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# *TRYPANOSOMA CRUZI:* EFFECT OF PHENOTHIAZINES ON THE PARASITE AND ITS INTERACTION WITH HOST CELLS

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*Phenothiazines were observed to have a direct effect on* Trypanosoma cruzi *and on its* in vitro *interaction with host cells. They caused lysis of trypomastigotes (50 uM/24 h) and, in axenic medium, dose-dependent inhibition of amastigote and, to a lesser extent, epimastigote proliferation. Treatment of infected peritoneal macrophages with 12.5 uM chlorpromazine or trifluprομazine inhibited the infection; this effect was found to be partially reversible if the drugs were removed after 24 h of treatment. At 60 uM, the drugs caused damage to amastigotes interiorized in heart muscle cells. However, the narrow margin of toxicity between anti-trypanosomal activity and damage to host cells mitigates against* in vivo *investigation at the present time. Possible hypotheses for the mechanism of action of phenothiazines are discussed.*

Key words: *Trypanosoma cruzi* – phenothiazines – calcium antagonists – parasite-host cell interactions

Phenothiazines are widely used in the treatment of psychiatric disorders. There is evidence that, by virtue of their cationic amphiphilic character, they may interact with acidic phospholipids accumulated in lysosomes (Drenckhahn et al., 1976; Hostetler, 1984), and with calcium-regulated modulators in such a way as to alter the activity of enzymes such as Ca2+–ATPase and cAMP-phophodiesterase (Levin & Weiss, 1979; Gietzen et al., 1982).

Chlorpromazine and other phenothiazinic derivatives have been tested on protozoa (Keegan & Blum, 1983), including *Leishmania* and *T. brucei* (Pearson et al., 1982; Seebeck & Gehr, 1983, El-On et al., 1986; Rice et al., 1987). Phenothiazines have been found to inhibit cAMP-phosphodiesterase in epimastigotes of *T. cruzi* (Tellez-Iñon et al., 1985) and to be active above 250 uM in a study that screened drugs for use in blood banks (Hammond et al., 1986).

Given the scarcity of published data on the effect of these licensed drugs on *T. cruzi*, and given our previous observations that inhibition of cAMP phosphodiesterase affects amastigote proliferation (de Castro et al., 1987), our aim was to analyze phenothiazine activity in an *in vitro* system (previously described in de Castro & Meirelles, 1987, 1990), and to assess the effect of these drugs on the parasite and on its interaction with host cells.

## MATERIALS AND METHODS

*Parasites and cell cultures* – Bloodstream trypomastigotes from infected mice and amastigotes from supernatant of infected J-774G-8 macrophage were obtained as previously described (Castro et al., 1987). The epimastigotes, maintained in LIT medium (Camargo, 1964), were harversted during the exponential growth phase. We used *T. cruzi* Y strain (Silva & Nussenszweig, 1953). Normal peritoneal mouse macrophages and embryonic mouse heart muscle cells were cultivated as described elsewhere (Meirelles et al., 1986; Araújo-Jorge et al., 1989).

*Direct effect on the parasites* – The three forms of T. cruzi ($5.10^6$ cells/ml) were incubated, in the presence or absence of 5-100 uM chlorpromazine (CP) or trifluprομazine (TF) (Sigma Chemical Co.), in LIT or DMES (Dulbecco modified Eagle medium plus 5%

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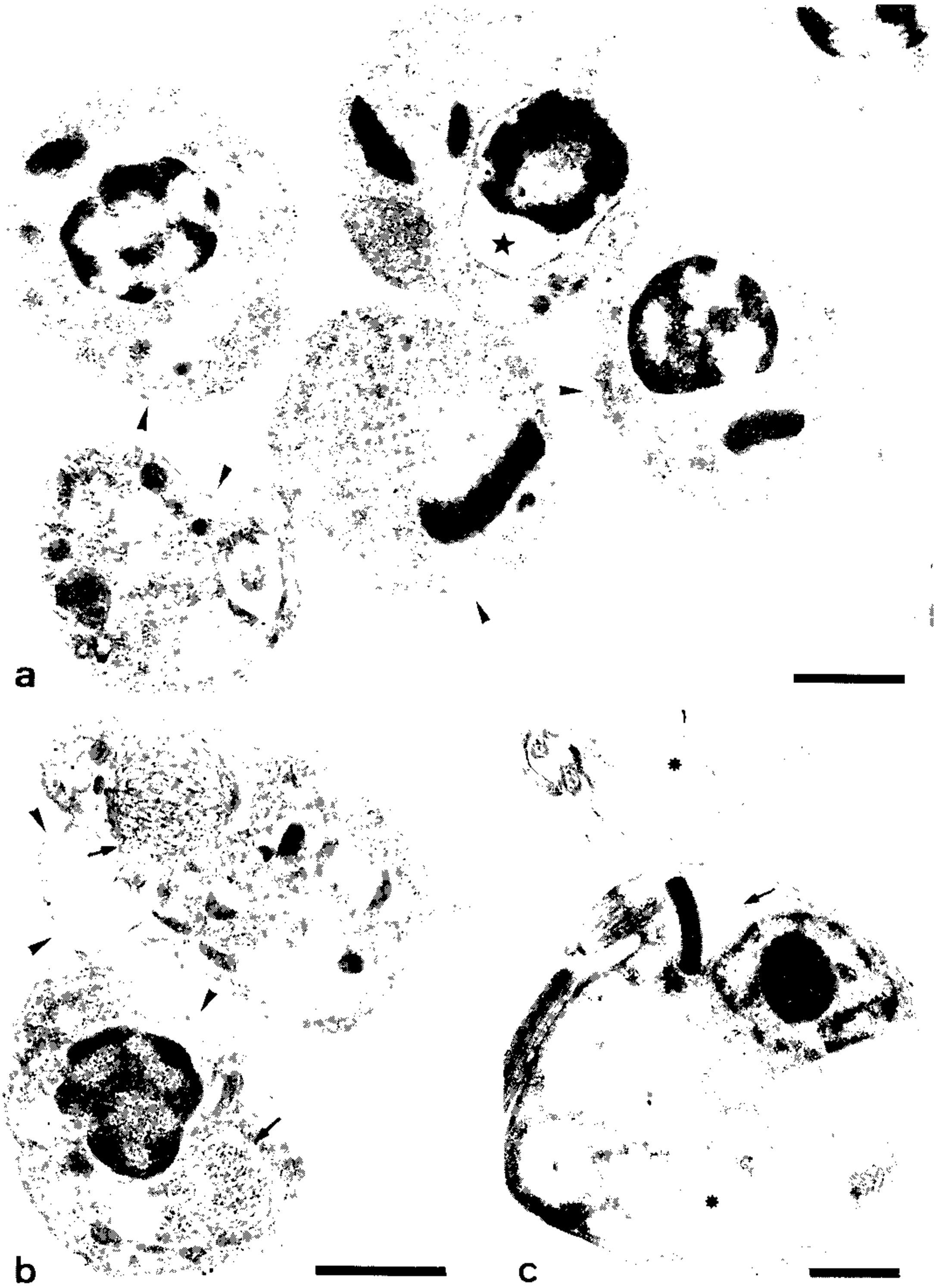


Fig. 1: effect of phenothiazines on *Trypanosoma cruzi*. The parasites were treated with the drug for 24 h. (a) amastigote forms (12.5 uM TF) with a pronounced swelling between the two nuclear membranes (star), alterations in the plasma membrane (arrow head) and an altered kinetoplast pattern and a swollen mitochondrion (arrow); (b) trypomastigote forms (12.5 uM TF) with alterations in the pattern of the kinetoplast, loss of cytoplasmic structures, and damage of the plasma membrane including disorganization of the subpellicular microtubes (arrow head); (c) epimastigote forms (25 uM CP) with swelling of mitochondria and cristae losses in the region of the kinetoplast (arrow), and with large vacuoles showing a well defined limiting membrane filling the cytoplasm (asterisk). Bars = 0.5 µm.

FCS) media at 29 °C. Cell counts were performed daily. The concentration of the drugs corresponding to 50% elimination of the parasites was expressed as ID50. Alternatively, the parasites were processed for electron microscopy after 24 h of treatment.

*Effect on* T. cruzi-*infected host cells* – Cell cultures were infected with trypomastigotes (10:1 parasite/cell ratio). After 1 h (in the case of peritoneal macrophages) and 18 h (heart muscle cells), the non-interiorized parasites were removed, and fresh DMES – with or without added drugs (6.3 – 100 uM) – was added and subsequently changed every two days. At specified intervals the cultures were processed for light or electron microscopy.

*Giemsa staining and counting* – Cells, cultured on glass coverslips, were fixed in Bouin's solution, stained with Giemsa, and counted using a Zeiss photomicroscope.

*Transmission electron microscopy* – The parasites or cell cultures were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h, rinsed in the same buffer, post-fixed with 1% osmium tetroxide, dehydrated in acetone, and embedded in Epon resin. Thin sections, stained with uranyl acetate and lead citrate, were examined in an EM10B Zeiss microscope.

## RESULTS

*Direct effect of phenothiazines on* T. cruzi – Treatment of the three forms of the parasite with CP or TF (12.5 and 25 uM/24 h) caused several ultrastructural alterations at plasma and nuclear membranes, at mitochondrion-kinetoplast complex and also vacuolization of the cytoplasm (Fig. 1). Swelling of trypomastigote forms was observed by light microscopy, and, after 24 h, at the concentration of 50 uM, both drugs caused total lysis of the parasite.

*Effect on* T. cruzi *proliferation in axenic media* – In both proliferative forms of *T. cruzi*, chlorpromazine caused a dose-dependent reduction in cell division (Fig. 2). Incubation of the parasites with 50 uM CP/24 h inhibited the proliferation of amastigotes by about 95%, and that of epimastigotes by about 25%. Triflupromazine gave similar results (data not shown).

*Effect on* T. cruzi-*infected macrophage cells* – Infected macrophages treated with 12.5 uM CP, 1 h or 24 h after interiorization of the parasites, showed a progressive decrease both in the percentage of infected macrophages and in the number of interiorized parasites; the effect was more pronounced when CP was added immediately after discarding the non-interiorized trypomastigotes (Table). This inhibitory effect could be partially reversed by the addition of drug-free medium after 24 h of treatment (data not shown).

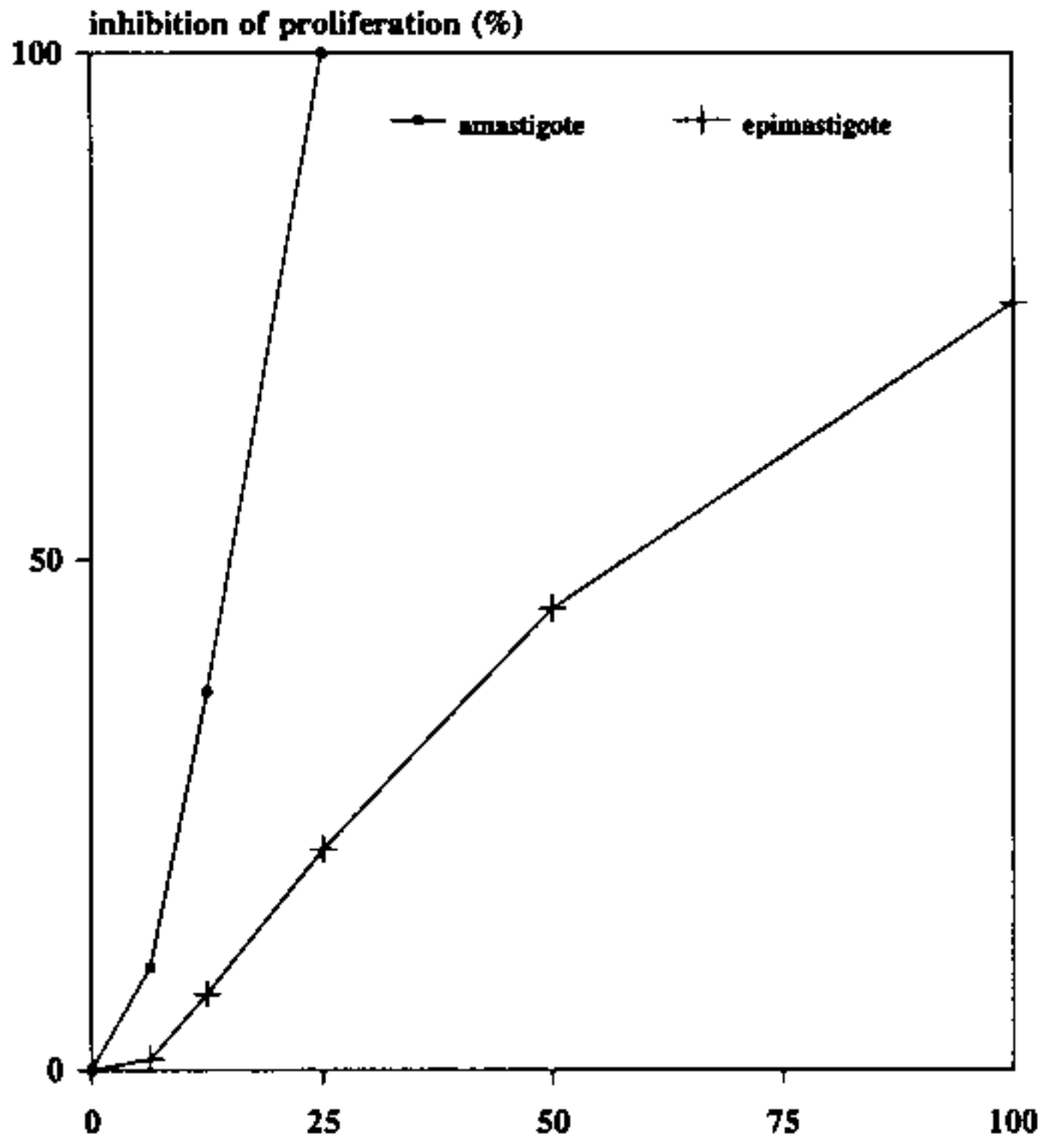


Fig. 2: inhibition of *Trypanosoma cruzi* proliferation in axenic medium chlorpromazine after 48 h. Amastigotes: ID50 = 15 uM and epimastigotes: ID50 = 57 uM.

TABLE

Effect of 12.5 uM chlorpromazine on *Trypanosoma cruzi*-infected peritoneal macrophages

| Days of infection | Addition of CP[a] | % inf.MO[b] | Par./inf.MO[b] |
|---|---|---|---|
| 1 | – | 12.9 ± 0.2 | 1.9 ± 0.1 |
| | 1 h | 1.7 ± 0.5 (86.6) | 1.2 ± 0.2 (36.8) |
| 2 | – | 16.3 ± 2.1 | 4.4 ± 0.8 |
| | 1 h | 3.0 ± 0.2 (81.6) | 2.4 ± 0.1 (45.5) |
| | 24 h | 13.5 ± 3.5 (17.2) | 4.2 ± 0.1 ( 4.5) |
| 3 | – | 16.7 ± 2.3 | 13.7 ± 1.1 |
| | 1h | 1.0 ± 0.0 (84.0) | 4.8 ± 0.5 (65.0) |
| | 24 h | 8.2 ± 0.2 (50.9) | 9.7 ± 0.0 (29.2) |
| 4 | – | 16.9 | 13.9 |
| | 1h | 1.2 ± 0.2 (92.9) | 2.9 ± 0.5 (78.8) |
| | 24 h | 5.7 ± 1.4 (66.3) | 10.1 ± 2.3 (26.3) |

The cultures were infected with trypomastigotes for 1 h; non-interiorized parasites were removed by washing.
*a:* 1 h – Immediately after washing extracellular parasites; 24 h – One day after the infection.
*b:* numbers inside parentheses indicate the % inhibition of each parameter.
The values are media ± standard deviation.

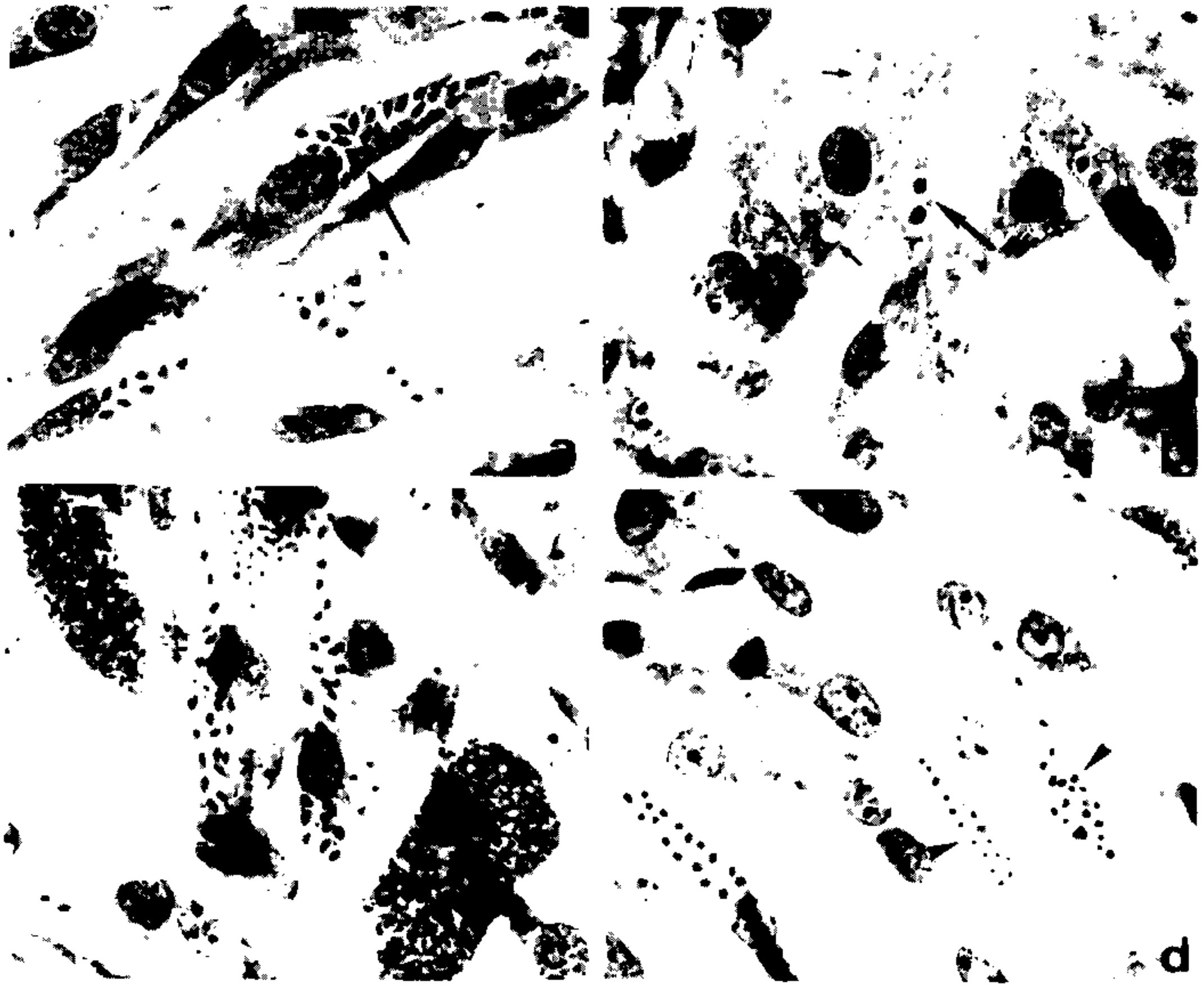


Fig. 3: effect of chlorpromazine on *Trypanosoma cruzi*-infected heart muscle cell cultures. The drug (60 uM) was added after the 24 h interiorization phase. Two days of infection: (a) control; (b) CP (one day of treatment), showing reduction in the parasite infection (arrows) and vacuolization of host cell cytoplasm (little arrow); four days of infection: (c) control; (d) CP (three days of treatment), showing damaged intracellular parasites (arrow head), and no differentiation to trypomastigotes unlike control.

At 6.3 uM, neither drug had any effect on the infection, whereas, above 25 uM, both drugs proved to be deleterious to the normal macrophage culture, causing intense vacuolization of the cytoplasm.

*Effect on* T. cruzi-*infected heart muscle cells* – At 60 uM, both drugs altered the course of infection in HMC. After two days of treatment with CP, the number of parasites/100 cells was reduced by 45%, and after three days by 81%. Amastigote proliferation and differentiation were both inhibited (Fig. 3). Treatment of infected cells with 60 uM CP for 24 h led to alterations in the plasma membrane of intracellular parasites, and even its rupture, with loss of cytoplasmic structures near these sites (Fig. 4a), and after 48 h, non-infected cardiac cells appeared normal except by the presence of numerous vacuoles containing myelin figures (Fig. 4b). At concentrations below 50 uM, CP and TF had no effect on the intracellular proliferation of amastigotes.

Normal HMC stopped contracting after treatment with 50 uM CP. However, when fresh medium without CP was added after 24 h, the cells resumed beating and vacuolization was reversed. At twice this concentration, severe damage to the host cell occurred.

## DISCUSSION

Our results show that chlorpromazine and triflupromazine were effective against *T. cruzi*, strongly inhibiting proliferation of the parasite. The ID50s, after 48 h, were 15 uM for amastigotes and 57 uM for epimastigotes. Amastigotes and trypomastigotes proved to be far more prone to the effect of phenothiazines than epimastigotes, giving further evidence that these vertebrate forms of the parasite need to

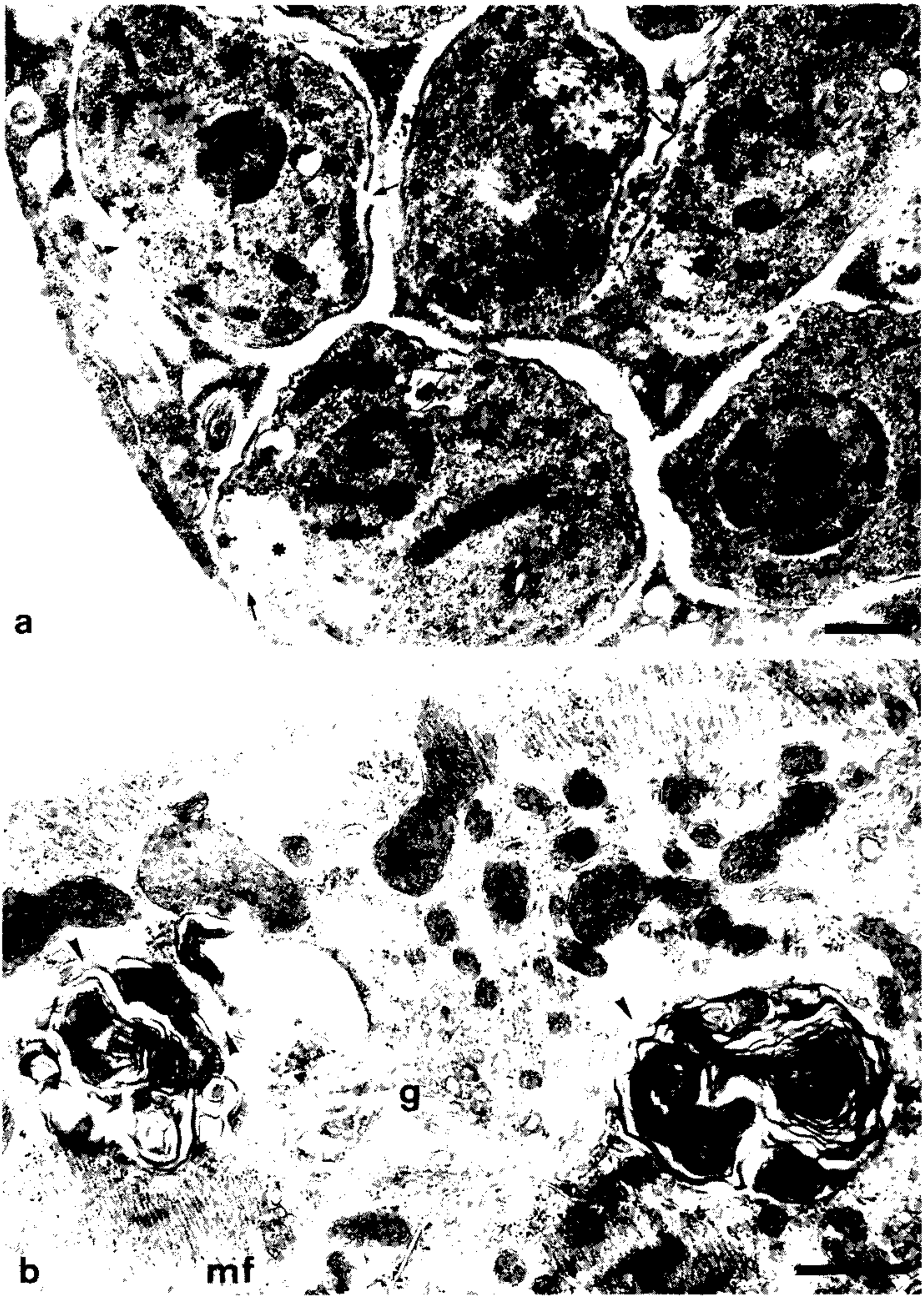


Fig. 4: effect of chlorpromazine on heart muscle cells: (a) cultures, after initial 48 h infection and treated with the drug (60 uM/24 h), showing parasites with ruptures in their plasma membrane (arrows) and with loss of cytoplasmic structures (asterisk); (b) uninfected HMC, treated with the drug (60 uM/48 h) and presenting cytoplasmic vacuoles containing lamellar inclusion bodies (arrow head). The cell still shows well preserved structures as myofilaments (mf), mitochondria (m) and Golgi cisternae (g) Bars = 0,5 μm.

be used when testing new drugs. With regard to the effect of chlorpromazine on the amastigote proliferation, there was a strong correlation between assays using axenic medium and those using macrophage cultures. In both protocols, after treatment with 12.5 uM CP/48 h, we observed a reduction of about 50% in the parasite proliferation. Similar levels of inhibition have been observed in studies of macrophage infection by *Leishmania* (Pearson et al., 1982; El-On et al., 1986).

The ultrastructural alterations observed in treated HMC were similar to those already detected in investigation on other mammalian cells (Drenckhahn et al., 1976, Fedorko et al., 1986). The alterations could be due to an intralysosomal accumulation of polar lipids resulting from their incomplete digestion following inhibition of lysosomal phospholipases A and C (Hostetler, 1984).

In epimastigote forms of *T. cruzi,* chlorpromazine caused a 48% inhibition of cyclic nucleotide phosphodiesterase in the presence of calmodulin and calcium ions (Telez-Iñon et al., 1985). Although our studies did not allow us to indentify the drug's mode of action, we suggest that, by inhibiting this enzyme, phenothiazines may induce an increase in cAMP levels, as already described by Oliveira et al. (1984) in epimastigotes, and by our group in amastigotes (de Castro et al., 1987), and that this may affect the proliferation and differentiation of *T. cruzi.*

Although phenothiazines are active against the parasite in axenic medium, our assays using cultured host cells showed that the margin between toxicity to the parasite and toxicity to host cells was narrow. This observation highlights the usefulness of our *in vitro* system (de Castro & Meirelles, 1987, 1990), in that it employs both approaches when testing new drugs on *T. cruzi.*

With regard to toxicity to the host, earlier work by Friebel & Kaestner (1955) showed that infected mice treated with chlorpromazine presented a reduced survival rate. In the light of these results and ours, animal experimentation would appear to be ill adviced, except in cases where phenothiazines are combined with other compounds in such a way as to reduce the required dosage; combination therapy of this type has already been performed successfully in studies on *Leishmania,* using phenothiazines together with pentavalent antimonials (El-On et al., 1986). Since the levels of chlorpromazine required to affect *T. cruzi* are about four times those tolerated by humans (Rivera-Calimlin, 1973), clinical trials should be ruled out until such time as successful combination therapy in animals has been achieved or new assays of its metabolites have been performed.

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