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NOTE

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Sequential changes in serum zinc concentrations in calves with experimentally induced endotoxin shock measured by the particle-induced X-ray emission method

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ABSTRACT. The aim of the present study was to measure changes in the serum concentrations of some elements in endotoxin-challenged calves using a particle-induced X-ray emission analysis and to screen for elements useful as diagnostic markers. The results obtained revealed that serum Zn concentrations were more accurate diagnostic markers for detecting endotoxin shock in calves than other elements. Serum Zn level in endotoxin-challenged calve was significantly lower from 8 to 12 hr after the endotoxin challenge than pre-challenge values. In addition, serum Zn concentrations in calves from 4 to 24 hr after endotoxin challenges were significantly lower than those of control. Our results indicate that serum Zn concentration has potential as diagnostic markers for detecting inflammation in calves with endotoxin shock.

KEY WORDS: endotoxin, particle-induced x-ray emission, zinc

Endotoxin is the primary virulence factor of Gram-negative bacteria responsible for damage to cows and is released from bacteria at the time of cell death, thereby initiating an inflammatory response [2]. Inflammation is a complex response to cell or tissue injury. Acute inflammation causes a non-specific systemic reaction denoted as the acute phase response. The host response to infection, the "acute phase response" is a highly conserved series of physiological reactions including marked changes in concentrations of plasma proteins. These proteins have been shown to participate in the immune response to infections. The acute phase protein (APPs) such as serum amyloid A and haptoglobin has been identified as markers of inflammation in cattle; however, C-reactive protein (CRP) has been designated as only a moderate (2–10 fold increase) APPs in ruminants [12].

Intracellular calcium (Ca), selenium (Se), and zinc (Zn) levels were previously suggested to contribute, at least partly, to the formation of free radicals in endotoxin-poisoned mice [15]. Iron (Fe) is known to play roles in many enzymatic activities and is an essential trace element for hosts and pathogens [13]. Significant decreases in Fe concentrations have been reported in the serum and/or plasma of patients and animals with acute inflammation [4, 5]. This reduction has been attributed to decreased intestinal absorption and reductions in the release of Fe by reticulo-endothelial cells [1, 4, 5]. Because measurement of some elements is individually available and easily applicable, an evaluation of the relationship between endotoxin and the status of trace and major elements may be useful for the diagnosis of endotoxemia. However, comparative studies have not yet been conducted on trace and major elements in serum collected from cattle with endotoxemia.

The particle-induced X-ray emission (PIXE) method used in the present study is a fast and reliable multi-element qualitative and quantitative analytical tool that is easy to use [6]. In this technique, a detector analyzes characteristic X-rays emitted as a result of the inner-shell ionization of target atoms. With this technique, a sample of a few micrograms is sufficient to analyze concentrations in the parts-per-million range [6]. Since the method does not involve complex sample preparation, the risk of contamination during the preparation of a sample for the PIXE method is markedly lower than that for other methods [20, 21]. Previous studies evaluated the relationships between trace element concentrations in biological samples, such as the liver [8, 10], bone [7], plasma [3, 18, 19], and serum [8, 11], under pathophysiological and, in particular, pathological conditions using the PIXE method. The aim of the present study was to measure changes in the serum concentrations of some elements in endotoxin-challenged calves using a

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Table 1. Results of the spike and recovery test for the particle-induced X-ray emission method

Samples		DW for injection[a] (triplecate)				Pooled bovine serum (triplecate)					Recovery (%)
Elements		Pre-spike (μg/m*l*)		Post-spike (μg/m*l*)		Pre-spike (μg/m*l*)		Post-spike (μg/m*l*)			
		Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	Actual[c]	CV	Standard[d]
Aluminum	Al	ND[b]		10.644 ± 0.617	0.133	1.050 ± 0.096	0.092	11.901 ± 1.701	10.851	0.143	101.9
Calcium	Ca	ND		10.439 ± 0.723	0.058	83.256 ± 9.299	0.133	93.469 ± 0.794	10.213	0.008	97.8
Cadmium	Cd	ND		8.312 ± 1.352	0.145	ND		8.578 ± 1.255	8.578	0.146	103.2
Cobalt	Co	0.000 ± 0.000	0.886	7.032 ± 0.284	0.040	0.027 ± 0.002	0.085	7.438 ± 0.286	7.411	0.038	105.4
Chromium	Cr	ND		7.037 ± 0.854	0.121	0.029 ± 0.011	0.393	7.042 ± 0.237	7.014	0.024	99.7
Copper	Cu	0.001 ± 0.001	0.814	7.921 ± 0.296	0.037	0.676 ± 0.108	0.160	8.620 ± 0.116	7.944	0.014	100.3
Iron	Fe	0.008 ± 0.002	0.194	7.341 ± 0.344	0.047	1.782 ± 0.115	0.064	9.678 ± 0.123	7.897	0.013	107.6
Gallium	Ga	0.000 ± 0.001	1.732	8.493 ± 0.399	0.047	0.013 ± 0.012	0.959	8.763 ± 0.153	8.750	0.017	103.0
Magnesium	Mg	0.167 ± 0.088	52.509	11.577 ± 4.115	35.543	7.363 ± 2.721	0.370	20.588 ± 14.258	13.225	0.693	114.2
Manganese	Mn	ND		8.130 ± 0.377	0.046	ND		8.733 ± 0.160	8.733	0.018	107.4
Nickel	Ni	0.001 ± 0.001	1.083	7.926 ± 0.292	0.037	0.007 ± 0.013	1.732	7.948 ± 0.256	7.941	0.032	100.2
Lead	Pb	0.008 ± 0.002	0.269	7.927 ± 0.229	0.029	0.848 ± 0.024	0.028	9.157 ± 0.467	8.309	0.051	104.8
Selenium	Se	0.001 ± 0.001	1.075	8.475 ± 0.363	0.043	0.116 ± 0.016	0.134	8.355 ± 0.123	8.238	0.015	97.2
Strontium	Sr	ND		9.270 ± 0.506	0.055	0.238 ± 0.040	0.167	8.351 ± 0.341	8.113	0.041	87.5
Zinc	Zn	0.013 ± 0.008	0.604	8.184 ± 0.402	0.049	1.110 ± 0.048	0.043	9.073 ± 0.357	7.963	0.039	97.3

a) DW (20 m*l* distilled water for injection, Otsuka Pharmaceutical Co.). b) ND: not detected. c) Actual: Spiked value of pooled plasma, d) Standard: Correction based on DW spike results

PIXE analysis and to screen for elements useful as diagnostic markers.

This animal study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the School of Veterinary Medicine at Rakuno Gakuen University (Approval#: VC16C1). Six Jersey breed calves aged two months old, weighing 140.9 ± 36.3 kg, were enrolled in the present study. All calves were clinically normal before the experiment based on vital signs, attrition, food and water intakes, and urine and feces production. Clinical signs, such as moist rales on auscultation, a moist cough, jugular vein congestion, exophthalmos, salivation, and arrhythmia, were not observed before the experiment. A complete and balanced growth diet consisting of pelleted concentrate rations and mixed grass hay was provided, and calves had unlimited access to fresh water.

All calves were fit with an indwelling jugular catheter immediately before the endotoxin was infused, and received 2.5 μg/kg bolus doses of O111:B4 LPS (L4391, Sigma-Aldrich, St. Louis, MO, U.S.A.) intravenously in 10 m*l* of autologous serum via the jugular vein. The median value of autologous serum endotoxin activity measured by the limulus amebocyte lysate turbidimetric assay (Endosafe® KTA2, Charles River, Charleston, SC, U.S.A.) was 152,641 endotoxin units (EU)/head (ranging between 117,968 and 229,296 EU/head). Blood samples (10 m*l*) were withdrawn from the contralateral jugular vein before and 1, 2, 4, 8, 12, 24, and 48 hr after the endotoxin challenge and stored in both serum separator and heparine-2K coated tubes. Serum and plasma were harvested after centrifugation at 3,000 rpm at room temperature for 15 min and was stored at −80°C until analyzed. As a control group, all calves were sampled at the same time interval two weeks before the endotoxin infusion.

The plasma endotoxin activities were also measured using the limulus amebocyte lysate turbidimetric assay. The mean concentrations of elements in serum were measured using the PIXE method [20, 21]. Briefly, 100 μ*l* of the serum supernatant was placed on a subtlety Mylar membrane and desiccated. Supernatants were directly irradiated with proton beams. A small (baby) cyclotron used for positron nuclear medicine at the Nishina Memorial Cyclotron Center (Iwate, Japan) provided a 2.9 MeV-proton beam on a target after passing through a graphite beam collimator.

Analytical precision was confirmed by comparing the results obtained with those from ICP-MS and a Neutron Activation Analysis [16, 17]. The PIXE System at the Nishina Memorial Cyclotron Center was maintained under the same conditions as the initial settings [16, 17]. The precision and accuracy of PIXE were confirmed using standard materials, such as bovine liver, tomato leaves, city ash, and human serum (National Institute of Standards and Technology, U.S. Department of Commerce, Gaithersburg, MD, U.S.A.), at regular intervals in accordance with the guidelines of the facility. Regarding the accuracy and precision of the PIXE method, a spike and recovery test using certified reference materials was conducted in addition to abovementioned regular maintenance performed at the Nishina Memorial Foundation. In addition, further verification step was included in this study to confirm that the bovine serum sample did not have any matrix effects. A certified multi-element standard (ICP multi-element standard VIII, Merck KGaA, Darmstadt, Germany) was added to pooled bovine serum and distilled water for injection (20 m*l* distilled water for injection, Otsuka Pharmaceutical Co., Tokyo, Japan) to achieve a final concentration of 10 μg/m*l* in each sample. Analyses of serum specimens before and after the addition of standard solutions were repeated three times by the PIXE method to measure the respective trace elements. The recovery ratios corrected based on spike results for distilled water for injection and coefficients of variation (CV) were calculated for 15 elements (Al, Ca, Cd, Co, Cr, Cu, Fe, Ga, Mg, Mn, Ni, Pb, Se, Sr, and Zn). Accuracy and precision values detected by the PIXE analysis are summarized in the Table 1. Apart from Mg, spike recovery values and CV were considered to be valid. However, since the recovery value and CV of serum Mg were high, Mg was not evaluated

in the present study. Therefore, 14 elements except Mg that were confirmed to be sufficiently accurate and precise were targeted.

Statistical analyses were performed using a commercial software package (IBM SPSS Statistics, v.21, IBM Co, Somers, NY, U.S.A.). Data were reported as the mean ± standard deviation (SD). We processed serum trace and major elements concentrations for each dependent variable (groups and times) with two-way repeated measures ANOVA. If interaction was detected by 2-way ANOVA, measured dependent variables among groups and within group were compared using the Student's *t* and Bonferroni test, respectively. The significance level was $P<0.05$.

The pre-challenge value of plasma endotoxin activity in control and LPS groups were 0.03 ± 0.003 and 0.03 ± 0.01 EU/m*l*, respectively. Based on the repeatedly measured ANOVA, the variation of plasma endotoxin activity was statistically evaluated. The results showed the significant difference was observed in interaction between groups and time ($P<0.001$). A significant increase was observed in plasma endotoxin activity in endotoxin challenged calves, reaching 1.24 ± 0.04 EU/m*l* at 0.5 hr after the endotoxin challenge ($P<0.001$), followed by a return to pre-challenge values after 4 hr (Fig. 1).

The PIXE method allowed for the detection of 28 elements: Na, Mg, Al, Si, S, Cl, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Br, Rb, Sr, Y, Zr, Nb, Mo, and Pb. In the 28 elements detected from plasma in calves by PIXE method, statistical analysis was performed in 14 elements that accuracy and precision were guaranteed by the recovery method. There were no significant changes during the observation period in these elements in control calves. In endotoxin-challenged calves, only the variation of serum Zn concentration showed the significant difference in interaction between groups and time ($P<0.01$). Serum Zn concentrations were 0.88 ± 0.37 μg/m*l* before the endotoxin challenge and then significantly decreased, reaching 0.18 ± 0.07 μg/m*l* 8 hr after the endotoxin challenge ($P<0.01$). Serum Zn levels in endotoxin-challenged calves were significantly lower from 8 to 12 hr after the endotoxin challenge than the pre-challenge values ($P<0.01$). As a result, serum Zn concentrations in endotoxin challenged calves from 4 to 24 hr after endotoxin challenge were significantly lower than those of control, respectively (Fig 1). It is well known that blood Zn concentration is influenced by inflammation [9, 14]. Peretz *et al.* [14] demonstrated that the plasma Zn concentration of patients with chronic inflammatory rheumatic diseases was low whereas mononuclear cell Zn content was elevated. In addition, Zn supplementation had no effect on inflammation [14]. It suggests that the decrease in Zn concentration in patients with inflammatory disease was result of redistribution of the element related to the inflammatory process rather than to a Zn-deficient state. Decreased serum Zn concentrations in endotoxin-challenged calves in this study may also be the result of redistribution related to the inflammatory process.

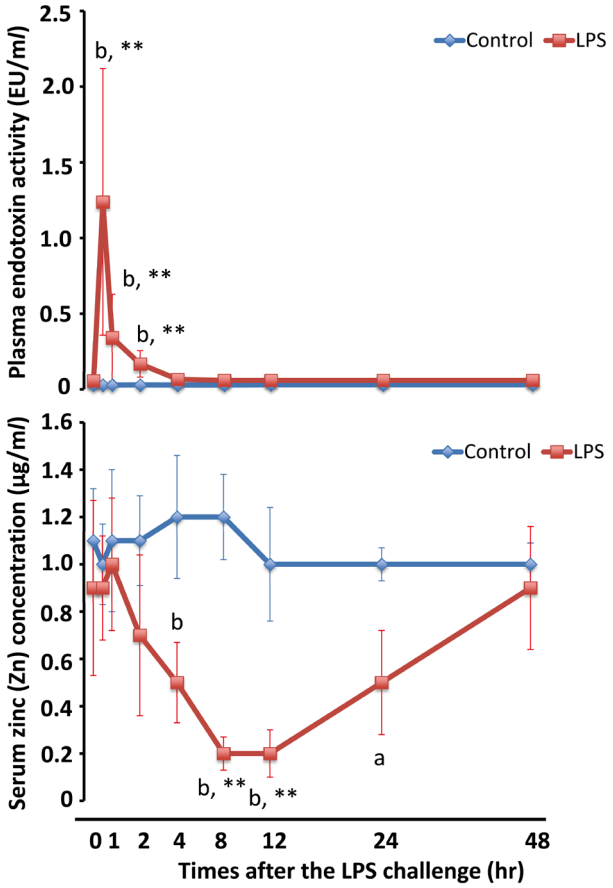

Fig. 1. Sequential changes in plasma endotoxin activity (upper) and serum zinc (Zn) concentration (bottom) in endotoxin-challenged calves. Levels of significance indicated; **: vs. before the endotoxin challenge $P<0.01$ by Bonferroni test and a: vs. control $P<0.05$, b: vs. control $P<0.01$ by Student's *t*-test.

In conclusion, we herein identified serum Zn levels as more accurate diagnostic markers for the detection of inflammation caused by endotoxin in cattle than other elements. The present results indicate the potential of serum Zn concentrations have potential as diagnostic markers for detecting inflammation in calves with endotoxin shock. Future studies need to focus on clarifying whether a relationship exists between the prognosis of naturally occurred endotoxin-related diseases and serum Zn concentrations.

CONFLICTS OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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