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Evaluation of the inhibitory effect of N-acetyl-L-cysteine on *Babesia* and *Theileria* parasites

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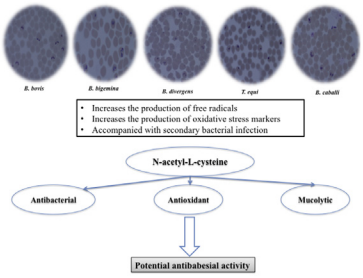
HIGHLIGHTS

- N-acetyl-L-cysteine has antioxidant activities.
- N-acetyl-L-cysteine potently inhibited *B. divergens* and *B. caballi*.
- N-acetyl-L-cysteine might be used as a treatment for *Babesia* and *Theileria* parasites.

GRAPHICAL ABSTRACT



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ABSTRACT

N-acetyl-L-cysteine is known to have antibacterial, antiviral, antimalarial, and antioxidant activities. Therefore, the in vitro inhibitory effect of this hit was evaluated in the present study on the growth of *Babesia* and *Theileria* parasites. The in vitro growth of *Babesia bovis*, *Babesia bigemina*, *Babesia divergens*, *Theileria equi*, and *Babesia caballi* that were tested was significantly inhibited ($P < 0.05$) by micromolar concentrations of N-acetyl-L-cysteine. The inhibitory effect of N-acetyl-L-cysteine was synergistically potentiated when used in combination with diminazene aceturate on *B. bovis* and *B. caballi* cultures. These results indicate that N-acetyl-L-cysteine might be used as a drug for the treatment of babesiosis, especially when used in combination with diminazene aceturate.

1. Introduction

Babesiosis is a tick-transmitted disease that causes great economic losses in the bovine and equine industries worldwide. *B. bovis* and *B. bigemina* are the main etiological agents of bovine babesiosis, which has a considerable impact on cattle health and productivity (Uilenberg, 2006). Moreover, the significance of *Babesia divergens* is almost certainly underestimated for the livestock industry in Europe in terms of the possibility of human infection (Zintl et al., 2003). Equine piroplasmosis caused by *T. equi* and *B. caballi* is considered one of the most important protozoan

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diseases affecting horses, mules, and donkeys (El-Sayed et al., 2015). Fever, hemolytic anemia, and hemoglobinuria are the principal clinical manifestations of such infection (Homer et al., 2000; Uilenberg, 2006). To date, newly developed antibabesial drugs such as epoxomicin, ciprofloxacin, thiostrepton, rifampicin (AbouLaila et al., 2010, 2012), pyronaridine tetraphosphate, luteolin, nimbolide, gedunin, and enoxacin (Rizk et al., 2015, 2016) are not available for use in the veterinary market. Moreover, some currently available antibabesial drugs in the veterinary field either have toxic side effects, as with imidocarb dipropionate, or have developed a resistance to *Babesia* parasites from prolonged use, as in the case of diminazene aceturate (Mosqueda et al., 2012). Therefore, the development of new antibabesial drugs with low toxic effects on animals and with no resistance from the parasite is urgently needed. To address this concern, the antimalarial effect of N-acetyl-L-cysteine (NAC) has been reported (Quadros Gomes et al., 2015). Additionally, many studies have validated the usage of N-acetyl-L-cysteine in the treatment of respiratory diseases (Grandjean et al., 2000; Stey et al., 2000; Poole and Black, 2001) and acute renal diseases (Tepel et al., 2000). This drug also has antioxidant (Quadros Gomes et al., 2015), antiviral (Geiler et al., 2010), and antibacterial effects (Buijtels and Petit, 2005). However, its potential as an antibabesial drug has not been examined. Therefore, the antibabesial effect of N-acetyl-L-cysteine was evaluated in the current study against the in vitro growth of bovine *Babesia* and equine *Babesia* and *Theileria* parasites.

2. Materials and methods

2.1. Chemical reagents

SYBR Green I (SGI) nucleic acid stain (Lonza, USA; 10,000x) was stored at −20 °C and thawed before use. A lysis buffer consisting of Tris (130 mM; pH 7.5), Ethylenediaminetetraacetic acid (EDTA) (10 mM), saponin (0.016%; W/V), and TritonX-100 (1.6%; V/V) was prepared in advance and stored at 4 °C. Diminazene aceturate (Novartis, Japan) was used as a positive control drug. N-acetyl-L-cysteine (Sigma-Aldrich, Japan) was prepared as a 100-mM stock solution and stored at −30° until use.

2.2. In vitro cultivation of Babesia parasites

A Texas strain of *B. bovis* (Hines et al., 1992), an Argentina strain of *B. bigemina* (Hotzel et al., 1997), a German bovine strain of *B. divergens* (Lengauer et al., 2006), a United States Department of Agriculture (USDA) strain of *B. caballi* (Avarzed et al., 1997), and *T. equi* (Bork et al., 2004) were cultivated in purified bovine or equine red blood cells (RBCs) using a microaerophilic stationary-phase culture system (Igarashi et al., 1998). Briefly, Medium 199 was used for *B. bovis*, *B. bigemina*, and *T. equi*, whereas RPMI 1640 medium was used for *B. divergens* and *B. caballi* (both from Sigma-Aldrich). Media were supplemented with 40% normal bovine serum (for bovine *Babesia* isolates) or 40% normal horse serum (for equine *Babesia* and *Theileria* isolates), 60 U/ml penicillin G, 60 μg/ml streptomycin, and 0.15 μg/ml amphotericin B (all three drugs from Sigma-Aldrich). Additionally, 13.6 μg of hypoxanthine (ICN Biomedicals, Inc. USA) per ml was added to the *T. equi* culture as a vital supplement. Cultures of parasitized RBCs (pRBCs) were incubated at 37 °C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

2.3. In vitro Babesia fluorescence assay

The in vitro *Babesia* fluorescence assay (*B*FA) was performed as previously mentioned by Rizk et al. (2015, 2016). *B. bovis*, *B. bigemina*, *B. divergens*, *T. equi*, and *B. caballi* pRBCs were diluted with non-parasitized bovine or equine RBCs to start the assay at 1% parasitemia. M199 medium was used for the culture of *B. bovis*, *B. bigemina*, and *T. equi* parasites; RPMI 1640 medium was used for *B. divergens* and *B. caballi*. The specific medium for each parasite was used alone or mixed with the indicated concentrations: 10, 5, 1, 0.5, and 0.25 μM for diminazene aceturate or 400, 100, 50, 10, and 0.5 μM for N-acetyl-L-cysteine were used for the cultivation of pRBCs in double 96-well plates (Nunc, Denmark) at 2.5% hematocrit (HCT) for *B. bovis* and *B. bigemina* parasites or 5% HCT for other *Babesia* and *Theileria* parasites for four days without daily replacement of the medium in triplicate wells for each concentration of the drug. Non-parasitized bovine or equine RBCs were loaded into each well in triplicate and used as blank controls. First, the culture plate was used to evaluate the antibabesial effect of NAC. On the fourth day of culture, a 100 μl lysis buffer containing 2 × SG I was added to each well on the first plate that contained either 97.5 μl or 95 μl of the specific medium in the assay with 2.5% or 5% HCT, respectively. Next, plates were incubated for 6 h in a dark place at room temperature, and fluorescence values were determined using a fluorescence plate reader (Fluoroskan Ascent; Thermo Labsystems, USA) at 485 nm and 518 nm excitation and emission wavelengths. Gain values were set to 100. Each experiment was repeated three times.

2.4. Viability test

The viability of bovine *Babesia* and equine *Babesia* and *Theileria* parasites was assessed on the second plate after four days of treatments, as previously described by Rizk et al. (2016). On the fourth day of culture treatment, 0.75 μl of the control or drug-treated infected RBCs from the culture with 2.5% HCT was mixed with 1.75 μl of parasite-free RBCs. For *Babesia* and *Theileria* cultures with 5% HCT, 1.5 μl of each of the control and drug-treated (at the various indicated concentrations) infected RBCs was mixed with 3.5 μl of parasite-free RBCs. Next, pRBCs were suspended in fresh growth-specific medium without drug supplementation. Then the plates were incubated at 37 °C for the next four days without daily replacement of the medium. Afterward, a 100 μl lysis buffer containing 2 × SGI was added to each well on the plate, and fluorescence values were determined as previously mentioned. The amount of parasite DNA was measured using a fluorescence spectrophotometer and used as an indicator of parasite recrudescence. Each experiment was repeated three times.

2.5. In vitro drug combination test

To examine the possible synergistic effect between N-acetyl-L-cysteine and diminazene aceturate, different concentrations of both drugs were tested in the in vitro cultures of *B. bovis* and *B. caballi* as bovine and equine *Babesia* models, respectively. Combinations of both drugs (M1, M2, M3, and M4) were based on the calculated IC_{50} values obtained from the in vitro *B*FA (Table 1) and prepared as

Table 1
Concentrations of N-acetyl-L-cysteine combined with diminazene aceturate applied to the cultures of *B. bovis* and *B. caballi* parasites.

	IC_{50}[a]	
	N-acetyl-L-cysteine	Diminazene aceturate
M1	¾	¾
M2	¾	½
M3	½	¾
M4	½	½

[a] Combinations were based on the calculated IC_{50} values obtained from the in vitro fluorescence-based assay.