Considerations on the mechanisms involved on the benefits of GM1 treatment of mice with experimental Chagas´ disease

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Abstract

Background: In previous papers, some of us reported that GM1 improved the condition of mice with acute Trypanosoma cruzi infection but the mechanisms proposed did not involve adrenergic or steroid receptors.

Methods: 160 three months old outbreed Albino Swiss mice were divided into four groups: a) non-infected, b) non-infected and treated with GM1 0,1 mg /day by intramuscular injection, for 30 days, c) infected with T. cruzi, 0.7×10^5 parasites, d) infected with T. cruzi, 0.7×10^5 parasites and treated with GM1 0,1 mg /day by intramuscular injection for 30 days. The animals were studied at 35 days from the beginning of treatment and at 120 days post treatment. Cardiomyocyte membranes were isolated and β adrenergic receptors, membrane fluidity and composition, plasma glucose, potassium, DHA, estradiol and cortisol were determined. Histological sections of heart and adrenal glands were examined.

Results: At day 35 receptor Kd (nM) was 3.6; 4.46; 1.89 for healthy, infected, and infected GM1 treated mice and Bmax (fmol/mg prot) was 71.9; 54.8 and 21.14 respectively. At 120 days Kd for each group was 4.6; 4.6; and 2.79 respectively and Bmax was 71.9; 54.89; 74.2 respectively. Fluorescence anisotropy was reduced by infection and GM1 treatment from 0.88; 0.56; 0.43 for healthy, infected, and infected GM1 treated mice. GM1 treatment increased plasma estradiol in uninfected mice of both sexes. Chronically infected mice with prolonged QT 59.5 ms presented 48.9 ms after GM1. Histology of cardiac muscle slices showed no fibrosis and adrenal glands remodeling.

Conclusions: GM1 improved the condition of acute lethally infected and chronically infected mice regulating beta adrenergic receptors directly or through the modulation of steroid hormones. The observed evolution was concomitant with structural tissue modifications.

1. Introduction

Chagas´ disease is an important endemic in Latin America. Its etiological agent, *Trypanosoma cruzi* (T.cruzi), is a flagellated protozoan that undergoes complex morphological changes throughout its life cycle in the insect vector and the vertebrate host. The acute infection is stated with detectable parasitemia, acheless eye swelling (the "Romaña sign"), and sometimes, with a pertinacious grippe condition: violent headaches, muscles pain, inappetence and ganglion (lymphatic nodes) acheless inflammation [1]. Heart disease is an outstanding point, since irreversible chagasic panmyocarditis is developed in almost 600.000 person out of 2,500,000 infected only in Argentina [2, 3]. Unfortunately, both antiparasitic drugs nifurtimox and benznidazole cause adverse drug-related reactions with frequency and severity that increase with patient's age and reduce treatment compliance [4, 5, 6]. Therefore, there is still need of experimental research to develop alternative drugs and chemotherapeutic programs. We already reported that mice infected with a lethal amount of T. cruzi to simulate an acute phase survived when treated with a mixture of total bovine brain gangliosides [7]. Treated mice diminished parasitemia, reaching undetectable levels by day 30 post infection (d.p.i). Another feature was that hearts from ganglioside treated mice presented only minor mononuclear infiltration and neither amastigotes nests nor fibrosis were observed. Parasite DNA in plasma was undetectable suggesting complete clearance of circulating parasites [8]. We supposed that GM1 was the active principle so we replaced whole brain mixture of gangliosides (TBBG) with GM1 and the evolution of parasitemia was similar to that observed in mice treated with TBBG. Cardiac frequency in the post-acute period of infected mice treated with GM1 was restored to normal values and no blockade conduction could be detected. Instead, high titers of antibodies (IgG) against T. cruzi were long lasting [9]. Although results were stimulating we were far from understanding at the molecular level the mechanism (why only one?) involved in GM1 protective effect. Regarding the molecular basis of Chagas' disease, Sterin-Borda et al 1988, Rosembaum et al 1994 [10, 11], presented evidence that pointed to β adrenergic receptors compromise. Normal behavior of cardiac β adrenergic receptors is modified to different degree depending on the stage of the infection. Isolated myocardium from acute and chronic chagasic mice reached contractile force levels similar to normal hearts. The addition of, norepinephrine led to lower contractile values only in acute ventricles. On the other hand, epinephrine lead to significantly higher contractile values on acute and chronic ventricles. Propranolol blocked this hyperreactivity to epinephrine in myocardium from the acute stage, but the β receptors antagonist was ineffective in myocardium from chronic phase [12]. In mice coursing the post-acute phase of chagasic infection, density of β adrenergic receptors was unchanged (78.591 +/- 3.125 fmol/mg protein and 73.647 +/- 2.194 fmol/mg protein for controls) but receptors' affinity was significantly diminished (Kd = 7.299 +/- 0.426 and Kd = 3.759 +/- 0.212 nM for the control $p < 0.001$). This result supported that alterations in pharmacological response reported in chagasic myocardium are related to a significantly reduced affinity of cardiac β adrenergic receptors [13]. This response in mice was also dependent on the parasite strain since when Tulahuen and SGOZ12 were compared, mice with Z12 strain increased receptor density but maintained normal contractile force [14]. When mice were infected with a reduced number of Tulahuen parasites, receptors´ affinity was

conserved while a substantial decrease in receptors' density was evident. [15]. The way these alterations are induced seems to be related to the immune response. Evidence accumulated over the last two decades concerning human and experimental models suggests that immunopathological mechanisms may be involved in heart disease [16, 17, 18, 19]. The pathogenesis of chronic Chagas' disease may fall into this scope [20, 21, 22, 23, 24]. Anti-beta 1 adrenergic receptor antibodies in patients with dilated cardiomyopathy recognized the first as well as the second extracellular loop of the receptor. The anti-β 1-adrenoceptor antibodies imitate the β-adrenergic agonist isoprenaline and induce a positive chronotropic effect in cultured rat cardiomyocytes [25]. Sterin-Borda L., et al 1988 demonstrated the existence of two different circulating IgG in chagasic patients which bind to myocardial β 1 and spleen cell β 2 adrenoreceptors, like non-competitive inhibitors. β 1 and β 2 adrenoreceptors were specific to the chagasic IgG. The prevalence of anti-β 1-adrenergic antibody is low in the acute stage, but it increases over time post infection, being higher in the group with more than 15 years of infection [26]. The anti-β 2-adrenoreceptor IgG appears during the acute stage, peaks on the group with less than 10 years of infection, and then decreases. In this report, we present unpublished results that could enlighten our understanding of GM1 action involving the modulation of β adrenergic receptors in membranes of infected mice cardiomyocytes and other unexplored possible mechanisms of regulation of heart pathogenesis.

2. Materials and Methods

2.1 *Chemicals*:

Ganglioside GM1 (under pharmaceutical name of Neurex) was obtained from Laboratorios Beta (Buenos Aires, Argentina). ^{3}H/dihydroalprenolol (^{3}H/DHA, specific activity 3.515×10^{15} Bq/mol) and Aquasol Universal LSC cocktail-were from NEN, USA. All other reagents were from Sigma.

2.2 *Animals:*

A set of 160 three months old outbreed Albino Swiss mice were used. They were divided into four groups: a) non-infected, b) non-infected and treated with GM1 0,1 mg /day by intramuscular injection, for 30 days, c) infected with T. cruzi, d) infected with T. cruzi and treated with GM1 0,1 mg /day by intramuscular injection for 30 days. The animals were studied at 35 days from the beginning of treatment and at 120 days post treatment. At this moment, the infected mice were in the sixth month of T. cruzi infection evolution.

2.3 *Parasites:*

Trypomastigotes forms of T. cruzi, Tulahuen strain were employed. The strain was maintained in 3-month-old Albino Swiss mice by weekly passages.

Experimental infection:

Parasites were obtained from infected mice. The number was determined by counting in a Thomas Chamber. Animals were injected intra-peritoneal with 0.5 ml of blood containing 1.4×10^{5} parasites/ml and they were used at 7 days p.i. Parasitemia was examined in fresh blood from tails of mice and found positive in all the animals used.

2.4 *Parasitemia evolution:*

Parasitemia checked in blood obtained by cutting the tip of the tail was weekly verified by microscopic observation, using a Neubauer hemocytometer and analyzing all its fields.

2.5 *Electrocardiographic records:*

Were obtained 35 and 150 days post-infection, under Ketamil anesthesia (10

mg/kg) in DI, DII, DIII, AVR, AVL and AVF leads in GM1 treated groups.

2.6 *Determination of cardiac β-adrenergic receptors binding:*

Right and left ventricles from all the experimental groups were used. The hearts were quickly removed and the ventricles were dissected, washed and immediately frozen in liquid N_2 until they were used β-adrenergic receptors binding was performed in ventricles from the four groups under study. A pool of 2 ventricles was homogenized in 10 volumes of ice cold homogenization buffer (mM composition: Sucrose 250; $MgCl_2$ 1 and TRIS HCl 20; pH 7.4). Homogenates were centrifuged at 2000 ×g for 10 min. Pellets were rehomogenized, centrifuged at 40,000 ×g for 30 minutes and washed twice with KCl 0.6 M in homogenization buffer only. The final pellet was suspended in incubation buffer (mM composition: $MgCl_2$ 12.5; EDTA 1.5; TRIS HCl 75; pH 7.65) in a volume of 1 ml/g of wet tissue.

^{3}H/dihydroalprenolol (^{3}H/DHA, specific activity 3.515×10^{15} Bq/mol from NEN, USA) was used as radioligand in β-adrenergic receptors binding assays. Experiments were performed in triplicate incubating 100 µl of membrane suspension (480 µg protein) at 37º C for 10 min with ^{3}H/DHA (2.4-11.5 nM) in a final volume of 1 ml. The incubation was stopped by adding 1 ml of cold incubation buffer to each tube and rapidly filtering the contents under reduced pressure through Whatman GF/B filters. The filters were dried and transferred to vials to count radioactivity in Aquasol Universal LSC cocktail-NEN.

Specific binding was defined as the difference in radioactivity bound in the absence or presence of propranolol 1 µM. Dissociation constant (Kd) and maximum ^{3}H/DHA binding (Bmax) were determined by a saturation curve and Scatchard analysis using GraFit (Erithacus Software Limited).

2.7 *Plasma glucose and potassium*

Samples of 200 µL of plasma from 24 healthy untreated and treated mice were collected two hours after 3.5 µg adrenaline subcutaneous to measure the impact of GM1 treatment alone on metabolic parameters. The samples were tested for glucose and creatinine with colorimetric techniques and with flame spectrometry for potassium.

2.8 *Fluorescence anisotropy measurement:*

Anisotropy measurements were performed according to [27]. DPH fluorescence emission anisotropy (1,6-diphenyl 1,3,5-hexatriene) in the cardiac membrane suspension was determined using a SLM 4800 C spectrofluorometer controlled by a T format microprocessor. The parameter r_{DPH} showed the degree of stiffness of rotation of fluorophore molecules inserted in the membranes, providing a notion of the relative fluidity of these membranes. The term "membrane microviscosity" was used to refer to the structural and dynamic properties that determined the order and relative movements of the lipids in the membrane. To determine fluorescence, the cardiac membranes suspension was diluted in 1000 µl of incubation "buffer" (pH: 7.65) to which 10 µl DPH solution was added (dissolved in chloroform) at the rate of 1% of the final volume of the incubation solution, which after being shaken was let to settle for an hour at 37 ºC. The sample was excited at 360 nm and the emission was measured at 420 nm. In all determinations, a 10x10x45 mm quartz cuvette was used. The cuvette was kept at a constant temperature. The DPH "r_{DPH}" anisotropy was calculated by the following equation: $r = [I_{VV} - (I_{HV} \div I_{HH})\, I_{VH}] \div [I_{VV} + 2\,(I_{HV} \div I_{HH})\, I_{VH}]$. Where the "I" represents the intensity of

fluorescence. The first and second subindexes indicate the position ($_V$: Vertical, $_H$: horizontal) of the excitation and emission polarizer respectively.

2.9 *Histopathologic studies*

The analyses were made 35 and 150 days post-infection for heart samples and at 10 days after GM1 treatment onset for suprarenal glands. The animals were killed by ether anesthesia and kidneys with suprarenal glands and hearts were dissected. Heart samples were sliced horizontally from the apex to the auricles. Cross-sectional slices 5 µm thick were stained according to the hematoxylin-eosin technique. A total of 50 slices from each group were analyzed. At least 30 areas from each slice were examined with a 40 x objective. The organs were fixed in buffered (pH 7.0) 10% formalin and embedded in paraffin. Cross-sectional slices 5 µm thick were stained according to the hematoxylin-eosin technique. A total of 50 slices from each group were analyzed. At least 30 areas from each slice were examined with a 40 x objective. Some 100x images were captured to appreciate with more detail.

2. 10 *Plasma Hormones*

To evaluate the possible correlation of tissue alteration with biochemical changes, 80 four month uninfected mice of the same age of different littermates were separated in four groups, male control, female control GM1 treated males and females. Both groups underwent daily injections of 100 µL of physiologic or 100µg/100 µL GM1 in physiologic solution intramuscular. After 20 days mice were immobilized by ether anesthesia and blood obtained for androgenic hormones levels.

2.11 *Statistical analysis:*

Data from β receptors´ binding were analyzed by a general linear model procedure for the analysis of variance and multiple comparison by REGWQ Test. Student "t" test was also applied in the other data. Significance level was set at <0.05.

3. Results

3.1 *Parasitemia, antibodies, ECG*

Figure 1 shows parasitemia curves for infected and infected GM1 treated mice. Infected mice showed a steady increase of parasite counts in blood to reach a maximum at day 14 p.i., a few hours before death. Parasitemia of GM1 treated mice was dropped down at days 8, 11 and 14 p.i. ($p< 0.05$, $p< 0.01$ and $p< 0.01$ respectively) and decreased progressively after the 14 th day to very low values by day 30. We interrupted the GM1 administration at day 30; however, no parasites were observed in the blood of these mice until the end of the experiments (120 days p.i.).

Antibodies responses were high all along the experiments and not different in GM1 treated and untreated mice, as can be seen in Table I. Pathological electrocardiographic tracing typical of chagasic myocardiopathy were not detected in GM1 treated groups at the end of the treatment, 35 days p.i., nor 4 months afterwards. ECG tracing from these animals were like that obtained from uninfected group (data not shown). In the case of mice with chronic infection, treatment with GM1 500 µg one dose at 120 and 100 µg at 150 d.p.i. restored partially QT interval to healthy values and in mice with severe bradycardia frequency was also normalized as can be seen in Table II for a representative experiment.

3.2 *Receptors Quantification*

Table III and IV show the analysis of β-adrenergic receptors on myocardium membranes obtained from uninfected mice, uninfected mice treated with GM1 and infected mice without or with GM1 treatment.

At 35 days, significant differences were found in cardiac β-adrenergic receptors' affinity among untreated and GM1 treated mice whether they were uninfected or infected as can be seen in Table II. The affinity in mice treated with GM1 was significantly higher with the best record for uninfected treated mice.

At 120 days p.i., in uninfected, and GM1 treated mice infected or not affinity decreased regarding the same groups at 35 days but, GM1 treated mice showed augmented receptor affinity compared to infected untreated mice at 120 dpi (Table III).

Receptor density among these groups was also different (Table IV, $p < 0.01$). The reduction in cardiac β-receptors´ density in chagasic membranes with respect to uninfected ones at 35 dpi. is typical of this phase of infection (Fernández et al, 1996). GM1 treatment induced a more pronounced drop in the number of receptors with or without infection. At 120 dpi. GM1 treated mice presented higher receptor density whether mice were infected or not. In the case of infected mice, receptor density equals that of uninfected untreated mice but GM1 uninfected mice showed the mayor value.

3.3 *Plasma glucose and potassim*

Plasma determinations show a slight increase in glucose and potassium in mice treated with GM1 although the differences are not significant given the size of the sample, Table V

3.4 *Fluorescence anisotropy measurement*

Although GM1 treatment conserved native rates of triacyclglycerols and cholesterol of cardiomyocytes plasma membranes, Trypanosoma cruzi infection and GM1 treatment diminished membrane microviscosity of cardiac myocytes plasma membranes, but GM1 effect was more pronounced., see Table VI. (results from [28])

3.5 *Histological examination*.

Chagasic heart disease is characterized by relentless progression of damage and of congestive symptoms. We examined heart tissue from infected mice treated with GM1. At the end of treatment (35 days p.i.) cardiac structure of infected GM1 treated mice showed mild infiltration with mononuclear cells; but amastigotes´ nests that characterize infected mice without treatment, were absent (Fig 2 a and b). Four months later mice infected and treated with GM1 presented cardiac structures similar to non-infected healthy animals (Fig 2 c).

Suprarenal glands from uninfected mice without and with 30 days treatment with GM1, were sliced, hematoxylin eosin stained and observed under light microscope to disclose any effect exclusively attributable to GM1. Treated animals presented a considerable enlargement of the medullar region when observed at 50x, and medulla seemed more tightly joined to the cortical region. With greater magnification, the medulla was more organized than control tissues. Cell nuclei of chromaffin cells were clustered in an ¨acinar¨ pattern surrounded by nerve projections. This kind of organization is important since normal mouse adrenal medulla is formed by a mesh of dispersed chromaffin cells without any structural pattern. Small-caliber reticular vascularization became trunk and of large caliber. The three zones of the cortex were less delimited with a thinning of the zona reticularis in favor of the zona fasciculata. Zona glomerulosa was poorly defined in GM1 treated mice. In the whole cortex but mainly in zona fasciculata cells were tightly packed. The slices allowed to investigate the nearby sympathetic ganglion. It was observed a predominance of nerve fibers

over somas of smaller granularities and larger number of satellites cells nuclei Fig 3.

3.5 *Plasma Hormones*

Results of plasma levels of androgens are presented in Table VII. Although the values were obtained from pooled mice plasmas, what explains the absence of means, it can be observed that there were no differences among untreated and GM1 treated mice with the exception of detectable estradiol in the later, particularly high in females.

4. Discussion

In a previous paper, we reported that GM1 treatment of mice infected with T. cruzi to develop an acute lethal phase, lowered parasitemia to undetectable levels by microscopic observation [9]. Parasitemia remained undetectable even if PCR technique was applied to blood screening for parasite DNA [8]. Mice survived and presented high levels of specific IgG. Electrophysiology of myocardium was restored to normal frequency and not sequels were evident [9]. So, mice recovery and survival induced with GM1 treatment involves parasitemia decay and myocardial recovery, two aspects that may be explained through different mechanisms. This does not mean that we think both aspects develop separately but they may be approached to the analysis and therapy in different ways. The results presented in this communication are concerned with and extend beyond myocardial recovery. Chagasic cardiopathy courses with decreased β adrenergic receptor function (bradycardia) and alterations in pharmacological response. In our model post-acute phase, the main feature was decreased receptor affinity when mice were inoculated with few parasites while lethal number of parasites produced diminished receptor density [13, 15]. In this communication GM1 treatment was carried out on mice with lethal parasitemia which were expected to die by day 14 p.i. without treatment. Treated mice survived and they were sacrificed at 35 and 120 d.p.i. to evaluate β adrenergic receptor affinity and density. At 35 d.p.i. GM1 induced a strong increase in affinity and decrease in receptor density. This compensatory mechanism is only attributable to GM1 since similar behavior was registered in treated uninfected mice. At 120 d.p.i., infected treated mice showed a slightly over control receptor affinity and receptor density equal to controls, rendering normal heart rates and electrocardiographic records. Uninfected treated mice showed no difference in receptor affinity compared to control mice but presented higher receptor density. So, it seems that GM1 treatment per se has a late effect that is to raise the number of receptors that are accessible to the ligand while increment in receptor affinity is immediate and slowly restored to normal values. This pattern of response resembles an oscillatory regulation as follows: in a first moment GM1 treatment augments receptors' affinity which is counterbalanced by decreased receptors´ density; in a second moment, affinity decreases and is counterbalanced augmenting receptors´ density.

Up to the moment information concerning receptor synthesis or turnover due a pathologic process comes from non-ischemic cardiomyopathy patients. During cardiac remodeling, β adrenergic receptor gene expression decreases. An increase in β adrenergic receptor density was observed when binding was measured at 120 days post infection in isolated membranes from infected mice after GM1 treatment. But GM1 had been stopped by day 30 post infection. It is not clear whether this resulted from a prolonged action of GM1 or due to GM1 clearance during three months after treatment interruption. Any way the result may be interpreted as remission of the

pathology. The interaction of GM1 with β1adrenergic receptor was characterized previously without distinction between binding and efficacy. Fan et al. 1994 [29] found that exogenous GM1 increased the open probability of maxi K+ channels and they point to an increase in receptor efficacy not affinity. The authors remark that the effect was specific for GM1 and excluded changes in membrane fluidity. In Sf9 insect cells in culture expressing β1 adrenergic receptor, the incubation with GM1 for 1 h caused a concentration-dependent inhibition of the isoproterenol-induced AMPc accumulation. Pre-incubation with GM1 significantly reduced the affinity of antagonist binding to β 1-adrenergic receptors [30] but the procedure is not fully detailed and affinity was estimated based on linear regression instead of nonlinear regression. This last point is important due to possible errors arising from incorrect modeling the zone where critical changes occur in the total vs bound curve. We do not know the reason of such discrepancy with our results but it is remarkable that we worked in vivo and systemic molecular interactions cannot be ruled out. Saito et al added GM1 to the culture medium and an interaction between not incorporated GM1 adsorbed to the exterior of the plasma membrane and the ligand may explain the decrease in affinity since both molecules could be removed by thoroughly washing.

If in our experiments GM1 was effectively incorporated to myocardial membranes, the possibility of a direct binding to β 1-adrenergic receptors as a constituent membrane lipid should be considered since other GPCRs have well documented allosteric interactions with GM1 through specific protein domains [31]. GM1 treatment also lowered membrane anisotropy parameter r_s meaning that membrane microviscosity decreased what in turn facilitates receptor stimulation independently of AMPc production [32]. GM1 presents long chain unsaturated fatty acids, so the increase in fluidity might be accounted by the big polar head groups producing irregularities in lipid distribution or by PLA2 inhibition upon incorporation to the plasma membrane. PLA2 are a wide family of proteins which vary considerably apart from their catalytic activity. Three mayor structural groups can be distinguished: secretory or sPLA2 with about 14 kD molecular weight calcium dependent (mM), found in snake and insects venom, cytosolic or cPLA2 which are also calcium dependent but with 80 kD and sensible to lower (μM) calcium concentration, and calcium independent iPLA2 which do not require or may even be inhibited by calcium. iPLA2 are the mayor PLA2 in myocardium with iPLA2β isoform that is cytosolic and can be recruited to the plasma membrane and a membrane iPLA2γ isoform associated with plasma and mitochondrial membranes. GM1 is a well documented non-allosteric non-competitive sPLA2 and cPLA2 inhibitor but at present we do not know whether it can inhibit iPLA2γ as well.

GM1 is a marker of rafts and increasing the concentration of GM1 in the plasma membrane modulates the formation of this domains. Activated β1-adrenergic receptors (β1-ARs), which commonly undergo clathrin-mediated endocytosis, switch their route of endocytosis to caveolae/rafts when phosphorylated by protein kinase A. Association with rafts enhances receptor activation and at the same time favors endocytosis [33]. PLA2ε have been involved in membrane budding and endosomes trafficking and fusion events in secretion, by changing the shape of vesicles through the generation of inverted cone shaped lysophospholipids [34]. Inhibition of iPLA2 added to clustering of receptors could interrupt the natural cycling of β1-ARs

favoring endocytosis and impairing exocytosis resulting in the decreased receptor density observed in GM1 treated infected mice at 35 d.p.i. compared to untreated infected mice at 14 d.p.i.. The increment in receptor density at 120 d.p.i. might be the result of suspension of GM1 treatment at 30 d.p.i..

Physiological regulation of adrenergic receptors in target organs for long periods are usually accompanied by alterations in the adrenal medulla. Histological examination of adrenal glands slices showed increased surface section of adrenal medulla with respect to the cortex and within the cortex a predominance of zona fasciculata in healthy mice treated with GM1. It is well documented that ACTH interacts with GM1 in a way that favors the interaction of the hormone with its receptor. The growth of zona fasciculata observed in GM1 treated mice would have resulted from an increased ACTH stimulation. The ACTH stimulation might have been also enhanced by GM1 inhibition of PLA2 in adrenal cortex since arachidonic acid blocks steroid synthesis [35]. The altered morphology of adrenal medulla cells in treated mice may be explained in the same way because these cells become rounded upon ACTH stimulation. Determination of plasma cortical hormones in the same mice resulted in two differences: detectable estradiol in GM1 treated samples from both sexes and lower cortisol in treated females regarding untreated counterparts. Both results are in accordance with an increased stimulation of zona fasciculata. We remark that these experiments were carried on uninfected mice. Estradiol has been shown to protect female and gonadectomized male rats' hearts from ischemic injury [36, 37]. In C. callosus it was found that males were more susceptible to T.cruzi infection and in the chronic phase presented higher anti T.cruzi lytic antibodies than females, suggesting the latter have better control of parasitemia [38]. GM1 inhibits PLA2 and decreases its product arachidonic acid which is an inhibitor of estradiol production. Thus, it seems that GM1 promoted estradiol synthesis also through PLA2 inhibition, and through this physiological loop adds another mechanism that improves the condition of mice with experimental infection.

The beneficial action of GM1 treatment is also supported if we consider that uninfected mice treated with GM1 developed a slightly enhanced response to a pulse of adrenaline and that in infected treated mice histological ventricle sections from animals 120 d.p.i. display no signs of fibrosis.

Moreover, when GM1 was administered as two single doses to mice with chronic infection that presented prolonged QT intervals and bradycardia, QT was reduced and frequency was restored to almost normal values (Table II shaded values). Cutrullis et all 2011 [39], reported that treatment of chronically infected mice with 5 mg / Kg of GM1 daily decreases the expression of TGFβ1 reducing the extent of fibrosis. This may be another way to enhance cardiac performance under the infection in addition to GM1 neuroprotective actions. GM1 presented wide spectrum of action in different organs and especially adrenal glands with increased estradiol serum levels in both gender. On the other hand the increased level of estradiol in these animals might underlie the immune-regulatory shift from Th1 to Th2 responses, that reduces the aggressive action of INFγ in favor of IL4, IL13 and immunoglobulin production. That feature, explains in part the trend to repair the aggressive reaction produced by the parasite.

The focus of molecular activity was PLA 2 and this is a very important feature in different pathologies. Our findings suggest strongly the importance of gangliosides in

metabolism of cellular membranes due to their regulatory properties on cell membrane receptors. This ability will probably turn GM1 into a pharmacologically relevant product with multiple actions on different pathologies, infectious, immunological, cardiovascular, hematological, endocrines.

ACKNOOWLEDGEMENTS:
This work was supported by Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECyT-UNC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Fondo Nacional para la Ciencia y la Tecnología (FONCyT)

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Table I: Antibody titers in unifected+GM1 treated mice and infected with and without GM1 administration (Test de Elisa)

Dilutions	Non infected+GM1	Infected+GM1	Infected
1/100	1,29	8,45	9,10
1/400	1,03	4,76	4,43
1/1600	1,14	4,05	4,57

ELISA test was done 15 days after the beginning of the experiments. n=10 for each group. This Table was previously published in **Acta Trop; 73(3):**295-302

Table II: QT interval and heart frequencies of four mice with chronic experimental Chagas Disease before and after two individual doses of 500 mg GM1 i.m. with two months interval. At the bottom in bold the average values ± SD. In grey frequencies of mice with severe bradycardia.

QT interval	(ms)	Frequency	Beats/min
Before	After	Before	After
61	49	606	657
53	53	653	654
54	50	374	652
70	43	422	547
59.5 ± 7.8	**48.75 ± 4.1**	**513.75 ± 136**	**627.5 ± 53.7**

Table III: Cardiac β receptors affinity (Kd) in uninfected mice with and without GM1administration and in T. cruzi infected mice with and without GM1administration 35 days post the beginning of the experiments

Days p.i.	35			120		
Groups	N	Kd (nM)	REGWQ	N	Kd (nM)	REGWQ
Infected	15	4.467±0.294	A	15	4.467±0.294	
Uninfected	15	3.610±0.050	A	15	4.693±0.210	
I+ GM1	15	1.895±0.083	B	15	2.791±0.140	
U+GM1	15	1.524±0.202	B	15	3.610±0.050	

Results are expressed as mean ± SE. Mean values with the same letter do not have statistic differences. (Multiple ranges test). Result of infected mice were determined by day 7th post infection in the maximum parasitemia value, because these mice died by day 12.

Table IV: Cardiac β receptors density (Bmax) in uninfected mice with and without GM1administration and in T. cruzi infected mice with and without GM1administration 35 days post the beginning of the experiments.

Days p.i.	35			120		
Groups	N	Bmax (fmol/mg prot.)	REGWQ	N	Bmax (fmol/mg.prot)	REGWQ
Infected	15	51.853±1.250	B	15	51.853±1.520	C
Uninfected	15	71.965±0.360	A	15	71.965±0.360	B
I+ GM1	15	27.148±0.404	C	15	74.279±1.043	B
U+GM1	15	30.378±1.228	C	15	89.935±1.422	A

Results are expressed as mean ± SE. Mean values with the same letter do not have statistic differences. (Multiple ranges test). Result of infected mice were determined by day 7th post infection in the maximum parasitemia value, because these mice died by day 12.

Table V: Plasma levels of glucose potassium and creatinine in mice treated or not with GM1. Samples were obtained two hours after one pulse stimulus with 3.5 µg adrenaline subcutaneous

	Treatment	
	Control	GM1
Variables		
Glucose (mg/dL)	139.4 ±24	153.12 ±26
Potassium (mmol/L)	4.85±1.2	5.15±0.8
Creatinine Kinase (mg/dL)	0.33±0.05	0.36±0.08

Table VI: Microviscosity of mice cardiomyocytes plasma membranes as a result of T. cruzi infection and GM1 treatment of healthy and infected mice

groups	**sample size**	**r**	**r_0/r**	**r_{DPH}**
Infected	25	0.13±0.0002	2.27846	0.56
Uninfected	25	0.17±0.00003	2.1294	0.88
Infected+ GM1	25	0.11±0.0002	3.2909	0.43
Uninfected+ GM1	25	0.12±0.00004	3.0166	0.49

Table VII: Plasma levels of sexual hormones in mice treated with GM1. ud: undetectable

Hormones / Group	Estradiol pg/mL	Androstenedione ng/mL	Cortisol µg/mL
FC	ud	>10.0	42.6
MC	ud	>10.0	22.3
FGM1	>2000.0	>10.0	37.0
MGM1	22.3	>10.0	22.7

Figures

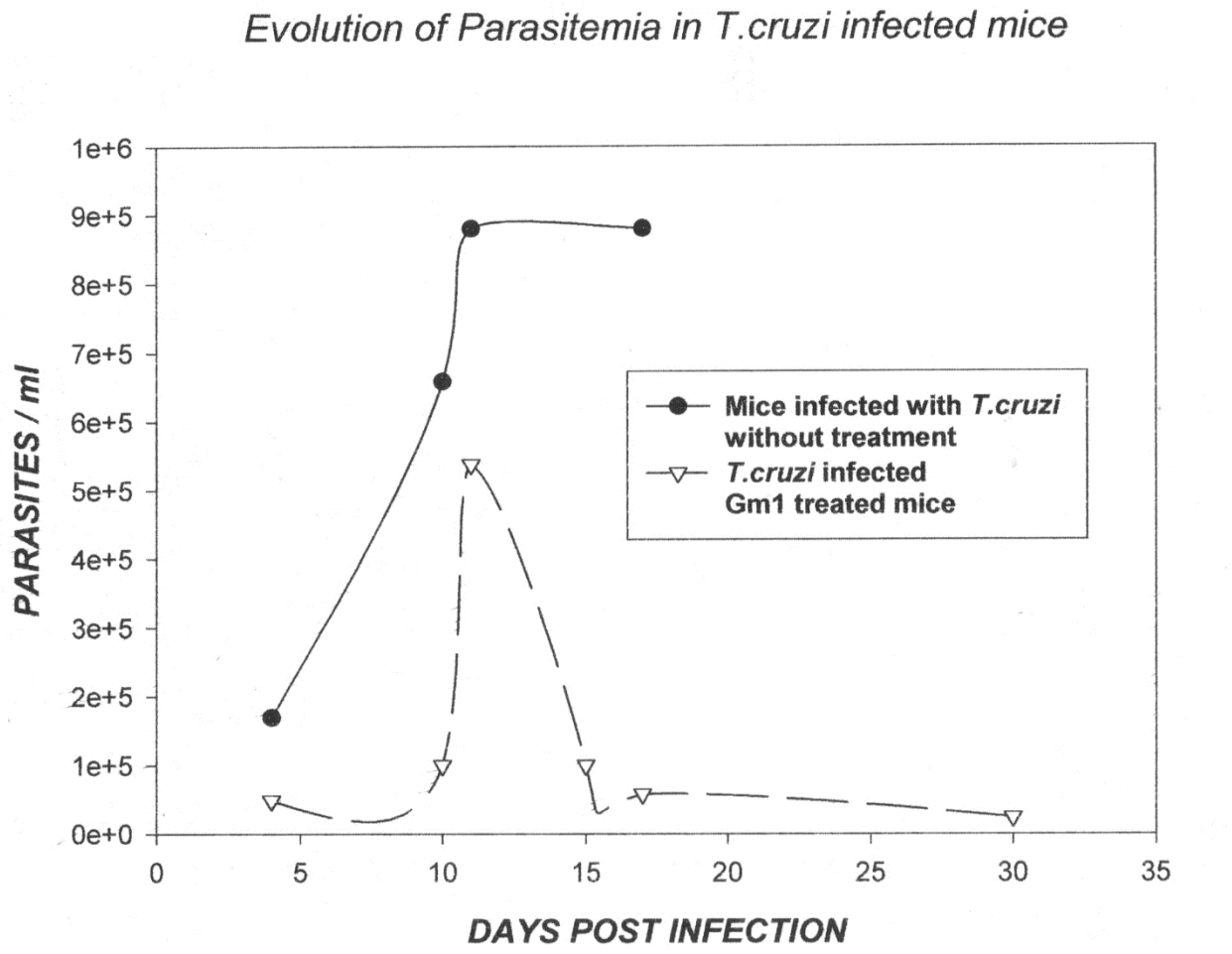

Fig. 1: Evolution of parasitemia in Trypanosoma cruzi infected mice
A more detailed graphic was published in Cossy Isasi et al 1999. Parasites were counted under microscopic observation of blood drops obtained cutting mice tail. Treated mice received 0.1 mg of GM1 by intramuscular injection daily during the first 30 days post infection. For details see Material & Methods

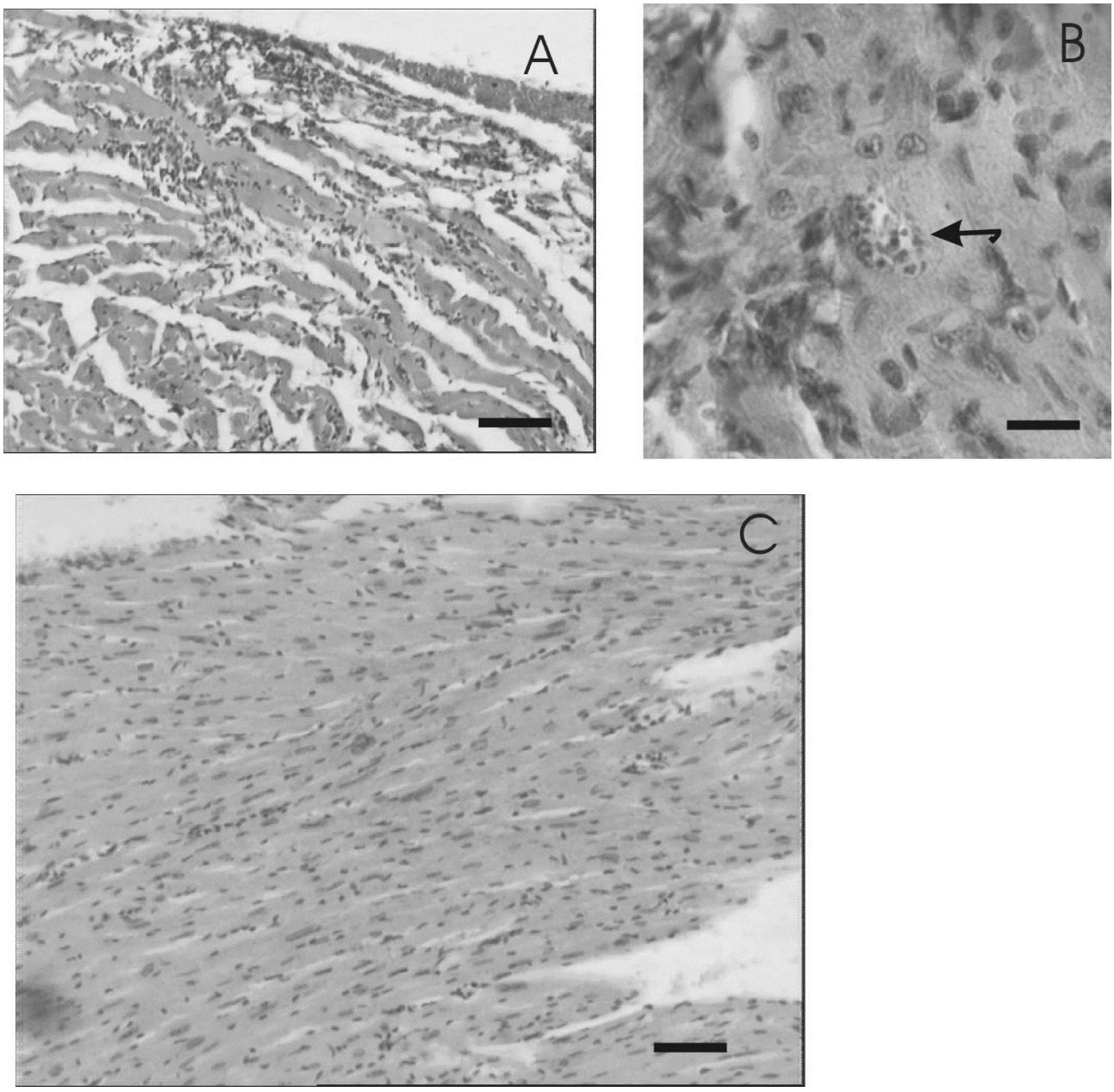

Fig 2: Histological sections of ventricles hematoxylin- eosin stained from infected GM1 treated mice at 35 (A) and 120 (C) days post infection. B corresponds to amastigote nest from untreated mice at 14 days post infection. Bar=1 μm.

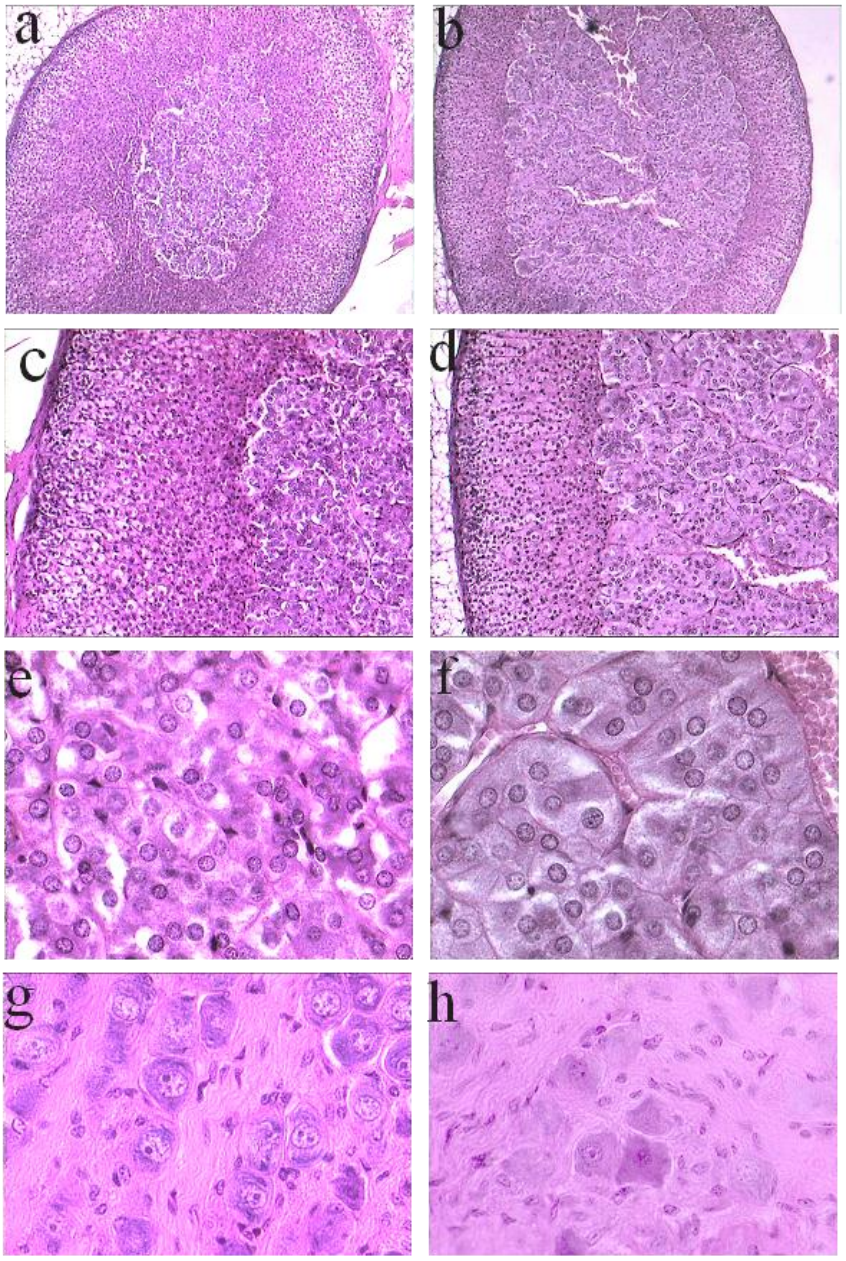

Fig 3: Hematoxylin eosin stained sections of suprarenal glands of uninfected mice without (a, c, e, g) and with GM1 treatment (b, d, f, h). a, b whole gland (50x), c, d cortex (100x); e, f, medulla 400x showing acinar organization in treated animals; proximal sympathetic ganglia showing inverse proportion soma/axon with GM1 treatment.

