Development of unstable resistance to diminazene aceturate in *Babesia bovis*

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**A B S T R A C T**

Diminazene aceturate (DA) is commonly used in the treatment of bovine babesiosis caused by *Babesia bovis*. In this study, we attempted to develop resistance in *B. bovis in vitro* to DA and clofazimine (CF, a novel antibabesial agent) using short- and long-term drug pressures. In the short term, we found that 6.7 ± 2.0 (0.54 ± 0.16 μM)-, 12.9 ± 8.6 (1.05 ± 0.7 μM)-, and 14 ± 5.9 (1.14 ± 0.48 μM)-fold increases in the half-maximal inhibitory concentration (IC50) of DA were demonstrated on *B. bovis* cultivated with 0.04 μM of DA pressure for 4, 8, and 12 days, respectively, as compared to that on parental culture (0.08 ± 0.0065 μM) before drug pressure was initiated. However, in *B. bovis* cultivated with 0.04 μM of DA pressure after 16 days, the parasites could not tolerate 0.8 μM of DA. In the long term, 7.6 ± 3.5-, 20.5 ± 0.1-, and 26.8 ± 5.5-fold increases in the IC50 of DA were demonstrated on parasites from subcultures at days 8, 3, and 5 post-cultivation, respectively, in a drug-free medium, where these subcultures were obtained from *B. bovis* cultivated with DA pressure with changing doses for 30, 60, and 90 days, respectively. However, the second and third times, no increase was demonstrated on *B. bovis* from these subcultures at days 15 and 30 post-cultivation in a drug-free medium. In addition, in *B. bovis* cultivated with drug pressure after 90 days, the parasites tolerate up to 0.64 μM DA. All findings demonstrated that DA resistance in *B. bovis* is unstable and lost within 15 days of drug withdrawal. However, treatment with subtherapeutic doses of DA in cattle might result in the development of resistance in *B. bovis*, which may not even respond to subsequent treatments with high doses of DA. Thus, if the bovine babesiosis caused by *B. bovis* is unresponsive to DA, treatment with other antibabesial agents might be recommended.

**1. Introduction**

The emergence of drug resistance in pathogens which include bacteria, protozoa, and fungi is a major challenge for the successful treatment of infectious diseases (Fairlamb et al., 2016; Yoshida et al., 2017). During last few decades, protozoan parasites have developed resistance to several antiprotozoal agents through various mechanisms, such as genetic changes in the target sites, the reduced uptake and increased efflux of drugs, and metabolic regulations (Fairlamb et al., 2016; Lemieux et al., 2016). The emergence of drug-resistant parasites has serious implications for treating clinical cases, leading to increased morbidity and mortality (Fairlamb et al., 2016). For instance, the emergence of *P. falciparum* strains resistant to almost all of the antimalarial agents has severely undermined malaria control programs (Corey et al., 2016).

The development of drug resistance in *Babesia* species, which are intraerythrocytic apicomplexan parasites, has been well documented (Hwang et al., 2010; Krause et al., 2008; Lemieux et al., 2016; Matsu et al., 2006). A combination therapy of atovaquone with azithromycin is recommended for the treatment of human babesiosis caused by *Babesia microti* (Krause et al., 2000), but *B. microti* strains resistant to both drugs have emerged recently (Krause et al., 2008; Lemieux et al., 2016; Wormser et al., 2010). In addition, a previous study found that treatment with atovaquone induces the development of resistance in *Babesia gibsoni* in dogs (Matsu et al., 2006). The possible mechanism of atovaquone resistance is suggested to be point mutations that result in amino acid substitution in cytochrome *b*, which is the molecular target of atovaquone (Lemieux et al., 2016; Matsu et al., 2006).

Diminazene aceturate (DA) is an antibabesial agent commonly used for the treatment of animal babesiosis, which is caused by a species of *Babesia* and has worldwide distribution (Bock et al., 2004; Mosqueda et al., 2012). Although different species of *Babesia* infect cattle, only three *Babesia* species including *B. bovis*, *B. bigemina*, and *B. divergens* are known to cause severe clinical babesiosis (Bock et al., 2004). In particular, *B. bovis* causes the most severe form of bovine babesiosis, as the parasite-infected erythrocytes adhere to the endothelial cells in the capillaries in internal organs, such as the brain and lungs, leading to neurological and respiratory signs (Everitt et al., 1986). Control strategies against *B. bovis* include tick control, vaccination, and chemotherapy (Bock et al., 2004; Mosqueda et al., 2012). Tick-control
strategies are often ineffective because of the development of acaricide resistance (Kunz and Kemp, 1994). Although live vaccines are used in several endemic countries, the wide use of such a vaccine is prevented by various factors, such as the risk of contamination with other blood pathogens, lack of protection due to strain variations, and time-consuming production procedures (Bock et al., 2004; Brown et al., 2006). Therefore, chemotherapy including DA is vital for minimizing the economic damage caused by Babesia parasites, including B. bovis. Unfortunately, however, recent studies have suggested the possible development of DA resistance in Babesia parasites (Hwang et al., 2010; Yamasaki et al., 2017). For instance, in vitro treatment with low to high doses of DA resulted in the development of DA-resistant B. gibsoni (Hwang et al., 2010). In addition, the low efficacy of DA against B. bovis in cattle has sometimes been documented, suggesting the possibility of the emergence of drug-resistant B. bovis in these cases (Mosqueda et al., 2012; Vial and Gorenflo, 2006). However, the development of DA-resistant B. bovis has never been investigated. Therefore, in the present study, we investigated whether continuous drug pressure could induce the emergence of DA-resistance in B. bovis in vitro. In parallel, we also investigated whether such drug pressure could result in B. bovis strains resistant to clofazimine (CF), an antibiotic that has recently been characterized as a potent antibabesial agent.

2. Materials and methods

2.1. Parasite and cultivation

The Texas strain of B. bovis was maintained in fresh cattle erythrocytes (from a mature Holstein cow) and serum-free GIT culture medium supplemented with 1 x antibiotic-antimycotic solution containing 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B, as previously described (Bork et al., 2005). Cultures were maintained in a multi-gas water-jacketed incubator in an atmosphere of 5% O2 and 5% CO2 at 37 °C. The culture medium was changed every day. All chemicals were purchased from Sigma-Aldrich (Tokyo, Japan). DA was purchased from Ciba-Geigy Japan Ltd. (Tokyo, Japan). CF was purchased from Sigma-Aldrich (Tokyo, Japan). DA and CF were dissolved in Milli-Q water (MQW) and dimethyl sulfoxide (DMSO), respectively, to prepare 10 mM stocks.

2.2. Drugs

DA was purchased from Ciba-Geigy Japan Ltd. (Tokyo, Japan). CF was purchased from Sigma-Aldrich (Tokyo, Japan). DA and CF were dissolved in Milli-Q water (MQW) and dimethyl sulfoxide (DMSO), respectively, to prepare 10 mM stocks.

2.3. Determination of the IC50 of DA and CF

The half-maximal inhibitory concentrations (IC50) of DA and CF were calculated on in vitro growth of B. bovis in serum-free GIT culture media using a fluorescence-based growth-inhibition assay as previously described (Rizk et al., 2015). Briefly, 97.5 μL of 0.01, 0.1, 0.5, 2.5, 5, 10, and 20 μM of DA and 0.025, 0.25, 0.125, 6.25, 12.5, and 50 μM of CF prepared in a culture medium was added in triplicate to a 96-well culture plate. To examine whether these solvents affect the growth of parasites, media containing 0.2% MQW and 0.5% DMSO (calculated based on their concentration in the highest dose of DA and CF, respectively) were also added in triplicate as untreated controls. When the percentage of parasitized erythrocytes (%PE) was calculated to be over 4% in previously maintained B. bovis in vitro cultures, the 4% PE were further diluted with fresh erythrocytes to achieve 1% PE, and then 2.5 μL of the 1% PE was added to each well. These 100 μL of cultures with 2.5% hematocrit concentration (HTC) and 1% initial PE were incubated as described in section 2.1 for 3 days without changing media. On day 3, 100 μL lysis buffer containing 2 × SYBR Green I (Lonza Rockland, Inc., Rockland, ME, USA) was added to each well, and then incubated at room temperature in the dark for 6 h. The fluorescence values were determined at 485 nm excitation and 518 nm emission wavelengths. Fluorescence values were corrected for background signals, converted to growth rates relative to untreated controls, and then the IC50 of DA and CF were calculated by a curve-fitting method. The calculated IC50 of DA and CF against B. bovis were 0.08 ± 0.0065 μM and 1.55 ± 0.045 μM, respectively. These IC50 of drugs were compared with the IC50 of DA and CF on parasites subjected to drug pressure, as described in sections 2.4 and 2.5.

2.4. Short-term DA and CF pressure with ½ × IC50 doses

B. bovis in vitro cultures were subjected to DA and CF pressure for 24 days (Suppl. Fig. 1). Briefly, in 24-well culture plates, 100 μL of bovine erythrocytes containing 1% PE was added to each well together with 900 μL of culture medium (10% HCT) containing ½ × IC50 doses of DA (0.04 μM) and CF (0.8 μM) and incubated as described in section 2.1. The culture medium was replaced every 24 h with fresh medium containing 0.04 μM of DA or 0.8 μM of CF. In addition, 0.2% MQW and 0.5% DMSO solvents of DA and CF, respectively, were used as respective untreated controls. On days 4, 8, 12, and 16 (every 4 days), Giemsa-stained erythrocyte smears were prepared to calculate the %PE, and then parasite-infected erythrocytes from each culture were used to prepare subcultures with 1% initial PE. The drug pressure with the ½ × IC50 dose was continued on the subcultures (1% initial PE) prepared on days 0, 4, 8, and 12, while the subcultures (1% or 4% initial PE) prepared from MQW-, DA-, DMSO-, and CF-treated cultures on day 16 were treated with 10 × IC50 doses of DA (0.8 μM) and CF (16 μM), respectively, for the next 8 days. On days 4, 8, and 12, parasites from the treated cultures were also used to determine the IC50 of both DA and CF in order to assess the potential development of drug resistance in B. bovis. The IC50 of DA and CF on B. bovis cultivated with drug pressure were calculated, essentially as described in section 2.5, using the fluorescence-based method. These IC50 were then compared with the IC50 of DA and CF as determined on previously untreated B. bovis cultures in section 2.3. All experiments were repeated 4 times.

2.5. Long-term DA and CF pressure with changing doses

Similar to short-term drug pressure, the cultures were initiated with 1% PE and 10% HTC, and the culture medium containing DA and CF was replaced every day (Suppl. Fig. 2). However, instead of preparing subcultures, fresh erythrocytes were added to the cultures, based on the HTC reductions, to maintain 10% HCT throughout the experiment. The drug pressure was initiated with a ½ × IC50 dose for DA (0.04 μM) or CF (0.8 μM). Monitoring of the %PE was performed by using Giemsa-stained erythrocyte smears. The drug doses were increased or decreased based on the levels of %PE. Subcultures were prepared and maintained in a drug-free medium after 30, 60, and 90 days of drug pressure. When over 4% PE were calculated in these subcultures (on days 8, 3, and 5 for subcultures prepared from B. bovis cultivated with drug pressure for 30, 60, and 90 days, respectively), parasites from these subcultures were used to determine the IC50 of both DA and CF for the first time. The second and third times, the IC50 of drugs on subcultures maintained in a drug-free medium were determined on days 15 and 30, respectively. The experiment was repeated twice.

2.6. DA and CF pressure with constant and increasing doses

After 90 days of drug pressure, as described in section 2.5, the cultures were treated with IC50, doses of DA and CF for another four days (Suppl. Fig. 2). After the %PE was calculated at day 94, subcultures (1% initial PE and 10% HTC) were prepared and subjected to treatment with the IC50 doses of DA and CF for three days. After this 3-day treatment, subcultures were prepared at day 97 from these cultures and treated with 2 × IC50 doses of DA (0.16 μM) and CF (3.2 μM) for 4 days. Subsequent subcultures prepared from the DA-treated cultures on days 101, 105, 109, 113, 117, and 121 were each treated for 4 days.
with 4 × 5 × 6 × 7 × 8 ×, and 9 × IC₅₀ doses of DA (0.32, 0.4, 0.48, 0.56, 0.64, and 0.72 μM, respectively). On the other hand, subcultures from CF-treated cultures prepared on days 97, 101, 105, 109, 113, and 117 were each treated for 4 days with a 2 × IC₅₀ dose of CF (3.2 μM), as the %PE in treated cultures did not increase. The experiment was repeated twice.

2.7. Statistical analysis

An unpaired Student's t-test with two-tailed method in Microsoft Excel (2016) was used to calculate the P values. A P value < 0.05 was considered to indicate significant differences between the IC₅₀s of drugs or the %PE in drug-treated cultures and their untreated controls.

3. Results

3.1. Short-term DA and CF pressure with ½ × IC₅₀ doses

As described in section 2.3, the calculated IC₅₀ of DA and CF on B. bovis in the parental culture were 0.08 ± 0.0065 μM and 1.55 ± 0.045 μM, respectively. We used ½ × IC₅₀ of DA (0.04 μM) and CF (0.8 μM) as drug pressure for 16 days because B. bovis was able to tolerate a ½ × IC₅₀ dose of the drugs (Suppl. Fig. 3A and B). On every 4th day of cultures, the IC₅₀ of DA were 0.54 ± 0.16, 1.05 ± 0.7, and 1.14 ± 0.48 μM in B. bovis cultivated with 0.04 μM DA pressure for 4, 8, and 12 days, respectively, demonstrating that the IC₅₀ of DA on the parental culture (0.08 ± 0.0065 μM) was increased by 6.7 ± 2, 12.9 ± 8.6, and 14 ± 5.9-folds, respectively (Fig. 1A, DA-line in the development of resistance). In the case of the untreated control, the determined IC₅₀ of DA were similar against parasites from parental and 0.2% MQW-treated cultures (Fig. 1A, MQW-line in the development of resistance). On day 16, 0.04 μM DA- and MQW-treated parasites in subcultures (1% or 4% initial PE) were subjected to treatment with 10 × IC₅₀ dose (0.8 μM) of DA for 8 days. Although the %PE in subcultures was significantly higher than that in the untreated MQW control during treatment with 0.8 μM DA, parasites cultivated with 0.04 μM DA pressure could not tolerate the 10 × IC₅₀ dose (0.8 μM) of DA for more than 8 days (Fig. 1B). On the other hand, the IC₅₀ of CF (1.55 ± 0.045 μM) increase of less than 5-fold was demonstrated against parasites cultivated with CF pressure for 4, 8, and 12 days and the untreated DMSO control as well (Fig. 1A, DMSO- and CF-lines in the development of drug resistance). Additionally, in B. bovis cultivated with CF pressure with a ½ × IC₅₀ dose of CF after 16 days, the parasites died on day 4 after treatment with CF at a 10 × IC₅₀ dose, as this result was similar to that of DMSO control (Fig. 1C). In addition, we found that parasites cultivated with CF and DA pressures were still sensitive to DA and CF, respectively, and the determined IC₅₀s of DA and CF were comparable to those against their untreated DMSO and MQW controls (Fig. 1A, DMSO-, CF-, MQW-, and DA-lines in cross-resistance).

3.2. Long-term DA and CF pressure with changing doses

After 30, 60, and 90 days of drug pressure with changing doses, three subcultures were obtained in drug-free medium (Suppl. Fig. 4A and B). Parasites from these 3 subcultures were then used to determine the IC₅₀ of drugs for the first time on days 8, 3, and 5, and then for a second and third time on days 15 and 30, respectively (Suppl. Fig. 5A, B, and C). The first time, 7.6 ± 3.5-, 20.5 ± 0.1-, and 26.8 ± 5.5-fold higher IC₅₀ of DA against B. bovis sourced from these 3 subcultures at 8, 3, and 5 post-cultivation in drug-free medium, respectively, as compared to those against their untreated DMSO and MQW controls (Fig. 1A, DMSO-, CF-, MQW-, and DA-lines in cross-resistance).
compared to the IC50 of DA (0.08 ± 0.0065 μM) determined before the drug pressure was initiated (Fig. 2A, DA-line in the development of drug resistance). However, the second and third times, no increase in the IC50 of DA was demonstrated on parasites from subcultures at days 15 and 30 post-cultivation in drug-free medium (Suppl. Fig. 6). In contrast, all IC50 of CF determined on subcultures obtained from B. bovis cultivated with 30, 60, and 90 days of CF pressure were comparable to the IC50 of CF determined before the initiation of drug pressure, except for the first IC50 of CF (increased 6-fold) on the subcultures obtained from parasites cultivated with 90 days of CF pressure (Fig. 2A, CF-line in the development of drug resistance, and Suppl. Fig. 6). In addition, all IC50 of DA and CF on subculture-obtained parasites cultivated with CF and DA over the long term, respectively, were similar to the IC50 of drugs determined on parental cultures when we attempted to expose drug resistance from the cultures with 16 days of drug pressure with doses determined based on the parasitemia dynamics; they were then maintained in drug-free medium. Parasites from the cultures maintained in a drug-free medium were then used to determine the IC50 of drugs for the first time on days 8, 3, and 5, and then for second and third times on days 15 and 30, respectively. The fold changes in the IC50 of drugs as compared with the IC50 of drugs on the parental lines were plotted. Note that the first IC50 of DA on B. bovis that had been subjected to drug pressure were significantly higher as compared to the IC50 of DA on the parental line, and that second and third determinations of the IC50 of DA were comparable to the IC50 of DA on the parental line. Also note that fold changes in the IC50 of CF on B. bovis cultivated with CF drug pressure were less pronounced. Additionally, the IC50 of DA and CF on B. bovis that had been subjected to CF and DA drug pressure, respectively, were comparable to those against the parental line (Nzila and Mwai, 2010). Therefore, this result suggests that the development of resistance to CF in B. bovis is unlikely with a short-term treatment. A previous study found that both DA and CF may bind AT-rich DNA regions in mitochondrial and plastid (apicoplast) genomes of B. bovis (Tuvshintulga et al., 2017). In general, parasite lines are considered to be resistant to a given drug if the IC50 of the drug increased by more than 10-fold as compared to the IC50 of DA on the parental line. B) Monitoring of %PE in culture treated with constant as well as increasing doses of DA, showing B. bovis was able to grow in an 8 × IC50 dose of DA (0.64 μM). C) Monitoring of %PE in culture treated with constant as well as increasing doses of DA, showing B. bovis was able to grow only 2 × IC50 of CF (3.2 μM).

3.3. DA and CF pressure with constant and increasing doses

After 90 days of drug pressure, we tested the effect of increasing doses of DA and CF on the development of drug resistance. After treating with fixed doses for 4 days, subcultures were prepared, and treatment was continued for several days with increased doses of DA and CF. The drug doses were increased until the parasites died. The parasites grew in an 8 × IC50 dose (0.64 μM) of DA, but all parasites died when treated with 9 × IC50 dose (0.72 μM) of DA (Fig. 2B). In contrast, all CF-treated parasites died when treated with CF at 2 × IC50 dose (3.2 μM) at day 121 (Fig. 2C).

4. Discussion

The emergence of drug-resistant parasites is one stumbling block for the effective treatment of babesiosis (Krause et al., 2008; Matsu et al., 2006; Vial and Gorenflo, 2006; Wormser et al., 2010; Yeruham et al., 1985). In general, parasite lines are considered to be resistant to a given drug if the IC50 of the drug increased by more than 10-fold as compared to the parental line (Nzila and Mwai, 2010). In the present study, we also found that a more than 10-fold increase in the IC50 of DA was determined on B. bovis cultivated with a ½ × IC50 dose (0.04 μM) of DA for 8 and 12 days (Fig. 1A). Therefore, the present findings suggest that short-term pressure with DA induces resistance in B. bovis. However, when we attempted to expose drug resistance from the culture with 16 days of pressure using a treatment with a 10 × IC50 dose (0.8 μM), parasites did not grow for more than 8 days (Fig. 1B). These observations suggest that DA-resistant parasites emerging after short-term drug pressure cannot tolerate high doses of DA. On the other hand, no more than a 5-fold increase in the IC50 of CF was demonstrated on B. bovis cultivated with a ½ × IC50 dose (0.8 μM) of CF. These slight changes in the IC50 of drugs may not indicate the development of resistance, as the fold changes were less than 10 (< 5-fold) (Corey et al., 2016; Nzila and Mwai, 2010). Therefore, this result suggests that the development of resistance to CF in B. bovis is unlikely with a short-term treatment. A previous study found a low efficacy of antibabesial agents, including clindamycin, doxycycline, and pentamidine, against DA-resistant B. gibsoni (Hwang et al., 2010). In addition, our previous study found that both DA and CF may bind AT-rich DNA regions in mitochondrial and plastid (apicoplast) genomes of B. bovis (Tuvshintulga et al., 2017). However, in the present study, DA-resistant B. bovis was not resistant to CF, indicating that resistance in B. bovis induced by DA pressure is
specific to DA.

Next, the stability and tolerable dose of drug-resistant *B. bovis* in a drug-free medium were investigated after long-term drug pressure with changing doses based on the levels of %PE. We found that after 30, 60, and 90 days of drug pressure, approximately 7.6, 20, and 26-fold increases, respectively, in the IC₅₀ of DA were demonstrated on B. bovis in subcultures at days 8, 3, and 5 post-cultivation in a drug-free medium, respectively, suggesting that DA resistance becomes more prominent with the duration of drug pressure. On the other hand, a previous study interpreted a possibility that a biphasic dose-response curve of drug indicates presence of two populations in a culture line such as drug-resistant and -sensitive parasites, whereas a monophasic dose-response curve indicates a single population (Nzila and Mwal, 2010). In the present study, we observed the monophasic dose-response curve of DA against parasites from parental culture and 0.04 µM DA-treated cultures in the short-term drug pressure, but the biphasic dose response curve of DA was only observed against B. bovis cultivated in a drug-free medium after the long-term drug pressure (Suppl. Fig. 7A and B). These results indicate that the DA resistance induced by drug pressure might be unstable when cultivated in a drug-free medium. Moreover, in the long-term drug pressure, no increase was demonstrated again on these parasites in a drug-free medium on days 15 and 30 post-cultivation, confirming that DA-resistant parasite is lost when cultivated in a drug-free medium for 15 days. The findings of present study collectively indicate that drug pressure with DA results in the development of unstable resistance in *B. bovis*. In previous studies, resistance to DA has been reported in *Trypanosoma* and *B. gibsoni* (de Koning et al., 2004; Hwang et al., 2010). The uptake of DA in *Trypanosoma brucei* and *T. equiperdium* is mediated by an adenosine transported known as P2, which is encoded by the *TbAT1* and *TevAT1* genes, respectively (de Koning et al., 2004; Witola et al., 2005). It has been proposed that the loss of activity of the P2 transporter in DA-resistant strains results in the low uptake of DA into *Trypanosoma*, leading to the reduced efficacy of DA (de Koning et al., 2004). In addition, upregulated mRNA expression of the TeDR40 gene contributes to increased resistance of *T. evansi* to DA (Witola et al., 2005). In a recent study, the reduced uptake of DA was observed in *B. gibsoni* resistant to DA, similar to DA-resistant *Trypanosoma* (Yamasaki et al., 2017). The same study found that the reduced uptake of DA is unlikely due to the loss of P2 transporter activity. Although the genes linked to DA-resistant *Babesia* are unknown, our previous study revealed that *B. bovis* mitochondrial (cob and cox3) and apicoplast (*nuA* and *clpC*) genes were upregulated during treatment with DA, while these genes were downregulated in CF-treated *B. bovis* (Tuvinintula et al., 2017). This may imply that temporally upregulated apicoplast and mitochondrial genes contribute to developing the unstable resistance in *B. bovis* to DA. Nevertheless, future investigation is necessary to clarify this assumption by using a quantitative analysis of mRNA in *B. bovis* withdrawal DA pressure. Although the mechanism of DA resistance in *B. bovis* is unclear, the present findings indicate that such mechanisms operate only during drug pressure, and parasites lose their resistance within 15 days of drug withdrawal. Therefore, the acquisition of DA-resistant *B. bovis* from cattle and its subsequent transmission to other cattle by tick vectors are highly unlikely. However, the unstable resistance of DA might potentially implicate the outcome of DA treatment in cattle with clinical babesiosis caused by *B. bovis*. On the other hand, our findings showed that the development of resistance to CF is unlikely in *B. bovis*. We also found that DA-resistant *B. bovis* is still sensitive to CF, suggesting that resistance is specific to DA only. Thus, in situations where bovine babesiosis caused by *B. bovis* is unresponsive to DA, treatment with alternative antibabesial agents, such as CF, might be recommended.

Authors' contributions

All authors conceived the present study. B.T. conducted all experiments in the present study and wrote the first draft of the manuscript, and all authors subsequently edited the manuscript.

Conflict of interest statement

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2019.02.001.

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