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Ginkgo biloba Extract: Experimental Model to Evaluate its Action on the Labeling of Blood Elements with Technetium-99m and on the Morphometry of Red Blood Cells

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Abstract: We have evaluated the influence of the *Ginkgo biloba* extract (infusion and crude extract) on the labeling of red blood cells (RBC) and plasma proteins with technetium-99m (Tc-99m). We also studied the morphometry of the RBC treated with *Ginkgo biloba* extract (EGb). Blood was withdrawn and incubated with EGb. Stannous chloride was added and, then, Tc-99m was added. Plasma (P) and RBC were isolated, also precipitated with trichloroacetic acid and soluble (SF) and insoluble fractions (IF) separated. The morphology of the RBC also was evaluated under optical microscope and morphometry. The analysis of the results shows that there is a decrease in the radioactivity on RBC and on IF of P and of RBC with the EGb. The study of the morphology of RBC showed important morphological alterations due to treatment with EGb. These observations were confirmed by morphometry. We suggest that the chemical agents presents in the *Ginkgo biloba* extract or its active metabolites could act, with: (i) a chelating action of the ions stannous/pertechnetate or (ii) by damages induced in plasma membrane; (iii) or by competition of the cited ions to the same bindings sites; or (iv) with possible generation of reactive oxygen species that could oxidize the stannous ion.

Key words: *Ginkgo biloba* effect, RBC labeling, technetium-99m, stannous chloride, morphology

Introduction

Ginkgo biloba is a gymnosperm considered a "living fossil" and is the phytoterapic most used in popular medicine in the treatment of Alzheimer's disease, cerebral ischaemia. *Ginkgo biloba* extract (EGb) has important antioxidant and free-radical scavenger properties due to probably to the presence of flavonoids (Yucheng *et al*., 1996; Diamond *et al*., 2000; Galluzzi *et al*., 2000). Therefore, this extract contains ginkgolide activities with confirmed antagonistic effects on platelet activating factor (PAF)-induced responses in several tissues (Galluzzi *et al*., 2000).

The use of medicinal plants or natural products has increased in the last decades all over the world. In nuclear medicine, red blood cells (RBC) are usually labeled with technetium-99m (Tc-99m) and used as radiopharmaceutical in studies of the cardiac function, volemia and detection of gastrointestinal bleeding sites. Plasma proteins are also labeled with Tc-99m and used for evaluation of lung perfusion and location of placenta (Early *et al*., 1999). These labeling techniques involve the pre-tinning of the blood constituents with stannous ions, followed by exposure to Tc-99m, as sodium pertechnetate, which is reduced within of the cell and remains trapped intra cellularly by the binding in the beta chain of hemoglobin (Early *et al*., 1999; Bernardo-Filho *et al.,* 1994). It is reported that many natural or synthetic substances can alter the labeling of blood elements with Tc-99m (Diré *et al.,* 2003; Sampson, 1996; Billinghurst and Jette, 1980; Vidal *et al*., 1998; Oliveira *et al*., 1997; Oliveira *et al*., 2002; Lima *et al*., 2002; Lima-Filho *et al*., 2003; Hladik *et al*., 1987; Hesslewood and Leung, 1994). There are some studies about the effect of the medicinal plants (*Thuya occidentalis, Nicotiana tabacum, Peumus boldus, Maytenus ilicifolia, Paullinia cupana,* cauliflower) on the labeling of RBC (Sampson, 1996; Billinghurst *et al*., 1980; Vidal *et al*., 1998; Oliveira *et al*., 1997; Oliveira *et al*., 2002; Lima *et al*., 2002; Hladik *et al*., 1987; Hesslewood and Leung, 1994).

Any chemical, physical or biological agent which alters the chemical identity of the tracer or modifies the physiological status of the organ of interest or modifies its binding capability to plasma proteins or other blood element or alters the labeling of the radiopharmaceuticals could be expected to alter the radio pharmacokinetics and the disposition of the

Table 1: Effect of *Ginkgo biloba* extract (infusion) on the labeling of red blood cells (RBC) and on the insoluble fraction of the red blood cells (IF-RBC) and plasma (IF-P) with Tc-99m

EGb (mg/ml)	RBC	P	IF-P	SF-P	IF-RBC	SF-RBC
0.0	97.7±0.7	2.3±0.7	73.8±6.6	26.6±6.6	87.1±2.8	12.9±2.8
0.004	97.4±0.3	2.6±0.3	69.0±3.2	31.0±3.2	88.0±3.5	12.0±3.5
0.04	96.3±0.1	3.7±0.1	75.3±0.5	24.7±0.5	88.7±1.8	11.3±1.8
0.4	*62.3±3.1	37.7±3.1	*27.9±0.7	72.1±0.7	*68.7±1.0	31.3±1.0
4.00*	49.5±3.9	50.5±3.9	*11.6±3.0	88.4±3.0	*32.8±10.7	67.2±10.7
20.00	*53.7±0.9	46.3±0.9	*8.8±0.1	91.2±0.1	*32.2±2.9	67.8±2.9
40.00	*48.1±15.5	51.9±15.5	*8.3±0.7	91.7±0.7	*23.5±12.1	76.5±12.1

Aliquots of heparinized blood were incubated in absence (control) or in presence of different *Ginkgo biloba* concentrations (infusion). The stannous chloride concentration used was 1.2mg/ml. The radioactivity incorporated in each fraction was determined. Results indicate the percent of the radioactivity, distribution in RBC and P, IF-P and SF-P and IF-RBC and SF-RBC. The ANOVA test (n=10) was employed to compare the results.
*$p<0.05$.

Table 2: Effect of *Ginkgo biloba* extract (crude extract) on the labeling of red blood cells (RBC) and on the insoluble fraction of the red blood cells (IF-RBC) and plasma (IF-P) with Tc-99m

Egb mg/ml	RBC	P	IF-P	SF-P	IF-RBC	SF-RBC
0.0	97.2±1.9	2.8±1.9	80.7±2.9	19.2±2.9	86.6±5.0	13.3±5.0
0.004	95.5±3.7	4.4±3.7	80.5±2.7	19.4±2.7	88.7±4.5	10.9±4.5
0.04	98.2±0.3	1.7±0.3	78.0±3.5	21.9±3.5	89.2±1.0	10.7±1.0
0.4	97.0±1.1	2.9±1.1	86.7±1.5	13.2±1.5	90.8±1.8	9.1±1.8
4.0	93.8±3.4	6.1±3.4	81.6±7.5	18.3±7.5	90.1±5.0	9.9±5.0
20.0	*71.0±7.5	28.9±7.5	*20.9±7.3	79.4±7.3	*83.1±6.0	16.9±6.0
40.0	*64.9±6.5	35.0±6.5	*20.2±2.2	79.8±2.2	*75.5±9.0	24.4±9.0

Aliquots of heparinized blood were incubated in absence (control) or in presence of different *Ginkgo biloba* concentrations (crude extract). The stannous chloride concentration used was 1.2mg/ml. The radioactivity incorporated in each fraction was determined. Results indicate the percent of the radioactivity distribution in RBC and P, IF-P and SF-P and IF-RBC and SF-RBC. The ANOVA test (n=10) was employed to compare the results.
*$p<0.05$

radiopharmaceuticals in the specific target (Srivastava *et al.*, 1990).

We have studied the effect of *Ginkgo biloba* (infusion and crude extract) on the labeling of blood constituents with Tc-99m and its action (crude extract) on the morphology (under an optical microscope and morphometry) of RBC.

Materials and Methods

Labeling Studies: Commercial *Ginkgo biloba L.* dry extract was obtained from China Jiangsu Medicines and Health Products (imported by Farmacutis RJ Brazil/Lot 001128, permission n° 00962/Ministério da Saúde, Brazil) under standardized extract EGb 761 form, w/w, what is due to contain 24% flavone glycosides (active principle) and 6% terpenoids.

Ginkgo biloba extract (40 mg) was weighted, mixture with 1ml of sodium chloride solution (0.9% NaCl), shaken by 20 seconds in the vortex, boiled by 2 minutes, refreshed in environmental temperature and centrifuged by 5 minutes. The supernatant solution (40mg/ml) obtained from infusion was also used to prepare the other dilutions (20, 4.0, 0.4, 0.04, 0.004 mg/ml) of the extract. The same procedure has done with the solution without boiling (crude extract).

Female *Wistar* rats (180-210 g) were obtained from *Laboratório* de Radiofarmácia Experimental, Departamento de Biofisica e Biometria, Universidade do Estado do Rio de Janeiro, RJ, Brazil. They were maintained under environmental conditions (22±5 ^{o}C, 12 h of light/dark cycle), water and normal diet.

The radio nuclide technetium-99m was obtained from Instituto de Pesquisas Energéticas e Nucleares, Comissão Nacional de Energia Nuclear, São Paulo, Brazil. It was recently milked from a ^{99}Molibdenium/^{99m}Technetium generator of Hospital Universitário Pedro Ernestro, Universidade do Estado do Rio de Janeiro, RJ, Brazil.

Heparinized whole blood was withdrawn from rats (n=10). An *in vitro* technique was employed to label the RBC (Bernardo-Filho *et al.*, 1994). Blood samples (0.5ml) were incubated and gently mixed, with 100µl of different dilutions of the infusion/crude extract *Ginkgo biloba* (0.004; 0.04; 0.4; 4; 20 and 40 mg/ml) for 60 minutes. After this period of time, 0.5ml of a recently prepared stannous chloride solution ($SnCl_2$, 1.2 µg/ml), (Sigma Chemical Co. St Louis, USA, Lot 65H26736) with 0.9% NaCl (Reagen, Rio de Janeiro, Brazil, Lot 970128)

Photomicrography of blood samples (incubated with saline solution 0.9%) that were withdrawn from animals (control)

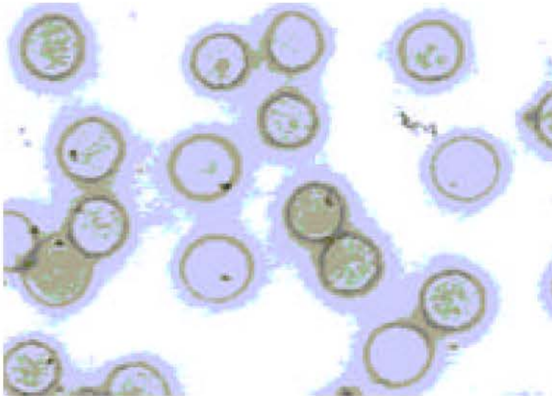

Fig. 1: Samples of whole blood were withdrawn from the animals incubated with NaCl 0.9% solution for 60 min. Stannous chloride and Tc-99m were added. Blood smears were prepared, dried, fixed and staining. After that, the morphology of RBC was evaluated under optical microscope (x1000).

Photomicrography of blood samples (incubated with *Ginkgo biloba* crude extract) that were withdrawn from animals

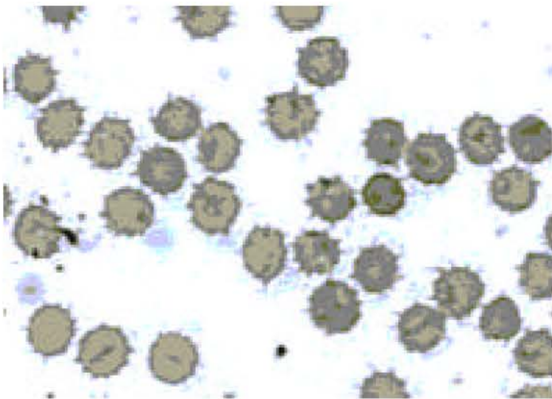

Fig. 2: Samples of whole blood were withdrawn from the animals and incubated with *Ginkgo biloba* (crude extract, above 4 mg/ml) for 60 min. Stannous chloride and Tc-99m were added. Blood smears were prepared, dried, fixed and staining. After that, the morphology of RBC was evaluated under optical microscope (x1000).

was added and the incubation continued for 60 minutes with this reducing agent. Then, 100µL of Tc-99m, were added and the incubation was continued for another 10 minutes. These samples were centrifuged by 5 minutes

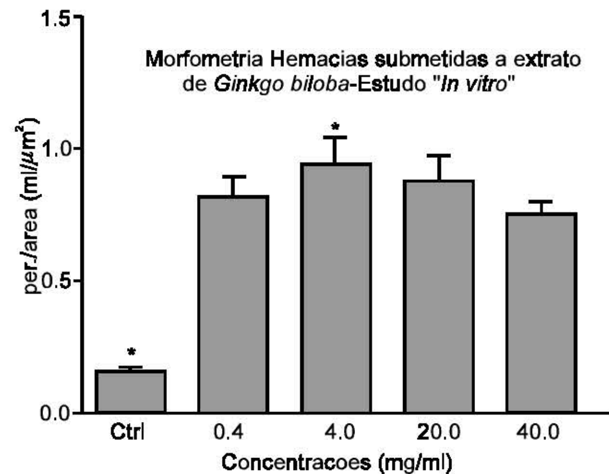

Fig. 3: Morphometry of red blood cells treated with different concentrations of *Ginkgo biloba* extract (mg/ml) employing the test Kruskal-Wallis com pós-teste Dunns - P =- 0.0216.

and plasma (P) and blood cells (RBC) were separated. Samples (20 µl) of P and RBC were precipitated with 1 ml of trichloroacetic acid (TCA, 5%) and soluble (SF) and insoluble fractions (IF) were separated. The radioactivity in P, RBC, IF-P, SF-P, IF-RBC and SF-RBC were determined in a well counter (Clinigamma, gamma counter, LKB, Wallac, Finland). After that, the percent of radioactivity (% ATI) was calculated, as previously described (Bernardo-Filho *et al.*, 1994; Sampson, 1996; Billinghurst *et al.*, 1980; Vidal *et al.*, 1998). The results are mean and standard diversion (S.D.).

Morphological evaluation: After the labeling reaction, histological preparations were carried out with blood samples treated with the EGb (crude extract). Blood smears were prepared, dried, fixed and staining (Junqueira and Carneiro, 1992; Oliveira *et al.*, 1992). After that, the morphology of the red blood cells was evaluated under optical microscope (x1000).
A statistical analysis (ANOVA test and Tukey-Kramer test and Kruskal-Wallis com pós-teste Dunns, $p<0.05$) was used to compare the experimental data, based on the morphometry evaluation.

Results and Discussion

The analysis of the results in the Table 1 (with extract of *Ginkgo biloba*/infusion) indicates that there were: (i) a significant decrease ($p<0.05$) on the uptake of Tc-99m by the red blood cells with the concentrations from 0.4 up to 40mg/ml of the extract and (ii) a significant decrease ($p<0.05$) in the fixation of Tc-99m in insoluble fractions of the blood cells and of the plasma, when the concentrations from 0.4 up to 40 mg/ml of the extract were used.
When the *Ginkgo biloba* (crude extract) was employed

(20 and 40mg/ml), there was a significant decrease ($p<0.05$) on the uptake of Tc-99m by the RBC, IF-P and IF-RBC (Table 2).

The Fig. 1 and 2 shows the optical microscopy of RBC and important morphological alterations were found (diameter/area rate: the treated RBC has the volume reduced, $p<0.05$) due to the treatment of the samples with *Ginkgo biloba* (crude extract), when compared with control samples (Fig. 1 and 2). The quantitative analysis using morphometry demonstrated that the morphological changes were significant only in the samples treated with EGb 4mg/ml (Fig. 3). We suggest that this result might be explained by: the cells that have received 20 and 40 mg/ml of the EGb have presented enhanced size (turgid aspect on the light microscopy), a characteristic more similar to controls.

Although the exact mechanism of the effect of *Ginkgo biloba* extract on the labeling of RBC, IF-RBC and IF-P is not elucidated, we suggest that it the chemical agents present in the EGb or could induce the generation of active metabolites or could act directly, with (i) a inhibition (chelating action) of the ions stannous/pertechnetate or (ii) by damages induced in plasma membrane; (iii) or by competition of the cited ions to the same bindings sites; or (iv) with possible generation of reactive oxygen species that could oxidize the stannous ion. These active metabolites or the direct action of the compounds present in the EGb could also to promote alterations in the morphology of red blood cells.

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