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The morphological analysis by optical and transmission electron microscopy of red blood cells membrane after incubation with a *Hypericum perforatum* aqueous extract.

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Abstract

The aim of this work was to evaluate the possible alterations on red blood cell (RBC) membrane induced by a *Hypericum perforatum* (hiperico) aqueous extract utilizing an optical (OM) and an electronic transmission microscopy technique (TEM) as seen in other publications done the author. Heparinized whole blood from Wistar rat was incubated with hiperico extract, stannous chloride and technetium-99 m, as sodium pertechnetate. Blood sample incubated with 0.9% NaCl was used as control. After that, the blood samples were smeared in slides and stained to be analysed by light field of the OM. Samples (control and treated) of RBC were also prepared to be analysed through TEM. The comparison of the shape of the RBC under OM revealed that the hiperico extract altered the morphology of RBC. The TEM images revealed a not integrity of the membrane with a not continuous electron density and an irregular surface and segmentations in all lengthy of the membrane. In conclusion, the effects observed with the hiperico extract may be due to the products presents in this extract that may alter the morphology of RBC possibly with an action in the membrane structure (lipid bilayers) and possible explain the effects in RBC when it was incubated with hiperico extract and modify the radiopharmaceutical capitation by RBC internal proteins. Moreover, the alterations on the membrane could have been seen with TEM that used very high magnification.

Keywords: *Hypericum perforatum*, Morphology, Optical microscopy, Transmission electron microscopy, Red blood cell.

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Introduction

Hypericum perforatum (hiperico, St John's wort) has been an herbal medicine used worldwide. Wurglics et al. [1] have well documented in an extensive review several hiperico chemical properties in *in vitro* studies, as well as actions of this natural product in humans. Several authors have showed that hiperico extracts are effective in patients with mild and moderate depression [2-5] and dysthymia [6,7]. This phytotherapic has also been used as antiviral and diuretic, and for diarrhea, dyspepsia, parasites, neuralgia, sciatica and rheumatism [6]. Clinical trials have shown that hiperico is about as effective as tricyclic (imipramine) and serotonin reuptake inhibitor antidepressants [8].

Components of hiperico can interfere with CYP3A4, one of the main cytochromes P450 isoenzymes. CYP3A4 is involved in the metabolism of many commonly used drugs [1,9]. In a study was reported that the metabolism of ethinylestradiol/ norethisterone was affected by hiperico extracts by an increase of the CYP3A4 activity. The breakthrough bleeding risk was also increased in the hiperico group. It is suggested that women taking oral contraceptives should use hiperico extracts with caution and these women should be counselled to expect breakthrough bleeding [1]. Hiperico reduces the efficacy of several pharmacological groups including immunosuppressant (risk of graft rejection), oral contraceptives (risk of pregnancy), oral anticoagulants (risk of thrombosis), and HIV protease inhibitors [5,10].

Hiperico extract induced a concentration-dependent increase of both specific transmembrane capacitance and conductance in phosphatidylcholine (PC) membranes in a dose-dependent

manner. The specific pattern of the hiperico extract interaction with lipid bilayers has possible consequences concerning its absorption and bioavailability, as well as its pharmacodynamic effects on neuronal excitability [11,12].

The hiperico extract reduced the fixation of the technetium-99 m on the red blood cells (RBC) and plasma and cellular proteins, probably by a biological action that could be related with alterations on the morphology of the RBC membrane or modifications on ions transport through the membrane [13].

In the early days of its application to biological materials, the electron microscope revealed many previously unimagined structures in cells [14]. The transmission electron microscopy (TEM) can be used to study the surface of a specimen at very high magnification, allowing individual macromolecules to be seen. TEM is an important hardware to observe and analyse ultra-structural and morphological designs [14,15].

Although the effect of the hiperico has been evaluated in several experimental models, it is not easy to find information in the literature (PubMed; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) about its effect on the RBC membrane, the aim of this work was to evaluate the possible alterations on erythrocyte membrane induced by a *Hypericum perforatum* aqueous extract utilizing an optical and an electronic transmission microscopy technique.

Material and Methods

The protocols of the experiments were performed without sacrificing the animals and was approved (CEA/113/2006) by the Ethical Committee of the Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro.

Heparinized whole blood was withdrawn by cardiac puncture from adult male Wistar rat, 3 months of age, 263 g of weight following the Ethical guidelines of our Institution.

The extract of hiperico was prepared with 400 mg of dust of *Hypericum perforatum* (Herbarium Laboratório Botânico LTDA, Brazil, lot 954661) in 10 ml of NaCl 0.9%. The preparation was homogenized in vortex mixer (30 s), centrifuged at 2000 rpm/15 min, the supernatant was separated and considered the hiperico aqueous extract. Afterwards, 1 ml of saline and 1 ml of the extract were weighted and the difference between both was considered the mass of the hiperico that it was dissolved in 1 ml of saline and the concentration of the extract was 10.72 mg/ml.

The tubes used in these experiments were previously closed with a rubber cap and a syringe was used to reduce the air atmosphere (vacuum) inside the vials. Samples of 0.5 ml were incubated with 100 µl of aqueous hiperico extract for 1 h at room temperature. A sample of heparinized whole blood was incubated with saline solution (NaCl 0.9%) as control. Then, 0.5 ml of a freshly prepared stannous chloride solution (1.2 µg/ml), as $SnCl_2$ (Sigma, USA) was added and the incubation continued for another 1 h. After this period of time, technetium-99 m (99mTc; 3.7 MBq; 0.1 ml), as sodium pertechnetate, recently milked from a 99Mo/99mTc generator (Instituto de Pesquisas Energéticas e Nucleares, Comissão Nacional de Energia Nuclear, Brasil), was added and the incubation continued for another 10 min as done before [13].

After the incubation with 99 mTc, one drop of the sample was smeared in slides (n=5) and the May-Grünwald-Giemsa (MGG) method was performed. The smear blood was fixed with methanol (Vetec, Brazil) for 5 min, then stained with Giemsa (azure eosin methylene blue solution, Isofar, Brazil) for 10 min and washed in methanol to remove excess of stain. The slides were stayed at room temperature to dry. The stained slides with MGG were analysed by light field optical microscope (OLYMPUS BH2, photographic ocular) and a total five fields per each slide were evaluated. The images were collected by CCD Sony DXC-151-A camera and processed by image pro plus (Cibernetics). A spherical shape and normal size distribution were assumed to RBC on control samples.

The samples, control and treated, were prepared for TEM of the RBC. Fixation was performed for 24 h with 2.5% glutaraldehyde and 4% paraformaldehyde in cacodylate buffer 0.1 M; pH 7.2, and post-fixed 1 h in 2% OsO_4. The samples were dehydrated under increasing concentrations of ethanol and embedded in EPON 812. Ultrathin sections (50-70 nm) were then collected on copper grids (300 mesh), stained with uranyl acetate and leak citrate and observed in TEM ZEISS model 906 in bright field at 80 kV. The images were collected by CCD camera Fview XS-Soft Imaging System GmB with 1300 × 1030 pixels of the resolution.

Results

The comparison of the shape of the RBC (no treated and treated with natural extract) under optical microscopy has revealed that the hiperico extract has altered the morphology (Figure 1) of the RBC.

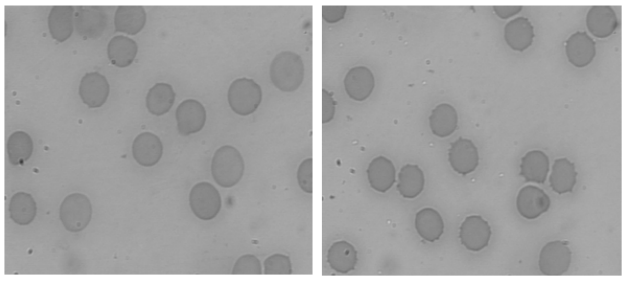

Figure 1. *Images of blood samples control (left side) and treated (right side) with hiperico in optical microscopy (1000X). In the right-side figure, it can be observed alterations in the shape of the RBC.*

The images of transmission electron microscopy (TEM) revealed details of the RBC membrane (Figures 2 and 3) that it was not showed by optical microscopy (Figure 1). Moreover, in Figure 3 ultra-structural alterations were detailed.

The analysis of the Figure 2 revealed the integrity of the RBC membrane with continuous electron density.

In other way, the analysis of Figure 3 showed a not integrity of the RBC membrane with a not continuous electron density. It

was possible to see that the membrane limits have an irregular surface and segmentations in all lengthy of the membrane. It is possible the explanation of the action of hiperico extract in membrane decrease the radiopharmaceutical effects observed in other experiments [13].

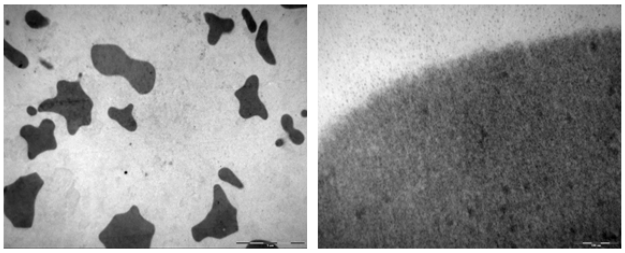

Figure 2. *Electronic transmission microscopy of an RBC sample control in different magnifications. In 1.670X (left side) and 60.000X (right side) where it can be observed that border of the cell has a linear morphology.*

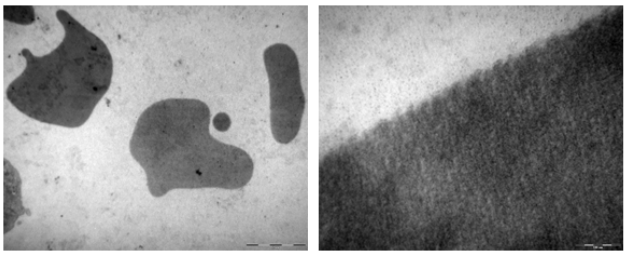

Figure 3. *Electronic transmission microscopy of an RBC sample (treated with hiperico) in different magnifications. In 3.597X showed variable forms (left side). Other way membrane border with 60000X, it can be observed irregular surface protrusions in border of the membrane.*

Discussion

Morphological analysis has been used in research in several methodologies, as to verify (i) changes with time in optic disc structure and thickness of retinal nerve fibre layer in chronic ocular hypertensive monkeys [3], (ii) the relationship between infarct-related artery stenosis and capillary density [16] and (iii) the effects of two sex hormones on normal mammary gland of female rats [17]. Some authors [18,19] show possible alterations in the morphological analysis of the RBC.

The hiperico extract has altered the morphology (Figure 1) of the RBC. Probably, the alterations on the RBC membrane could not have been seen due to the magnification used in the optical microscopy was not enough to a correct conclusion.

The analysis of the results in the Figure 2 showed a normal integrity and continuous electron density membrane of the RBC. Furthermore, the Figure 3 showed cell membranes with altered morphology in function of the treatment with a *Hypericum perforatum* aqueous extract. The image with 60.000X of magnification revealed that membrane limits have an irregular surface protrusions and segmentations in all lengthy of the membrane. These results could also, in part, to be related to the capability of the interaction of the chemical components of the hiperico extract with the lipid membrane [13]. Neagoe et al. [11] have demonstrated that the specific pattern of the hiperico extract interaction with lipid bilayers has possible consequences concerning its absorption and bioavailability, as well as its pharmacodynamic effects on neuronal excitability. Moreover, it would be due to the physiological/pharmacological properties of the hyperforin, the major active constituent of hiperico in the RBC membrane. This fact could be justified by an effect in another kind of cells [20], as neuronal cells that the hyperforin affects several neurotransmitter systems in the brain putatively by modulation of the physical state of neuronal membranes. Other authors have also used the TEM to evaluate some characteristics of the RBC membrane. Kanna et al. [21] and Li et al. [22] have suggested that the membrane bilayer and the network of membrane-associated proteins together regulate the characteristic shape and elastic properties of RBC. Eber et al. [23] and Shin et al. [24] have reported that the primary cellular defect is loss of membrane surface area relative to intracellular volume, leading to spheroidal shape and decreased deformability. Moreover, Majunder et al. [25] have described that TEM studies revealed presence of pores with diameters ranging from 100 to 200 nm on the RBC membrane surface of myeloid leukaemia with acute myeloid leukaemia being the most prominent among others. Such pathophysiological alterations of the RBC morphology in leukemic patients could be identified as characteristic signature of the onset of anaemia associated with the disease. Kumar et al. [26] have indicated that the TEM of infected RBC with Plasmodium berghei ANKA revealed variable and irregular surface protrusions and deep surface indentations on infected RBC.

In conclusion, the effects observed with the hiperico extract may be due to the specific chemical compounds presents in this extract that may alter the morphology of RBC possibly with an action in the RBC membrane structure (lipid bilayers). Moreover, the alterations on the membrane could have been seen with TEM because it is used to study the surface of a specimen at very high magnification.

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