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An *In vitro* Study of a Natural Product (chayotte): An Analysis on the Labeling of Blood Components with Technetium-99m and on the Morphology of DNA

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Abstract: The evidence that natural and synthetic drugs can affect radiolabeling or biodistribution of red cells in setting of nuclear medicine clinic has come to light only recently. A therapeutic drug can modify the nature/amount of technetium-99m (99mTc) radiopharmaceutical bound to blood elements and this may result in a unexpected behavior of the radiopharmaceutical. Once chayotte is used as food and also in folk medicine, we decided to evaluated the influence of this natural product on the labeling of blood elements with 99mTc and in the structural conformation of a plasmid pUC 9.1 trough gel electrophoresis analysis. Blood withdrawn from *Wistar* rats was incubated with the extract, (100% v/v).The blood samples were incubated with stannous chloride and with 99mTc. Plasma (P) and blood cells (BC) were isolated, also precipitated with trichloroacetic acid and soluble (SF) and insoluble fractions (IF) separated. A sample of blood was treated with ficoll-hypac, centrifuged and white cells were isolated. A solution of lymphocytes (2.5 mL) was obtained. After that, 0.2mL of a solution of lymphocytes was incubated with 0.1 mL of the vegetable extracts. A solution of stannous chloride and 99mTc were added. Lymphocytes and aqueous solution were separated. The %ATI bound to blood components was evaluated. The analysis of the results showed that there is not a decrease in %ATI in the blood constituents with chayotte extract. Although chayotte extract is not capable to alter the radiolabeling of. blood proteins and cells with 99mTc it was observed that the referred extract is capable to induce lesions in the plasmid DNA showing their possible oxidant properties. The studied natural product was not capable to oxide the stannous ions sufficiently to reduce the labeling of blood elements with 99mTc.

Key words: Chayotte, blood elements, lymphocytes, technetium-99m

Introduction

Since its introduction in 1976 as an imaging procedure, the use of labeled leukocyte scintigraphy has continued to increase. At the present time it is a routine procedure in most nuclear medicine departments for the investigation of different inflammatory pathologic involving leukocyte infiltration (Sinzinger and Thakur, 1989; Martín-Comín *et al.*, 1994; Becker, 1995 and Chianelli *et al.*, 1997).

Many authors prefer *in vitro* methods for the technetium-99m (99mTc) labeling of leukocytes in whole blood, based on phagocytosis. For instance, the stannous fluoride colloid method is apllicable to granulocytes and monocytes (Schroth *et al.*, 1981; Martindale *et al.*, 1990 and Bartholomeusz, 2001). However, the mechanism of labeling is shown to be surface adherence rather than phagocytosis (Mock and English, 1987).

The usefulness of indium-111 labeled white cells in acute infections is well documented, but it has several limitations, among them a long preparation time, poor count rate due to the low allowable administered dose, and relatively high splenic dosimetry (Thrall and Ziessman, 1995). The high cost and availability of this radionuclide have diminished its attractiveness (Spinelli *et al.*, 1989; Barbar *et al.*, 1989 and Vorna *et al.*, 1989). It has been reported that Indium 111 labeled white cell scintigraphy is an unreliable indicated of malignant external otitis resolution (Redleaf *et al.*, 1994). Labeled lymphocytes have not been widely used clinically due to the fact that radiation kills or damage the cells. The risks of tumor formation in chromosomal damaged lymphocytes have been well documented and are particularly relevant if the cells are labeled with 111In (Berge and Natarajan, 1983). A number of workers have proposed 99mTc as a suitable label for lymphocytes, as chromosomal damage is likely to be considerably less than that with 111In (Merz, 1986).

99mTc has been the most utilized radionuclide in diagnostic nuclear medicine procedures. It is not only relatively cheap but readily available (Hladik *et al.*, 1987; Saha, 1998 and Mattos *et al.*, 2001). 99mTc-labeled infection-seeking radiopharmaceuticals have been actively sough (Early and Sodee, 1995 and Sampson, 1996). A number of new 99mTc-labeled radiotraceres with various mechanisms of uptake are under investigation and may become

available for clinical use (Thrall and Ziessman, 1995).
Technetium-99m-hexamethylpropyleneamine oxime (99mTc-HMPO) and Indium-111-oxinate (111In oxinate) have been largely compared in the literature for their relative advantages and disadvantages regarding: quality of imaging characteristics; radiation dose; efficiency of granulocyte labeling and the uptake by the infection site (Bertrand-Caix, 1995).
99mTc-HMPO is now being widely used for leukocyte scintigraphy and is used in white cell scanning in Crohn´s disese (Malcolm, 1994).
Red blood cells (RBC) labeled with 99mTc are also used for several evaluations in nuclear medicine, including imaging of the cardiovascular system, detection of gastrointestinal hemorrhage and localization of intramuscular hemangioma (Sampson, 1996 and Al Haider *et al.,* 2000). The labeling of RBC, leukocytes and blood proteins with 99mTc requires a reducing agent, and stannous chloride ($SnCl_2$) is widely used with this purpose (Hladik *et al.,* 1987; Gutfilen *et al.,* 1993; Bernardo-Filho *et al.,* 1994 and Saha, 1998). Gutfilen *et al.,* 1993 reported in their experiments with white cells labeled with 99mTc that the viability of the cell is great in the presence of low $SnCl_2$ concentration. A low labeling efficiency is observed in the presence of plasma (Ecclestone *et al.,* 1990 and Gutfilen *et al.,* 1993) and may be due to plasma protein competition for this labeling, as indicated for RBC (Bernardo-Filho *et al.,* 1990).
$SnCl_2$ is known to (i) inhibit immune response in rodents, (ii) alter gene expression and (iii) induce tumor generation in thyroid gland. There is no general agreement regarding its genotoxicity and it was discussed that the effects of. this salt might depend on the physicochemical conditions and the route of. its administration. This salt is directly administrated to human beings, endovenously, when it is used as a reducing agent to prepare 99mTc-radiopharmaceuticals (Saha, 1998). Stannous chloride is capable of inducing the generation of reactive oxygen species (ROS) that are responsible for oxidative stress. It was reported that the survival of *Escherichia coli* cultures was improved by the presence of the extracts of *Cymbopogon citratus, Baccharis genistelloides, Matrenus ilicifolia* and *Peumus boldus* due the fact of these extracts have been capable of reducing the lethal effect induced by $SnCl_2$ (Silva *et al.,* 2002).
Some authors have described that synthetic or natural products in the blood as well as labeling conditions can have an effect on the labeling of RBC with 99mTc (Hesslewood and Leung, 1994; Sampson 1996; Braga *et al.,* 2000; Capriles *et al.,* 2002; Gomes *et al.,* 2002 and Oliveira *et al.,* 2003). Thus, the presence of the disease may be missed and or underestimated (Hladik *et al.,* 1987; Hesslewood and Leung, 1994).
Natural products, as medicinal plants, have been widely used by human beings in all the world. Chayotte (*Sechium edule*) are used as food or as medication in folk medicine (Albuquerque, 1989 and Flores, 1989). Chayotte a subtropical vegetable with potent diuretic action, is a cucurbitaceus species which is used as food or as medication in popular medicine. It was reported a case of severe hypokalemia pregnancy and that a chayotte preparation was implicated, as the potassium level returned to normal, without recurrence of hypokalemia, once the ingestion of this vegetable was stopped. The biological effect of *Sechium edule* has been studied by different researchers (Flick *et al.,* 1978; Rodrigues *et al.,* 1984; Jensen, 1986 and Flores, 1989).Then, we have evaluated the influence of chayotte extract (macerated) on the labeling of blood elements with 99mTc and in the structural conformation of a plasmid pUC 9.1 trough gel electrophoresis analysis.

Materials and Methods

Radiolabeling: These experiments were performed without sacrificing the animals. Heparinized whole blood was withdrawn from *Wistar* rats. Samples of 0.5 mL of blood were incubated with 100 μL of a chayotte extract (macerated, 100%v/v). *Sechium edule* was purchased in a local market in Rio de Janeiro, RJ, Brazil. To prepare the macerated of the referred vegetable, it was used 50 g of the skin of the chayotte with 500 mL of saline solution 0.9% which was triturated with a domestic electric extractor. This macerated was filtered and the watery extract was obtained. The presence of toxic compounds was tested and we did not find them in the preparations of chayotte used in our experiments. In the used technique, brain acethylcholinestarase was utilized as an *in vitro* detector of organophosphorus and carbamate insecticides (Cunha Bastos *et al.,* 1991).
The referred vegetable used *in vitro* was incubated with samples of blood (0.5 mL) for 1 hour at room temperature. Samples of heparinized blood (0.5 mL) which were incubated with saline solution were utilized as control. Then, 0.5 mL of stannous chloride (1.2 μg mL^{-1}), as $SnCl_2.2H_2O$ (Reagen, Quimibrás Indústrias Químicas SA, Brazil) was added and the incubation continued for another 1 hour. After this period of time, 99mTc (0.1 mL), as sodium pertechnetate, recently milked from a 99Mo/99mTc generator (Instituto de Pesquisas Energéticas e Nucleares, Comissão Nacional de Energia Nuclear, Brazil), was added and the incubation continued for another 10 min. These samples were centrifuged in a clinical centrifuge (used in the routine in a clinical laboratory) with a small speed (1500 rpm) and plasma (P) and red blood cells (BC) compartments were separated. Samples (20 μL) of P and BC were also precipitated with 1 mL of trichloroacetic acid (TCA) 5% and soluble (SF) and insoluble fractions (IF) were separated. The radioactivity in P, BC, IF-P, SF-P, IF-BC and SF-BC were determined in a gamma well counter with a NaI (Tl) crystal (Clinigamma, gamma counter, LKB, Wallac, Finland). After that, the % of radioactivity (%ATI)

was calculated. The %ATI in P was calculated by dividing the radioactivity in P by the sum of the radioactivity in P and BC. The %ATI in BC was calculated by dividing the radioactivity in BC by the sum of the radioactivity in P and BC. The %ATI in SF-P was calculated by dividing the radioactivity in SF-P by the sum of the radioactivity in SF-P and IF-P. The %ATI in IF-P was calculated by dividing the radioactivity in IF-P and SF-P. The %ATI in SF-BC was calculated by dividing the radioactivity in SF-BC by the sum of the radioactivity in SF-BC and IF-BC. The %ATI in IF-BC was calculated by dividing the radioactivity in IF-BC and SF-BC.

In order to isolate white blood cells 3mL of heparinized whole blood was withdrawn from *Wistar* rats. The Ficoll-Hypaque (d = 1.076) technique was followed according to Phelan and Mickelson (1995). Blood treated with Ficoll-Hypaque was centrifuged and white cells were isolated. Then, lymphocytes were washed by centrifugation in a saline solution (0.9%NaCl) to remove the Ficoll-Hypaque. The pellet was resunpended in saline solution. A solution of lymphocytes (2.5 mL) was obtained. After that, 0.2mL of a solution of lymphocytes was incubated with 0.1 mL of the vegetable extracts (macerated) for 60 min at room temperature. After this period of time, a solution (0.2 mL) of stannous chloride (1.2 μg ml^{-1}) as $SnCl_2.2H_2O$ was added and the incubation continued for another 60 min. 99mTc, as sodium pertechnetate, was also added and the incubation continued (10 min). Lymphocytes and aqueous solution were separated. The %ATI bound to lymphocytes was determined in a well counter. A statistical analysis (Tukey tests) was utilized to compare the experimental data.

Nucleic Acid Manipulations: Preparations of plasmids were performed using the alkaline method described by Sambrock *et al.* (1989). Plasmid samples were further purified from high molecular weight RNA contaminants, performing LiCl precipitation (2.5 M final concentration), while the residual RNA contaminants, were disgested by RNAase (20 μg mL^{-1}) treatment for 30min at room temperature.

For incubation with $SnCl_2$, plasmids were diluted, dispensed into eppendorf tubes (200 ng per tube) and incubated with 200 μg mL^{-1} of $SnCl_2$ in 10mM Tris-HCl buffer at pH 7.4. To evaluate the influence of the chayotte extract in DNA breakage induced by $SnCl_2$ an extract of chayotte (100%) were added before the treatment of DNA with the reducing agent. In all cases, reaction mixtures were incubated at 37°C for 60 min.

The analysis of SSB formation was performed using 0.8% agarose gel electrophoresis in order to separate different conformations of plasmid DNA: form I supercoiled (SC) native conformation, form II open circle (OC) resulting form single strand breaks. Aliquots from each sample (10μL) were mixed to 2 μL of 6 x concentrated loading buffer (0.25% xylene cyanol FF; 0.25% bromofenol blue, 30% glycerol in water), and applied in a horizontal gel electrophoresis chamber in Tris acetate-EDTA buffer at pH 8.0. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg mL^{-1}) and the DNA bands were visualized by fluorescence in an ultraviolet (UV) transiluminator system. Permanent records were performed using a Polaroid MP-3 system.

Results

The presence of toxic compounds was tested and we did not find them in the preparations of the extract used in our experiments. In Table 1 is shown the distribution of radioactivity in lymphocytes. The analysis of the results shows that chayotte extracts do not alter the 99mTc fixation in lymphocytes.

Blood withdrawn from *Wistar* rats was treated with Ficoll-Hypaque, centrifuged and white cells were isolated. A solution of lymphocytes (2.5 mL) was obtained. After that, 0.2mL of a solution of lymphocytes was incubated with 0.1 mL of the vegetable extract. A solution of stannous chloride and 99mTc were added. Lymphocytes and aqueous solution were separated. The %ATI bound to lymphocytes was evaluated.

In the Fig. 1 is shown the effect of *Sechium edule* (macerated *in vitro*) on the labeling of blood elements with 99mTc. The results indicated that the extract was not capable of altering the radiolabeling on red blood cells (BC) (from 92.62 ± 4.88 to 89.14 ± 3.56), on the insoluble fraction of the red blood cells (IF-C) (from 87.10 ± 2.74 to 89.84 ± 5.49) and on the insoluble fraction of the plasma (IF-P) (from 71.43 ± 9.37 to 72.70 ± 10.43).

Samples of Heparinized blood were incubated for 1 hour with *Sechium edule* macerated extract (100% v/v). A sample of Heparinized whole blood was incubated for 1 hour with saline solution (NaCl 0.9%) as control. Then, stannous chloride (1.2 μg mL^{-1}) and 99mTc, as sodium pertechnetate were added. These samples were centrifuged and plasma (P) and blood cells (BC) were separated. Samples (20 μL) of BC were precipitated with trichloroacetic acid (TCA) 5% and soluble (SF) and insoluble fractions (IF) were separated. The radioactivity in P, BC, SF-BC, IF-BC, SF-P and IF-P was determined in a well counter and the % of radioactivity (% ATI) was calculated. A statistical analysis (Kruskal Wallis test, n = 5) was used to compare the results.

In the Fig. 2 is shown the electrophoresis in 0.8% agarose gel of pUC 9.1 plasmid treated with $SnCl_2$ and an extract of chayotte.

Table 1: Effect of chayotte on the labeling of lymphocytes with 99mTc

Cells	Control	Chayotte
Lymphocytes	92.62 ± 4.88	92.84 ± 5.49

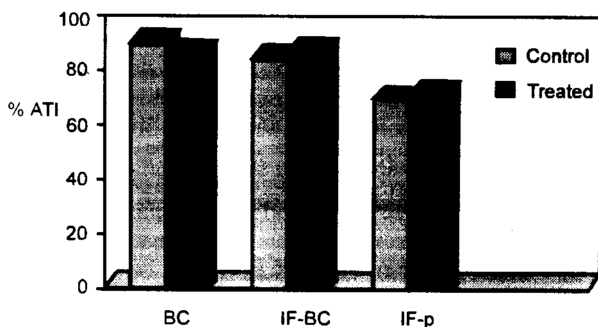

Fig.1: The effect of *Sechium edule* (macerated *in vitro*) on the labeling of blood elements with 99mTc.

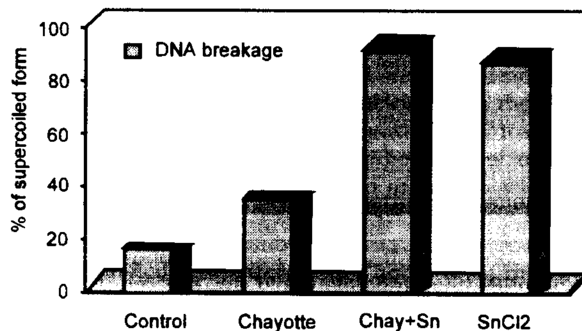

Fig. 2: Electrophoresis in agarose gel of pUC 9.1 plasmid treated with $SnCl_2$ and/or the extract. Column 1: (Control: DNA + water), Column 2: (Chayotte100%), Column 3: (Chayotte 100% + Sn 200μg mL^{-1}), Column 4: ($SnCl_2$-200μg mL^{-1}). Photos of the gels were scanned and the supercoiled form was quantified

Discussion

A number of surveys have been undertaken to assess the possibility of drug interference as a cause of problems with labeling or biodistribution (Sampson, 1996). Extracts of medicinal plants can also alter the labeling of blood elements with 99mTc (Braga *et al.*, 2000; Oliveira *et al.*, 2002 and Santos-Filho, 2002) and the biodistribution of radiopharmaceuticals (Mattos *et al.*, 1999 and Gomes *et al.*, 2002). It has been reported that an extract of chayotte is capable to alter the biodistribution of the radiopharmaceutical sodium pertechnetate in *Wistar* rats (Diré *et al.*, 2001), this fact could be due to the metabolic transformation of this extract which could be capable to induce the generation of active metabolites with hurtful properties in specific biological systems. However, this fact was not observed with an extract of cauliflower (leaves) related to biodistribution although the referred extract was capable to induce lesion in the plasmid DNA showing its possible oxidant properties (Lima *et al.*, 2001). In other study, Bernardo *et al.*, (2002) demonstrated that rutin extract was not capable of damaging the plasmid DNA. In a *in vivo* study Diré *et al.* (2002) eyed that chayotte extract were capable to alter the radiolabeling of blood elements. Lima *et al.* (2002) observed in an *in vivo* study that the cauliflower extract was not capable of altering the radiolabeling of blood constituents with 99mTc, a similar result was obtained in a *in vitro* study with the *Peumus boldus* (Reiniger *et al.*, 1999) and *Sechium edule* (Diré *et al.*, 2002) extracts We agree with Hesslewood and Leung (1994) that many reports on drug interactions with radiopharmaceuticals are anecdotal and in some instances a direct cause and effect relationship has not been unequivocally established. This fact could be diminished with the development of *in vitro* tests to evaluate the drug/radiopharmaceuticals interactions and the consequence for the bioavailability of the radiopharmaceuticals and the labeling of blood constituents.

One of the difficulties of using labeled lymphocytes in nuclear medicine is the problem getting sufficient activity for imaging the small number of cells available (Sampson, 1996). According to Gutfilen *et al.* (1993) the leukocytes labeling not depends on the stannous chloride concentration as red blood cells do. However the presence of plasma proteins and platelets can reduce the radiolabeling. In the labeling process of blood elements with 99mTc needs a reducing agent, and probably the stannous ion would be oxidized. In *in vitro* studies was verified that extracts of *Thuya ocidentallis* (Oliveira *et al.*, 1997), *Nicotiana tabacum* (Vidal et al, 1998), *Maytenus ilicifolia* (Oliveira *et al.*, 2000), *Paullinia cupana* (Oliveira *et al.*, 2002), *Solanum melongena* (Capriles *et al.*, 2002), *Mentha crispa L.* (Santos-Filho *et al.*, 2002) and *Fucus vesiculosus* (Oliveira *et al.*, 2003) possibly, would have oxidants compounds, and the labeling of blood elements decrease in the presence of these extracts. In this work, we isolated white blood cells from the other blood elements and plasma and we could observe that the efficiency of the lymphocytes labeled with 99mTc was well succeeded. In the present study, we have observed that the studied natural product is not capable to reduce the labeling of lymphocytes and the blood red cells and blood proteins with 99mTc. The genotoxic effect related to *Paullinia cupana* (Fonseca *et al.*, 1994) and *Brassica oleracea* (cauliflower) (Lima *et al.*, 2001), a natural products, could be associated to the generation of reactive oxygen species (ROS) that are oxidant agents. It was reported that *Sechium edule* extract was capable of altering the biodistribution of 99m-Tc-radiopharmaceutical (Diré *et al.*, 2001) as it was observed with *Solanum melongena* (Capriles *et al.*, 2002) and *Ginkgo biloba* (Moreno *et al.*, 2002) extracts. It was remarked that chayotte extract has induced lesions on the DNA molecule. This result would probably be due to the generation of ROS, which could be oxidizing the stannous to stannic ion. Qualitative alterations on the shape of the red blood cells were found with blood treated with tobacco (Vidal *et al.*, 1998), *Maytenus ilicifolia* (Oliveira *et al.*, 2000), *Thuya ocidentallis* (Braga *et al.*, 2000),

Sechium edule (Diré *et al.*, 2001), *Paullinia cupana* (Oliveira *et al.*, 2002), *Mentha crispa L.* (Santos-Filho *et al.*, 2002), *Piper methysticum* (Santos-Filho, Ribeiro *et al.*, 2002), *Ginkgo biloba* (Moreno *et al.*, 2002), *Fucus vesiculosus* (Oliveira *et al.*, 2003). Then, we can speculate that this fact could be associated with the alteration on the morphology of red blood cells related by Diré *et al.*, 2001 in an *in vivo* qualitative study.

Conclusion

Although chayotte extract is not capable to alter the radiolabeling of bood constituents with 99mTc it was capable to induce lesions in the plasmid DNA showing their possible oxidant properties. The studied natural product was probably not capable to oxide the stannous ions sufficiently to reduce the labeling of blood elements with 99mTc.

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