Pathogenesis of liver lesions in *Theileria orientalis*-inoculated cattle and severe combined immunodeficiency mice with bovine erythrocyte transfusion

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ABSTRACT

*Theileria orientalis* (*T. orientalis*) is a bovine protozoal disease similar to malaria in humans. Although the common outcome of malaria in humans and *T. orientalis* infection in cattle is hepatic disorder, the mechanisms of its development remain unknown. In this study, we investigated hepatocyte injury characterized by accumulation of macrophages with ingested erythrocytes in sinusoid and extramedullary hematopoiesis in cattle and mice experimentally infected with *T. orientalis* (*T. orientalis*-infected cattle and *T. orientalis*-infected mice). Vacuolization of hepatic cells was frequently observed in the vicinity of the aggregated macrophages in the liver sinusoids of *T. orientalis*-infected mice. A significant percentage of the macrophages accumulated in the liver sinusoids of the severely infected cattle and mice (14.6% and 24.2 to 53.2%, respectively) reacted positively with interleukin-1, interleukin-6 and TNF-α antibodies. Increase in the production of these cytokines was confirmed in *T. orientalis*-infected cattle and mice by real-time RT-PCR. These findings strongly suggest that increased cytokine production by the macrophages that have phagocytosed *T. orientalis*-infected erythrocytes causes hepatic disorder in *T. orientalis*-infected animals.

INTRODUCTION

Malaria is one of the most common infectious diseases caused by protozoans. In 2018, there were 228 million estimated cases (95% confidence interval: 206–258 million) of malaria worldwide, resulting in an estimated 405,000 deaths (WHO 2019). In animals, malaria typically causes hemolysis, blood cytokine response, fervescence and microvascular occlusion, resulting in multiple organopathy (Miller et al. 2002; WHO 2019). Bovine piroplasmosis is a mite-borne infectious blood disease caused by the protozoan *Theileria orientalis* (*T. orientalis*). Similar to malaria, the infected animals exhibit anemia, fervescence, hepatic disorder and anorexia (James et al. 1984; Yagi et al. 2003; McFadden et al. 2011). The stress caused by delivery or transportation can worsen the disease condition of the infected animals and lead to large economic losses when the animals die. While anemia is mostly caused by hemolysis/phagocytosis of the infected erythrocytes by macrophages in the spleen or liver (Yagi et al. 2003; Razavi et al. 2010; Yamaguchi et al. 2010), it has been shown that the hepatocyte injury caused by anemia-induced oxygen-deprivation is a major cause of the hepatic disorder. However, other factors causing hepatocyte injury remain unknown.
Bovine erythrocytes escape immune rejection and circulate in the body of severe combined immune deficiency (SCID) mice, allowing SCID-Bovine (SCID-Bo) mice to be produced by frequent bovine erythrocytes transfusion. The experimental procedure for producing *T. orientalis*-infected SCID-Bo mice was developed in 1992 (Tsui et al. 1992). This enabled *T. orientalis* maintenance in an animal body other than cattle (Hagiwara et al. 1993; Terada et al. 1995; Terada et al. 1998). Thus, using *T. orientalis*-infected SCID-Bo mice as a model to pathologically analyze the liver damage, we aimed to clarify the developmental mechanisms of hepatocyte injury in *T. orientalis*-infected animals.

MATERIALS AND METHODS

*Animals and treatment. T. orientalis*-infected cattle No. 1: A splenectomized 14-month-old female Holstein breed cattle was transfused with 500 mL of physiological saline containing 8.7 × 10^9 *T. orientalis*-infected erythrocytes. The infected erythrocytes were pooled from 8 piroplasmic cattle which were infected with major surface protein type II (MPSP type II) *T. orientalis* strain. The infected erythrocyte solution was instilled intravenously into the cattle No. 1 over 30 min. Blood counts and necropsy were performed 9 months after transfusion.

*T. orientalis*-infected cattle No. 2: A splenectomized 14-month-old male Holstein breed cattle was transfused with 1 L physiological saline containing 8.2 × 10^9 MPSP type II *T. orientalis*-infected erythrocytes collected from cattle No. 1 (5.1% infection rate at the time of inoculation). The infected erythrocyte solution was instilled intravenously into the male cattle over a period of 2 h. Blood counts and necropsy were performed 5 months after transfusion. Post autopsy and sampling by the Kyoritsu Pharmaceutical Advanced Technology Development Center/ Tsukuba Plant, the organs and blood of cattle No. 1 and No. 2 were transferred to Azabu University.

Generation of *T. orientalis*-infected SCID-Bo mice: Splenectomy was performed on 9-week-old female SCID mice (C.B-17 scid/ scid; CLEA, Tokyo, Japan) anaesthetized with Somnopentyl (Kyoritsu Pharmaceutical, Tokyo, Japan) and stored in 20% neutral buffered formalin, serially dehydrated using graded alcohol, and embedded in paraffin for histochemical observations. Hematoxylin eosin (HE), Giemsa and Berlin blue staining were used to study the 3-μm-thick liver sections.

Transmission electron microscopy observation. Each liver lobe of cattle No. 1, cattle No. 2, *T. orientalis*-infected mice and non infected mice were used for transmission electron microscopy. Cubes of 1 mm^3 were fixed for 6 h in 2.5% glutaraldehyde solution diluted with 0.1 M phosphate buffer (PB) (pH 7.4). They were rinsed in PB sufficiently, then post-fixed in 1% osmium tetroxide in PB followed by standard procedure of dehydration with alcohol and displacement with QY-1 before being embedded in epoxy resin. Semi-thin (1 μm) sections were stained using 1% toluidine blue. Ultra-thin sections
Hepatic disorder in Protozoa

performed using F(5'-GCTATGTTGTCCAAGAGA-3') and R(5'-TGTGAGACTCAATGCGCC-3') as the first primer, while F(5'-CAGTCAATGCAACACAAT-3') and R(5'-GTGCAAATCCTTGTTTG-3') were used as the second primer. For these reactions, 35 cycles of Thermal Cycler (PERKIN ELMER, GeneAmp™ PCR System 9600) were used. An initial 5 min denaturation step at 95°C was followed by cycles consisting of 30 min denaturation at 94°C, 45 s annealing at 58°C, and 1 min extension at 72°C, followed by an additional 7 min at 72°C. The amplified PCR products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide (FUJIFILM Wako PURE Chemical Corporation, Osaka, Japan), and then visualized under an ultraviolet light.

Real-time RT-PCR. cDNA was synthesized using total RNA extracted from 0.1 g of cattle and mouse livers collected at the time of dissection using Sepa-M® RNA I (Nakarai, Kyoto, Japan). Specific primers for cattle and mouse IL-1β, IL-6, TNF-α and glyceral-3-phosphate dehydrogenase (GAPDH) are shown in Table 2. mRNA expression was measured by real time RT-PCR and shown as relative values of GAPDH mRNA.

RESULTS

Infection of cattle and mice by transfusion of T. orientalis-infected erythrocytes

Observation of blood smears of cattle and mice in -

(80 nm) stained with uranyl acetate and lead citrate were then examined under a JEOL JEM 1400 electron microscope (Tokyo, Japan).

Immunohistochemistry. Immunohistochemical studies were performed on liver tissue specimens. The primary antibody and antigen retrieval methods used for immunostaining are shown in Table 1. Briefly, the sectioned liver tissues were incubated with primary antibody at 4°C for 18 h, washed with PBS, and then reacted with 0.3% H₂O₂ in methanol for 20 min to remove endogenous peroxidase. Subsequently, the tissues were incubated with En Vision + System-HRP Labelled Polymer Anti-Rabbit (Dako, Glostrup, Denmark) secondary antibody; this reaction was carried out at room temperature for 30 min. 3-amino-9-ethyl-carbazole (Wako Pure Chemical Industries, Osaka, Japan) was used for color development. Such immunohistochemically treated liver tissue specimens from cattle No. 1, cattle No. 2, T. orientalis-infected mice and non infected mice were observed at 400 × magnification and positive cells in 10 fields were enumerated. The image analysis was performed by the method of calculating the average five times for each sample.

DNA extraction. Genomic DNA was extracted and purified from 200 μL of blood and 50 mg of liver tissue using the QIAamp®DNA blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instruction. The extracted DNA was stored at −20°C until further use.

Nested PCR assays. MPSP II gene product was detected in the DNA extracted from cattle and mice by nested polymerase chain reaction (nested PCR) assay using primers derived from the T. orientalis specific gene (Tanaka et al. 1993). Amplification was performed using F(5'-GCTATGTTGTCCAAGAGA-3') and R(5'-TGTGAGACTCAATGCGCC-3') as the first primer, while F(5'-CAGTCAATGCAACACAAT-3') and R(5'-GTGCAAATCCTTGTTTG-3') were used as the second primer. For these reactions, 35 cycles of Thermal Cycler (PERKIN ELMER, GeneAmp™ PCR System 9600) were used. An initial 5 min denaturation step at 95°C was followed by cycles consisting of 30 min denaturation at 94°C, 45 s annealing at 58°C, and 1 min extension at 72°C, followed by an additional 7 min at 72°C. The amplified PCR products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide (FUJIFILM Wako PURE Chemical Corporation, Osaka, Japan), and then visualized under an ultraviolet light.

Real-time RT-PCR. cDNA was synthesized using total RNA extracted from 0.1 g of cattle and mouse livers collected at the time of dissection using Sepasol® RNA I (Nakarai, Kyoto, Japan). Specific primers for cattle and mouse IL-1β, IL-6, TNF-α and glyceral-3-phosphate dehydrogenase (GAPDH) are shown in Table 2. mRNA expression was measured by real time RT-PCR and shown as relative values of GAPDH mRNA.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). The significance of differences was analyzed using the Welch’s t test (Table 4, Figs. 7 and 8). A value of P < 0.05 and P < 0.01 was considered to be significant.

RESULTS

Infection of cattle and mice by transfusion of T. orientalis-infected erythrocytes

Observation of blood smears of cattle and mice in-

MW: Micro wave

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Host species</th>
<th>Isotype</th>
<th>Peroxidase blocker</th>
<th>dilution</th>
<th>Antigen retrieval method</th>
<th>source</th>
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<tr>
<td>Lysozyme</td>
<td>Rabbit</td>
<td>IgG</td>
<td>0.3% H₂O₂</td>
<td>1:100</td>
<td>sodium citrate buffer, 0.25% trypsin at room temperature</td>
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<tr>
<td>IL-1β</td>
<td>Rabbit</td>
<td>IgG</td>
<td>0.3% H₂O₂</td>
<td>1:100</td>
<td>sodium citrate buffer (10 mM pH 6.0)</td>
<td>Bioss, Inc, Boston, USA</td>
</tr>
<tr>
<td>IL-6</td>
<td>Rabbit</td>
<td>IgG</td>
<td>0.3% H₂O₂</td>
<td>1:100</td>
<td>sodium citrate buffer</td>
<td>Bioss, Inc, Boston, USA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Rabbit</td>
<td>IgG</td>
<td>0.3% H₂O₂</td>
<td>1:100</td>
<td>sodium citrate buffer</td>
<td>ABBIOTEC, Inc, San Diego, USA</td>
</tr>
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</table>

Table 1 Antibodies tested for immunohistochemical reactivity in tissues
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Edited vacuolar degeneration, and an accumulation of macrophages with ingested erythrocytes in the sinusoids (Fig. 2A) along with extramedullary hematopoiesis. Protozoa were identified in erythrocytes found in the sinusoid by Giemsa staining (Fig. 2A inset). In Berlin blue staining, hemosiderin deposits were observed in hepatocytes and macrophages/Kupffer cells in sinusoids (Fig. 2B). In cattle No. 2, hepatocytes had vacuolar degeneration, and an accumulation of macrophages that phagocytosed erythrocytes in sinusoids was slightly observed. Similarly, T. orientalis-infected mouse liver tissues exhibited vacuolar degeneration in hepatocytes and accumulations of macrophages with ingested erythrocytes in the sinusoids (Fig. 3A) along with extramedullary hematopoiesis by HE staining. Protozoa were identified in erythrocytes found in the sinusoid by Giemsa staining (Fig. 3A inset). Extramedullary hematopoiesis was found in the sinusoids of occluded with T. orientalis-infected erythrocytes confirmed the presence of T. orientalis protozoa (Fig. 1 A and B). All 4 mice inoculated with T. orientalis-infected erythrocytes were infected with the protozoa and had the highest rate of protozoan infection being observed (24.2 to 53.2%) 47 days after erythrocyte inoculation. Moreover, in the PCR assay, an amplified band of 592 bp identical to that of the positive control was observed in the samples obtained from the peripheral blood of the infected mice.

Protozoan infection rate of erythrocyte and hematological and biochemical tests

The infection rate in cattle No. 1 was 14.6%, and the hematocrit value was 23.3%. The infection rate in cattle No. 2 was 0.5% and the hematocrit value was 34.6%. Both the cattle showed elevated AST and LDH levels (Table 3). The protozoa infection rate in T. orientalis-infected mice was 24.2 to 53.2%, and the hematocrit value was 19.6 to 32.9%, but in non-infected mice the hematocrit value was 40.7 to 43.7%. Anemia was observed in all the T. orientalis-infected mice (Table 4). As a result of comparing T. orientalis-infected mice and non-infected mice, there was a significant difference in hemoglobin concentration, but other items were not observed. In LDH, the mean value was 2914 IU/L in T. orientalis-infected mice and 1074 IU/L non-infected mice, but no significant difference was observed.

Pathological findings

In the liver tissue of cattle No. 1, hepatocytes exhibited vacuolar degeneration, and an accumulation of macrophages with ingested erythrocytes in the sinusoids (Fig. 2A) along with extramedullary hematopoiesis. Protozoa were identified in erythrocytes found in the sinusoid by Giemsa staining (Fig. 2A inset). In Berlin blue staining, hemosiderin deposits were observed in hepatocytes and macrophages/Kupffer cells in sinusoids (Fig. 2B). In cattle No. 2, hepatocytes had vacuolar degeneration, and an accumulation of macrophages that phagocytosed erythrocytes in sinusoids was slightly observed.

Similarly, T. orientalis-infected mouse liver tissues exhibited vacuolar degeneration in hepatocytes and accumulations of macrophages with ingested erythrocytes in the sinusoids (Fig. 3A) along with extramedullary hematopoiesis by HE staining. Protozoa were identified in erythrocytes found in the sinusoids by Giemsa staining (Fig. 3A inset). Extramedullary hematopoiesis was found in the sinusoids of
Transmission Electron Microscopy observation

In cattle No. 1, abundant mitochondria were observed in the cytoplasm of hepatocytes, and vacuoles were scattered (Fig. 4). Furthermore, in *T. hepatica* infection of the liver (Fig. 3B), in Berlin blue staining, hemosiderin deposits were observed in macrophages/Kupffer cells in sinusoids, but not in hepatocytes (Fig. 3C).

### Table 3
**Protozoan infection rate and blood test results in cattle at autopsy**

(Normal value: Niinuma *et al.* 1991)

<table>
<thead>
<tr>
<th></th>
<th>Normal value</th>
<th>Cattle No. 1</th>
<th>Cattle No. 2</th>
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<tr>
<td>Protozoan infection rate (%)</td>
<td></td>
<td>14.6</td>
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<tr>
<td>RBC (10^4/μL)</td>
<td>812</td>
<td>485</td>
<td>770</td>
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<tr>
<td>Hb (g/dL)</td>
<td>11.3</td>
<td>7.1</td>
<td>10.7</td>
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<tr>
<td>Ht (%)</td>
<td>34.7</td>
<td>23.3</td>
<td>34.6</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>58.9</td>
<td>687</td>
<td>650</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>16.8</td>
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<td>18.0</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>443</td>
<td>1023</td>
<td>870</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>1574</td>
<td>4040</td>
<td>3920</td>
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</table>


### Table 4
**Protozoan infection rate and blood test results in mice**

<table>
<thead>
<tr>
<th>Mouse No.</th>
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<th>Non-infected group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Protozoan infection rate (%)</td>
<td>24.2</td>
<td>53.0</td>
</tr>
<tr>
<td>RBC (10^4/μL)</td>
<td>596</td>
<td>729</td>
</tr>
<tr>
<td>Hb (g/dL)*</td>
<td>7.9</td>
<td>10.2</td>
</tr>
<tr>
<td>Ht (%)</td>
<td>26.3</td>
<td>32.9</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>405</td>
<td>348</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>224</td>
<td>173</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>169</td>
<td>185</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>4521</td>
<td>2963</td>
</tr>
</tbody>
</table>

RBC: red blood cell, Hb: hemoglobin concentration, Ht: hematocrit, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, LDH: lactate dehydrogenase, *P < 0.05

**Fig. 2** Liver tissue of cattle No. 1. **A:** Hepatocytes showing vacuole degeneration (▶), accumulation of macrophages that phagocytose erythrocytes in the sinusoids (▶). (HE staining) Inset: Protozoa in erythrocytes in sinusoid (▶). (Giemsa staining). **B:** Hemosiderin deposits in hepatocytes and macrophages/Kupffer cells in sinusoids. (Berlin blue staining) Bars = 20 μm.
orientalis-infected mouse liver tissues, abundant mitochondria were observed in the cytoplasm of hepatocytes, and swelling mitochondrial structures and lipid were scattered (Fig. 5).

Immunohistochemistry
Macrophages positive for lysozyme, IL-1β, IL-6 and TNF-α were identified in the cattle and mouse liver sinusoids. The number of immunostaining-positive macrophages in cattle No. 1, which had high protozoan infection rate, and in T. orientalis-infected mice with high protozoan infection rate (Fig. 6). Significantly higher numbers of macrophages were positive for lysozyme, IL-1β, IL-6 and TNF-α immunostaining in liver tissue of cattle No. 1 and T. orientalis-infected mice having high infection rate (Fig. 7).

Real-time RT-PCR expression of IL-1β, IL-6 and TNF-α
In analysis by real-time RT-PCR, cattle No. 1 and T. orientalis-infected mice displayed high expression of mRNA of IL-1β and IL-6. Regarding TNF-α, both cattle No. 1 and cattle No. 2 had extremely low values. On the other hand, the expression amount of mRNA of IL-1β of cattle No. 2 was about 1/8 compared to cattle No. 2, and about 1/6.5 in IL-6. In non-infected mice, the expression levels of TNF-α, IL-1β and IL-6 were hardly observed (Fig. 8).

DISCUSSION
Histopathological findings of the liver of cattle No. 1 and T. orientalis-infected mice showed macrophages infiltration in the sinusoids derived from phagocytosis of protozoan-infected erythrocytes, deposition of hemosiderin, extramedullary hematopoietic appearance in the sinusoids due to anemia, and cytoplasmic vacuolar degeneration; histopathological findings common to hepatocyte injury were noted. On the contrary, in cattle No. 2 and non infected mice whose infection rate was as low as 0.5%, infiltration of macrophages in the sinusoid, deposition of hemosiderin, extramedullary hematopoiesis, and cytotoxicity were rarely observed or absent. This suggested that if there was no infection or only mild...
infection, no lesion was found in the liver. Transmission electron microscopic findings showed swelling mitochondria in cattle No. 1 hepatocytes. This result suggested that it was involved in vacuole formation. In *T. orientalis*-infected mice, not only swelling mitochondria in hepatocytes but also lipids were observed. This result suggested that in infected mice, the vacuoles in hepatocytes were swelling mitochondria and lipids.

From the lysozyme and inflammatory cytokine immunohistochemistry and real-time RT-PCR expression results, an increased macrophage release and high levels of inflammatory cytokines (IL-1β, IL-6 and TNF-α) produced by macrophages in relation to the protozoan infection rate was confirmed. Therefore, it was suggested that the mechanisms of liver injury not only involve the influence of anemia but also result from the over-release of cytokines by macrophages, resulting in liver cell injury (Fig. 9).

SCID-Bo mice were prepared by intraperitoneal inoculation of 0.5 mL of non-infected cattle erythrocytes once a week in each SCID mouse. Tsuji et al. (1992) and Terada et al. (1995) proposed a protocol to inoculate 1.5 mL of non-infected cattle erythrocytes intraperitoneally twice a week for the production of SCID-Bo mice; however, blood transfusion volume required for our method is only 1/6 of that required for their method. Even with this blood transfusion volume, *T. orientalis* infection was confirmed, and persistent protozoan infection was observed. Thus, the results here indicate that the number of blood transfusions and the experimental period should be considered carefully to minimize the influence of xenotransfusion of cattle erythrocytes on the liver of SCID mice. The SCID-Bo mouse system produced by this method could be a good model for cattle piroplasmosis infection.

Recent studies have revealed that hepatic cells in
Fig. 7 Image analysis of immunohistochemistry result. As a result of image analysis, a significantly higher number of immunostaining positive macrophages (a: Lysozyme, b: IL-1β, c: IL-6, d: TNF-α) in liver tissue of cattle No. 1 and *T. orientalis*-infected mice with high infection rate were observed. *P < 0.01 vs. No. 2 cattle, **P < 0.01 vs. non-infected mouse.

Fig. 8 Comparison of real-time RT-PCR expression level of cytokines. High levels of IL-1β and IL-6 cytokines were observed in cattle No. 1 and *T. orientalis*-infected mice with high infection rates. TNF-α was highly expressed in infected mice. *P < 0.05, **P < 0.01 1-4: Infected group 5, 6: Non infected group.
sinusoids including Kupffer cells, are stimulated by chemicals that cause hepatic injury and are involved in the development of the injury (MacDonald et al. 1987). In the case of liver damage initiated by several chemicals, it has been shown that in addition to Kupffer cells, macrophages from the blood infiltrate into the liver and accumulate locally prior to the damage. While in rats, macrophages infiltrate the central vein area in response to acetaminophen and carbon tetrachloride administration (Thompson et al. 1980; Laskin et al. 1986), they infiltrate entire hepatic lobules when endotoxin and galactosamine are administered (Pilaro et al. 1986; MacDonald et al. 1987); moreover, the inflow site and the necrotic area of hepatocytes agree well. In a manner similar to the response to administration of chemicals such as acetaminophen and endotoxins, the livers of T. orientalis-infected cattle and SCID-Bo mice showed hepatocyte degeneration in line with local infiltration of macrophages. When macrophages are stimulated by the substances that cause liver injury, their cytoplasmic volumes increase, and the secretion of functional molecules such as active oxygen (Yamato et al. 2000), proteolytic enzymes, and inflammatory cytokines is enhanced; these factors play an important role in hepatocyte injury and subsequent tissue regeneration. In this study, elevated expression of genes involved in the secretion of inflammatory cytokines, such as TNF-α, IL-1β and IL-6, was confirmed by immunohistochemistry and real-time RT PCR.

Acknowledgments
We thank the Kyoritsu Pharmaceutical Advanced Technology Development Center/Tsukuba Plant for providing the cattle blood and livers used for the experiment. We also appreciate the support of late Dr. Michi Kodama.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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Lee JW, Bannerman DD, Paape MJ, Huang MK and Zhao X...


