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In vitro growth inhibitory effect of (-)-Epigallocatechin-3-gallate from green tea on the growth of equine *Babesia* parasites

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ABSTRACT

(-)-Epigallocatechin-3-gallate (EGCG) accounts for 50-80% of the total catechin in green tea. (-)-Epigallocatechin-3-gallate has antioxidant, anti-inflammatory, antimicrobial, anticancer, and antitrypanocidal activities. In this study, an inhibitory effect of (-)-Epigallocatechin-3-gallate was revealed on the *in vitro* growth of equine *Babesia* parasites. The *in vitro* growth of the equine *Babesia* species was significantly ($P < 0.05$) inhibited in the presence of micromolar concentrations of EGCG (IC_{50} values = 4.42 and 3.64 μM for *Babesia caballi* and *Babesia equi,* respectively). The parasites showed no regrowth at the concentration of 100 μM for *B. caballi* and 50 μM for *B. equi* in the subsequent viability test. These findings highlight the potentiality of (-)-Epigallocatechin-3-gallate as a chemotherapeutic drug for the treatment of equine babesiosis.

Keywords: (-)-Epigallocatechin-3-gallate; *in vitro*; *Babesia caballi*; *Babesia equi.*

INTRODUCTION

Equine piroplasmosis is a well-recognized tick-transmitted disease of horses, donkeys, mules and zebras in many regions of the world; the disease is caused by two intraerythrocytic apicomplexan parasites, *Babesia equi* (*Theileria equi*) (Melhorn and Schein, 1998) and *Babesia caballi* (Ristic, 1988). The parasites induce severe symptoms, including hemolytic anemia, icterus, fever, edema, loss of body weight, poor performance in the infected animals, and produce enormous losses in the horse industry (Kuttler, 1988).

Several babesicidal drugs that have been in use for years have proven to be ineffective owing to problems related to their toxicity and the development of resistant parasites (Bork *et al*., 2005a; Vial and Gorenflot, 2006). Therefore, there is a need to develop new drugs that have a chemotherapeutic effect against babesiosis with high specificity to the parasites and low toxicity to the hosts.

Tea (*Camellia sinensis*) is one of the most popular beverages in the world, and its beneficial effects on health have attracted great attention (Yang and Landau, 2000). The most significant groups of tea components are polyphenols, especially the catechin group called flavonols. (-)-Epigallocatechin-3-gallate (EGCG) is the major tea catechin and EGCG accounts for 50-80% of the total catechin (Graham, 1992). EGCG has antitumorigenic (Chen and Zhang, 2007), anti-inflammatory (Lin and Lin, 1997), antioxidative (Fraga *et al*., 1987), antiproliferative (Shammas *et al*., 2006), antibacterial (Mabe *et al*., 1999), and antiviral (Fassina *et al*., 2002; Yamaguchi *et al*., 2002; Williamson *et al*., 2006) effects. EGCG also has trypanocidal activity against *Trypanosoma cruzi* (Paveto *et al*., 2004). EGCG has strong inhibitory effect on bovine *Babesia* (AbouLaila *et al*., 2010). In this study, we present the inhibitory effect of EGCG on *B. equi* and *B. caballi* in *in vitro* cultures.

MATERIALS AND METHODS

Chemical compound

(-)-Epigallocatechin-3-gallate (EGCG) was purchased from Sigma-Aldrich (USA) and used as a test drug. A working stock solution of 1 mM dissolved in PBS was prepared and stored at -30°C until use.

In vitro growth inhibition assay

United States Department of Agriculture strains of *B. equi* and *B. caballi* were grown in a micro-aerophilous stationary phase culture system using equine erythrocytes (RBCs) and sera as described previously (Bork *et al.*, 2004a). A serum-free GIT medium also was used to study the effect of serum-free medium on the drug inhibition of *B. caballi* (Bork et al., 2005b). The *in vitro* growth inhibition assay for EGCG followed a method previously described (Bork *et al.*, 2003b, 2004a) with some modifications. Parasite-infected RBCs were diluted with uninfected RBC to obtain 1% parasitemia. Twenty μl of RBC with 1% parasitemia was dispensed to a 96-well microtiter plate together with 200 μl of the culture medium containing the indicated concentration of EGCG (5, 25, 50 and 100 μM) and then incubated at 37°C in a humidified multi-gas water-jacketed incubator. For the control, cultures without the drug and another culture containing only PBS in a similar concentration to the highest drug concentration were prepared. The experiments were carried out in triplicate for each drug concentration for 3 separate trials for a period of 4 days. The culture medium was replaced daily with 200 μl of a fresh medium containing the appropriate concentration of the drug. Parasitemia was monitored daily by counting the parasitized RBC to approximately 1,000 in Giemsa-stained thin blood smears. The values of a 50% inhibitory concentration (IC_{50}) of the tested drug against all parasites were calculated based on parasitemia observed at day 3 after drug treatment by interpolation after curve fitting.

Viability test

After the fourth day of the treatment, 6 μl of each of the control and drug treated (at the various concentrations) RBC was mixed with 14 μl of parasite-free RBC and suspended in a 200 μl fresh growth medium without drug supplementation. The plates were incubated for the next 10 days. The culture medium was replaced daily, and parasite recrudescence was determined by light microscopy to evaluate the parasite viability (Bork *et al.*, 2004a).

Statistical analysis

JMP statistical software version 5.1(SAS Institute Inc., USA) was used to compare the means in the *in vitro* and *in vivo* experiments by independent Student's *t*-test and considered to be significantly different when $P < 0.05$.

RESULTS

The growth of *B. caballi* (Fig. 1A) and *B. equi* (Fig. 1B) from an initial parasitemia of 1% was significantly (Student's *t*-test, $P < 0.05$) inhibited at 5 μM and significantly suppressed in the presence of 100 μM of EGCG. Complete clearance of *B. caballi* and *B. equi* (Fig. 1A and B) was observed on the 4th day of drug treatment. The 50% inhibitory concentration (IC_{50}) values of EGCG on the third day of culture were 4.42 μM (*B. caballi*) and 3.64 μM (*B. equi*). Subsequent cultivation of the parasites without the drug for a 10-day period showed no regrowth of the parasites at 100 μM for *B. caballi* and 50 μM for *B. equi* (Fig. 1A and B). Parasites exposed to lower drug concentrations started to grow again, as shown by light microscopy. There was no difference in the growth inhibition of EGCG for *B. caballi* with GIT medium (data not shown); therefore, serum had no effect on the activity of EGCG. EGCG affected the morphology of the parasites in treated cultures as indicated by light microscopy. Some parasites appeared to degenerate (*B. equi* (Fig. 2B) and *B. caballi* (Fig. 2D)). Based on light microscopic observations of the changes in the

host cell shape, size, and color and the appearance of perforations, EGCG was non-toxic to erythrocytes.

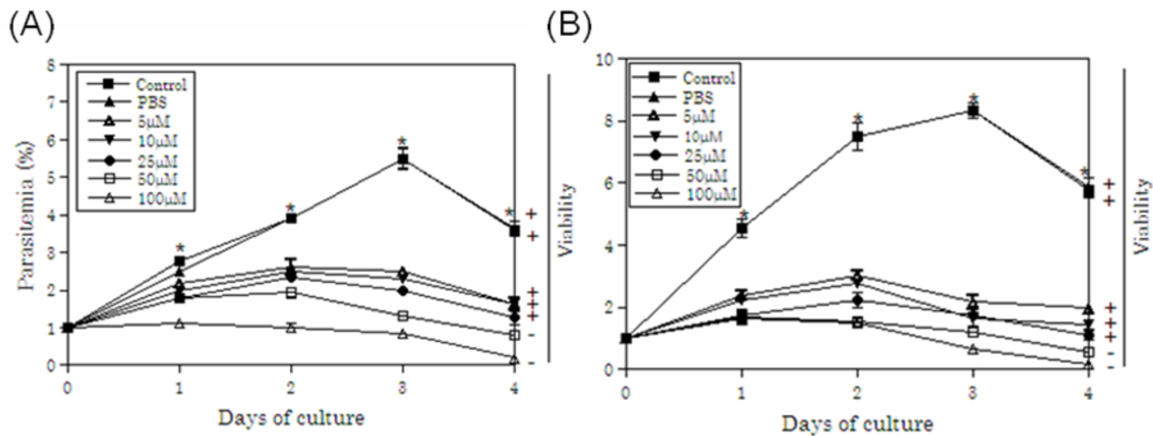

Figure 1. Inhibitory effect of EGCG on the *in vitro* growth of *B. caballi* (A) and *B. equi* (B). Each value represents the mean ± standard deviation of three separate experiments carried out in triplicate. Asterisks indicate a significant difference (Student's *t*-test; * $P<0.05$) between the 5 μM EGCG-treated and the control cultures of *B. caballi* and *B. equi*, respectively.

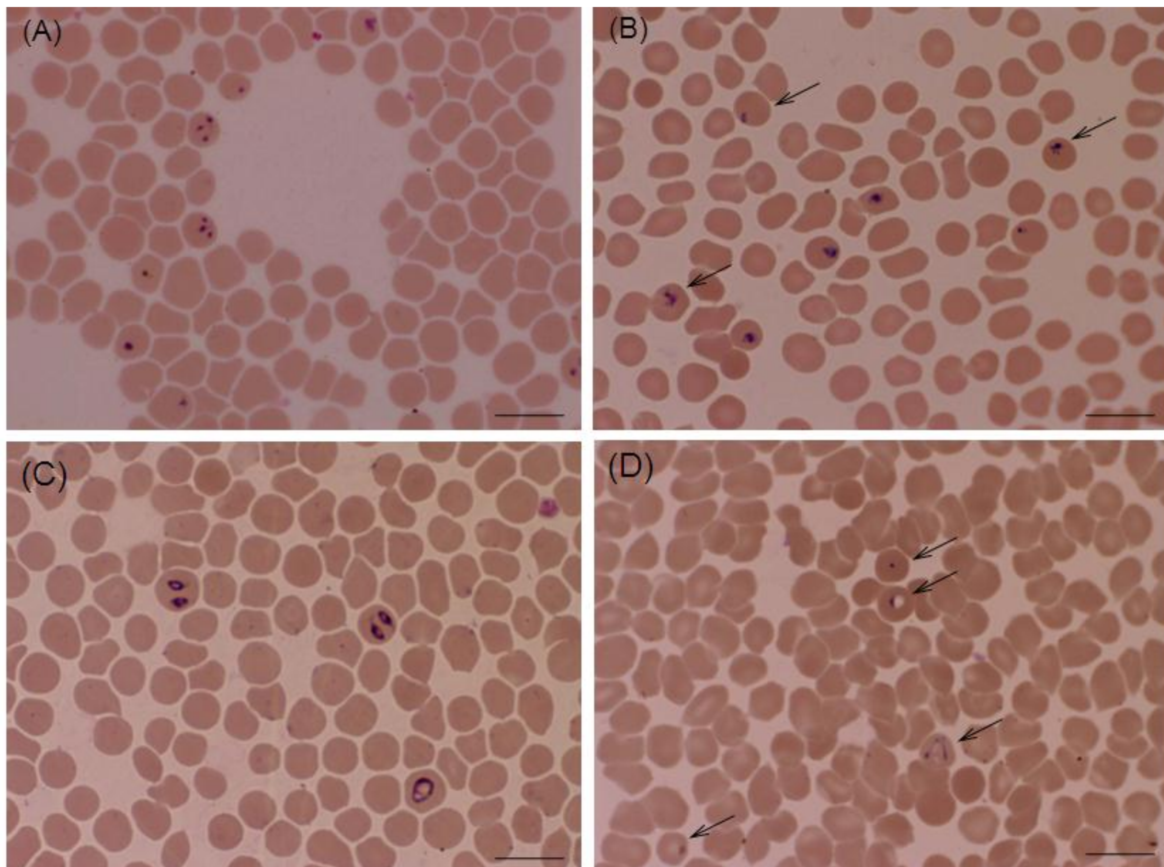

Figure 2. EGCG-treated equine *Babesia* parasites in an *in vitro* culture. *B. equi*: control (A), treated (B). *B. caballi*: control (C), treated (D). Drug-treated cultures showed a higher number of degenerated parasites. Bars, 10 μm.

DISCUSSION

In the present study, the inhibitory effect of EGCG on the *in vitro* growth of equine *Babesia* was evident. The presence of a higher concentration of EGCG in the culture completely suppressed the growth of all parasites tested in this study. As the presence of the solvent alone did not affect the growth of the parasites, the growth inhibition observed in this study was due to the effects of EGCG. The IC_{50} values of EGCG for equine *Babesia* parasites were lower than its IC_{50} values for bovine *Babesia* (AbouLaila *et al*., 2010) and lower than those of other drugs used in previous studies: ketoconazole (Bork *et al*., 2003b), gossypol (Bork *et al*., 2004b), pamaquine (Nagai *et al*., 2003), heparin (Bork *et al*., 2004a), EGTA (Okubo *et al*., 2006), chloroquine diphosphate (Matsuu *et al*., 2008), triclosan (Bork *et al*., 2003a), clotrimazole (Bork *et al*., 2003b), tetracyclines (Matsuu *et al*., 2008), staurosporine (Bork *et al*., 2006), purvalanol A (Nakamura *et al*., 2007), ALLN (Okubo *et al*., 2007) and clindamycin phosphate (Matsuu *et al*., 2008). The IC_{50} values of EGCG were higher than other babesicidal drugs: quinuronium sulfate (Brockelman and Tan-ariya, 1991), imidocarb dipropionate (Rodriguez and Trees, 1996; Brasseur *et al*., 1998), Clindamycin phosphate (Brasseur *et al*., 1998), artesunate and pyrimethamine (Nagai *et al*., 2003), and atovaquone (Pudney and Gray, 1997; Matsuu *et al*., 2008). The IC_{50}s values of EGCG were very high compared to those reported for *T. cruzi* (Paveto *et al*., 2004) indicating a higher sensitivity of *T. cruzi* than *Babesia* parasites to EGCG. The IC_{50} values of EGCG for *Babesia* were very low when compared with those reported for the normal mammalian cells of H9 (174.8 μM) and THP-1(440.3 μM) types (Yamaguchi *et al*., 2002); furthermore, the concentration of 20 μM had no effect on normal mammalian cells (Shammas *et al*., 2006). Thus, EGCG is non-toxic to mammalian cells.

EGCG was used as an antioxidative (Fraga *et al*., 1987), anti-inflammatory (Lin and Lin, 1997), antiproliferative (Ramirez-Mares *et al*., 2004; Shammas *et al*., 2006), anticancer (Chen and Zhang, 2007), antibacterial (Mabe *et al*., 1999), antiviral (Fassina *et al*., 2002; Yamaguchi *et al*., 2002; Williamson *et al*., 2006), and trypanocidal (Paveto *et al*., 2004). EGCG inhibited *B. bovis* hexose transporter I (Slavic *et al*., 2009); although, the mechanism of inhibition of EGCG for *Babesia* parasites is not fully known. On the other hand, there are several reported mechanisms of inhibition in the previous research on other microorganisms. EGCG inhibits the arginine kinase of *T. cruzi* (Paveto *et al*., 2004), while this enzyme was not found in the genome sequence database of either *B. bovis* or *B. bigemina* (http://www.sanger.ac.uk), indicating a different mechanism of action for *Babesia* parasites. EGCG has been shown to be an inhibitor for the dihydrofolate reductase (DHFR) of *Stenotrophomonas maltophilia* (Navarro-Martínez *et al*., 2005); therefore, EGCG may inhibit the homologous enzyme of *Babesia* parasites, but this requires further investigation. EGCG binds to the hemagglutinin (HA) spike proteins of the influenza virus which resulted in blocking the viral attachment to the receptors of target cells (Nakayama *et al*., 1993); furthermore, EGCG inhibited the binding of HIV1-gp120 to $CD4^{+}$T cells in a dose-dependent manner (Williamson *et al*., 2006). However, EGCG only affected on the morphology of the intracellular parasites, and the percentages of the extracellular parasites in treated cultures were not different from those of the control. Therefore, EGCG has no effect on the parasite invasion and further studies are required to identify the potential mechanism of action.

In summary, EGCG inhibited the *in vitro* growth of two equine *Babesia* species. EGCG may have potential for practical use in the *in vivo* therapy of babesiosis; however, further studies will be required to confirm its mechanism of inhibition to *Babesia* parasites.

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