NOTE *Laboratory Animal Science*

Repression of Hepatic Cytochrome P450 2D Expression in Mice during *Babesia microti* Infection

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ABSTRACT. To examine the effect of *Babesia* infection on the level of the drug-metabolizing enzyme hepatic cytochrome P450 (CYP) 2D, we intraperitoneally inoculated *Babesia microti* into male ICR mice. CYP2D protein and CYP2D9 mRNA were significantly decreased at 12 days after infection with *B. microti*. The activity of bunitrolol 4-hydroxylase, which is catalyzed by CYP2D, was also significantly decreased. The mRNA levels of transcriptional regulators of CYP2D9, hepatocyte nuclear factor 4α and signal transducer and activator of transcription 5b, were markedly suppressed. These results suggest that *Babesia* infection represses CYP2D expression in the mouse liver. The decline in CYP2D-dependent drug metabolism might be involved in the incidence of adverse drug reactions in patients with babesiosis.
KEY WORDS: *Babesia microti*, CYP2D, cytochrome P450

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Babesiosis is a parasitic infection caused by hemotropic protozoa of the genus *Babesia*. It is a well-recognized disease of veterinary importance in both wild and domestic animals and has gained attention as an emerging zoonotic disease. Infection with *Babesia* leads to a host-mediated pathology and hemolysis, resulting in anemia, hyperbilirubinuria, hemoglobinuria and possibly organ failure [11]. Chemotherapy against babesiosis is indicated in only moderately to severely ill cases, and side effects associated with the drug used for treating babesiosis in humans have been observed in clinical studies [8, 14]. As most drugs are partially or completely biotransformed by hepatic metabolism prior to their elimination from the body, it is of clinical importance to evaluate alterations in drug metabolism during *Babesia* infection.

Hepatic cytochromes P450 (CYPs) are heme proteins, existing in several isoforms, that function as monooxygenases, which play a key role in the metabolism of clinically used drugs, chemical compounds and steroids, leading to detoxification or activation of the parent compounds [20]. We previously reported that the levels and activities of hepatic CYP3A were down-regulated during anemia caused by *Babesia* infection [22]. However, it remains unclear whether *Babesia* infection has any effect on other CYPs in the mouse liver. Nine CYP2D isoforms have been identified in mice, with CYP2D9 being a well-studied isoform that is specifically expressed in the male mouse liver [18]. CYP2D6 in humans mediates the oxidative metabolism of drugs, such as tricyclic antidepressants, selective serotonin reuptake inhibitors, antipsychotics, antirrythmics, β-blockers, opioid analgesics, antiemetics and antihistamines [5]. CYP2D9 is postulated to have drug-metabolizing characteristics equivalent to CYP2D6 [5]. Therefore, CYP2D9 could be a suitable model isoform of CYP2D6 in humans. In this study, we examined the effects of *Babesia* infection on CYP2D9 mRNA, CYP2D protein and CYP2D activity in the male mouse liver. Furthermore, to identify the mechanism underlying CYP2D regulation during *Babesia* infection, the mRNA levels of hepatocyte nuclear factor 4α (HNF4α) and signal transducer and activator of transcription 5b (STAT5b) were also examined as these act transcriptional regulators of CYP expression, including that of the CYP2D9 gene in the male mouse liver [13].

Male ICR mice (aged 6 weeks, Charles River, Yokohama, Japan) were intraperitoneally inoculated with red blood cells (RBCs) containing 1×10^6 of *B. microti*(Munich strain) obtained from infected mice or RBCs from normal donor mice. The percentage of parasitemia was determined by counting the number of parasitemic RBCs in tail blood smears stained with Giemsa. Hematocrit in the blood was measured using Celltac α (NIHON KODEN, Tokyo, Japan). In our preliminary experiment, in which we examined parameters at 3 time points (10, 12 and 21 days after the infection, corresponding to most severe anemia phase, early phase in recovery from anemia and recovery phase, respectively), the only significant change in CYP2D activity was observed at 12 days after infection. Therefore, mice were sacrificed by cervical dislocation 12 days after infection. Liver samples and hepatic

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Table 1. Primer pairs used in RT-qPCR

	NCBI Accession No.	Forward primer (5′- 3′)	exon (s)	Reverse primer (5′- 3′)	exon (s)	Product size (bp)
CYP2D9	NM_010006.2	TGCTCATGGTGGTGCGTGACCT	6	CTTGTTGGACTCTGCGCTGCACA	6/7	117
HNF4α	NM_008261.2	TTGCCGGCATGGATATGGCCGA	1	AGATGGGGACGTGTCATTGCCCA	1/2	110
STAT5b	NM_011489.3	TCGCGAAGCCAACAACGGCA	4	TCAGGCGCAGCTCCTCAAACGT	5	101
ACTB	NM_007393.3	CACCCGCGAGCACAGCTTCTTT	1	TTGTCGACGACCAGCGCAGCGATA	2	99

microsomes were prepared, and protein concentrations were determined as previously described [15, 19]. All the animal experiments were conducted in a biosafety level 2 containment laboratory with clearance from the Animal Ethic Committee of Kitasato University.

Extraction of total RNA and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were performed as previously reported [22]. The specific primers used in the present study are listed in Table 1. Primers spanning at least one intron were chosen. A standard curve for each transcript was constructed using the purified PCR product generated for each specific primer pair. All samples were amplified in duplicate. For each RT-qPCR reaction, the absence of genomic DNA was confirmed by a reverse transcription negative control. To normalize expression data, β-actin (ACTB) was used as an internal control. The thermal cycling program consisted of 2 min at 95°C for enzyme activation and 40 cycles of denaturation for 20 sec at 95°C, and annealing for 30 sec at 60°C.

The activity of bunitrolol 4-hydroxylase (BTL), which is catalyzed by CYP2D, was measured according to the method of Ishizuka *et al.* [12]. Western blot analysis was performed as previously described [22]. In brief, liver microsomal proteins (15.6 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After immunoreaction using a polyclonal rabbit antibody against human CYP2D6 (Daiichi Pure Chemical Co., Tokyo, Japan), as the primary antibody, followed by horseradish peroxidase conjugated anti-rabbit goat antibody (Cell Signaling Technology, Danvers, MA, U.S.A.), the bands of CYP2D protein were detected using an ECL Plus Western Blotting Detection System (GE Healthcare Japan, Tokyo, Japan). An image of the bands was captured using Lumicube (Liponics, Tokyo, Japan). Band intensity was analyzed using ImageJ [1].

All the data are shown as the mean ± SD. Statistical comparisons were made by Student's *t*-test. A P-value<0.05 was regarded as statistically significant.

B. microti infection caused parasitemia and anemia. Parasitemia reached a peak (83 ± 4.0%) at 10 days after infection and then began to decline (25 ± 5.5%) at 12 days (Fig. 1A). Conversely, the hematocrit values were lowest (13 ± 2.4%) at 10 days after infection and then recovered slightly (19 ± 2.8%) at 12 days (Fig. 1B).

CYP2D9 mRNA was significantly decreased (Fig. 2A). Western blot analyses and Bunitorol metabolism revealed that the CYP2D protein and activity levels were also decreased at the same point in time (Fig. 2B and 2C). The decreases in CYP2D9 mRNA, CYP2D protein and CYP2D

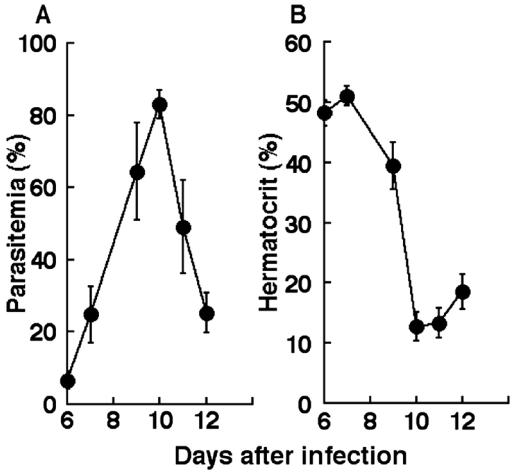

Fig. 1. Timecourses for parasitemia (A) and hematocrit (B) after *Babesia microti* infection. Representative data for two sets of experiments are shown. Data are shown as the mean ± SD of 4 mice from the infected group.

activity were 16.3, 64.0 and 58.2% of the control, respectively. These results suggest that *Babesia* infection has an inhibitory effect on the expression and activity of hepatic CYP2D in mice.

We previously reported an increase in tumor necrosis factor α (TNFα) mRNA in the mouse liver after *Babesia* infection [22]. Pro-inflammatoty cytokines, such as interleukin (IL)- 1, TNFα and interferon gamma, are increased in mouse serum during babesiosis [2, 24]. These cytokines act to decrease the expression and activity of hepatic CYP2D in rodents [17]. Therefore, we speculate that the downregulation of CYP2D caused by *Babesia* infection may be caused through the action of pro-inflammatory cytokines.

A concomitant decrease in the level of BTL activity, which is catalyzed by CYP2D, was observed with that of CYP2D protein, although the extent of the decrease in CYP2D9 mRNA was greater than that of the decrease in the activity and protein level of CYP2D in this study. Recently, a global mass spectrometry-based proteomics approach has demonstrated the expression of four CYP2D proteins, CYP2D9, 2D10, 2D22 and 2D26, in the male mouse liver [9]. BTL activity is mostly catalyzed by CYP2D in mouse liver microsomes [16]. In addition, polyclonal anti-CYP2D6 antibody used in this study has the potential to cross-react with these CYP2D proteins in mice as CYP2D6 displays high amino acid sequence homology with CYP2D9 (71%),

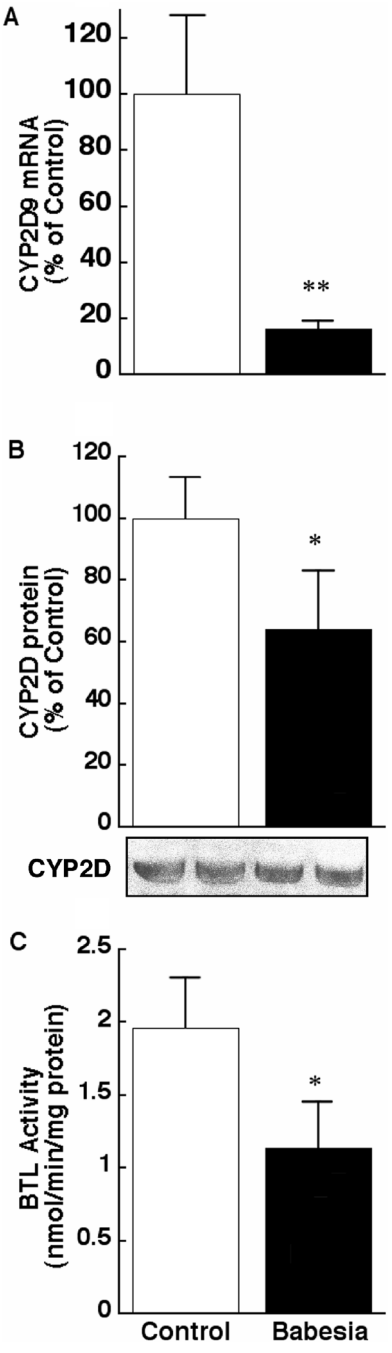

Fig. 2. Effects of *Babesia microti* infection on CYP2D in the mouse liver at 12 days after infection: (A) CYP2D9 mRNA, (B) CYP2D protein and a representative image of the bands after immunoreaction with the anti-CYP2D6 antibody, (C) bunitrolol (BTL) activity. Data are shown as the mean ± SD of 4 mice from each treatment group. Representative data for two sets of experiments are shown. Significant differences: * $P<0.05$ and ** $P<0.01$, respectively, from the control performed concurrently.

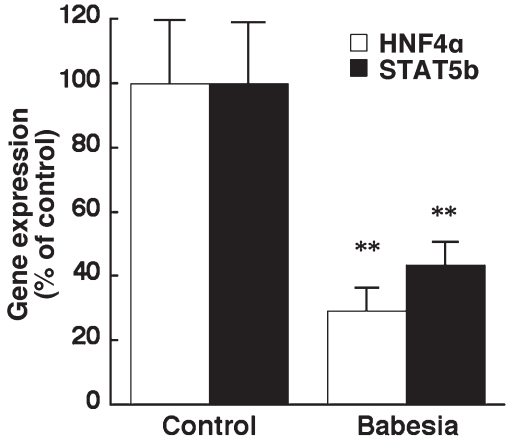

Fig. 3. Effects of *Babesia microti* infection on the mRNA levels of HNF4α and STAT5b in the mouse liver at 12 days after infection. Representative data for 2 sets of experiments are shown. Data are shown as the mean ± SD of 4 mice from each treatment group. Significant differences: ** $P<0.01$ from the control performed concurrently.

2D10 (70%), 2D22 (76%) and 2D26 (71%) (protein blast; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Therefore, the disparity in the decreases in level between CYP2D9 mRNA and the protein level and activity of CYP2D may be explained by the participation of CYP2D proteins other than CYP2D9 in BTL activity and immunoreactivity.

We observed that the level of HNF4α mRNA was significantly decreased in the infected mouse liver (Fig. 3). Since HNF4α acts as a positive regulator of the liver-specific transcription of CYP genes [13], the decrease in expression of this regulator may lead to a down-regulation of CYP2D9 as a result of *Babesia* infection. TNFα decreases the amount of HNF4α protein in HepG2 cells through the nuclear factor κB and MEK1/2 pathways [21]. In addition, a TNFα-induced reduction in sex hormone-binding globulin expression is mediated by the downregulation of HNF4α [23]. Therefore, a quantitative alteration in HNF4α could be involved in the repression of CYP2D9 expression resulting from *Babesia* infection. In addition, suppression of the DNA-binding ability of HNF4α by nitric oxide (NO) contributes to a down-regulation of CYP2D6 during inflammation or cytokine stimulation [7]. Considering the induction of NO synthase (NOS) 2 in the mouse liver during *Babesia* infection [22], we speculate that the functional inhibition of HNF4α by NO might also contribute to the down-regulation of CYP2D9.

We also observed the repression of STAT5b mRNA in the infected mouse liver (Fig. 3). The expression of CYP2D9 requires the growth hormone (GH) pulse-activated transcription factor STAT5b [13]. The decrease in STAT5b mRNA could contribute to the repression of CYP2D9 during *B. microti* infection. Moreover, endotoxin lowers the levels of nuclear phosphorylated STAT5b and reduces the binding of phosphorylated STAT5b to DNA along with a marked increase in TNFα and IL-6 mRNA in the liver [4]. IL-6 medi-

ates hepatic GH resistance by the time-dependent inhibition of GH-inducible promoter activity which is associated with reductions in STAT5 DNA binding [3]. The down-regulation of CYP2D9 might be caused by the inhibition of the DNA binding of STAT5b through the action of inflammatory cytokines.

We previously reported that hepatic CYP3A as well as mRNA levels of nuclear receptors, that participate in CYP3A expression, pregnane X receptor (PXR), constitutive androstane receptor (CAR) and retinoid X receptor α (RXRα), were down-regulated after *B. microti* infection [22]. The down-regulation of CYP2D and CYP3A and the reduction in the mRNA levels of their transcriptional regulators appear to be due to insufficient liver function after *B. microti* infection, but we also observed that the hepatic expression of CYP2E1 mRNA was up-regulated (267% of control, $P<0.05$) at 12 days after infection (unpublished data). These results suggest that the down-regulation of CYP2D and CYP3A does not arise from hepatic dysfunction after infection, indicating that hepatic CYPs could be differentially regulated during *B. microti* infection.

The results of this study demonstrated the repression of CYP2D expression during anemia resulting from *Babesia* infection. The decline in CYP2D-dependent metabolism can lead to an increase in the concentration of substrate drugs, such as antihypertensive agents and antidepressants, in the blood, leading to an increase in the incidence of adverse drug reactions. Therefore, careful selection of drug dosage is needed in the treatment of hypertensive or depressive patients with babesiosis.

Unfortunately, we could not clarify the mechanism underlying the down-regulation of hepatic CYP2D and CYP3A caused by *B. microti* infection in this study. We now put forward TNFα as a major candidate for the mediator of the decrease in CYP2D and CYP3A after *B. microti* infection. TNFα is released from macrophages or Kupffer cells after *B. microti* infection [10]. Nuclear factor κB (NFκB) in hepatocytes, after its activation by TNFα, then lowers the DNA binding of transcriptional regulators of CYP2D and CYP3A, such as PXR-RXRα complex and HNF4α [6, 21]. A NO-mediated pathway also exists in the NFκB-induced reduction in the binding of HNF4α to CYP2D regulatory sites in the DNA [7]. TNFα could reduce the binding of phosphorylated STAT5b to DNA [4]. Therefore, we hypothesized that the TNFα-NFκB axis plays a pivotal role in the down-regulation of CYP2D and CYP3A during *B. microti* infection (Fig. 4). A further study is needed to elucidate the mechanism underlying the alteration in CYPs in *Babesia* infection.

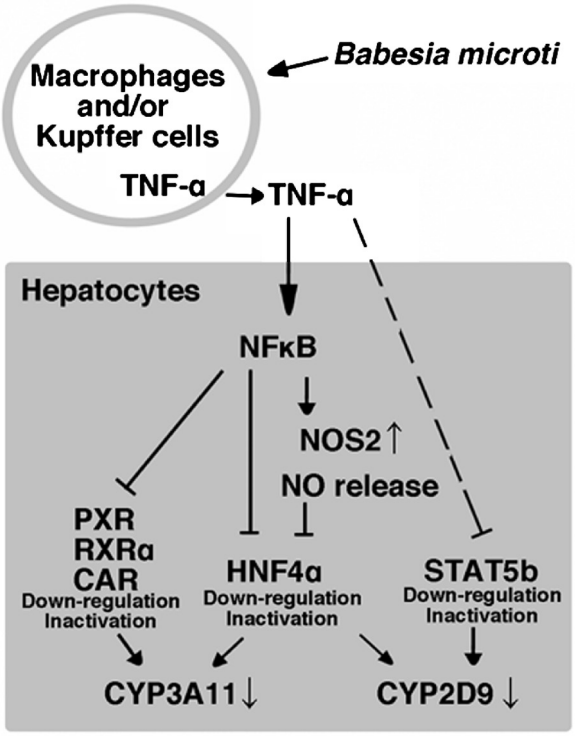

Fig. 4. Proposed mechanism of down-regulation of hepatic CYP2D and CYP3A in mice infected with *Babesia microti*. TNFα; tumor necrosis factor α, NFκB; nuclear factor κB, NOS2; nitric oxide synthase 2, NO; nitric oxide, PXR; pregnane X receptor, CAR; constitutive androstane receptor, RXRα; retinoid X receptor α, HNF4α; hepatocye nuclear factor 4α, STAT5b; signal transducer and activator of transcription 5b.

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REFERENCES

1. Abramoff, M. D., Magelhaes, P. J. and Ram, S. J. 2004. Image processing with imageJ. *Biophotonics Int.* **11**: 36–42.
2. Aguilar-Delfin, I., Wettstein, P. J. and Persing, D. H. 2003. Resistance to acute babesiosis is associated with interleukin-12- and gamma interferon-mediated responses and requires macrophages and natural killer cells. *Infect. Immun.* **71**: 2002–2008. [Medline] [CrossRef]
3. Ahmed, T. A., Buzzelli, M. D., Lang, C. H., Capen, J. B., Shumate, M. L., Navaratnarajah, M., Nagarajan, M. and Cooney, R. N. 2007. Interleukin-6 inhibits growth hormone-mediated gene expression in hepatocytes. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**: G1793–G1803. [Medline] [CrossRef]
4. Chen, Y., Sun, D., Krishnamurthy, V. M. and Rabkin, R. 2007. Endotoxin attenuates growth hormone-induced hepatic insulin-like growth factor I expression by inhibiting JAK2/STAT5 signal transduction and STAT5b DNA binding. *Am. J. Physiol. Endocrinol. Metab.* **292**: E1856–E1862. [Medline] [CrossRef]
5. Gardiner, S. J. and Begg, E. J. 2006. Pharmacogenetics, drug-metabolizing enzymes, and clinical practice. *Pharmacol. Rev.* **58**: 521–590. [Medline] [CrossRef]
6. Gu, X., Ke, S., Liu, D., Sheng, T., Thomas, P. E., Rabson, A. B., Gallo, M. A., Xie, W. and Tian, Y. 2006. Role of NF-kappaB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. *J. Biol. Chem.* **281**: 17882–17889. [Medline] [CrossRef]
7. Hara, H. and Adachi, T. 2002. Contribution of hepatocyte nuclear factor-4 to down-regulation of CYP2D6 gene expression by nitric oxide. *Mol. Pharmacol.* **61**: 194–200. [Medline]

[CrossRef]
8. Hatcher, J. C., Greenberg, P. D., Antique, J. and Jimenez-Lucho, V. E. 2001. Severe babesiosis in Long Island: review of 34 cases and their complications. *Clin. Infect. Dis.* **32**: 1117–1125. [Medline] [CrossRef]
9. Hersman, E. M. and Bumpus, N. N. 2014. A targeted proteomics approach for profiling murine cytochrome P450 expression. *J. Pharmacol. Exp. Ther.* **349**: 221–228. [Medline] [CrossRef]
10. Hopkins, S. J. 2003. The pathophysiological role of cytokines. *Leg. Med. (Tokyo)* **5** Suppl. 1: S45–S57. [Medline] [CrossRef]
11. Hunfeld, K. P., Hildebrandt, A. and Gray, J. S. 2008. Babesiosis: recent insights into an ancient disease. *Int. J. Parasitol.* **38**: 1219–1237. [Medline] [CrossRef]
12. Ishizuka, M., Lee, J. J., Masuda, M., Akahori, F., Kazusaka, A. and Fujita, S. 2006. CYP2D-related metabolism in animals of the Canoidea superfamily − species differences. *Vet. Res. Commun.* **30**: 505–512. [Medline] [CrossRef]
13. Jover, R., Moya, M. and Gomez-Lechon, M. J. 2009. Transcriptional regulation of cytochrome p450 genes by the nuclear receptor hepatocyte nuclear factor 4-alpha. *Curr. Drug Metab.* **10**: 508–519. [Medline] [CrossRef]
14. Krause, P. J., Lepore, T., Sikand, V. K., Gadbaw, J. Jr., Burke, G., Telford, S. R. 3rd., Brassard, P., Pearl, D., Azlanzadeh, J., Christianson, D., McGrath, D. and Spielman, A. 2000. Atovaquone and azithromycin for the treatment of babesiosis. *N. Engl. J. Med.* **343**: 1454–1458. [Medline] [CrossRef]
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275. [Medline]
16. Masubuchi, Y., Iwasa, T., Hosokawa, S., Suzuki, T., Horie, T., Imaoka, S., Funae, Y. and Narimatsu, S. 1997. Selective deficiency of debrisoquine 4-hydroxylase activity in mouse liver microsomes. *J. Pharmacol. Exp. Ther.* **282**: 1435–1441. [Medline]
17. Morgan, E. T. 1997. Regulation of cytochromes P450 during inflammation and infection. *Drug Metab. Rev.* **29**: 1129–1188. [Medline] [CrossRef]
18. Nelson, D. R., Zeldin, D. C., Hoffman, S. M., Maltais, L. J., Wain, H. M. and Nebert, D. W. 2004. Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics* **14**: 1–18. [Medline] [CrossRef]
19. Omura, T. and Sato, R. 1964. The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. *J. Biol. Chem.* **239**: 2370–2378. [Medline]
20. Porter, T. D. and Coon, M. J. 1991. Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J. Biol. Chem.* **266**: 13469–13472. [Medline]
21. Shavva, V. S., Mogilenko, D. A., Dizhe, E. B., Oleinikova, G. N., Perevozchikov, A. P. and Orlov, S. V. 2013. Hepatic nuclear factor 4alpha positively regulates complement C3 expression and does not interfere with TNFalpha-mediated stimulation of C3 expression in HepG2 cells. *Gene* **524**: 187–192. [Medline] [CrossRef]
22. Shimamoto, Y., Sasaki, M., Ikadai, H., Ishizuka, M., Yokoyama, N., Igarashi, I., Hoshi, F. and Kitamura, H. 2012. Downregulation of hepatic cytochrome P450 3A in mice infected with Babesia microti. *J. Vet. Med. Sci.* **74**: 241–245. [Medline] [CrossRef]
23. Simó, R., Barbosa-Desongles, A., Lecube, A., Hernandez, C. and Selva, D. M. 2012. Potential role of tumor necrosis factor-alpha in downregulating sex hormone-binding globulin. *Diabetes* **61**: 372–382. [Medline] [CrossRef]
24. Wood, P. R. and Clark, I. A. 1984. Macrophages from Babesia and malaria infected mice are primed for monokine release. *Parasite Immunol.* **6**: 309–317. [Medline] [CrossRef]