+}. These effects led to greater deposition of Pb^{2+} in all major organs in the overweight mice compared with that in normal weight mice. Co-administration of ZNPs and $Pb(Ac)_2$ at doses that produced little toxicity in the normal weight mice induced subacute toxicity only in the overweight mice. Specifically, co-administration of ZNP and $Pb(Ac)_2$ increased the levels of ROS and inflammation, induced severe vacuolar degeneration in the liver, and increased the plasma levels of ALT and AST only in overweight mice. The findings that the overweight mice are more susceptible to nanoparticle- and $Pb(Ac)_2$-induced toxicity argue that more caution is needed regarding exposure to nanoparticle/heavy metal adducts in overweight populations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b05200.

Composition of the high-fat diet (Table S1) and dissolution analysis of ZNPs in water and acidic gastric fluid (Figure S1) (PDF)

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The authors declare no competing financial interest.

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