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Mitochondrial Cardiac Energy Metabolism after One Treatment with Benzonidazole-Rochagan®

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Abstract: It was studied the mitochondrial parameters that characterized the mitochondrial cardiac energy metabolism after one treatment with benzonidazole-Rochagan®. The drug was given orally (100 mg benzonidazole/kg body weight) adult male Sprague-Dawley rats during nine consecutive days. The assayed mitochondrial parameters, using glutamate/malate and succinate as oxidative substrates, were the state 4, the state 3, the respiratory control, the efficiency of oxidative phosphorylation, the transmembrane electrical potential and the activity of the mitochondrial ATPsynthase. The results showed that all these mitochondrial parameters were not altered statistically after the above mentioned treatment, indicating that after this benzonidazole administration the mitochondrial cardiac energy metabolism was not altered.

Key words: Benzonidazole, mitochondria, heart, respiration, oxidative phosphorylation

INTRODUCTION

At the present the benzonidazole-Rochagan® is the unique drug used against Chagas´ disease (Turrens *et al*., 1996; Marr and Docampo, 1986). Chagas´ disease, caused by *Trypanosoma cruzi* infection, affects several millions of Latin Americans (Weir, 2006) and is the leading cause of infectious myocarditis (Feldman and McNamara, 2000). Benzonidazole-Rochagan® is effective for treating acute stage of Chagas´ disease, but its effectiveness for treating chronic stage remains uncertain (Viotti *et al*., 2006). Recently, it has been published some investigations (Viotti *et al*., 2006; Garcia *et al*., 2005; Toledo *et al*., 2004; de Souza *et al*., 2000), concerning to Benzonidazole-Rochagan®´ effectiveness at the chronic stage of Chagas´ disease. Given the lack of therapeutic options for Chagas´ disease, the potential benefits of benzonidazole treatment in the chronic phase of the disease should be carefully studied (Garcia *et al*., 2005). Within this kind of ideas it is also very important to clarify if benzonidazole-Rochagan® treatment alone does not cause myocardium cellular lesions, because the clinic treatment with benzonidazole has very toxic effects for different organs (Rodrigues and de Castro, 2002; Castro and Díaz de Toranzo, 1988).

Mitochondria are very important organels within cardiomyocites, because they transforme and store, at physiological conditions, more than 90% of the cellular energy, which is utilized by cardiomyocytes for their functions (Harris and Das, 1991). Additionally, the importance of mitochondria for cardiac cells is reflected by the fact that 25-35% of the volume of cardiomyocites is occupied by them. For this reason, pathophysiological events that alter mitochondrial cardiac energy production may imply severe myocardium cellular lesions (Jennings *et al*., 1978). Several investigations have already showed that cardiac mitochondria are the target of the action of some drugs and toxic agents (Scott *et al*., 1970; Moreno-Sanchez *et al*., 1999; Sanchez *et al*., 2000, 2001). Based on these facts, it is very important to know if benzonidazole administration alone implies negative alterations on cardiac mitochondrial functionality, compromising normal cellular energy supply for different biological processes at myocardium. Then, the current investigation was aimed at studying the functional state of cardiac mitochondria after one treatment with benzonidazole-Rochagan®. The studied mitochondrial bioenergetics parameters were the state 4, the state 3, the respiratory control (RC), the efficiency of oxidative phosphorylation (ADP:O), the mitochondrial transmembrane electrical potential ($\Delta\Psi$) and the activity of the mitochondrial ATPsynthase.

MATERIALS AND METHODS

Benzonidazole administration: Benzonidazole (N-benzyl-2-nitroimidazoyl acetamide, Rochagan®, Roche) was prepared daily by trituration and suspension of the tablet in distilled water. The therapeutic scheme with benzonidazole was according to de Souza *et al*. (2000), in which the drug was given orally (100 mg benzonidazole/kg body weight) male adult Sprague-Dawley rats for nine

consecutive days. Control animals receive only distilled water (the same daily volume than treated animals) for nine consecutive days. All animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA).

Preparation of mitochondria and assay of mitochondrial bioenergetics parameters: Rat heart mitochondria were isolated from adult male Sprague-Dawley rats, fasted overnight according to Mela and Seitz (1979). State 4, state 3, RC and ADP:O were assayed by means of the rates of oxygen consumption, which was determined with a Clark oxygen electrode in the basal medium 100 mM Sucrose, 75 mM KCL, 10 mM HEPES, 0.1 mM EGTA, 10 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.4; the substrate was 4 mM glutamate plus 1 mM malate or 5 mM succinate-2.5 μM rotenone. $\Delta\Psi$ was measured by monitoring the fluorescence of safranine O (520 nm/580 nm) according to Lemeshko (2002), using the above mentioned basal medium in which 10 mM KH_2PO_4 was replaced by 20 mM potassium acetate in order that measured $\Delta\psi$ accounts for the entire electrochemical potential (Δp) generated by mitochondria. The activity of mitochondrial ATPsynthase was estimated from state 3 and ADP:O parameters. All experiments were carried out at 30°C using 1 mg of mitochondrial protein × mL^{-1}. Protein was determined by the biuret method (Gornall *et al*., 1949). All reagents were of analytical grade and were purchased from Sigma. The quality of the current isolated mitochondria is reflected by their good obtained RC (7.61±0.19; n = 7), using glutamate/malate as substrate.

Statistical analysis: All values were means±SEM of seven independent experiments and their statistical significance ($p<0.05$) was evaluated using Students t-test. Statistical analysis was performed by GraphPad PRISM-version 2.0 software (San Diego-CA, USA).

RESULTS AND DISCUSSION

At the present benzonidazole-Rochagan® is the only drug that is used in Latin America for the treatment of Chagas´ disease, a parasitic disease that is endemic in Latin America, affecting 16-18 million people, with more than 100 million exposed to the risk of infection. The parasite *Trypanosoma cruzi* is the etiological agent of Chagas disease. Benzonidazole-Rochagan® is effective for treating acute stage of Chagas´ disease, but its effectiveness for treating chronic stage remains uncertain (Viotti *et al*., 2006). Recently, it has emerged scientific works that focus on the prophylactic effect of benzonidazole treatment during the chronic phase of Chagas´ disease (Viotti *et al*., 2006; Garcia *et al*., 2005; Toledo *et al*., 2004; de Souza *et al*., 2000). Because benzonidazole treatment has very toxic effects (Rodrigues and de Castro, 2002; Castro and Díaz de Toranzo, 1988), it is very pertinent to study the possible myocardial cellular lesions due to this treatment. Mitochondria play crucial role at the physiology of myocardium because they produce more than 90% of the cellular energy (ATP), which is utilized in the myocardium (Harris and Das, 1991). To our knowledge, at the present, no studies have investigated the mitochondrial cardiac energy-converting system after one benzonidazole administration.

In the current investigation it was studied heart mitochondrial respiration, using the oxidative substrate glutamate/malate as well succinate. When mitochondria are respiring using glutamate/malate or succinate their respiratory chain is feeding with electrons from Krebs cycle or from complex II, respectively. All these data are complementary and reflect the overall state of the functionality of mitochondrial respiratory chain coupled with Kreps cycle, for production of mitochondrial cellular energy (ATP).

In Fig. 1, it was showed the ratio of the heart weight, divided by the body weight from normal and benzonidazole-Rochagan® treated rats after the 9th day of the treatment; this treated ratio was statistical equal to the control ones, showing that after this benzonidazole treatment the heart does not reflect hypertrophy.

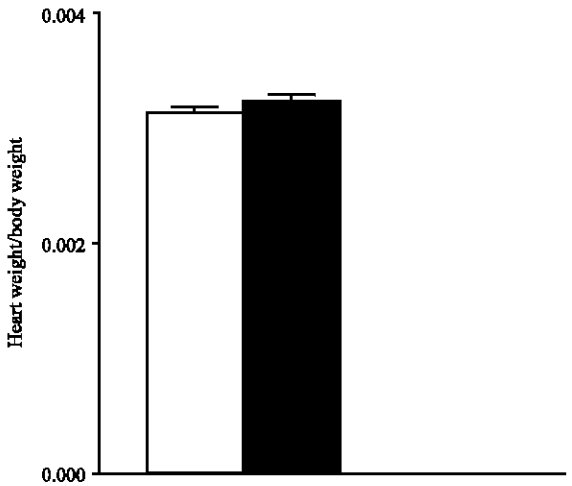

Fig. 1: The ratio of heart weight, divided by body weight from normal and benzonidazole-Rochagan® treated rats after the 9th day of the treatment. Normal animals (white bar) and treated animals (black bar); all data were means±SEM (n = 11). There is no statistical difference between groups

Table 1 and 2 showed the current studied mitochondrial bioenergetics parameters of heart mitochondria after one treatment with benzonidazole-Rochagan®, using glutamate/malate and succinate as substrate, respectively.

Table 1 and 2 showed that benzonidazole-Rochagan® administration did not decrease $\Delta\Psi$ with respect to that of the control when mitochondrial respiration was supported by glutamate/malate or succinate, indicating that it did not induce an increase in the passive membrane permeability to protons. The activity of the mitochondrial ATPsynthase did not decrease upon benzonidazole-Rochagan® administration when mitochondrial respiration was supported by glutamate/malate or succinate, i.e., the mitochondria after benzonidazole-Rochagan® treatment were able to rephosphorylate ADP with a rate sufficient to preserve the mitochondrial cellular energy supply. Indeed, the ADP/O ratio did not statistically decrease after benzonidazole administration when mitochondrial respiration was supported by glutamate/malate or succinate, reflecting any important energy dissipation. These tables also show that the state 4 and 3 from control and treated groups, with glutamate/malate or succinate, are practically equal; additionally, these data also display no statistical difference, with respect to the control, in RC (a mitochondrial parameter used for assessing the functional integrity of a mitochondria preparation) when mitochondrial respiration was supported by glutamate/malate or succinate. Taken together, all these data indicated that the functional state of heart mitochondria was not altered after the current benzonidazole-Rochagan® administration, warranting normal supply of cellular mitochondrial energy for the biological processes taking place at myocardium after this benzonidazole-Rochagan® treatment.

Table 1: Functional state of heart mitochondria after one treatment with benzonidazole-Rochagan®, using glutamate/malate as oxidative substrate

Assayed parameter	Control	Treated
State 4	20.07±1.82	21.10±2.14
State 3	151.20±13.75	154.90±12.23
RC	7.61±0.19	7.47±0.23
$\Delta\Psi$	0.99±0.07	0.89±0.08
ADP:O	3.22±0.11	3.06±0.09
ATPsynthase	491.80±37.49	476.80±36.72

State 3 and State 4 represent the oxygen consumption rates measured in the presence of 300 μM of exogenous ADP and in its absence, respectively. Oxygen consumption rates expressed as nmol O/min. x mg of mitochondrial protein. RC is the ratio between the oxygen consumption rates measured in the presence and in the absence of exogenous ADP. $\Delta\Psi$ is the mitochondrial transmembrane electrical potential expressed in fluorescence a.u. ATPsynthase indicates the activity of mitochondrial ATPsynthase expressed as nmol Pi/min. x mg of mitochondrial protein. The oxidative substrate was 4 mM glutamate plus 1 mM malate. Values are the means (±SEM) of seven independent experiments in duplicate. There is no statistical difference between groups

Table 2: Functional state of heart mitochondria after one treatment with benzonidazole-Rochagan®, using succinate as oxidative substrate

Assayed parameter	Control	Treated
State 4	76.00±3.77	76.60±4.54
State 3	267.40±16.40	258.70±15.53
RC	3.52± 0.07	3.39±0.10
$\Delta\Psi$	0.64±0.09	0.71±0.05
ADP:O	1.79±0.02	1.72±0.04
ATPsynthase	481.70±32.19	455.70±24.22

State 3 and State 4 represent the oxygen consumption rates measured in the presence of 300 μM of exogenous ADP and in its absence, respectively. Oxygen consumption rates expressed as nmol O/min. x mg of mitochondrial protein. RC is the ratio between the oxygen consumption rates measured in the presence and in the absence of exogenous ADP. $\Delta\Psi$ is the mitochondrial transmembrane electrical potential expressed in fluorescence a.u. ATPsynthase indicates the activity of mitochondrial ATPsynthase expressed as umol Pi/min. x mg of mitochondrial protein. The oxidative substrate was 5 mM succinate. Values are the means (± SEM) of seven independent experiments in duplicate. There is no statistical difference between groups

The results of the present investigation correlated with the results obtained by de Souza *et al.* (2000) in which it was employed the current scheme of benzonidazole-Rochagan® treatment (100 mg benzonidazole × kg^{-1} body weight, for nine consecutive days). They observed an increase of both creatine kinase and its cardiac creatine kinase isoenzyme (enzymes that are useful indicators of myocardium cellular lesion), in the plasma, after one *T. cruzi* infection, which was reversed by this benzonidazole treatment. In this study, it was also tested if benzonidazole-Rochagan® administration alone could induce any change in both above mentioned enzymes, determined in plasma. Their results showed that benzonidazole-Rochagan® administration alone did not alter both creatine kinase and its cardiac creatine kinase isoenzyme plasma levels, indicating that the current benzonidazole-Rochagan® treatment did not cause myocardium cellular lesions tested by creatine kinase and its cardiac creatine kinase isoenzyme plasma release.

The results of the current investigation and the observation of de Souza *et al.* (2000) do not imply that the used benzonidazole-Rochagan® treatment alone does not alter the heart physiology. It is possible to take myocardium cellular lesions, e.g., abnormalities of mechanical properties of cardiac fibers, associated with negative alterations at heart physiology without alterations of mitochondrial cardiac energy processes and without the loss of membrane cardiomyocite integrity accompanying with creatine kinase and its cardiac creatine kinase isoenzyme drain into blood vessels. In this

sense it will be very worthy to focus future investigations on the effect of benzonidazole treatment on diverse biological parameters characterizing heart physiological state. For example, the investigators (Garcia *et al.*, 2005) have shown alterations in ECG records (heart rate, PR interval, P-wave duration, QT interval, QT_c, atrioventricular block and intraventricular block) at heart from *T. cruzi* infected mice, which were decreased when *Trypanosoma cruzi* infected mice were treated by benzonidazole-Rochagan® (100 mg × kg^{-1} of body weight daily for 1 week, followed by weekly administrations for an additionally 8 months). Unfortunately, these investigators did not study the effect of benzonidazole treatment alone on the ECG records of heart from uninfected mice; but exactly this kind of study will be worthy to carry out.

Finally, it is very important to point out something about the therapeutic scheme with benzonidazole. The investigations on molecular and cellular mechanisms of tissue lesions after one benzonidazole treatment are few. Being at the beginning of this kind of investigations, the most appropriated approach is to carry out this future studies using the same therapeutic scheme with benzonidazole, in order to be able to integrate the all new information that it will be appearing.

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