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ORIGINAL PAPER

Effects of a putrescine analog on *Giardia lamblia*

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Abstract The protozoan *Giardia lamblia* is the most frequent intestinal parasite of first-world countries and a major cause of waterborne disorder often referred to as traveler's diarrhea. We have previously noticed that the putrescine analog 1,4-diamino-2-butanone (DAB) remarkably inhibits the growth of anaerobic trichomonad and *Trypanosoma cruzi* parasites. Here, we examined the role of polyamines in *Giardia* cells using this putrescine analog. DAB impaired parasite proliferation dose-dependently. The analog induced increased flagella numbers and sometimes four ventral disks as well as asymmetrical division, indicating truncated or deregulated cytokinesis. Electron microscopy analysis revealed that DAB also triggered the encystment process. Oxidative stress was evaluated by measuring lipid peroxidation by thiobarbituric acid reactive substances (TBARS) detection. Trophozoites incubated either with 1 mM of DAB or putrescine for 18 h displayed increased lipoperoxide levels. Addition of 200 μM aminoguanidine, a polyamine/diamine oxidase inhibitor, partially reverted the DAB, but not the putrescine effects, indicating that the DAB effects are due, at least in part, to DAB oxidation end products. These data indicate that polyamines play a role in *Giardia* cell division, differentiation, and antioxidant defenses.

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Abbreviations

DAB	1,4-diamino-2-butanone
AG	aminoguanidine
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances

Introduction

The microaerophilic lumen-dwelling protozoan *Giardia lamblia* (syn. *G. intestinalis*, *G. duodenalis*) is the major cause of parasitic waterborne diarrheal outbreaks with about 280 million clinical annual cases worldwide reported by WHO (Upcroft and Upcroft 2001; Lloyd et al. 2002). Despite comprising a common cause of outbreaks in developed countries such as the US and Canada (Petri 2000),

giardiasis is regarded as a "neglected disease" (Savioli et al. 2006). The importance of this disease of global incidence is further highlighted by the malabsorption syndrome which causes stunting and cognitive impairment particularly among children (Berkman et al. 2002; reviewed by Farthing 2002; Ali and Hill 2003; Partovi et al. 2007).

Infections may range from asymptomatic, mainly in adults, to severe steatorrhoea mostly in children (Lengerich et al. 1994). The pathophysiological processes of giardiasis was not fully elucidated, but previous studies have reported shortening of the intestinal villi and covering of epithelium microvilli by rapidly proliferating trophozoites, resulting in reduced absorption area with impaired electrolyte and nutrient transport (Gorowara et al. 1992; Scott et al. 2002).

The 5-nitroimidazole metronidazole is first-line drug of choice not only for giardiasis, but also for amebiasis, trichomoniasis, and infections caused by different anaerobic bacteria (Nash 2001; Bansal et al. 2006; Nanda et al. 2006). It is among the most commonly prescribed drugs, extensively employed since the 1950s, therefore refractory cases are rising, and *circa* 21% of the French giardial isolates present some degree of resistance (Lemée et al. 2000; Upcroft and Upcroft 2001). In addition, metronidazole is mutagenic and potentially carcinogenic for humans (Goldman 1980; Bendesky et al. 2002) and therefore search for new giardicidal compounds is required.

Polyamines such as putrescine, spermidine, and spermine are low molecular weight organic polycations involved in cell multiplication and differentiation, as cofactors for the macromolecule synthesis and functioning (Stimac and Morris 1987; Taylor et al. 1988;). These molecules also play important roles protecting membranes and nucleic acids from oxidative stress (Tabor and Tabor 1984), which may be caused by prooxidant compounds (Bellé et al. 2004; Turrens 2004).

Putrescine reaches high levels in anaerobic parasites such as *G. lamblia*, *Trichomonas vaginalis*, (Yarlett 1988) and *Entamoeba histolytica* (Gillin et al. 1984). In most cells, synthesis occurs by the decarboxylation of ornithine to putrescine by ornithine decarboxylase (ODC). Ornithine may also be used for the polyamine synthesis, but the *Giardia* ODC activity is relatively low, compared to the arginine dihydrolase pathway, and little is known about the biological roles of putrescine, spermidine, or spermine in this parasitic protozoan (Schofield et al. 1990). The DL-α-difluoromethylornithine (DFMO; Eflornithine®), an irreversible ODC inhibitor, was developed for cancer therapy (Tabor and Tabor 1976) and shown to have microbicidal activity against African trypanosomes, becoming the drug of choice for sleeping sickness (Bacchi and Yarlett 2002). DFMO also inhibits in vitro growth of *G. lamblia*, but was ineffective against other extracellular parasites such as *T. vaginalis* and *E. histolytica* (Gillin et al. 1984).

Polyamine biosynthetic pathways furnish important chemotherapy targets, and its analogs comprise a powerful arsenal against parasitic diseases (Muller et al. 2001; Bacchi and Yarlett 2002).

Polyamine analogs have been developed and tested against different pathogens, but most studies focused on trypanosomatid parasites, and the role of polyamines in anaerobic protozoa remains largely overlooked. The putrescine analog, 1,4-diamino-2-butanone (DAB), was reported to be effective against DFMO-resistant parasites even in complex media (Calvo-Mendez et al. 1993; Reis et al. 1999).

We have previously noticed that DAB inhibits the growth of anaerobic trichomonad parasite *Tritrichomonas foetus* (Reis et al. 1999) and *T. cruzi* (Menezes et al. 2006), but little is known about the role of putrescine in *Giardia* cell biology. Here, we examined the effects of DAB in *G. lamblia* trophozoite proliferation, differentiation, ultrastructure, and oxidative stress.

Materials and methods

Chemicals 1,4-Diamino-2-butanone aminoguanidine and the polyamine putrescine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions were stored at −20°C. All reagents were of analytical grade.

Parasites The Portland-1 (ATCC no. 30888) strain of *G. lamblia* was cultivated in TYI-S-33 medium supplemented with 10% bovine serum at 37°C (Keister 1983). Parasites were harvested by centrifugation after chilling on ice for approximately 10 min. Cell density was determined by counting under light microscopy on Neubauer chamber. Growth assays were performed with a 2.0×10^5 cells/mL inoculum.

Transmission electron microscopy Parasites were fixed in 4% paraformaldehyde (Polysciences), 1% glutaraldehyde (Polysciences), 4% sucrose in 0.1 M sodium cacodylate buffer, pH 7.2, postfixed in 1% osmium tetroxide (Polysciences) and 0.08% potassium ferricyanide in the same buffer, dehydrated in acetone series, and embedded in Polybed resin (Polysciences). Thin sections were stained with uranyl acetate and lead citrate and observed under a Zeiss 900 transmission electron microscope (TEM).

Scanning Electron Microscopy Samples were fixed and postfixed as described above, dehydrated in ethanol series, dried by the critical point method in a Balzers apparatus, mounted on stubs, and covered with a 20 nm-thick gold layer. Specimens were observed in a JEOL 5310 scanning electron microscope (SEM).

Lipid peroxidation Trophozoites were washed twice by centrifugation at 500×*g* for 15 min in phosphate-buffered saline (PBS), pH 7.2. After centrifugation, 200 μL of 0.1% thiobarbituric acid (TBA) were added to 200 μL of cell suspension and incubated at 100°C for 3 h. Thiobarbituric acid reactive substances (TBARS) produced were measured at 532 nm using TBA in PBS as standard.

Results

Parasite proliferation

A remarkable and sustained (Fig. 1a) dose-dependent (Fig. 1b) inhibition of parasite proliferation was observed in DAB-treated parasites. Drug-induced alterations in cell architecture were analyzed by SEM. Contrary to control dividing cells (Fig. 1c), mitotic trophozoites exposed to DAB presented increased flagella numbers, and sometimes four ventral disks could be observed (Fig. 1d). Abnormal ventral disk disposition was commonly observed after DAB treatment. The large number of cells undergoing incomplete (Fig. 1) and asymmetric (Fig. 2a–d) division processes indicate that the antigiardial effect may be attributed, at least in part, to the impaired or deregulated cytokinesis.

Giardial differentiation

In the early phase of culture, the parasite cytoplasm often presented encystment clefts and vesicles (Fig. 3a,b). Surface protrusions assessed by SEM suggest extrusion of such vesicles at parasite surface (Fig. 3c). Ventral disk internalization was also observed by SEM (Fig. 3d). TEM analysis of the DAB-treated trophozoites revealed the disassembly and internalization of flagella (Fig. 3e) and ventral disk cytoskeleton (Fig. 3f) after 24 h of treatment. Formation of a loose, slender cyst wall was detected in some DAB-treated cells (Fig. 3e,f).

Oxidative stress

To evaluate the oxidative stress in DAB-treated trophozoites, we measured lipid peroxidation by the TBARS method. Parasites incubated with 1 mM of DAB for 18 h displayed significantly increased lipoperoxide levels (Fig. 4). Putrescine not only did not revert this effect, but also increased the levels of peroxidation in *Giardia*, (not shown). The addition of 200 μM aminoguanidine, a diamine/polyamine oxidase inhibitor, partially reverted the DAB (Fig. 4), but not the putrescine effects (not shown).

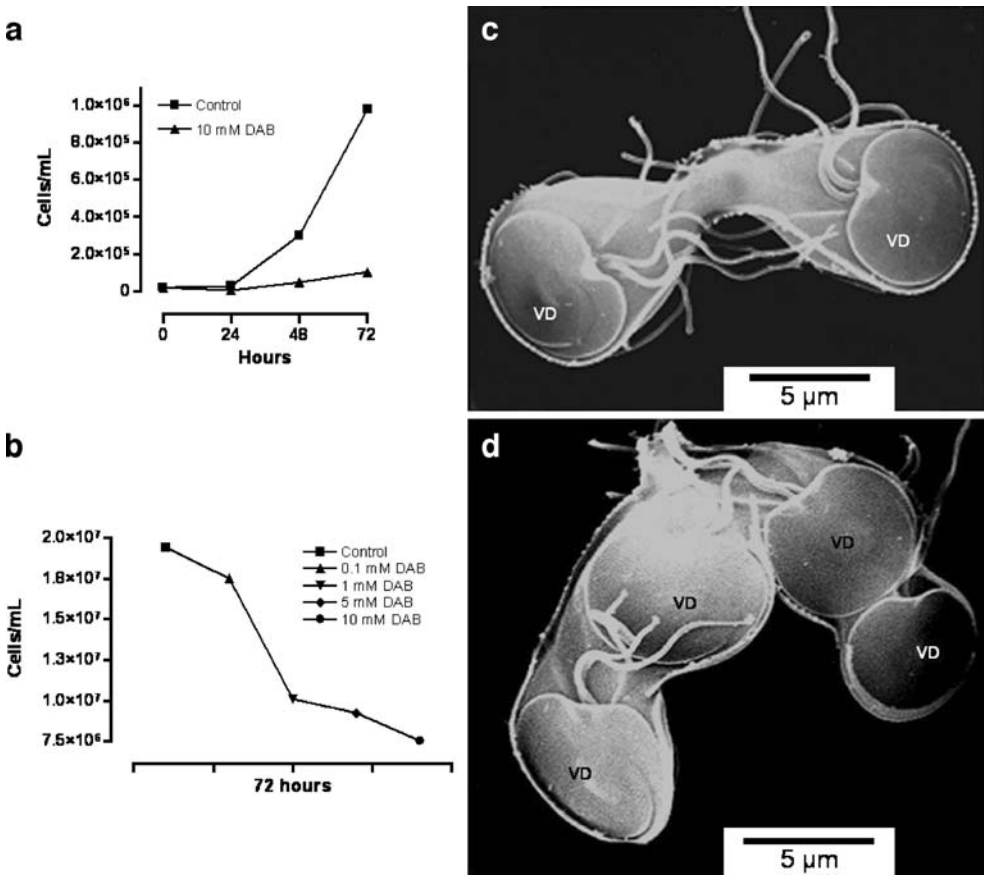

Fig. 1 1,4-diamino-2-butanone (DAB) remarkably inhibited *G. lamblia* P1 strain trophozoites in vitro proliferation in TYI-S33 medium supplemented with 10% FCS (**a**). Parasites were incubated with increasing DAB concentrations (0.1 to 10 mM). The inhibitory effect of the analog was dose-dependent in the concentration range tested (**b**). Contrary to the stepwisely dividing untreated control cells (**c**), DAB-treated parasites observed under scanning electron microscopy presented gross alterations in morphology including increased flagella number (*arrowheads*) and impaired septation process as trophozoites presenting four ventral disks (*VD*) were observed (**d**). Note that DAB-treated dividing cells formed an angle of roughly 60′ compared to the nearly 180′ in control cells. ×5,000

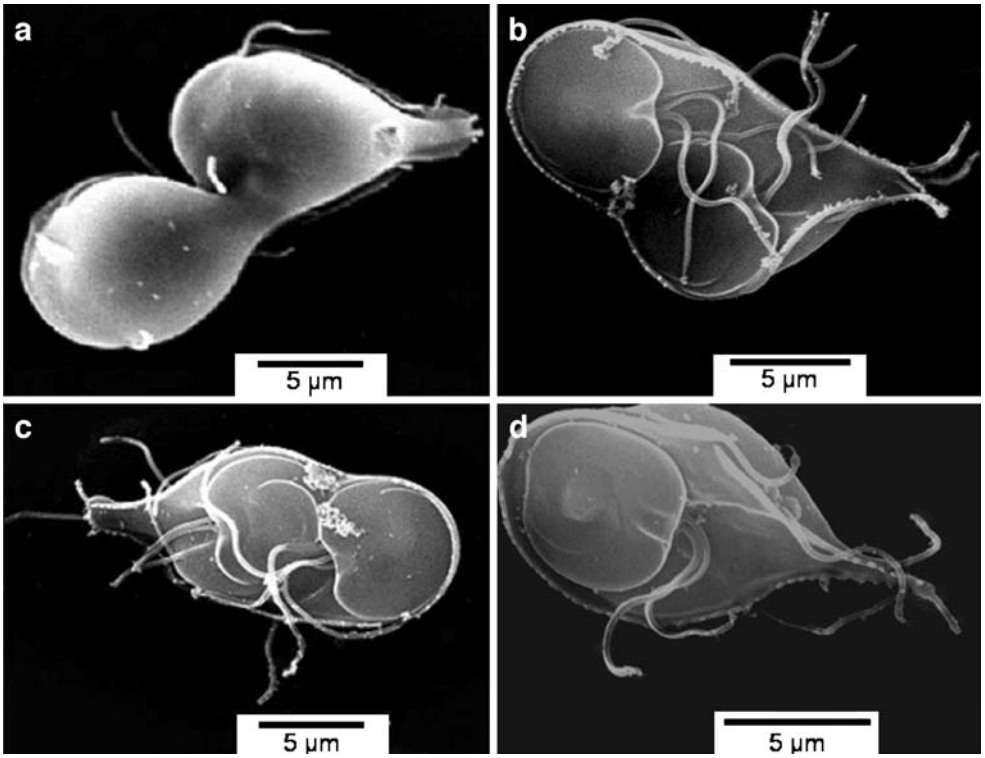

Fig. 2 Contrary to symmetrically dividing untreated control cells (*vide* Fig. 1c), DAB-treated parasites displayed asymmetrical mitosis both laterally (**a**–**c**) and dorsally (**d**)

Discussion

Giardia trophozoites colonize the small intestine, and trophozoites rapidly take up arginine, which is converted to citrulline and then to ornithine, which is extruded in an arginine–ornithine antiport system (Knodler et al. 1995). Arginine depletion from the extracellular millieu down-modulates NO production by intestinal epithelial cells, comprising an escape mechanism (Eckmann et al. 2000). Furthermore, polyamines play a protective role in stress conditions (Jung et al. 2003; Rider et al. 2007). Therefore, the detailed understanding of the aminoacid/polyamine metabolism in *Giardia* can lead not only to the elucidation of giardiasis pathophysiology but also to the discovery of new chemotherapy targets.

Gillin et al. (1984) previously showed that growth inhibition by DFMO is reverted by addition of exogenous spermidine, but not putrescine and spermine, which were toxic to parasites. Little is known about the biological role of putrescine, spermidine, or spermine in this parasitic protozoan.

G. lamblia, ODC activity is significantly higher than that in *T. vaginalis* and *E. histolytica*, but these parasites present several-fold more putrescine than the former (Gillin et al. 1984). In *Giardia*, the putrescine and spermidine concentrations are nearly equimolar, whereas the other ones display remarkably higher diamine levels. The *Giardia* ODC activity is relatively low, compared with the arginine dihydrolase pathway (Schofield et al. 1992).

Moreover, putrescine may be recognized by the arginine transport system, possibly interfering with the arginine–ornithine antiport and significantly diminishes the arginine deiminase activity (Knodler et al. 1995). DAB may act in a similar way, diminishing the proliferation capacity of *G. lamblia*. This effect is presumably multifactorial, as parasite cytokinesis was both truncated and deregulated, and differentiation was also triggered. Polyamines are involved in mammalian cell nuclear division and cytokinesis (Sunkara et al. 1979; Knuutila and Pohjanpelto 1983), and their deprivation may lead to multinucleate *Trypanosoma brucei* (Bacchi et al. 1983) and mammalian cells (Sunkara et al. 1981; Kramer et al. 2001). The giardial cell division is accepted to be rather symmetrical (Ghosh et al. 2001; Sagolla et al. 2006), but diverse cell division patterns were also reported (Benchimol 2004a). These discrete results may be caused, at least in part, by different experimental conditions. It is noteworthy that under the conditions employed here, parasites generally divide rather symmetrically as previously reported, but many DAB-treated trophozoites were observed during asymmetrical cell divisions. The detection of impaired cytokinesis and asymmetrically dividing cells suggest that putrescine/polyamines regulate the parasite mitotic process, as reported in mammalian cells (Sunkara et al. 1983).

Polyamines were also shown to regulate cell differentiation in several cell types (Fernandez-Pinilla and Pestana 1987; Gavin et al. 2004; Pietila et al. 2005; Maeda et al. 2006). The encystment-specific vesicles (ESV) and clefts

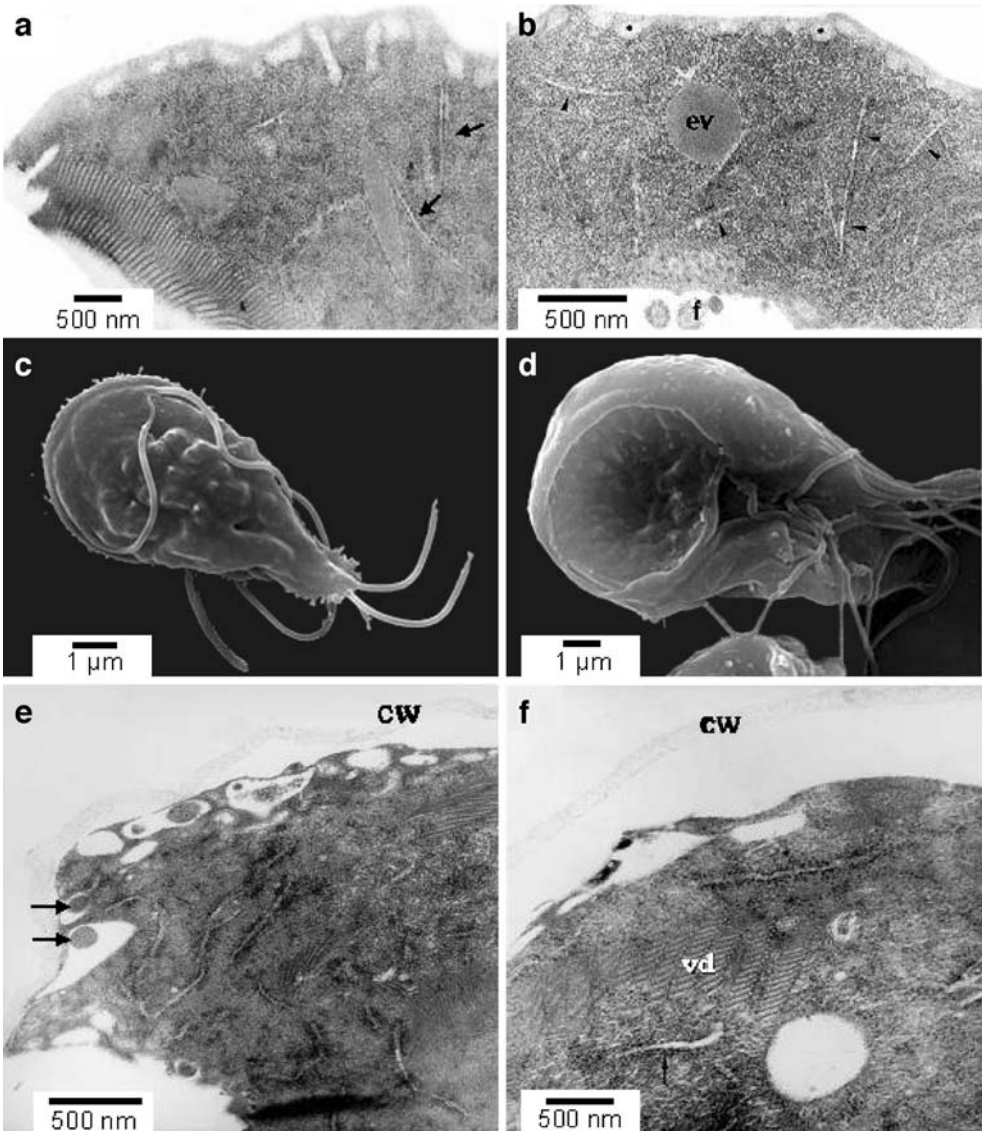

Fig. 3 After 24 h DAB treatment, trophozoites presented cytoplasmic clefts (**a**) and encystment-specific vesicles (**b**). Using SEM, we observed dorsal surface protrusions (**c**), presumably formed by the discharge of encystment-specific vesicles, and ventral disk internalization (**d**). Disassembly and internalization of flagella (**e**, *arrows*) and ventral disk cytoskeleton (**f**, *VD*) were detected after 72 h. The formation of thin loose cyst walls (CW) was also observed (**e**, **f**)

take part in the giardial secretory machinery that releases cyst wall components (Lanfredi-Rangel et al. 2003; Hehl and Marti 2004;). The surface protrusions detected by SEM in DAB-treated trophozoites were presumably formed by the fusion of ESV (Lanfredi-Rangel et al. 2003), discharging cell wall material at the transforming parasite surface, as reported previously (Benchimol 2004b). The observation of cytoplasmic clefts, ESV, and internalization of flagella and ventral disk as well as the formation of cyst walls indicate that DAB is an encystment-triggering agent.

Differentiation to cyst forms which are predominant in the jejunum is triggered by stimuli such as elevated pH and

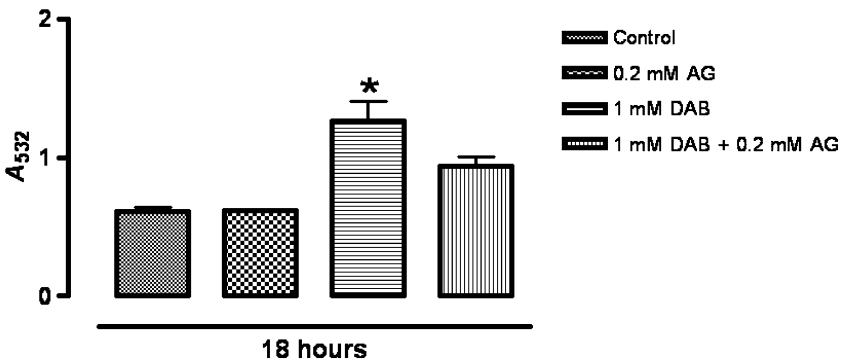

Fig. 4 Detection of TBARS. 1 mM DAB treatment significantly ($P<0.05$—ANOVA and *a posteriori* Tuckey) enhanced lipid peroxidation, and this effect was partially reverted by 200 μM aminoguanidine. Basal lipid peroxidation level (as in untreated control) were unaffected by aminoguanidine

high bile concentration (Gillin et al. 1987; Schupp et al. 1988; Reiner et al. 1993; Campbell and Faubert 1994). ODC activity may regulate microbial differentiation, and DAB was reported to remarkably inhibit ODC activity in fungi (Martinez-Pacheco et al. 1989) and bacteria (Tkachenko et al. 2001). In this regard, the encystment process in *Entamoeba invadens* is associated with decreased ODC activity (Calvo-Méndez et al. 1993). Interestingly, DAB induced the formation of pseudocysts in *T. foetus* (unpublished observation).

Furthermore, as cysts are resistant forms, encystment may comprise a response to stress conditions (Luján et al. 1996). Although cyst viability was not evaluated, the fact that only a few cells formed cyst walls, and these were loose and thin as compared to normal cysts (e.g. Chávez-Munguía et al. 2007), even employing optimal microscopy procedures, suggests that the encystment maybe not be effectively producing viable cysts.

We have previously noticed that DAB leads to the destruction of redox organelles such as the trichomonad hydrogenosomes (Reis et al. 1999) and trypanosomatid mitochondria (Menezes et al. 2006). Because polyamines (Tabor and Tabor 1984) and putrescine (Tkachenko and Nesterova 2003) display antioxidant activity, we decided to assess the oxidative stress using TBARS detection. The DAB-induced lipid peroxidation reported here may be due at least in part to diminished polyamine concentrations as reported in *T. foetus* (Reis et al. 1999). Besides down modulating polyamine transport/metabolism, DAB might be oxidized by amine oxidases present in the serum and/or the parasite, which catalyze the oxidation of biogenic amines, including polyamines to the corresponding aldehyde, with the release of NH_3 and H_2O_2, leading to the increased lipid peroxidation (Agostinelli et al. 2004).

To approach the DAB prooxidant effects, we tested whether polyamine oxidation could be involved, using a well-known antagonist. The addition of 200 μM aminoguanidine, a diamine/polyamine oxidase inhibitor, partially reverted the DAB (Fig. 4), but not the putrescine effects (not shown).

Aminoguanidine acts as an antioxidant in vivo, preventing the formation of oxygen-free radicals and lipoperoxides (Soška et al. 1997; Giardino et al. 1998). Therefore, aminoguanidine probably inhibits the DAB oxidation, reducing lipoperoxidation. In this regard, a polyamine oxidase activity was recently demonstrated in *Toxoplasma gondii*, a parasitic protozoan that also transports arginine and ornithine, which are metabolized via arginine dehydrolase pathway (Cook et al. 2007). In addition, DAB may be less effective than putrescine in protecting membranes, and as it is not metabolized as the native diamine, it is presumably accumulated, resulting in the prooxidant effect.

Taken together, these data indicate that the detailed understanding of polyamine biology in *Giardia* parasite will provide a new arsenal for therapeutic intervention.

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