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Oxidative injury in the mouse spleen caused by lanthanides

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ABSTRACT

The organ-toxicity of high-dose lanthanides on organisms had been recognized, but very little is known about the injury of immune organ such as spleen induced by Ln. In order to understand the splenic toxicity of Ln, various biochemical and chemical parameters were assayed in the mouse spleen. Abdominal exposure to $LaCl_3$, $CeCl_3$, and $NdCl_3$ at dose of 20 mg/kg body weight caused splenomegaly and oxidative stress to the spleen. Evident Ln deposition, congestion, mitochondria swelling, and apoptosis in the spleen could be observed, followed by increased generation of reactive oxygen species, lipid peroxidation and SOD activity, and decreased GSH-Px activity as well as nonenzymatic antioxidants such as glutathione and ascorbic acid content. In addition, the high amount of NO and increased NOS activities caused by Ln were measured. Furthermore, both Ce^{3+} and Nd^{3+} exhibited higher oxidative stress and toxicity on spleen than La^{3+}, and Ce^{3+} caused more serve splenocyte injuries and oxidative stress than Nd^{3+}, implying that the difference in the splenic injuries caused by Ln was related to the number of 4f electrons of Ln.

1. Introduction

With their widespread application in agriculture, industry, culture, medicine, and daily life, lanthanides (Ln) compounds are being brought into the ecological environment and human body through food chains [1–3]. It is important to know the acute and chronic effects of Ln on the environment, nature balance, and the human body after their entry into bodies and the environment. The studies on the toxicology of Ln showed that lanthanide ions (Ln^{3+}) had adverse effects on organs such as the liver, kidney and lung as well as the nervous system of animals, e.g., the lesion caused by Ln showed oxidative stress, disturbance of the homeostasis of essential elements and enzymes as well as histopathological changes [4–7,14]. However, the studies focused the biological and toxic effects of Ln primarily on single Ln and their mixtures in animals. In the study of the effects on vigor of aged spinach seeds caused by $LaCl_3$, $CeCl_3$ and $NdCl_3$, Liu et al. showed that the effects of Ce^{3+} were most significant, then followed by Nd^{3+} while La^{3+} was not as effective as Ce^{3+} and Nd^{3+} [8]. Li et al. found an increase in ketone bodies, creatinine, lactate, succinate and various amino acid in the serum of rats intraperitoneally exposed to La^{3+} and Ce^{3+} at doses of 10 and 50 mg/kg body weight after 48 h by MAS ^{1}H NMR spectroscopic-based metabonomic approach, together with a decrease in glucose in the serum from Ce^{3+}-treated groups, thus they thought that both La^{3+} and Ce^{3+} at high doses impaired the specific region of liver and Ce^{3+} exhibited a higher toxicity than La^{3+} at the same dose [9]. Liao et al. also demonstrated that Ce^{3+} caused lesions on liver and kidney in rats while La^{3+} only caused liver injury at the same dose [10], and Nd^{3+} had similar acute toxicity to Ce^{3+} [11]. These studies suggested that La^{3+}, Ce^{3+} and Nd^{3+} had different biological effects on organisms and Ce^{3+} or Nd^{3+} had a stronger effect than La^{3+} at the same dose.

As we know, Ln belong to the IIIB family in the periodic table of elements. The special electronic configuration in Ln is the occupation of 4f orbitals: the outer-shell 5s, 5p and 6s orbitals are occupied completely in the closed-shell (no electron or only one electron in 5d orbital); while the inner-shell seven 4f-orbitals are occupied one by one incompletely in the open shell according to the increase of the atomic number (0–14). And all Ln form stable triple-charged state when they lose outer electrons and the electron configuration of Ln^{3+} ions extends from f^0 (La^{3+}) to f^{14} (Lu^{3+}) regularly. Thus La^{3+} has no f electron, Ce^{3+} has one and Nd^{3+} has three f electron, respectively. Moreover, according to the Hund's rule, the empty (f^0), half-filled (f^7) and the completely filled shell (f^{14}) are in stable state. So Ce^{3+} (f^1) can easily lose an electron to be oxidized to Ce^{4+} (f^0) [1,12,13]. Ln as the 4f group elements varied only in the number of 4f electrons, their chemical properties are similar. Based on the small amount of available data, Kostova et al. proposed that the difference in the number of 4f electrons leads to quite different biological properties of Ln [15]. Do Ln behave differently in biological effects determined by the 4f electron? It deserves to be investi-

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gated. In addition, the previous studies on bio-toxicity of Ln were generally concentrated on organs such as lung, liver, and kidney and suggested that the damages were related to the oxidative stress caused by Ln [5,14,16]. The researches on splenic toxicity of Ln are rarely reported. Spleen is the largest immune organ in humans, participating in immune response, generating lymphocytes, eliminating aging erythrocytes and storing blood. Is the bio-toxicity of Ln on spleen also related to the oxidative injuries? The Ln-induced toxicity on spleen needs investigation.

In this paper, spleen indices, the deposition of Ln, the changes of histopathological and cellular ultrastructure, the level of nitric oxide and nitric oxide synthase as well as antioxidant system in the mouse spleen were investigated to understand the splenic injury in mice caused by Ln.

2. Materials and methods

2.1. Reagent

$LaCl_3$, $CeCl_3$, and $NdCl_3$ were purchased from Shanghai Chem. Co. and were analytical-grade.

2.2. Animals and treatment

Male CD-1 (ICR) mice (~25 g) were obtained from the Animal Center of Soochow University, Suzhou, China. They were 4–6 weeks old upon arrival and allowed to acclimatize in an environment-controlled animal room (temperature, 26 ± 1 °C; relative humidity, 50 ± 5%; photoperiod, 12 h light/dark cycle) for 7 days prior to treatment. Distilled water and sterilized food were provided *ad libitum*. All animal procedures were performed in compliance with the international ethics committee regulations and guidelines on animal welfare. Animals were randomly divided into four groups: control group and Ln^{3+}-treated groups (La^{3+}, Ce^{3+}, Nd^{3+}) ($n = 15$ in each group). Ln^{3+}-treated groups were given $LaCl_3$, $CeCl_3$, and $NdCl_3$ dissolved in saline at a dose of 20 mg/kg body weight (BW)/day by intraperitoneal injection, respectively; and control group was treated with the equivalent saline. The symptom and mortality were observed and recorded carefully everyday. After 14-day intraperitoneal injection, all animals were weighed and anaesthetized prior to spleen collection. The spleens were removed immediately, blotted, weighted and stored at −80 °C until further analysis. Spleen indices was expressed as the spleen weight (mg) relative to body weight (g). And portions of the spleen were fixed in 10% neutral buffered formalin and 4% glutaraldehyde for tissue fixation, respectively.

2.3. Ln content analysis in spleen

About 0.1–0.2 g of spleen were weighed, digested, and analyzed for Ln content. Briefly, prior to elemental analysis, the tissues of interest were digested in nitric acid (ultrapure grade) overnight. After adding 0.5 ml of H_2O_2, the mixed solutions were heated at about 160 °C using high-pressure reaction container in an oven chamber until the samples were completely digested. Then, the solutions were heated at 120 °C to remove the remaining nitric acid until the solutions were colorless and clear. At last, the remaining solutions were diluted to 3 ml with 2% nitric acid. ICP-MS (Thermo Elemental X7, Thermo Electron Co.) was used to determine the REE concentration in the samples. Data are expressed as microgram per gram of fresh spleen.

2.4. Histological and transmission electron microscope (TEM) observation

For histological observations, the formalin-fixed splenic tissue samples were embedded in paraffin, thin-sectioned, and then mounted on glass microscope slides using the standard histopathological techniques. The mounted sections were stained with hematoxylin–eosin (H&E) and examined by light microscopy (Nikon U-III Multi-point Sensor System, USA).

Additionally, blocks of fresh spleen were fixed with 4% glutaraldehyde, rinsed in 0.2 mol/l phosphate buffer, postfixed in osmium tetroxid, dehydrated through a series of graded acetone, replaced in propylene oxide and embedded in Epon 812, ultrathin sections were made and double stained with uranium acetate and lead citrate and examined under a TEM (JEM-1200EX, JEOL Ltd., Tokyo, Japan).

2.5. ROS and lipid peroxidation levels assay

Superoxide ion ($O_2^{\bullet -}$) in the spleen tissue was measured as described by Oliveira et al. [17], by monitoring the reduction of XTT in the presence of $O_2^{\bullet -}$, with some modifications. The spleen was homogenized with 2 ml of 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 5000 × *g* for 10 min. The reaction mixture (1 ml) contained 50 mM Tris–HCl buffer (pH 7.5), 20 μg spleen protein, and 0.5 mM sodium, 3′-[1-[phenylamino-carbonyl]-3,4-tetrzolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT). The reaction of XTT was determined at 470 nm for 5 min. Correction was made for the background absorbance in the presence of 50 units of SOD. The production rate of $O_2^{\bullet -}$ was calculated using an extinction coefficient of $2.16 \times 10^4\ M^{-1}\ cm^{-1}$.

The detection of H_2O_2 production in the spleen tissue was carried out by flow cytometry using 2′-7′-dichlorofluoroscein diacetate (DCFH-DA; Sigma). DCFH-DA was added (10 μM final concentration) to spleen and the mixture was incubated for 30 min at 37 °C. After the incubation, cells were subjected to flow cytometry analysis (FACScan; Becton Dickinson) [18].

Splenic lipid peroxidation was determined as the concentration of malondialdehyde (MDA) generated by the thiobarbituric acid (TBA) reaction as described by Buege and Aust [19], with the introduction of a isobutanol extraction step for the removal of interfering compounds. For analysis, a subsample of the tissue was thawed, homogenized, and cells lysed using a 4% TBA solution in 0.2 M HCl. The reaction mixture was then incubated at 90 °C for 45 min. The resulting TBA–MDA adduct was phase-extracted using isobutanol. The isobutanol phase was then read at a wavelength of 535 nm on a UV-3010 spectrophotometer. MDA standard curves were prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane (TMP).

2.6. Antioxidant assay

The spleen was homogenized in 1 ml of ice-cold 50 mM sodium phosphate (pH 7.0) that contained 1% polyvinyl polypyrrolidone (PVPP). The homogenate was centrifuged at 30,000 × *g* for 30 min and the supernatant was used for assay of activity of SOD. The SOD activity in spleen was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 3 ml reaction mixture contained 50 mM sodium phosphate (pH 7.8), 13 μM methionine, 75 μM NBT, 2 μM riboflavin, 100 μM EDTA, and 200 μl of the enzyme extract. Monitoring the increase in absorbance at 560 nm followed the production of blue formazan [20]. One enzyme unit of SOD activity was defined as the amount of SOD inhibiting the reduction of NBT by 50% in 1 mg protein of tissue. In the remaining aliquot, proteins were assayed according to the method of Lowry [21].

Spleen samples were homogenized in ice-cold 0.9% saline and centrifuged at 3000 × *g* for 10 min at 4 °C. The measurement of GSH-Px activity in spleen homogenate was performed according to the kit protocol (Nanjing Jiancheng Bioengineering Institute, China) and based on the following principle: GSH-Px catalyzed the reduced glutathione to oxidized glutathione by H_2O_2-induced oxidation. A yellow product was produced by reduced glutathione reacting with dithiobisnitrobenzoic acid and had absorbance at 412 nm. One GSH-Px unit was defined as the amount of the enzyme which lowered the concentration of reduced glutathione 1 μM/min at 37 °C in 1 mg protein of tissue.

Ascorbic acid (AsA) determination in spleen was performed as described by Jacques-Silva et al. [22]. Protein was precipitated in 10 volumes of a cold 4% trichloroacetic acid (TCA) solution. An aliquot of homogenized sample (300 ml), in a final volume of 1 ml of the solution, was incubated at 38 °C for 3 h, then 1 ml H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml).

In order to perform the reduced glutathione (GSH) assay, the spleen homogenized in 1 ml of 25% H_3PO_4 and 3.5 ml of PBS (0.1 mol/l, pH 8.0). The homogenate was centrifuged at 5000 × *g* for 30 min at 4 °C and the supernatant was used for assay. GSH contents were estimated using the method of Hissin and Hilf [23]. The reaction mixture contained 100 μl of supernatant, 100 μl *o*-phthaldehyde (1 mg/ml), and 1.8 ml phosphate buffer (0.1 M sodium phosphate, 0.005 M EDTA, pH 8.0). Fluorometry was performed using a F-4500 fluorometer (F-4500, Hitachi Co., Japan) with excitation at 350 nm and emission at 420 nm.

2.7. Nitric oxide (NO) and nitric oxide synthase (NOS) measurement

Spleen samples were homogenized in ice-cold 0.9% saline and centrifuged at 3000 × *g* for 10 min at 4 °C. The supernatants were taken for the determination of NO concentration and NOS activity. Assays were performed according to the kit protocols (Nanjing Jiancheng Bioengineering Institute). The OD value was determined by a spectrophotometer (U-3010, Hitachi, Japan). Results of NO were read with OD value at 550 nm. The result was calculated using the following formula: $NO\ (\mu mol/l) = (A_{sample} - A_{blank})/(A_{standard} - A_{blank}) \times 20\ (\mu mol/l)$. Results of NOS were read with OD value at 530 nm. Activities of total NOS (TNOS), inducible NOS (iNOS) and structural NOS can be evaluated at the same time by U/mg protein. The content of protein was determined following the Lowry method [21]. Each parameter was determined in five animals.

2.8. Statistical analysis

Results were analyzed by analysis of variance (ANOVA). When analyzing the variance treatment effect ($p \leq 0.05$); the least standard deviation (LSD) test was applied to make a comparison between means at the 0.05 levels of significance.

Table 1
The weight gain and spleen indices of ICR mice after intraperitoneal injection with Ln^{3+} solutions for 14 days.

Indexes	Experimental group (20 mg/kg BW)			
	Control	La	Ce	Nd
Weight gain (g)	8.37 ± 1.35	7.64 ± 1.67	7.46 ± 1.74	7.97 ± 2.40
Spleen/BW (mg/g)	3.73 ± 0.83	4.88 ± 0.49**	5.10 ± 0.85**	5.19 ± 0.73**

Values are represented as means ± S.D., $n = 15$. Ranks marked with double stars were different from various groups in that panel at the 1% confidence level.

3. Results

3.1. Body weight and spleen indices in mice

During the treatment, animals were all at growth state. The daily behaviors such as feeding, drinking and activity in Ln^{3+}-treated groups were as normal as the control group. Table 1 shows the weight gain and spleen indices of mice after the 14-day administration. The weight gain of mice by Ln^{3+} treatments were lower than the control, but the differences were not statistically significant ($p > 0.05$). The spleen indices in Ln^{3+}-treated groups was significantly higher than the control ($p < 0.001$), i.e., Nd^{3+} treatment was most significant, next came Ce^{3+} treatment and then La^{3+} treatment.

3.2. Histopathological and ultrastructure evaluation

The histological photomicrographs of the spleen sections are shown in Fig. 1. The congestion of the spleen tissue was showed in the La^{3+}-treated group (Fig. 1b) and lymph nodule proliferation was observed in the Nd^{3+}-treated group (Fig. 1d), while no severe damages of spleen tissue were reflected in the Ce^{3+}-treated group (Fig. 1c). The changes of splenocyte ultrastructure in the mice spleen are presented in Fig. 2. Control group presented a normal ultrastructure (Fig. 2a). The erythrocytosis was clearly observed in the La^{3+}-treated group (Fig. 2b), and the Ce^{3+}-treated group exhibited apoptotic cells and the apoptotic body (Fig. 2c), and then the Nd^{3+}-treated group displayed significant mitochondria swelling (Fig. 2d). Taken together, Ln indeed damaged the spleen tissue, and the splenocyte injury in the mouse caused by Ce^{3+} was most severe; the injury caused by Nd^{3+} was slighter than Ce^{3+} but more severe than La^{3+}.

3.3. Ln deposition

The contents of administered Ln in the mouse spleen after the 14-day intraperitoneal injection are shown in Fig. 3. With Ln injection, the Ln contents in the spleen were significantly elevated, but the deposition of the three lanthanides was different, i.e., the order of the deposited concentration was $Nd^{3+} > Ce^{3+} > La^{3+}$.

3.4. Lipid peroxidation and ROS accumulation

The effects of Ln^{3+} on the production rate of $O_2^{\bullet -}$ and H_2O_2, and the level of lipid peroxidation (MDA content) in the mouse spleen are shown in Table 2. The generating rate of $O_2^{\bullet -}$ and H_2O_2 and the MDA contents from the Ln^{3+}-treated groups were significantly increased, and the order was Ce > Nd > La > control ($p < 0.05$ or 0.01). The result demonstrated that Ln at a dose of 20 mg/kg BW caused lipid peroxidation and oxidative stress in the mouse spleen.

3.5. Antioxidant systems

The activities of SOD and GSH-PX and the contents of AsA and GSH in the mouse spleen were listed in Table 3. The SOD activity was significantly increased by Ce^{3+} and Nd^{3+} treatments compared to the control ($p < 0.05$ or 0.01), while there was no statistical difference in La^{3+}-treated group ($p > 0.05$). However, the obvious

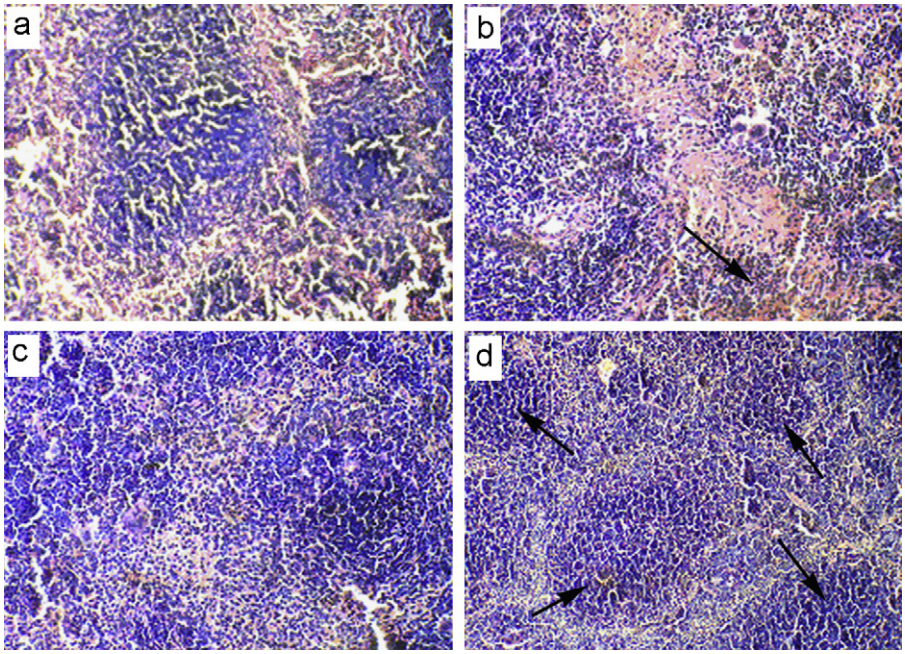

Fig. 1. Photomicrographs of the mouse spleen after intraperitoneal injection with Ln^{3+} solutions for 14 days (H&E, × original magnification). (a) Control group (100×); (b) La^{3+}-treated group (200×) presents congestion (arrow shows); (c) Ce^{3+}-treated group (200×) presents no abnormal; (d) Nd^{3+}-treated group (100×) presents lymph nodule proliferation (arrow shows).

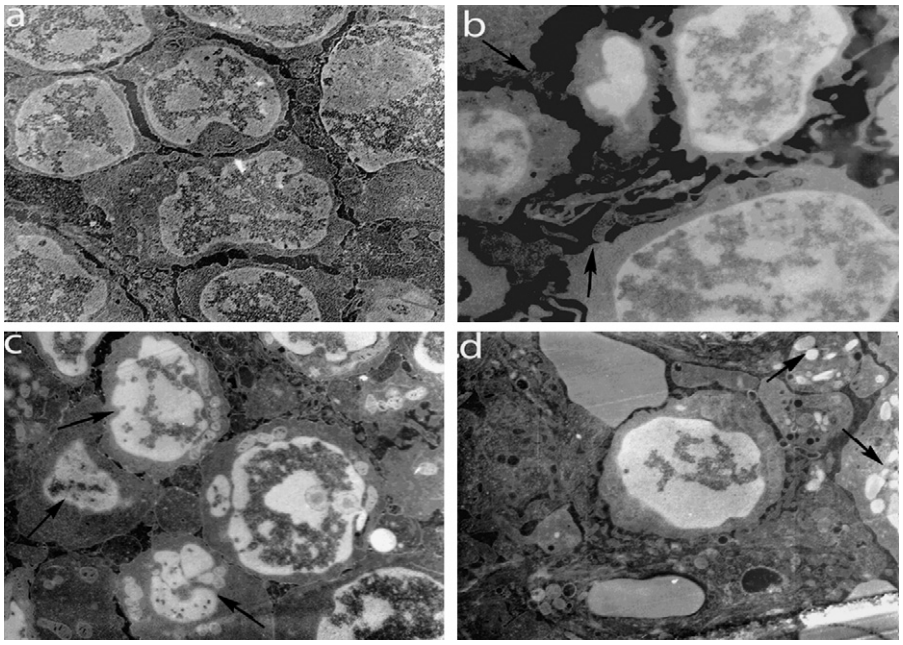

Fig. 2. TEM images of the mouse splenocyte after intraperitoneal injection with Ln^{3+} solutions for 14 days. (a) Control group (6000×); (b) La^{3+} group (8000×) (arrows indicate erythrocyte aggregation); (c) Ce^{3+} group (6000×) (arrows indicate apoptotic cells); (d) Nd^{3+} group (10,000×) (arrows indicate mitochondria swelling).

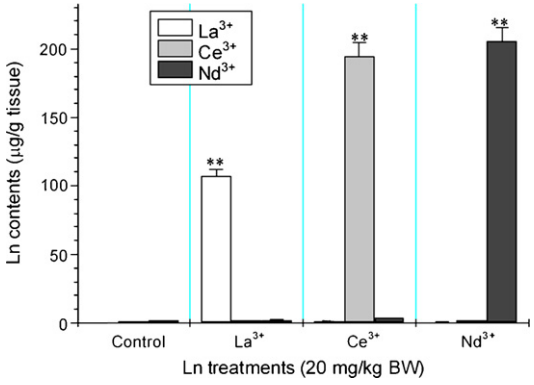

Fig. 3. Ln contents in the mouse spleen after intraperitoneal injection with Ln^{3+} solutions for 14 days. Bar marked with double stars means it is significantly different from the control at the 1% confidence level, respectively. Values are represented as means ± S.D., $n = 5$.

Table 2
The lipid peroxidation level abd the ROS accumulation in the mouse spleen after intraperitoneal injection with Ln^{3+} solutions for 14 days.

Indexes	MDA (μmol/g tissue)	$O_2^{\bullet -}$ (μmol/g tissue* min)	H_2O_2 (μmol/g tissue)
Control	1.83 ± 0.08	16.50 ± 0.83	0.53 ± 0.03
La^{3+}	2.13 ± 0.13*	20.01 ± 1.00*	0.76 ± 0.4*
Ce^{3+}	2.91 ± 0.09**	36.88 ± 1.84**	0.87 ± 0.04**
Nd^{3+}	2.45 ± 0.09**	35.82 ± 1.79**	0.81 ± 0.04**

Values are represented as means ± S.D., $n = 5$. Ranks marked with a star or double stars were different from various groups in that panel at the 5% or the 1% confidence level.

reduction of GSH-PX activities caused by Ln^{3+} was observed, and the decrease caused by Ce^{3+} treatment was most significant, next came from Nd^{3+} and then La^{3+}. The contents of AsA and GSH in the mouse spleen caused by Ln^{3+} were significantly lower than the control ($p < 0.05$ or 0.01) and the contents of AsA and GSH in Ce^{3+}-treated group was the lowest, next was in Nd^{3+}-treated group and then in La^{3+}-treated group (Table 3). All of the results indicated Ln caused oxidative stress in the mouse spleen by inducing lipid peroxidation and reducing antioxidant capacity.

3.6. NO and NOS

The change of NO concentration in the splenic tissue was showed in Fig. 4. It can be seen that NO concentrations were significantly increased by Ln^{3+} treatments compared to the control ($p < 0.01$) and ranked in the order of Ce, Nd, La and control. Fig. 5 shows that Ln^{3+} treatments significantly elevated the activities of iNOS and TNOS in the mouse spleen ($p < 0.05$), while no significant change in cNOS activity was observed ($p > 0.05$).

4. Discussion

In this experiment the mice growth was not obviously inhibited by Ln^{3+} at dose of 20 mg/kg, while the spleen is clearly sensitive to Ln action, where an increase in spleen weight/body weight ratio took place. Liu et al. indicated that the ratios of spleen to body weight of rats or mice were not modified after feeding mixture of $La(NO_3)_3$ and $Ce(NO_3)_3$ by oral administration for a month compared with the control [24,25]. As nonessential metal elements, overdose Ln^{3+} entering abdominal cavity must have adverse effects

Table 3
The changes of antioxidative enzyme activities and nonenzymatic antioxidants contents in the mouse spleen after intraperitoneal injection with Ln^{3+} solutions for 14 days.

Indexes	SOD (U/mg protein* min)	GSH-PX (U/mg protein* min)	AsA (mg/g tissue)	GSH (mg/g tissue)
Control	1.3 ± 0.67	626.92 ± 19.63	0.38 ± 0.01	1.32 ± 0.07
La^{3+}	13.38 ± 0.61	586.59 ± 40.09*	0.29 ± 0.02*	1.12 ± 0.06
Ce^{3+}	18.68 ± 0.49**	555.58 ± 31.22**	0.18 ± 0.01	0.78 ± 0.04**
Nd^{3+}	15.75 ± 0.48*	574.72 ± 23.11**	0.23 ± 0.01**	0.98 ± 0.05*

Values are represented as means ± S.D., $n = 5$. Ranks marked with a star or double stars were different from various groups in that panel at the 5% or the 1% confidence level.

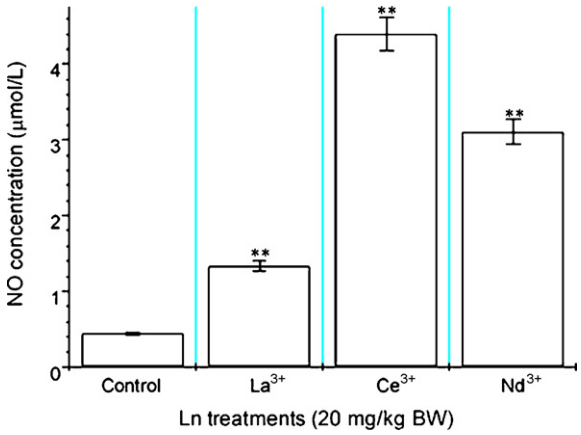

Fig. 4. NO concentration in the mouse spleen after intraperitoneal injection with Ln^{3+} solutions for 14 days. Bar marked with double stars means it is significantly different from the control at the 1% confidence level. Values are represented as means ± S.D., $n = 5$.

on spleen. However the effect of Ln on spleen differs with the routes of administration. By oral administration Ln goes on to the gastrointestinal tract where the liver and kidney are the fundamental target organs and it mainly eliminated by faeces. In the case of the intraperitoneal administration, Ln initially goes on to the peritoneal cavity and later to the blood where there no elements to eliminated Ln, and spleen becomes the target organ through blood stream. Thus intraperitoneal administration appeared to cause more pronounced effects on spleen than the oral administration.

Ln^{3+} were shown to enter the cells via multiple pathways and accumulated in some types of cells [26,27]. Our data suggested that the deposition of Ln in the mouse spleen (107–205 μg/g) was remarkable by intraperitoneal injection with Ln at dose of 20 mg/kg. The concentrations of Ln in liver, lung and kidney were also measured, finding concentrations in liver (2.66–19.5 μg/g), in lung (2.36–10.77 μg/g) and in kidney (1.23–9.08) were much lower than that in spleen. Thus it appears that the spleen is the major target organ for Ln deposition by intraperitoneal injection. Shinohara et al. observed the highest concentrations of Ln in the mouse spleen (179–310 μg/g) by intravenous injection with Tb, Sm and Yb at dose of 10 mg/kg BW, respectively [28]. Kawagoe et al. also reported that there was more cerium distributed in the spleen compared with the liver and the lung at 1 or 3 days after tail vein injection at a dose of 10 mg/kg in mice [29]. It is concluded that Ln were more easily deposited in spleen than liver, lung and kidney either by intraperitoneal injection or intravenous injection. It

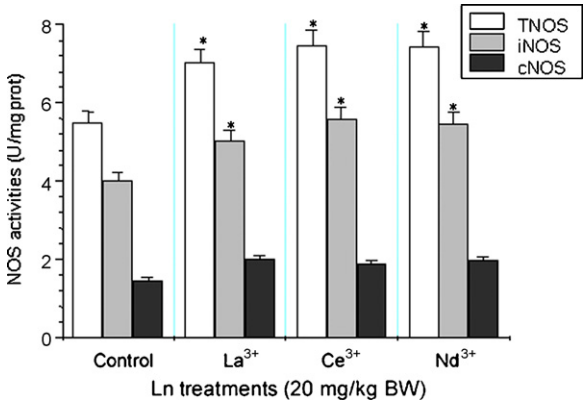

Fig. 5. NOS activities in the mouse spleen after intraperitoneal injection with Ln^{3+} solutions for 14 days. Bar marked with a star means it is significantly different from the control at the 5% confidence level. Values are represented as means ± S.D., $n = 5$.

is also interesting that the accumulation of administered elements was element-dependent in the spleen, e.g., Ce and Nd were more easily accumulated in the the mouse spleen than La. The accumulation of Ln might be closely related to 4f electron and the lanthanide contraction regularity of Ln, which needs further investigation.

Figs. 1 and 2 showed that Ln induced histopathological changes and ultrastructure damage of spleen such as MT swelling and cell apoptosis. Ln ions were known to induce MT swelling in several types of cells. The MT swelling suggested that Ln ions enter the cell, bind to the MT, and result in structural changes of MT and subsequent effects. Early studies have found that Ln ion such as Ce^{3+} and Gd^{3+} could induce cell apoptosis [30,31]. But the mechanisms underlying the apoptosis induced by Ln is complicated. Liu et al. suggested that Ln-induced apoptosis was associated with MT swelling and elevated cellular ROS levels [32]. Moreover the increased intracellular Ca^{2+} concentration which activates apoptosis-related gene expressions is considered to play an important role. And Ln can increase the intracellular Ca^{2+} level by increasing the Ca influx, which indirectly induced expressions of apoptosis-related gene [33].

ROS generation is the influencing factor to induce tissue injury. The interaction between H_2O_2 and $O_2^{\bullet -}$ can create $^{\bullet}OH$ and O_2, which are far more destructive and can peroxide the unsaturated lipid of the cell membrane [34]. Our data showed that the production rate of ROS (such as $O_2^{\bullet -}$, H_2O_2) in the spleen of mice by intraperitoneal injection of Ln^{3+} at dose of 20 mg/kg was significantly elevated (Table 2), indicating that the spleen suffered oxidative stress. It is interesting that Ln caused elevated level of cellular ROS although Ln ions were previously thought to be scavengers of free radicals in vitro [35–37]. The reasons for the generation of ROS are not understood. However, these ROS might be either from Ln-damaged MT or other Ln-triggered signal pathways [38]. As one of the most important products of lipid peroxidation, MDA can intensively react with various cellular components, seriously damaging enzymes and membranes and inducing the decrease of membranous electric resistance and fluidity, and this eventually leads to the destruction of the membrane structure and physiological integrality [39]. In this study, membrane lipid peroxidation in spleen demonstrated by the enhancement of MDA content was due to the production of overall free radicals induced by Ln (Table 2). Lipid peroxidation and oxidative damage of DNA were shown be induced by Ce^{3+} at high-concentration and reduced by Ce^{3+} at low-concentration [40]. It implied that the effect of Ln on ROS was related with the concentration of Ln, which still needs further studies both in vivo and vitro.

Furthermore, the overall ROS and lipid peroxidation in the spleen could be associated with the decrease of antioxidant defense. Organisms use a diverse array of enzymes like SOD, CAT, and GSH-PX as well as nonenzymatic antioxidants like ascorbate and GSH to decrease oxidative stress. SOD converts $O_2^{\bullet -}$ into H_2O_2 and O_2; moreover, CAT, APx, and GSH-Px reduces H_2O_2 into H_2O and O_2 [19,20]. Therefore, SOD, CAT, APx, and GSH-Px can keep a low level of ROS and prevent ROS from poisoning cells. Marubashi et al. found that YCl_3 significantly induced Mn-SOD in the rat lung by intratracheal instillation and thought that the induction of Mn-SOD was associated with the protection against oxidative stress in the rat lung [14]. Yang et al. reported that the concentrations of protein and MDA in the rat liver were increased, but the concentration of GSH and the activities of SOD, CAT, GSH-Px and GSH-ST were decreased after Ce^{3+} administration [41]. Shimada's research indicated that 200 mg/kg BW of Tb^{3+} treatment accelerated lipid peroxidation and inhibited activities of SOD and CAT in the mouse lung [16]. In this article, we observed that SOD activity was enhanced, while GSH-PX activity in the mouse spleen was significantly inhibited by Ln^{3+} (Table 3). It is well known that SOD is an inducible enzyme by $O_2^{\bullet -}$, and its activation in spleen is helpful

to scavenge excessive $O_2^{\bullet -}$, which is a protection against oxidative damage of spleen. And the decrease of GSH-PX activity is probably due to the inhibition of GSH-PX synthesis by Ln, but it needs to be further investigated. Additional evidence pointing to the possibility of oxidative stress was provided by the reduction in ascorbate and GSH contents in the spleen treated with Ln (Table 3). Ascorbate and GSH as effective nonenzymatic active oxygen scavengers can directly interact with and detoxify oxygen free radicals. The depletion of ascorbate and GSH in the mouse spleen caused by Ln was associated with the increases of ROS and MDA, accounting for that spleen utilized antioxidant defense system to prevent oxidative stress. The antioxidant assay also showed that the accumulation of ROS, the increase of lipid peroxidation level and the decrease of antioxidant capacity of spleen caused by Ce^{3+} was most significant, next came Nd^{3+}, and then by La^{3+}.

NO, identified in 1987 as a vasodilator of blood vessels, is an important intercellular mediator that regulates several physiological and pathophysiological processes (i.e. blood pressure and immune response) of higher organisms. In mammals, NO is generated by NOS, which consists of two principal forms: constitutive NOS (cNOS) and inducible NOS (iNOS). And the iNOS plays more important pathological role. The content of NO generated by iNOS is 1000 times as much as that generated by cNOS [42]. Our data showed that 20 mg/kg dose Ln could elevate significantly NO content and iNOS activity in the mouse spleen. Yang et al. treated rats with $Ce(NO_3)_3$ of 1 mg/kg and 50 mg/kg BW by intraperitoneal injection for 15 days, showing that NO level and NOS activity were significantly increased in the rat liver and kidney [43]. Shen et al. also demonstrated that the NO content and the NOS activity of hepatocytes were enhanced after exposure to Ce^{3+} [44]. One physiological effect of NO is that small amounts of NO kill tumour cells and regulate apoptosis. In contrast, high amounts of NO produced by iNOS would change cytosolic Ca^{2+} concentration, and thus activate iNOS and increase NO [45]. Both activation of cNOS and induction of iNOS are dependent on cytosolic Ca^{2+} concentration. As analogs to Ca^{2+}, Ln^{3+} could occupy or substitute for the position of Ca^{2+} after entering the body, and play a role of calcium channel blocker. The imbalance of Ca level can disturb the ion homeostasis and cause a series of physiological disorders in the immune system [43]. In this paper, Ln^{3+} uptaked by splenocyte could affect the homeostasis of cytosolic Ca^{2+} and induced iNOS to produce high amounts of NO. Superfluous NO is cytotoxic, and increases oxidative stress, leading to cell apoptosis. The chemical properties of Ce^{3+} among the three lanthanide elements is most similar to Ca^{2+}, i.e. Ce^{3+} radius is at 101–120 pm when its coordination number is at 6–9, and Ca^{2+} radius is at 100–118 pm when its coordination number is at 6–9; and after entering body Ce^{3+} could more easily occupy or substitute for the position of Ca^{2+} than La^{3+} and Nd^{3+} [1]. Thus Ce^{3+} exhibits more special biological effect compared to La^{3+} and Nd^{3+}.

5. Conclusion

Our study demonstrated that abdominal exposure to La^{3+}, Ce^{3+}, and Nd^{3+} at dose of 20 mg/kg BW for 14 days caused evident deposition of Ln, increased spleen indices, histopathological changes and ultrastructure lesion as well as oxidative stress in the mouse spleen. Among the three treatments, the Ce^{3+}-treated group exhibited the most severe splenic injury and oxidative stress, next was the Nd^{3+}-treated group, and than the La^{3+}-treated group. The difference of splenic injuries caused by Ln^{3+} was probably determined by the 4f electron of Ln.

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