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# EFFECT OF DRUGS ON *TRYPANOSOMA CRUZI* AND ON ITS INTERACTION WITH HEART MUSCLE CELL "IN VITRO"

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*Megazol, nifurtimox, benznidazol and allopurinol were investigated, by light and electron microscopy, for their action on* T. cruzi. *Both the direct effect upon amastigote and trypomastigote forms and the effect upon the interaction of heart muscle cells (HMC) with bloodstream trypomastigotes were studied.*

*The proliferation of amastigotes in Warren medium was inhibited in a dose-dependent manner by megazol, nifurtimox and benznidazol. Treatment of amastigotes (25-50 μM/24h) and trypomastigotes (25 μM/24h) led to several ultrastructural alterations in the parasites. These three drugs also had a potent effect on the treatment of infected heart muscle cells when added at the begining of the interaction or after one or three days of infection. The interiorized parasites showed a similar pattern of ultrastructural alterations as observed by the direct effect on the amastigotes. The primary heart muscle cell culture proved to be a suitable model for the study of drugs on intracellular parasites. Likewise, the amastigote proliferation in axenic medium was shown to be an adequate assay for an initial trial of drugs. These parameters seem very reliable to us for a systematic investigation of the mechanism of action of new drugs.*

Key words: *Trypanosoma cruzi* – Chagas' disease – chemotherapeutic agents – amastigotes – trypomastigotes – heart muscle cells

The chemotherapy of Chagas' disease is still an open field, with few substances reaching clinical trial. A better understanding of the action of the currently used drugs and of newly synthesized compounds is necessary in order to identify the mechanisms of their selective toxicity for the parasite, so that more effective and safer chemotherapeutic agents can be obtained (Cançado & Brener, 1979; Avila, 1983; Stoppani, 1983; Gutteridge, 1985).

Before clinical tests can be started, the intracellular nature of the life cycle of *T. cruzi* requires "in vitro" studies for the analysis of the efficacy of drugs, as well as experimentation in animals. The use of cell cultures is important in this process. The absence of nervous connections and humoral factors in cell cultures and the possibility of adding drugs in precise concentrations are the advantages of this approach (Silva & Kirchner, 1962).

Several cell types have already been used for "in vitro" studies of the action of drugs upon *T. cruzi* (Silva & Kirchner, 1962; Brener, 1966; Bayles et al., 1966; Gutteridge, et al., 1969; Gönert & Bock, 1972; Dvorak & Howe, 1977; Alves & Rabinovitch, 1983; Murray et al., 1983; McCabe et al., 1983). Our laboratory has developed a primary culture of mouse heart muscle cells (HMC), which has been ultrastructurally characterized, showing the same basic pattern as mammalian heart cells found "in situ" (Meirelles et al., 1985, 1986). In this paper, results are given of two different approaches: a) the study of the direct effect of drugs upon vertebrate forms of *T. cruzi*-trypomastigotes and amastigotes and b) the effect upon the interaction of trypomastigotes and heart muscle cells. We tested two drugs which are used clinically with chagasic patients, nifurtimox (Lampit) and benznidazol (Radanil). Two other compounds: megazol, a very potent drug in experimental Chagas' disease (Filardi & Brener, 1982) and allopurinol, a pyrazolopyrimidine derivative currently used in the treatment of hyperuricemia and also assayed in *T. cruzi* infections (Berens et al., 1984; Avila et al., 1981, 1984), were also tested.

Our initial approach was to investigate the effects of these drugs by monitoring the timing of their action in different concentrations and schemes, in order to establish the best conditions for a subsequent analysis of the biochemical processes involved in their action.

## MATERIAL AND METHODS

*Parasites* – *T. cruzi* trypomastigotes, Y strain (Silva & Nussenszweig, 1953) and the Colombiana strain (Andrade et al., 1977 were obtained from albino mice at the peak of parasitaemia (7 and 22 days after inoculation, respectively). The parasites were isolated by differential centrifugation of the blood.

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*T. cruzi* amastigotes, Y strain, were obtained from the supernatant of a J774G-8 macrophage cell line, after 6-7 days of infection with bloodstream trypomastigotes, as described by Carvalho & de Souza (1983).

*Cell Culture* – Primary heart muscle cell cultures (HMC) were obtained from hearts of 18 days old mouse embryos. They were dissected, minced and treated with trypsin and collagenase, as described previously (Meirelles et al., 1985, 1986).

*Treatment of the isolated parasites* – The parasites were incubated, in the presence or absence of the drugs (12.5 to 50 $\mu$M) in Dulbecco's modified Eagle medium plus 10% fetal calf serum (DMES) from 2 to 72 h at 29°C, for amastigotes, and at 37°C for trypomastigote forms, and then processed for electron microscopy.

*Inhibition of amastigote proliferation* – Amastigotes at a concentration of $5.10^6$ cells $ml^{-1}$, were grown in Warren (Warren, 1960) or DMES medium, at 29°C, in the presence or absence of the drugs (3.1 to 50 $\mu$M). Cell counting was performed daily using a Neubauer chamber.

*Treatment of the heart muscle cell culture* – Three to five days-old cultures were infected with bloodstream trypomastigotes using a parasite: cell ratio of 10:1. After 24 h of interaction the cells were washed and fresh medium was added and changed every two days. Drugs in the range of 6.3 to 50 $\mu$M were added following different schemes: a) during interaction or b) after 1 or 3 days of infection, and the drugs were maintained in the cultures throughout the experiment. After addition of the drugs, the cells were fixed for observation with the light microscope or processed for electron microscopy at different times of interaction.

*Giemsa staining and counting* – The cultures were fixed in Bouin's fixative, stained with Giemsa and the cells were counted using a photomicroscope Zeiss. The percentage of infected cells and the mean number of parasites per infected cell were calculated.

*Transmission electron microscopy* – The parasites (amastigote and trypomastigote forms) or cultures were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 hour and rinsed in the same buffer. They were left overnight at 4°C, and were then gently scraped off with a "rubber policeman" and collected by centrifugation. They were then post-fixed with 1% $OsO_4$, dehydrated in acetone and embedded in Epon resin. Thin sections were stained with uranyl acetate and lead citrate and observed in an EM 10B Zeiss microscope.

*Drugs* – Stock solutions of nifurtimox (3-methyl - 4 - (5' - nitrofurfurylideneamino) - tetrahydro-4H-1,4-thiazine)-1,1-dioxide-Lampit, Bayer, W. Germany), benznidazol (N-benzyl-2-nitro-1 - imidazoleacetamide - Radanil, Roche, Switzerland) and megazol (2 - amino - 5 - (1 - methyl - 5 - nitro - 2 - imidazolyl) - 1, 3,4-thiadiazole - CL 64855, American Cyanamid Co.) were prepared in a mixture of PBS: DMSO (1:4 v/v). The final concentration of the solvent in the experiments was always less than 0.1%. The stock solution of allopurinol (4-hydroxy-pyrazolo - (3,4-d) - pyrimidine-Sigma Chemical Co.) was prepared in PBS with the addition of a few drops of NaOH 0.1M. No alteration in the pH occurred when this solution was added to the medium. All these drugs were kindly provided by Dr. Zigman Brener.

## RESULTS

*Effect on amastigote proliferation* – In control conditions, amastigotes are capable of proliferation in axenic medium, where they begin, after 6-8 days, a process of differentiation into epimastigotes (de Castro et al., 1987). Up to the fourth day in culture, only amastigote forms are present. In the presence of the drugs, a dose-dependent inhibition of their proliferation can be noted in the range of 3.1 to 50 $\mu$M. After four days, at a concentration of 3.1 $\mu$M, nifurtimox and megazol appeared more potent than benznidazol in the inhibition of the proliferation (62.8, 65.1 and 39.5%, respectively). After the 4th day, 50 $\mu$M of the three drugs caused death of all the parasites. In identical conditions, allopurinol showed only a slight effect upon the amastigotes (Fig. 1).

*Ultrastructural analysis of the parasites* – Treatment of amastigotes for a few hours with the four drugs, at a concentration of 50 $\mu$M, caused no morphological alterations in the parasite. After 24 h-treatment, with 25-50 $\mu$M of benznidazol, nifurtimox and megazol, the amastigotes showed variable degrees of alteration, such as cytoplasmic vacuolization, loss of structure in the cytoplasm, swollen kinetoplasts, and less frequently, rupture of the membrane (Fig. 2).

Treatment of trypomastigotes with 25 $\mu$M/24 h with nifurtimox and megazol showed a similar pattern of cytoplasmic disorganization, presenting dilatation of perinuclear area, disruption of kinetoplasts with swollen mitochondria and loss of structures. The maintainance of benznidazol for 48 h at a concentration of 25 $\mu$M led to a higher degree of ultrastructural disorganization in the parasites of both, Y and Colombiana strains as compared with a 24 h treatment (Fig. 3). With these three drugs, at

a concentration of 50 $\mu$M, after 24 h, total lysis of the trypomastigotes (Y and Colombiana strain) occurred.

*Effect on T. cruzi-heart muscle cell interaction-Light and Electron Microscopy* – We studied the effect of the drugs in the process of controlling the ability of the parasites to infect the cells and also their fate after interiorization, analysing the results by light and electron microscopy. In all experiments, after 24 h, the non-interiorized trypomastigotes were removed by washing the cultures. The drugs were then added, at different times: a) at the begining of the interaction, b) after 24 h of infection, and c) after 72 h, when the cells were already heavily infected. The drugs were maintained throughout the duration of the experiments.

Addition of benznidazol or nifurtimox (50 $\mu$M) during the interaction of bloodstream trypomastigotes with HMC, caused a 84% inhibition of the penetration of the parasites. When the drugs were maintained in the cultures, the development of interiorized parasites stopped (Fig. 4).

Benznidazol and nifurtimox (12.5 $\mu$M) had also a potent effect when added 24 h after infection of the cultures. After one day of treatment with benznidazol, we observed a 84% reduction in the number of parasites per infected myocyte; and after 48 h, the amastigotes presented a high degree of destruction (Table I). Comparing the effects of benznidazol and nifurtimox after three days of treatment, the effect of the nitrofuran derivative appeared more drastic than that of benznidazol (Fig. 5).

In order to test the effect upon heavily infected cultures, the four drugs were added 72 h after the infection. In one experiment, comparing benznidazol and nifurtimox (12.5 $\mu$M), this second drug had a somewhat stronger effect, but with both drugs, destruction of the parasites occurred after four days of treatment.

TABLE I

Effect of benznidazol on the interaction of heart cells with trypomastigotes (Y strain)

| Benznidazol * 12.5 $\mu$M | Myoblasts | | | |
|---|---|---|---|---|
| | % infection | | par/infected cell | |
| | control | treated | control | treated |
| 24 h | 24.0 | 15.0 | 8.0 | 1.3 |
| 48 h | 29.0 | 0.0 | 22.0 | 0.0 |

**The drug was added 24 h after infection.**

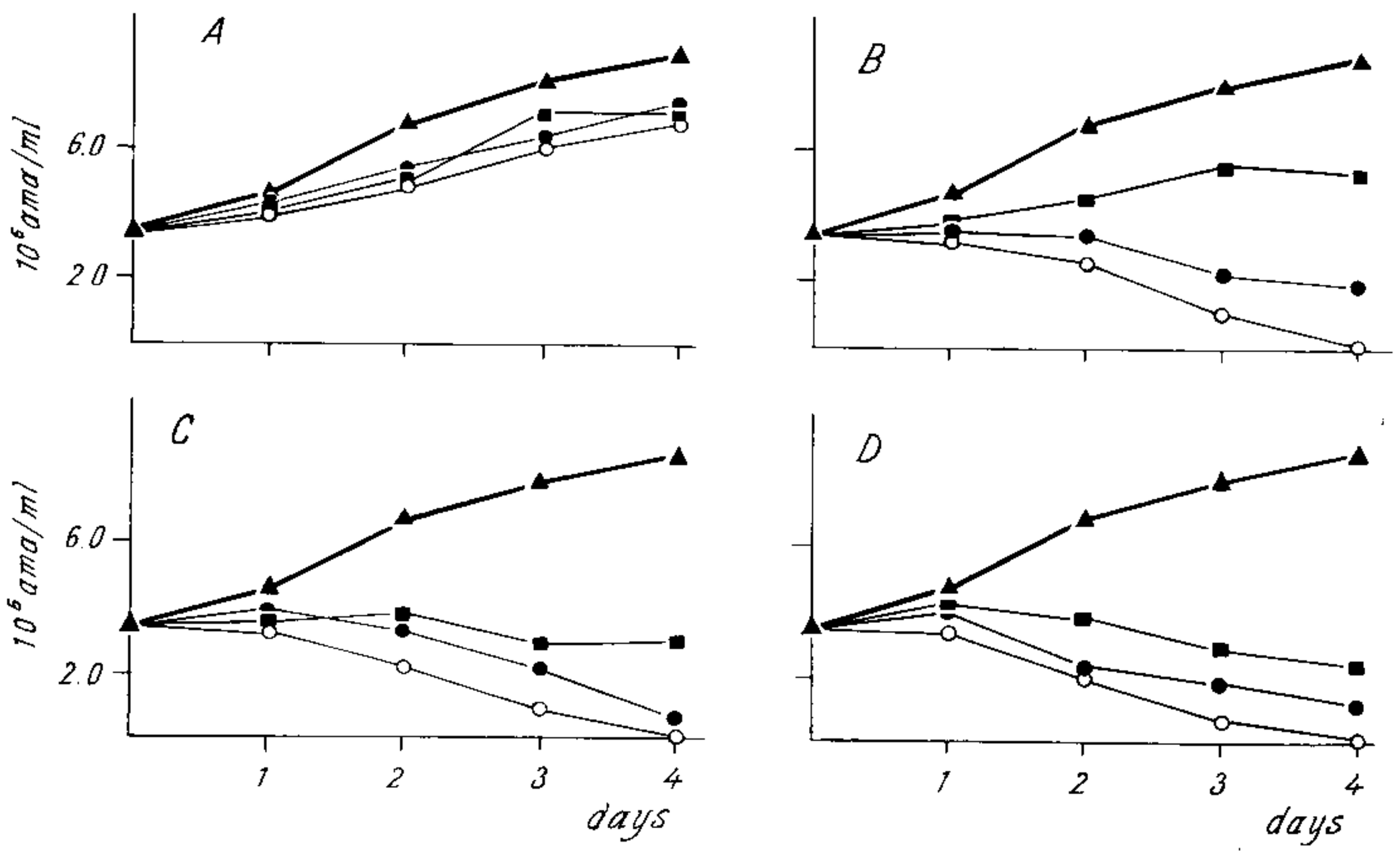


Fig. 1: Effect of the drugs on the proliferation of amastigotes of *T. cruzi* (Y strain) in Warren medium, at 29°C. (A) Allopurinol; (B) Benznidazol; (C) Megazol; (D) Nifurtimox; (▲) Control; (■) 3.1 $\mu$M; (●) 12.5 $\mu$M; (O) 50 $\mu$M.

The effect of megazol, in the range of 6.3 to 25 $\mu$M is also very potent. On treating the infected cultures with 12.5 $\mu$M megazol, we noticed a gradual degeneration and reduction of the number of interiorized parasites (Fig. 6). In the case of allopurinol, even after 8 days of treatment at a concentration of 25 $\mu$M, we observed only a very slight effect on the intracellular amastigotes.

The experiments with the Colombiana strain followed the same pattern. Heavily infected heart muscle cultures treated with nifurtimox, benznidazol and allopurinol (6.3 $\mu$M), showed that after only one day of treatment with the first two drugs, all parasites presented various degrees of degeneration with a reduction of the cellular volume. On the other hand, allopurinol showed effect only after 7 days of treatment,

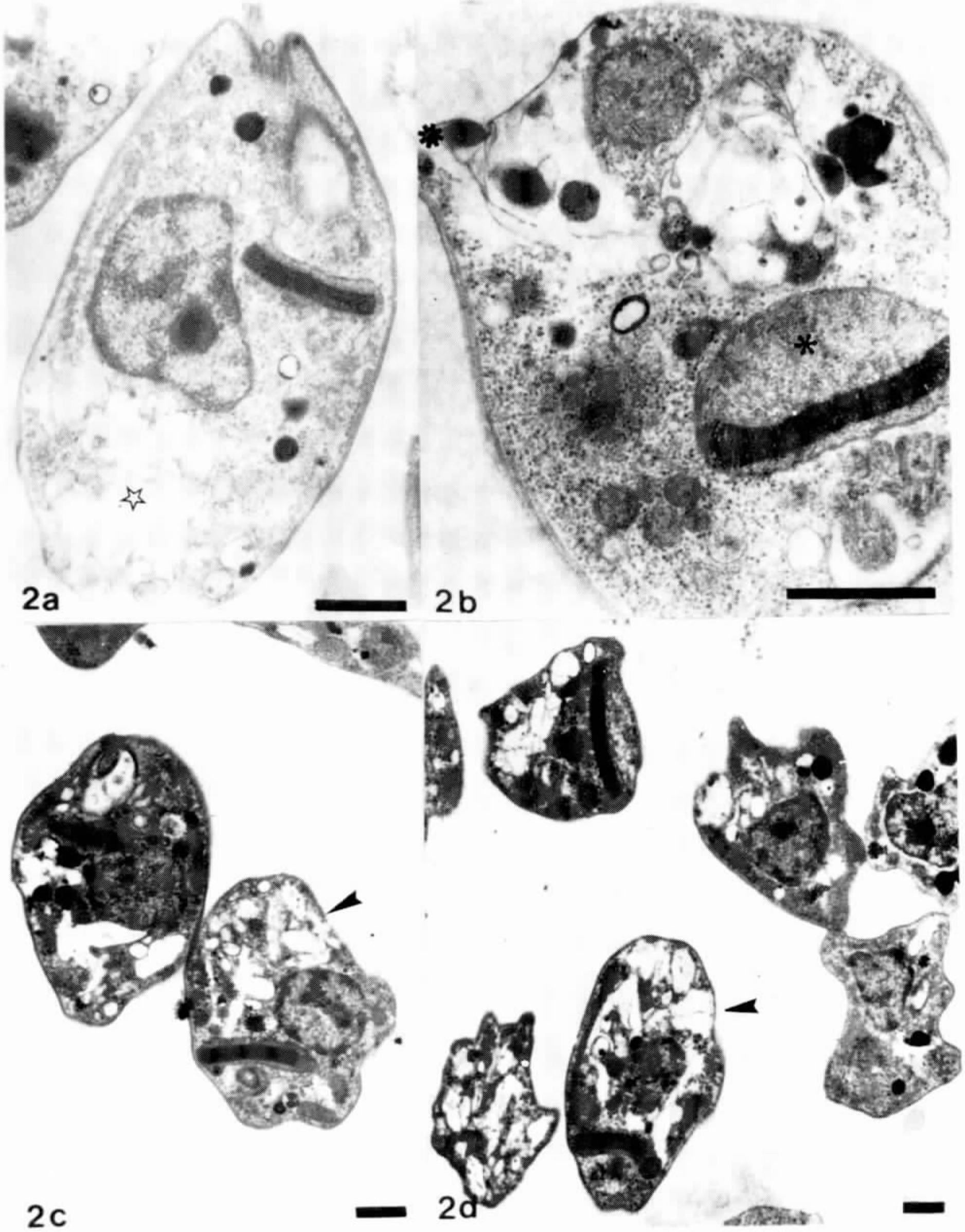


Fig. 2: Effect of the drugs on the amastigotes of *T. cruzi* (Y strain). The parasites were treated, at 29°C with 25-50 $\mu$M of the drugs for 24 h: (a) benznidazol, 25 $\mu$M (b) nifurtimox, 25 $\mu$M (c–d) megazol, 50 $\mu$M. The amastigotes present cytoplasmic vacuolization ( ➤ ), loss of structure of cytoplasm, (☆) swollen mitochondria ( ✱ ), and rupture of membrane ( ✸ ). Bars = 1 $\mu$m.

leading to a certain degree of alteration of the intracellular parasites (Table II). Megazol in the range of 6.3 to 25 $\mu$M had a very potent effect upon this strain of *T. cruzi*.

We also tested the effect of drugs upon the non-infected heart muscle cell. Allopurinol had no deleterious effect, even at 350 $\mu$M, but benznidazol, megazol and nifurtimox were toxic above 150 $\mu$M (data not shown).

At the ultrastructural level, the treatment of infected cultures for one day with nifurtimox 25 $\mu$M, showed extensive destruction of the amastigotes, in a way similar to that detected in the case of the direct treatment of the amastigotes (Fig. 7).

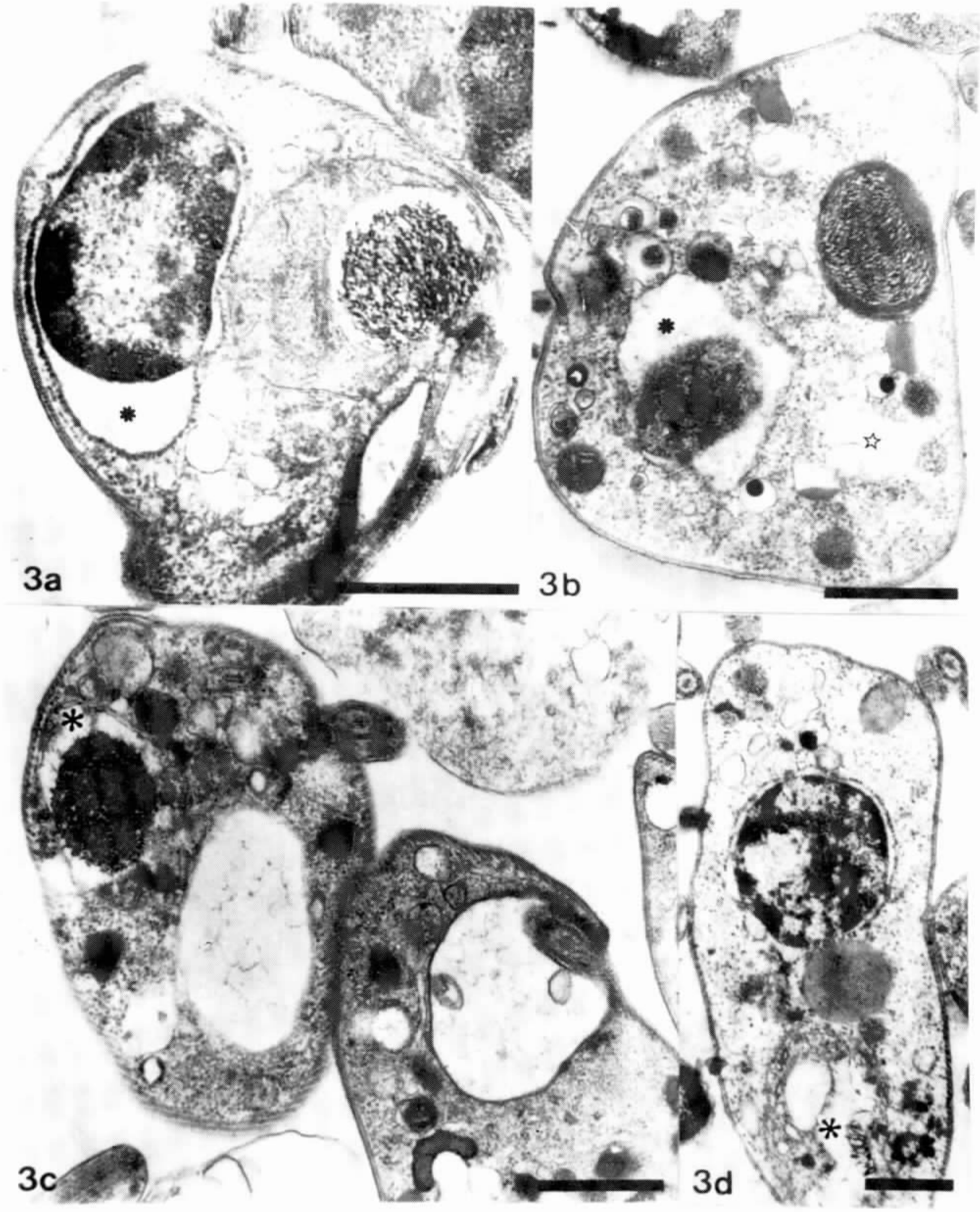


Fig. 3: Effect of the drugs of the trypomastigotes of *T. cruzi*. The parasites were treated at 37°C with 25 $\mu$M of the drugs for 24 or 48 h: (a) nifurtimox, 24 h, Y strain (b) megazol, 24 h, Y strain (c) benznidazol, 48 h, Y strain, (d) benznidazol, 48 h, Colombiana strain. An extensive effect is observed on nuclei, kinetoplasts and cytoplasm of the parasites with dilatation of perinuclear area (✸), disruption of kinetoplasts with swollen mitochondria ( * ) and loss of structures ( ☆). Bars = 1 $\mu$m.

TABLE II

Effect of several drugs on the interaction of heart muscle cells with trypomastigotes (Colombiana strain)

| Action of drugs * (days) | Control | | Allopurinol | | Benznidazol | | Nifurtimox | |
|---|---|---|---|---|---|---|---|---|
| | % inf. | par/cel | % inf. | par/cell | % inf. | par/cell | % inf. | par/cell |
| 1 | 66.0 | 18.9 | 70.0 | 12.6 | 66.0 | 13.7 | 60.0 | 8.2 |
| 2 | 66.0 | 21.1 | 66.0 | 22.2 | 48.0 | 14.0 | 60.0 | 6.6 |
| 3 | 58.0 | 24.1 | 66.0 | 20.2 | 47.0 | 8.4 | 14.0 | 8.1 |
| 4 | 58.0 | 23.7 | 62.0 | 15.5 | 41.0 | 7.4 | 5.0 | 2.4 |
| 7 | 57.0 | 21.7 | 9.0 | 4.8 | 1.0 | 5.0 | 0.0 | 0.0 |

* The drugs (6.3 $\mu$M) were added 3 days after infection.

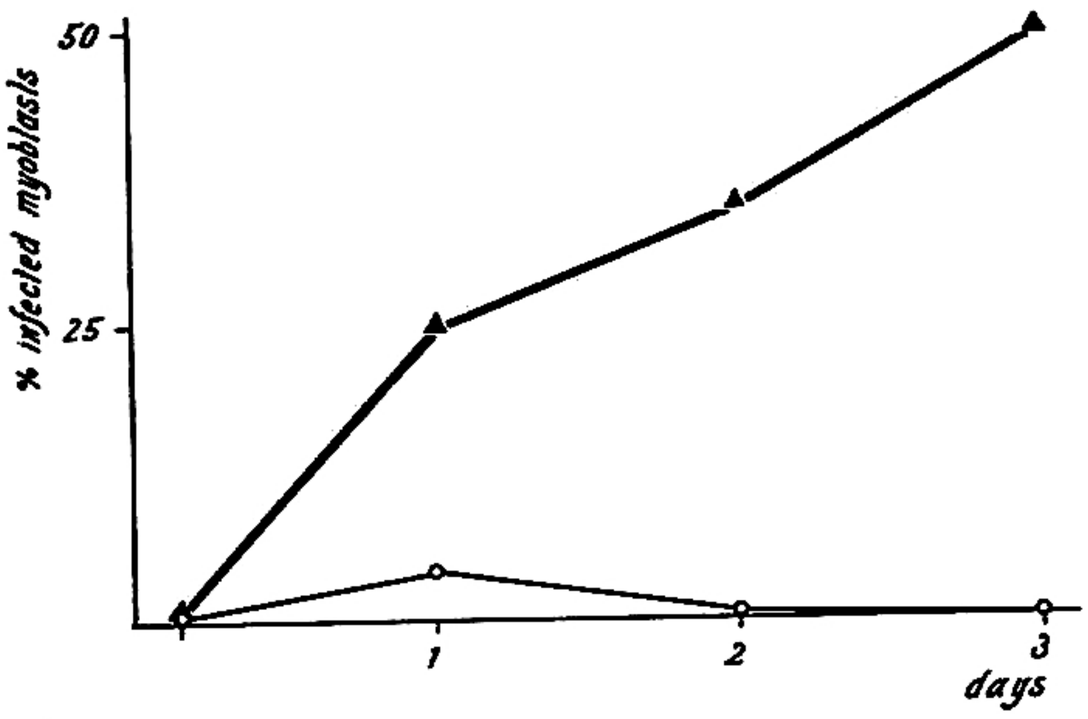


Fig. 4: Effect of benznidazol on the interaction of HMC with trypomastigotes (Y strain). The drugs was added at the begining of the interaction and maintained throughout the experiment (▲) control, (O) benznidazol 50 $\mu$M.

## DISCUSSION

Since most vertebrate cells "in vitro" can be infected with *T. cruzi*, several systems have been employed to test the efficacy of drugs. It has been shown that "in vivo" *T. cruzi* preferentially infects heart and skeletal muscle cells (Andrade, 1974; Zingales & Colli, 1985). Recently we developed, in our laboratory, an experimental system of primary heart and skeletal muscle cell cultures for the study of the interaction of the cells with *T. cruzi* (Meirelles et al., 1985, 1986). This has become a suitable model for a systematic investigation of the efficacy and mode of action of drugs. The possibility of growing amastigotes in axenic medium (de Castro et al., 1987) led us to complement our studies of cell culture with the direct investigation of amastigotes and also of bloodstream trypomastigotes. We found good correlation between the results of the direct effect upon amastigotes and the effect on the intracellular forms in heart muscle cells. We believe that the amastigote proliferation assay in axenic medium is more reliable than the use of epimastigotes cultures, for an initial trial of new drugs. Several authors had already pointed out discrepancies in the results obtained with epimastigotes comparing them with studies of vertebrate forms (Schlemper et al., 1977; Polak & Richle, 1978; Avila, et al., 1981; McCabe et al., 1985).

Amastigotes seem to be more resistant than trypomastigotes when the treatment was performed with 50 $\mu$M of the three effective drugs. Total lysis of trypomastigotes was found after 24 h, whereas amastigotes were destroyed only after four days in axenic culture. Ultrastructural examination of the direct treatment of amastigotes with benznidazol, nifurtimox and megazol showed cytoplasmic vacuolization, reduction of the number of ribosomes, as has been also described by several other authors referring to intracellular amastigotes in different systems (Voigt et al., 1972; Brener et al., 1969; Villalta et al., 1979; Maria et al., 1984). Similar alterations were observed during the study of blood trypomastigotes (Y and Colombiana strain) treated with 25 $\mu$M of these drugs.

After using different schemes, all the drugs tested in the range of 6.3 to 50 $\mu$M with the exception of allopurinol, revealed a similar potent effect upon the interaction of *T. cruzi* and heart muscle cells. At light microscopy level, the sequence of parasite destruction begins with a decrease of the volume of amastigotes with nuclear and kinetoplastic alterations. Finally, no more parasites were found, but only some loose kinetoplasts, with an aspect similar to that described by Voigt et al. (1972). These findings are in accordance with ultrastructural studies that indicate the fibrilar network of the kinetoplast as one of the more resistant structures to the action of drugs (Villalta et al., 1979, Maria et al., 1984). At ultrastructural level, the treatment with nifurtimox led to alterations of the intracellular amastigotes in heart muscle cells similar to those observed in amastigotes in axenic medium.

In experimental Chagas' disease using the rodent model, several authors described dif-

ferent behaviors of different strains of *T. cruzi* to treatment with drugs (Brener & Chiari, 1967; Habekorn & Gönert, 1972; Brener et al., 1976; Andrade et al., 1975; Andrade & Figueira, 1977; Avila et al., 1981). Our results so far showed, in the two strains tested, Y and Colombiana, no differences in the susceptibility to the drugs during the interaction with the HMC.

The study of the effect of drugs on amastigote proliferation and on interaction of cultured heart muscle cells with trypomastigotes seems to us a very reliable approach for a systematic investigation of new drugs. This system also allows a more detailed investigation of the mechanism by which these selected drugs act, through cytochemical ultrastructural methods and biochemical assays of macromolecule synthesis and enzyme determinations, studies which have already been undertaken in our laboratory.

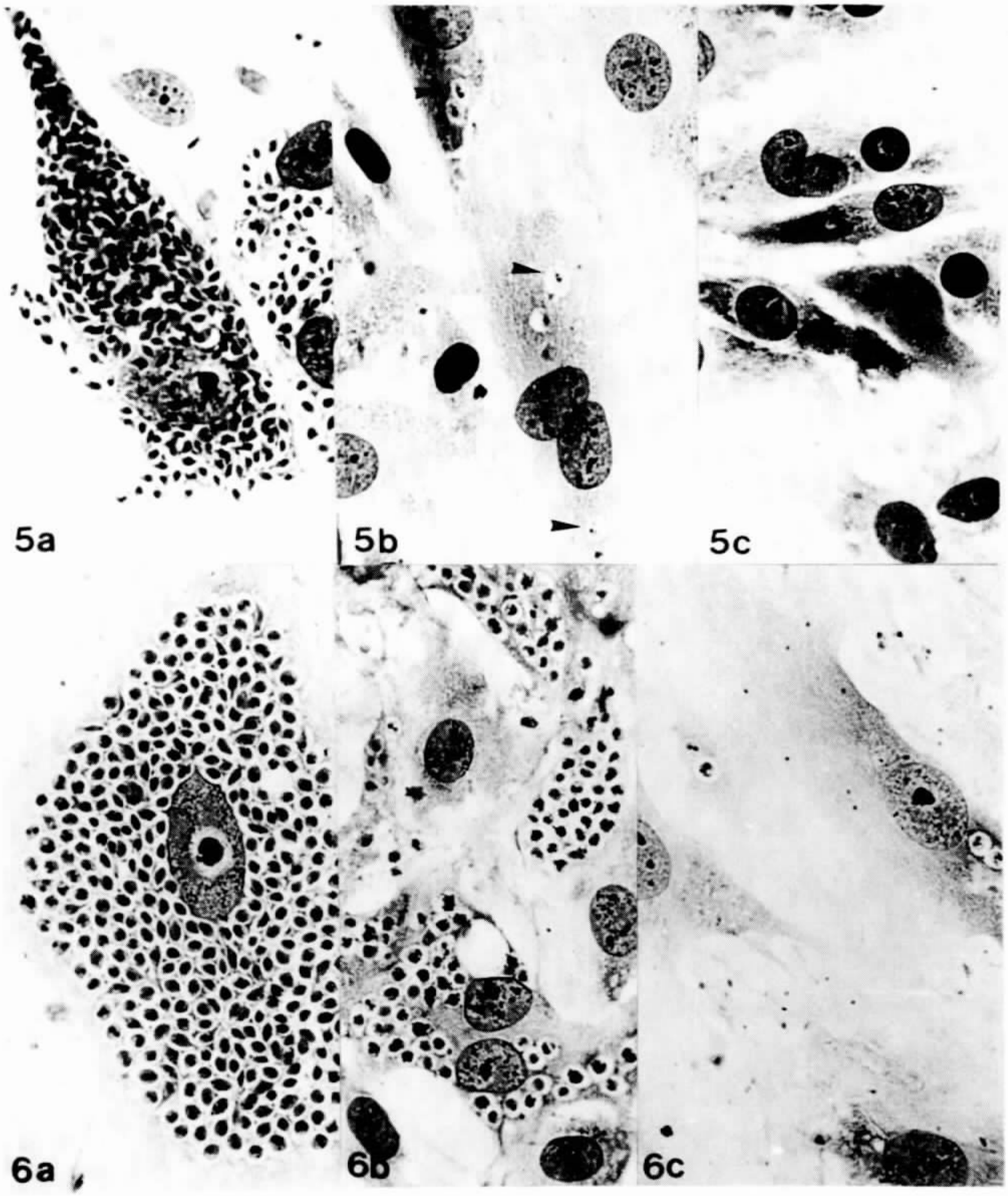


Fig. 5: Effect of nifurtimox and benznidazol on the interaction of HMC with trypomastigotes (Y strain). The drugs (12.5 $\mu$M) were added 24 h after the infection and maintained for 3 days. (a) control, (b) benznidazol, (c) nifurtimox. Fig. 5b: benznidazol causes a reduction in the number of parasites, that presented several degrees of degeneration ( ➤ ). Fig. 5c: no parasites can be seen in the cultures. Fig. 6: Effect of megazol on the interaction of HMC with trypomastigotes (Y strain). The drug (12.5 $\mu$M) was added 3 days after infection. (a) one day, (b) two days and (c) three days of treatment. A progressive effect of the drug is noticed with reduction in the number of parasites and different patterns of degeneration of the intracellular forms.

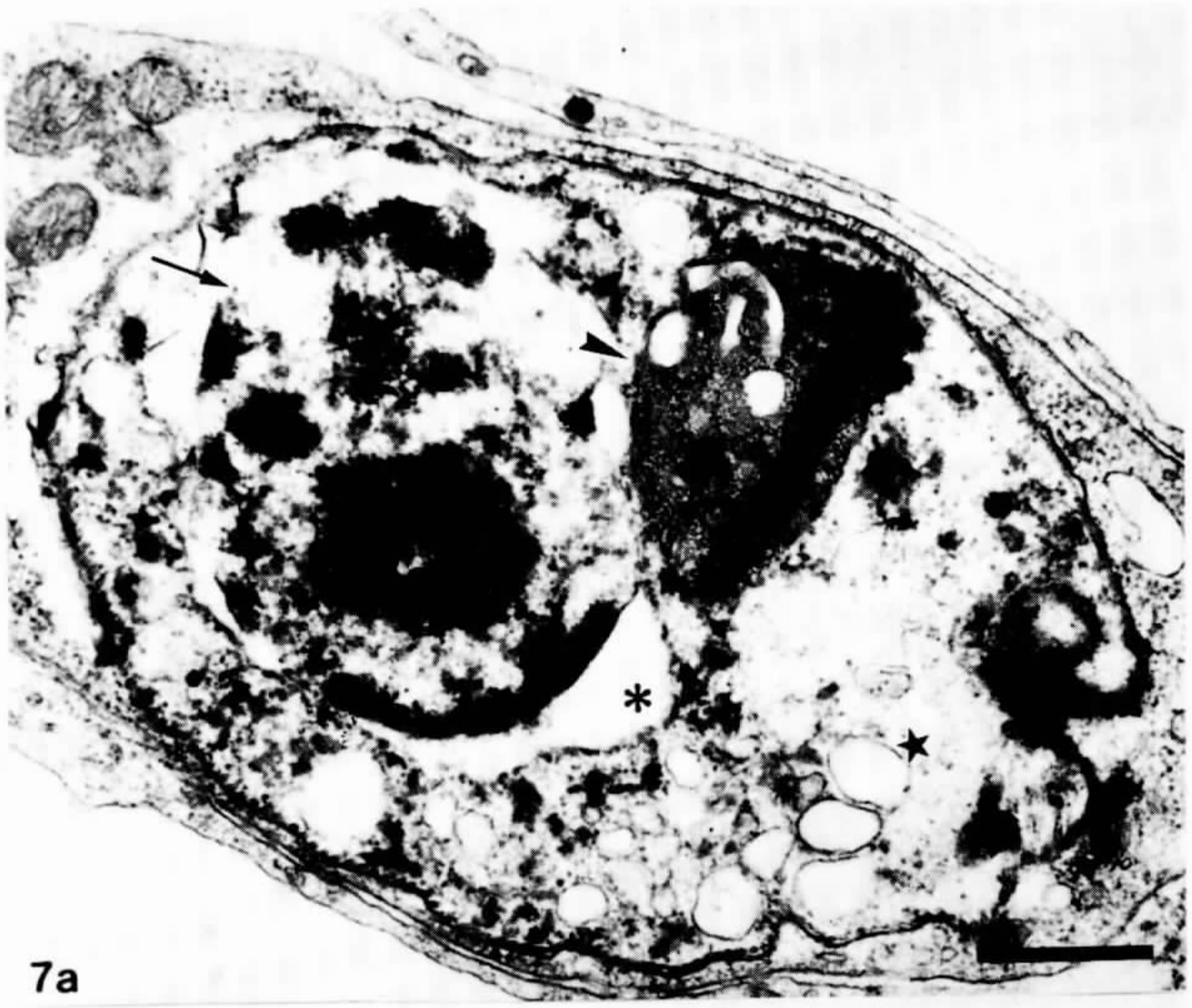



Fig. 7: Effect of nifurtimox on the interaction of HMC with trypomastigotes (Y strain). The drug (25 $\mu$M) was added 3 days after infection and the cultures fixed after 24 h of treatment. Parasites with heavily damaged structures are seen inside HMC cultures, with strong dissociation of cytoplasmic content ( ✸ ), dilatation of perinuclear area ( * ), nuclear alterations ( → ), swollen mitochondria ( ➤ ) and vacuolization ( ★ ). Bars = 1 $\mu$m.

RESUMO

**Efeito de drogas sobre o *Trypanosoma cruzi* e sua interação com células musculares cardíacas "in vitro"** — As ações de megazol, nifurtimox, benznidazol e allopurinol sobre o *T. cruzi* foram investigadas, através de microscopia ótica e electrônica, pela análise do efeito direto sobre formas amastigotas e tripomastigotas e do efeito sobre a interação de cultura de célula muscular cardíaca com tripomastigotas sangüíneos. A proliferação de amastigotas em meio Warren foi inibida de modo dose-dependente por megazol, nifurtimox e benznidazol. O tratamento de amastigotas (25-50 $\mu$M/24 h) e de tripomastigotas (25 $\mu$M/24 h) levou a várias alterações ultraestruturais nos parasitas. Estas três drogas tiveram também um efeito potente no tratamento de culturas de células cardíacas infectadas, quando adicionadas desde o início da interação ou após um ou três dias de infecção. Os parasitas interiorizados mostraram um padrão de alterações ultraestruturais semelhante ao observado no tratamento direto de formas amastigotas. A cultura primária de célula muscular cardíaca mostrou ser um modelo adequado para o estudo de drogas sobre formas intracelulares de *T. cruzi* e o ensaio de proliferação de amastigotas em meio axênico, indicado para uma triagem inicial de drogas. Estes parâmetros nos parecem muito confiáveis para uma investigação sistemática do mecanismo de ação de drogas.

Palavras-chave: *Tryponosoma cruzi* – doença de Chagas – agentes quimioterápicos – amastigotas tripomastigotas – células musculares cardíacas

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