



THE EFFECT OF THE ROCHAGAN™ ON RADIOLABELING WITH ^{99m}Tc

C. M. C. X. HOLANDA^{1,2,3}, M. F. SILVA-JÚNIOR², R. C. ALVES², V. S. A. BARBOSA³, R. P. SILVA³, L. G. ROCHA¹ AND A. C. MEDEIROS^{2,3}

¹Universidade Federal do Rio Grande do Norte (UFRN), Centro de Biociências, Departamento de Microbiologia e Parasitologia. Av. Salgado Filho, 3000, Natal, RN 59078-970, Brasil -Fax: +55 84 3211 9210; E-mail: cechol@ufrnet.br

²Hospital Universitário Onofre Lopes (UFRN), Av. Nilo Peçanha, s/n, Natal, RN 59012-300, Brasil

³Programa de Pós-Graduação em Ciências da Saúde, Centro de Ciências da Saúde (UFRN), Av. Nilo Peçanha, s/n, Natal, RN, Brasil

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Abstract – Radionuclides are used in nuclear medicine by variety of diagnostic procedures. The labeling of red blood cells (RBC) with ^{99m}Tc is a current method applied in clinical nuclear medicine. Drugs can alter this labeling and modify the disposition of the radiopharmaceuticals. The influence of Rochagan on the labeling of blood constituents with ^{99m}Tc was reported. Samples of blood were incubated with different concentrations of Rochagan (0%; 6.25%; 12.5%; 25%; 50%; 100%). Stannous chloride and ^{99m}Tc (3.7MBq/mL) were added. Plasma (P) and (RBC) were isolated and precipitated with trichloroacetic acid 5%. The insoluble (IF) and soluble fractions (SF) were separated. The %ATI in RBC, IF-P and IF-RBC were calculated. The %ATI on RBC decreased significantly ($p<0.05$) from control to all concentrations of Rochagan, respectively: 90.15±0.14(control) to 70.80±4.21; to 64.36±0.33; to 57.30±1.56; to 50.28±2.71; to 42.41±2.24; on IF-RBC, respectively: 84.70±0.87(control) to 67.16±4.38; to 63.63±2.92; to 59.02±3.17; to 43.75±1.00; to 24.15±0.94 and also on IF-P, respectively: 83.46±1.09(control) to 50.90±3.36; to 35.46±4.13; to 35.78±2.31; to 28.74±3.09; to 19.66±1.34. The analyses were performed by *T*-Student and Mann Whitney tests, $p<0.05$. This effect was probably due to products present in Rochagan that may complex with ions or have a direct/indirect effect on intracellular stannous ion concentration.

Key words: Rochagan, Chagas' disease, technetium-99m, blood constituents.

INTRODUCTION

Trypanosoma cruzi is a flagellate protozoan and the etiological agent of American Trypanosomiasis, known as Chagas' disease, an illness that is widespread on Latin America where 16–18 million inhabitants are infected and with more than 100 million exposed to the risk of infection (21). It is one of the most important public health problems in this area. The currently available treatment for Chagas' disease, which

involves chemotherapy with nitrofurantoin (NFX) and 2-nitroimidazole benzimidazole (BZ), is unsatisfactory as these drugs have toxic side effects and the efficacy of these drugs in treating the chronic phase of the disease is very low (18). This disease is characterized by an acute phase with detectable parasitemia and a long-lasting asymptomatic phase (14). The immune system is also involved in the parasite persistence (24), the lymphoid system being a possible target and an active component of the *Trypanosoma cruzi* infection.

The treatment of Chagas's disease depends on the use of the nitroderivative *N*-benzyl-2-nitro-1-imidazole acetamide (benznidazole, commercially known as Rochagan™). Benznidazole seems to act via a reductive stress which involves covalent modification of macromolecules by nitroreduction intermediates. This compound can eliminate acute phase symptoms and shorten the infection course and

Abbreviations: % ATI, Percentage of the Total Activity Injected; BZ, Benznidazole; IF, Insoluble Fractions; IF-RBC, Insoluble Fractions of Red Blood Cells; IF-P, Insoluble Fractions of Plasma; NaCl, Sodium Chloride or Saline Solution; Na^{99m}TcO₄, Sodium Pertechnetate; NFX, Nifurtimox; P, Plasma; RBC, Red Blood Cells; SF, Soluble Fractions; SF-RBC, Soluble Fractions of Red Blood Cells; SF-P, Soluble Fractions of Plasma; Sn⁺², Stannous Ions; SnCl₂, Stannous Chloride; TCA 5%, Trichloroacetic acid 5%; ^{99m}Tc, Technetium-99m; ^{99m}TcO₄, Pertechnetate Ion

cure at least 50% of recent infections (4, 6). In the chronic phase the treatment is less effective (8, 20). Besides its restricted efficacy, it has been also reported that benznidazole may cause severe side effects in patients and resistance in the parasite, probably due to heterogeneity of *Trypanosoma cruzi* populations (7).

Nuclear medicine as a diagnostic modality has grown to such an extent that it is practiced in almost all hospitals nationwide. Nuclear physicians interpret scintillation images of diseases and pathologies basing on a known normality pattern (11, 16). Radionuclides provided a way of selectively studying the distribution and metabolism of exogenous and endogenous materials in biological matter. Technetium-99m (^{99m}Tc), in the form of sodium pertechnetate ($\text{Na}^{99m}\text{TcO}_4$) was the first convenient radionuclide for labeling a variety of molecules, cells (16, 17), biological species as *Schistosoma mansoni* (2) and cellular structures such as leukocytes and red blood cells (3, 9).

Red blood cells labeled with ^{99m}Tc have come into wide use in clinical nuclear medicine for several important applications, including imaging of the cardiovascular system, detection and localization of gastrointestinal hemorrhage, measurement of red cell volume and spleen imaging. This labeling method depend on the presence of a reducing agent, and stannous chloride (SnCl_2) is widely employed for this purpose (3, 5).

The presence of medication (synthetic or natural drugs) in the patient's blood, as well as labeling conditions, can have an effect on the labeling of blood elements or can alter the characteristic and/or labeling process of the radiopharmaceuticals, changing their biological behavior and/or labeling efficiency (3, 5, 9, 17, 22, 23).

The aim of this study was evaluate the effect of RochaganTM on the radiolabeling of blood cells, plasma and cellular proteins with ^{99m}Tc using an *in vitro* procedure, since there is no reported *in vitro* study of RochaganTM interaction with radionuclides.

MATERIALS AND METHODS

The experiments were carried out with total blood of three *Wistar* rats and the guidelines established by the *Centro de Ciências da Saúde, Universidade Federal do Rio Grande do Norte (UFRN)* were followed. This study was approved by Ethical Committee for Animal Experimentation of *UFRN*.

Heparinized whole blood was withdrawn from three *Wistar* rats. Samples of 500 μL of the blood were gently mixed and incubated with 100 μL of different concentrations (0.0%; 6.25%; 12.5%; 25.0%; 50.0% and 100.0%) of Rochagan solution. All these different concentrations were prepared using a solution pattern of Benznidazole (Rochagan, Roche, Rio de Janeiro, Brasil). This solution pattern of benznidazole was prepared by pulverization of one tablet containing 100mg of active principle, followed by suspension in distilled water containing 3% Tween 80 and sonication for 15min. The control samples were prepared using 100 μL of a saline solution (0.9% NaCl). All the samples were incubated for 60 min at room temperature. After this incubation period, a freshly prepared stannous chloride solution (500 μL of 1.2 $\mu\text{g/mL}$), in the form of $\text{SnCl}_2 \cdot \text{H}_2\text{O}$ (Reagen, Quimibrás Indústrias Químicas SA, Brasil), was added and the incubation continued for another 60 min. Then, 100 μL of ^{99m}Tc (3.7MBq/mL), in the form of sodium pertechnetate, recently milked from a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (*Instituto de Pesquisas Energéticas e Nucleares, Comissão Nacional de Energia Nuclear, São Paulo, Brasil*) was added and the incubation was continued for another 10 min. These samples were centrifuged (1500rpm/5min) and plasma (P) and red blood cells (RBC) were separated. Aliquots of 20 μL of P and RBC were also precipitated with 1.0 mL of trichloroacetic acid (TCA) 5% and soluble fraction (SF) and insoluble fraction (IF) were isolated.

The radioactivity in P, RBC, SF-P, IF-P, SF-RBC and IF-RBC was determined in a gamma counter (Wallac, 1470-Wizard, Perkin Elmer, Finland). Thus, the percentage of radioactivity (%ATI) in each fraction was calculated, as previously described elsewhere (1). Briefly, to determine %ATI, the radioactivity present in RBC, IF-P and IF-RBC was divided, respectively, by the radioactivity in P+ RBC, IF-P+SF-P and IF-RBC+SF-RBC. The obtained values were multiplied by 100. The experiments were repeated nine times (samples of whole blood withdrawn from three different *Wistar* rats) and the means and standard deviations were determined. Statistical analysis was performed by T-Student and Mann Whitney tests, $p < 0.05$.

RESULTS

Table 1 shows the distribution of radioactivity in RBC and P from blood treated with different concentrations of rochagan. Analysis of the results indicates that there was a significant ($p < 0.05$) decrease in radioactivity uptake by RBC in the presence of rochagan from control (without Rochagan) to all the concentrations of rochagan (0.00; 6.25; 12.5; 25.0; 50.0 and 100.0%, respectively): 90.15 ± 0.14 (control) to 70.80 ± 4.21 ; to 64.36 ± 0.33 ; to 57.30 ± 1.56 ; to 50.28 ± 2.71 ; to 42.41 ± 2.24 .

Table 2 shows the distribution of radioactivity in soluble fraction (SF-RBC) and insoluble (IF-RBC) fraction of red blood cell proteins from blood treated with different concentrations of rochagan. Analysis of the results indicates that there was a significant ($p < 0.05$) reduction in radioactivity fixation in IF-RBC from control to all the concentrations of

Table 1. Effect of RochaganTM on the labeling of red blood cells (RBC) and plasma (P) with sodium pertechnetate in the total blood.

Concentrations of Rochagan (%)	(%ATI)	
	RBC	P
0.00(control)	90.15 ± 0.14	9.84 ± 0.14
6.25	70.80 ± 4.21	29.20 ± 4.21
12.5	64.36 ± 0.33	35.64 ± 0.33
25.0	57.30 ± 1.56	42.69 ± 1.56
50.0	50.28 ± 2.71	49.71 ± 2.71
100.0	42.41 ± 2.24	57.58 ± 2.24

Samples of heparinized blood were incubated with Rochagan solution (0.00; 6.25; 12.5; 25.0; 50.0 and 100.0%). Then, stannous chloride and ^{99m}Tc were added. These samples were centrifuged and plasma (P) and red blood cells (RBC) were separated. The %ATI in P and RBC was calculated. The values are the mean ± standard deviation. A statistical analysis (T-Student and Mann Whitney tests $p < 0.05$) was used to compare the results.

Table 2. Effect of RochaganTM on the labeling of insoluble fraction (IF-RBC) and soluble fraction (SF-RBC) of red blood cells proteins with sodium pertechnetate in the total blood.

Concentrations of Rochagan (%)	(%ATI)	
	IF-RBC	SF-RBC
0.00(control)	84.70 ± 0.87	15.29 ± 0.87
6.25	67.16 ± 4.38	32.83 ± 4.38
12.5	63.63 ± 2.92	36.36 ± 2.92
25.0	59.02 ± 3.17	40.97 ± 3.17
50.0	43.75 ± 1.00	56.24 ± 1.00
100.0	24.15 ± 0.94	75.84 ± 0.94

Samples of heparinized blood were incubated with Rochagan solution (0.00; 6.25; 12.5; 25.0; 50.0 and 100.0%). Then, stannous chloride and ^{99m}Tc were added. These samples were centrifuged and plasma (P) and red blood cells (RBC) were separated. Aliquots of RBC were precipitated with TCA 5% and soluble fraction (SF) and insoluble fraction (IF) of RBC were separated. The %ATI in IF-RBC and SF-RBC was calculated. The values are the mean ± standard deviation. A statistical analysis (T-Student and Mann Whitney tests, $p < 0.05$) was used to compare the results.

Table 3. Effect of RochaganTM on the labeling of insoluble fraction (IF-P) and soluble fraction (SF-P) of plasma proteins with sodium pertechnetate in the total blood.

Concentrations of Rochagan (%)	(%ATI)	
	IF-P	SF-P
0.00 (control)	83.46 ± 1.09	16.53 ± 1.09
6.25	50.90 ± 3.36	49.09 ± 3.36
12.5	35.46 ± 4.13	64.53 ± 4.13
25.0	35.78 ± 2.31	64.21 ± 2.31
50.0	28.74 ± 3.09	71.25 ± 3.09
100.0	19.66 ± 1.34	80.34 ± 1.34

Samples of heparinized blood were incubated with Rochagan solution (0.00; 6.25; 12.5; 25.0; 50.0 and 100.0%). Then, stannous chloride and ^{99m}Tc were added. These samples were centrifuged and plasma (P) and red blood cells (RBC) were separated. Aliquots of P were precipitated with TCA 5% and soluble fraction (SF) and insoluble fraction (IF) of P were separated. The %ATI in IF-P and SF-P was calculated. The values are the mean ± standard deviation. A statistical analysis (T-Student and Mann Whitney tests, $p < 0.05$) was used to compare the results.

Rochagan (0.00; 6.25; 12.5; 25.0; 50.0 and 100.0%, respectively): 84.70±0.87 (control) to 67.16±4.38; to 63.63±2.92; to 59.02±3.17; to 43.75±1.00; to 24.15±0.94.

Table 3 shows the distribution of radioactivity in soluble fraction (SF-P) and insoluble fraction (IF-P) of plasma proteins from blood treated with different concentrations of Rochagan. Analysis of the results indicates that there was also a significant ($p < 0.05$) reduction in radioactivity fixation in IF-P from control to all the concentrations of rochagan (0.00; 6.25; 12.5; 25.0; 50.0 and 100.0%, respectively): 83.46±1.09 (control) to 50.90±3.36; to 35.46±4.13; to 35.78±2.31; to 28.74±3.09; to 19.66±1.34.

DISCUSSION

Drug resistance is a particularly important problem in Chagas' disease chemotherapy, but relatively few reports have been published concerning this matter. *In vitro* and *in vivo* studies have shown a tendency of *Trypanosoma cruzi* stocks to increase resistance to clinical drugs (19).

Current knowledge of the biochemistry of *T. cruzi* has led to the development of new drugs and the understanding of their mode of action. Some trypanocidal drugs such as nifurtimox and benznidazole (RochaganTM) act through free radical generation during their metabolism. The nitro group of both drugs is reduced to an amino group by the action of nitroreductases, with the

formation of various free radical intermediates and electrophilic metabolites (15).

A number of researchers have turned their attention to *in vitro* testing of drugs with labeled red cells. The evidence that drugs can affect either radiolabeling or biodistribution of red blood cells in the context of nuclear medicine is indisputable. A therapeutic drug can also modify the nature/amount of the ^{99m}Tc-radiopharmaceutical bound to blood elements and this may result in unexpected behavior of the radiopharmaceutical (3, 10, 13, 16, 17, 22, 23).

The presence of drugs and normal dietary intake in the blood stream may interact with blood elements or any component of the labeling process. In this case, they may: act as an oxidant or a reducing agent; change the permeability of the cellular membrane or occupy the pertechnetate (^{99m}TcO₄⁻) and/or stannous ions (Sn⁺²) binding sites (2, 3, 13, 17). The oxidation effect would impair reduction of ^{99m}Tc by the stannous ion (stannous to stannic ion), compromising its binding and fixation of the radionuclide to the β-chain of hemoglobin. In contrast, the anti-oxidant effect would increase the red blood cell and plasma protein labeling efficiency, protecting the stannous ion from the oxidative process (13, 16, 17). The cellular membrane permeability could impair the labeling process by compromising the active transport of the pertechnetate ion by blocking the anionic transport of band-3 protein, responsible for chloride/bicarbonate exchanges and possibly by the transport of the pertechnetate ion by blocking

the calcium channels, impairing the stannous ion flow through the membrane (5), or causing morphological changes in the cellular membrane, hindering the free diffusion of the pertechnetate ion (2, 3, 13, 17). An *in vitro* method of labeling red blood cell and plasma proteins is a practical way of evaluating these effects.

In the labeling process of red blood cells with ^{99m}Tc , the stannous and pertechnetate ions pass through the plasma membrane. Sequential stages of the intracellular labeling process include reduction of ^{99m}Tc -pertechnetate by Sn^{2+} , subsequent binding of the reduced ^{99m}Tc to hemoglobin (5, 13, 16) and transmembrane transport of Sn^{2+} and $^{99m}\text{TcO}_4^-$ into the internal compartment of the red blood cells. The band-3 anion transport system and calcium channels also may be involved in this transport (5).

We have previously shown that glucantime (anti-*Leishmania*) can alter the labeling of red blood cells and cellular proteins with $\text{Na}^{99m}\text{TcO}_4$ by *in vitro* process (12). Now, in this work, we have studied the effect of rochagan (anti-*T.cruzi*) on the labeling of red blood cells, plasma and cellular proteins with sodium pertechnetate by also *in vitro* method.

Red blood cell proteins can be labeled with ^{99m}Tc , mainly in the β -chain of the hemoglobin molecule (5, 13). The fixation of this radioactivity was likely modified by rochagan in all the concentrations. When these concentrations of rochagan were incubated with whole blood, there was a significant decrease in the fixation of ^{99m}Tc in the red blood cell proteins. This probably occurred due to the oxidation effect that would impair reduction of ^{99m}Tc by the stannous ion affecting binding and fixation of the radionuclide to the β -chain of the hemoglobin molecule (3, 5, 9, 12, 13).

Plasma proteins were also labeled with technetium-99m. ^{99m}Tc -labeled plasma proteins have been used to locate the placenta, to evaluate cardiac function and pulmonary perfusion, to determine blood volume and to study gastrointestinal protein loss (10, 16, 17). The fixation of this radioactivity does seem to be modified by rochagan solution. This fact possibly also indicates the presence of an oxidative effect, increasing the valence of stannous ion to +4 and reducing the fixation of the ^{99m}Tc (17).

In conclusion, our results show that the labeling of red blood cell with technetium-99m and the fixation of this radionuclide to insoluble fractions of cellular and plasma proteins can be decreased in the presence of rochagan when an *in*

vitro technique to label red blood cells is employed. We suggest that this effect may be due to products present in rochagan solution that may complex with ions (Sn^{+2} and $^{99m}\text{TcO}_4^-$) or have a direct or indirect effect on intracellular stannous ion concentration. Furthermore, this study allows for the assessment of the effect of Rochagan on direct or indirect oxidant properties.

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