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In vitro and in vivo safety and efficacy studies of amphotericin B on *Babesia gibsoni*

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ABSTRACT

Babesia gibsoni is a causative pathogen of canine babesiosis, which is commonly treated with anti-babesial drugs; however, the development of novel, more effective anti-babesial drugs is necessary because the currently used drugs cannot remove the parasites from dogs. Therefore we investigated the anti-babesial effect of amphotericin B (AmB), a membrane-active polyene macrolide antibiotic. The interaction of such compounds with sterols in bilayer cell membranes can lead to cell damage and ultimately cell lysis. AmB exhibits in vitro activity against *B. gibsoni* in normal canine erythrocytes within 12 h. We also studied liposomal AmB (L-AmB), a liposomal formulation of AmB that required a longer incubation period to reduce the number of parasites. However, L-AmB completely inhibited the invasion of free parasites into erythrocytes. These results indicated that free parasites failed to invade erythrocytes in the presence of L-AmB. Both AmB and L-AmB induced mild hemolysis of erythrocytes. Moreover, the methemoglobin level and the turbidity index of erythrocytes were significantly increased when erythrocytes were incubated with AmB, suggesting that AmB induced oxidative damage in erythrocytes. Finally, the anti-babesial activity of AmB in vivo was observed. When experimentally *B. gibsoni*-infected dogs were administered 0.5 and 1 mg/kg AmB by the intravenous route, the number of parasites decreased; however, recurrence of parasitemia was observed, indicating that AmB did not eliminate parasites completely. Blood urea nitrogen and creatinine of dogs were abnormally elevated after the administration of 1 mg/kg AmB. These results indicate that AmB has in vivo activity against *B. gibsoni*; however, it does not eliminate parasites from infected dogs and affects kidney function at a high dose.

1. Introduction

Babesia gibsoni is a causative pathogen of canine babesiosis, which is commonly treated with diminazene aceturate. Other anti-babesial drugs such as atovaquone, clindamycin, metronidazole, doxycycline, and pentamidine are also used for the treatment of canine babesiosis; however, these drugs do not eliminate the parasites from the infected dogs (Fowler et al., 1972; Farwell et al., 1982; Wulansari et al., 2003; Suzuki et al., 2007; Sakuma et al., 2009). Accordingly, the development of novel, more effective anti-babesial drugs is necessary.

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We previously reported that nystatin, an ionophorous antibiotic, exhibited in vitro effects against *B. gibsoni* (Yamasaki et al., 2011). Nystatin modifies the intracellular concentrations of monovalent cations and can markedly injure cells that maintain their intracellular potassium concentration via active transporters such as Na,K-ATPase (Yamasaki et al., 2011). Nystatin can destroy *B. gibsoni* through its ionophorous activity (Yamasaki et al., 2011).

Some dogs have erythrocytes containing high concentrations of potassium (high kalium, HK), GSH and free amino acids as a result of inherited high Na,K-ATPase activity (i.e., canine high kalium [HK] erythrocytes) (Inaba and Maede, 1984). Maede et al. (1983) reported that some dogs have canine HK erythrocytes containing high concentration of potassium. In addition, canine HK erythrocytes are useful host cells for in vitro culture of *B. gibsoni* because the parasites proliferate better in canine HK erythrocytes than in canine normal erythrocytes with low potassium (LK) concentrations (i.e., LK erythrocytes) (Yamasaki et al., 2000). Nystatin cannot reduce the number of *B. gibsoni* in HK erythrocytes, although it exhibits in vitro anti-babesial effects on the parasites in LK erythrocytes (Yamasaki et al., 2011). The anti-babesial activity of nystatin seems to be counteracted by Na,K-ATPase activity in HK erythrocytes. In addition, canine HK erythrocytes are mildly hemolysed by nystatin (Yamasaki et al., 2011); therefore, it is possible that nystatin could cause hemolytic anemia in dogs with HK erythrocytes, although it does not affect LK erythrocytes or peripheral polymorphonuclear leukocytes (Yamasaki et al., 2011). Moreover, nystatin cannot currently be intravenously administered to dogs. It is therefore not useful as a therapeutic treatment against *B. gibsoni* infection.

Amphotericin B (AmB) is a membrane-active polyene macrolide antibiotic and an antifungal compound that is similar in structure to nystatin; therefore, it might also induce hemolysis of canine HK erythrocytes. However, it can be intravenously administered to dogs. AmB and nystatin are membranolytic due to their lipid-binding activities and the target membrane is thought to be osmotically fragile by binding AmB to beta-ergosterol, the principal fungal and protozoal sterol (Wiehart et al., 2006). Wiehart et al. (2006) also found that erythrocytes infected with the trophozoite stage of *Plasmodium falciparum* were particularly susceptible to lysis by AmB and nystatin; however, AmB and nystatin can also bind cholesterol in mammalian cell membranes, albeit with lower affinity. Thus AmB and nystatin induce tissue injury to the kidney and erythrocytes. Recently, therefore, a liposomal formulation of AmB was developed for intravenous use. Liposomal AmB (L-AmB) displays specificity for *P. falciparum*-infected erythrocytes, but complete lysis requires a longer incubation period than for AmB (Wiehart et al., 2006). These results imply that AmB and L-AmB may be effective in the treatment of severe malaria caused by *P. falciparum* (Wiehart et al., 2006). The piroplasms *Babesia rodhaini* and *Theileria parva* are inhibited by nystatin and AmB in vitro (McColm and McHardy, 1984), suggesting that AmB and L-AmB might be useful as therapeutic agents against *Babesia* spp. parasites. However, it is possible that AmB and L-AmB could cause hemolysis of canine HK erythrocytes as described above; therefore, the effects of AmB and L-AmB on canine erythrocytes should be examined. In addition, their effects on canine leukocytes should be determined.

In the present study, we examined the effects of AmB and L-AmB on *B. gibsoni*, canine erythrocytes, and peripheral polymorphonuclear leukocytes in vitro. Additionally, we treated experimentally *B. gibsoni*-infected dogs with AmB in vivo to evaluate its therapeutic activity.

2. Materials and methods

2.1. Preparation of canine erythrocytes

Canine HK erythrocytes containing a high concentration of potassium (high kalium, HK) and a low concentration of sodium as a result of inherited high Na,K-ATPase activity (Inaba and Maede, 1984) were obtained from three mongrel male dogs that had inherited high kalium (HK) erythrocytes. Dogs with HK erythrocytes have been maintained since 1986 in our laboratory. Canine normal erythrocytes having low potassium (low kalium, LK) and high sodium concentrations (i.e., low kalium [LK] erythrocytes) were obtained from three genetically unaffected male beagle dogs. Peripheral polymorphonuclear leukocytes were also prepared from peripheral blood of three beagle dogs with LK erythrocytes. Canine LK and HK erythrocytes were identified by measuring the intracellular concentrations of potassium and sodium (Yamasaki et al., 2005). The dogs used had body weights of 8–12 kg and were 2–3 years old. In the experimental protocols for animal care and handling, the investigators adhered to the guidelines of Hokkaido University, which basically conform to those of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The present study was approved by the Committee for Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University (approval number: 1022).

2.2. Cultivation of B. gibsoni

The *B. gibsoni* used in the present study originated from a naturally infected dog from Nagasaki, Japan in 1973. Since then this isolate has been maintained in experimentally infected dogs and in culture (Yamasaki et al., 2003). For in vitro assay, the parasites maintained in culture with LK erythrocytes were utilized. To prepare erythrocytes for culture, blood samples were collected into sterile disposable syringes, immediately moved into blood collection tubes with ethylenediaminetetraacetic acid disodium salt (EDTA-2Na; Wako Pure Chemical Co., Osaka, Japan), and washed according to the method of Yamasaki et al. (2000). Briefly, blood samples were centrifuged at 900 g for 5 min at room temperature (ca. 25 °C). After removal of the plasma and buffy coat, packed cells were resuspended in dog plasma to yield a packed cell volume (PCV) of 50% (v/v) and filtered through an alpha-cellulose/microcrystalline cellulose column to remove leukocytes and platelets. Filtered cells were washed three times with 10 mM phosphate-buffered saline (PBS, pH 7.4) and washed twice with RPMI-1640 with L-glutamine and 25 mM HEPES