

Study of the Mechanisms of Action of Medicines in the Immune System Using Microscopy

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Our research group has been evaluating in the last years the action of several substances extracted from algae, lichens, and mushrooms, as well as other medicines in neoplastic and inflammatory cells. More specifically in macrophages, bone marrow cultures, and tumor cells. The *in vivo* treatment evaluation was performed in mice, bearing or not a tumor. For this evaluation we used several tools, as light, confocal, or interference microscopy, flow cytometry, and mainly, as the best tools, the transmission and scanning electron microscopy. The results are very interesting since some of these substances and medicines activate the immune system allowing a natural defense against the tumor cells.

Keywords: cell culture; ultrastructural cytochemistry; Canova medication.

1. Introduction

Brazilian popular culture is particularly rich and the medicinal plants are used non-specifically for fitness and to improve the body function. In Brazil the population utilizes several plants, commonly prepared by infusion (tea), or decoction, that are generically referred as tonics. The most common popular uses are as fortifiers, as aphrodisiacs, and to counteract weakness in general. Juice, bottled brew, and consumption of the plant as food, are other frequently forms of utilization. There is no predominance in the use of any specific part of the plant; in some cases, the whole plant is employed. Essential oils and ethanol extracts from the leaves and/or roots of medicinal plants are also commonly used. Brazil is an example of country that has a diverse flora and a rich tradition in the use of medicinal plants for both antibacterial and antifungal applications, such as the treatment of tropical diseases, including leishmaniosis, malaria, schistosomosis, fungal, and bacterial infections [1]. However the development of phytotherapeutic agents using the Brazilian huge biodiversity allied to the popular knowledge is not satisfactory yet. Nowadays the research in this field is increasing with satisfactory results. Our group decided to investigate if the biological model used in our laboratory (cell culture), and microscopy techniques, would be useful to make an evaluation of the mechanisms of action of some products.

2. Experimental details

All recommendations of the National Law (number 6.638, November, 5th 1979) for scientific management of animals were respected and the Institutional Animal Care Committee at UFPR approved all related practices. Experiments were carried out at the Neoplastic and Inflammatory Research Laboratory, UFPR, which has a management program for produced residues. All experiments were performed at least three times with three control groups and the results analyzed in a double blind way.

2.1 Cell culture preparation

Primary cultured cells were obtained from washing mice peritoneal cavities (macrophages) or femurs (bone marrow cells). Lineage cells were purchased from specialized agencies. The cells were maintained

in culture, incubated at 37°C under 5% CO₂ with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 50 µg/ml penicillin and 100 U/ml gentamicin, and handled according to the procedures required by each experiment.

2.2 Light microscopy

Cells were plated into culture plates with cover slips, maintained as described for each specific protocol, and then rinsed with phosphate buffer solution (PBS), fixed in Bouin, stained with Giemsa, dehydrated, and mounted with Entellan®. Adhered cells were observed by light microscopy using a Nikon Eclipse E200 microscope. The morphological characteristics of each cells was analyzed. For each cover slip about 100 cells were counted from the total cells plated, and the data obtained was used in statistical analysis.

2.3 Transmission electron microscopy

After each experiment the cells were fixed with 2% glutaraldehyde, 4% paraformaldehyde, 5 mM CaCl₂, in 0.1 M cacodylate buffer (pH 7.2); post-fixed in 1% osmium tetroxide (OsO₄), dehydrated in acetone and embedded in Epon [2-3]. Ultrathin sections were stained with lead citrate and uranyl acetate and observed with a Jeol-JEM 1200 EX II transmission electron microscope at the Electron Microscopy Center of UFPR. A GATAN CCD camera and GATAN digital micrograph software were used to obtain the digital images.

2.4 Scanning electron microscopy

The cells were fixed with 2.5% glutaraldehyde (0.1 M cacodylate buffer, pH 7.2), washed and post-fixed in 1% OsO₄ for 30 min in the dark at room temperature [2-3]. After washing, the cells were dehydrated using increasing ethanol concentrations. Cells were CO₂ critical point dehydrated, metalized and observed using a JEOL JSM-6360 LV SEM scanning electron microscope in the Electron Microscopy Center at UFPR.

2.5 Transmission electron microscopy

Immunostaining was performed according to standard protocols using commercially available antibodies for surface markers. The cells were maintained on ice, blocked with 1% PBS/BSA (bovine serum albumin), and incubated with 1 µg biotinylated antibody in 1% PBS/BSA for 40 minutes. After washing, they were fixed in 2% paraformaldehyde for 30 minutes and the aldehyde radicals were blocked with 0.1M glycine in PBS. The cells were incubated with phycoerythrin (PE) labelled secondary antibody in PBS for 40 minutes. The nuclei were stained with 300 nM DAPI (4,6 – diamidino – 2 – phenylindole, dihydrochloride) (Molecular Probes, Eugene, OR, USA), which was added 15 minutes before cell observation. The cells were washed with PBS, mounted with fluormont-G and the fluorescence was analyzed with a Radiance 2001 laser scanning confocal microscope (BIO-RAD) coupled to an Eclipse E-800 (Nikon).

Acridine orange: after treatment, the cells were incubated with 5 µg/ml acridine orange solution for 20 minutes at 37°C 5% CO₂. The coverslips were placed in a PBS drop and observed in the Radiance 2001 laser scanning confocal microscope (BIO-RAD) using the argon laser (488nm).

2.6 Flow cytometry

Immunophenotyping was performed using specific antibodies for each cell. Leukocytes were labeled using monoclonal antibodies anti-CD45. Among them monocyte/macrophage lineage (CD11b), granulocytes (Ly-6G), B lymphocytes (CD45R), dendritic cells (CD11c), T lymphocytes (CD3), erythrocytes (TER-119) were labeled. Cells (1×10^6) were incubated with 0.5 $\mu\text{g/ml}$ PE or FITC labeled antibodies in PBS for 40 minutes in the dark. After incubation, the cells were washed, resuspended in PBS and the fluorescence was analyzed according to standard procedures on a FACSCalibur flow cytometer (Becton Dickinson – BD), equipped with an argon ion laser (488 nm). Data were analyzed in Cell Quest program (BD) and submitted to analysis of variance (ANOVA) and Tukey test ($p < 0.05$) to determine the statistical significance.

2.7 Ultrastructural cytochemistry

All ultrathin sections from ultrastructural cytochemistry were observed without stain using a Jeol 1200 EXII transmission electron microscope. A GATAN image analyzer was used to acquire images. Acid Phosphatase (AcPase) and Mg^{++} ATPase activity techniques are based on the reaction of the phosphate generated by cells with cerium chloride, which results in an electrondense precipitate of cerium phosphate. The cells were prefixed with 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C for 10 min, then rinsed and washed in 0.05 M tris-maleate buffer, pH 5.0. The cells were then rinsed and preincubated in a medium containing 2 mM CeCl_3 , 5% sucrose, and the substrate of the specific enzyme in the same buffer. The reaction control was incubated in the same medium but without the substrate. After incubation, the cells were rinsed, fixed, and processed routinely. NADPH oxidase activity technique is based on the reaction of H_2O_2 generated by cells with cerium chloride, which results in a precipitate of cerium perhydroxide ($\text{Ce}[\text{OH}]_2\text{OOH}$). The cells were washed with 0.1 M TRIS maleate buffer (pH 7.5) containing 7% sucrose, at 4°C. After washing, the cells were incubated for 10 min at 37°C with the same buffer containing 1 mM 3-amino-1,2,4 triazole-catalase inhibitor (AT), and subsequently incubated with a new solution for 20 min at 37°C in 0.1 M TRIS-maleate buffer (pH 7.5) supplemented with 7% sucrose, 0.71 mM NADH as the substrate, 2 mM CeCl_3 , as the capture agent, and 10 mM AT. The solution used as enzyme control lacked the enzyme substrate. After incubation, the cells were processed routinely for transmission electron microscopy. The peroxisomal enzyme marker catalase and the cytochrome oxidase activity were detected based upon the oxidative polymerization of 3,3'-diaminobenzidine (DAB) to an osmiophilic reaction product (DAB precipitation). Cells were rinsed at 4°C with 0.1 M cacodylate buffer (pH 7.5) containing 5% sucrose and pre-fixed for 1 h at 25°C in a solution with 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 5% sucrose. The cells were then rinsed and incubated in the reaction medium containing 5 mg 3,3'-diaminobenzidine (DAB) in 0.05 M TRIS-HCl buffer (pH 7.6), and subsequently incubated for 10 min with 0.05 M TRIS-HCl buffer (pH 7.6). Then the cells were processed according transmission electron microscopy protocol as described above.

3. Results and discussion

Polysaccharides from various sources have been shown to have anti-tumoral activity and low toxicity and Electron Microscopy is one of the best tools for this kind of investigation. The polysaccharide α -D-glucan from the lichen *Ramalina celastri* has been studied against HeLa cells (in vitro) and against Sarcoma 180 (in vivo). It was shown that α -D-glucan was cytotoxic to HeLa cells with a dose of 80 $\mu\text{g/ml}$, although the cell monolayer was similar to the control; at ultrastructural levels a large number of microvilli were substituted by cytoplasm blebs, indicating cellular injury. The treatment of Sarcoma 180 bearing mice indicated that α -D-glucan could inhibit 36% of tumor growth and affect host defense and cell responses [4-5].

The laboratorial researches with homoeopathic medicines are very scarce; most of them are clinical reports, with varied methodologies, controversial and doubtful results. Even so the number of people that

uses this therapeutic system is huge. In Europe, homeopathy is the most frequent complementary and alternative medical therapy. In Brazil, the general data of homoeopathy use is unknown. In the medical school community 60% of the teachers admitted a low knowledge on this therapeutics, and 80% admitted a reasonable or a considerable usefulness of homoeopathy [6]. Since 1997, our group has been founding many important results with a study focused on a homeopathic medicine called Canova (CA). Its manipulation is based on Hahnemann's ancient homoeopathic techniques that use highly diluted substances that are vigorously shaken (succussed) during the preparation. Clinical observation of patients confirmed the success of this treatment that seems to enhance the individual's own immunity to trigger a particular immunologic response against several pathological conditions. Several patients and doctors relate the same results: increase of the appetite, reduction of pains, and return to the daily activities.

The CA medication is a commercial product that represents a new form of immunomodulatory therapy. It is an aqueous, colorless and odorless solution produced and sold in authorized Brazilian drugstores. Mother tinctures are purchased from authorized agencies indicated by the Brazilian Health Ministry. These agencies assure the quality (endotoxin free) and physico-chemical composition of its products. Starting from the original mother tincture (in the case of a plant this is an ethanol extract) several dynamizations – succussion (shaking) and dilution in distilled water – are performed. The final commercial product is composed of *Aconitum napellus* (Ranunculaceae), *Thuja occidentalis* (Cupresaceae), *Bryonia alba* (Curcubitaceae), Arsenicum (arsenic trioxide), *Lachesis* (Viperidae) and less than 1% ethanol in distilled water (www.canovadoBrasil.com.br). In our experiments we used the commercial product purchased from Canova do Brasil.

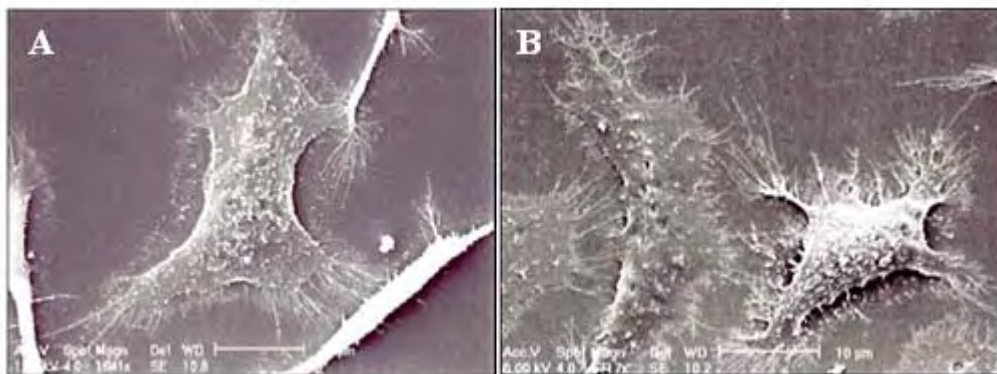


Fig. 1 - Scanning electron microscopy: A and B – Canova treated macrophages showing an activated morphology with innumerable cytoplasmic projections.

Several microscopy techniques were utilized to demonstrate that CA activates macrophages. Piemonte and Buchi, in 2002, demonstrated both *in vivo* and *in vitro* that mice macrophages treated with CA were activated according to morphologic (Fig.1 and 2), biochemical and molecular criteria, namely $\alpha 5$ - $\beta 1$ integrins, FC receptors and α -actin filaments distribution were altered and TNF- α production was decreased [7]. Over production of TNF- α plays an important role in a number of pathological conditions, including cachexia, septic shock, and autoimmune disorders. Our results showed that TNF- α release decreased after repeated doses of CA, justifying, in part, the clinical improvements of many patients. Our assays have shown that the increase in nitric oxide (NO) production is accompanied by an increase in inducible nitric oxide syntase (iNOS) detection. The enzyme was found on the cytoplasm located mainly near vesicles and mitochondria. NO and reactive oxygen species (ROS) are produced, under a variety of biological conditions and they are critical in host defense not only because they can damage pathogens and tumoral cells but also since they are immunoregulatory. Ultrastructural cytochemical detection of NADPH oxidase activity was performed and characterized by a local cerium precipitate. Ultrathin sections of the material were observed without stain, so that electrondense markers are indicative of a

positive enzyme reaction. NADPH oxidase is normally dormant in resident macrophages, but can be rapidly activated by a variety of stimuli. When the phagocyte is activated, the cytosolic subunits migrate to the membranes, where they bind to the membrane-associated subunits to assemble the active oxidase resulting in the delivery of its products into vesicles and extracellular environment. The control group showed electrondense products in the few activated macrophages found. Canova, in some way, activates this pathway because in the treated cells we observed, mainly in vesicles, stronger positive reaction products indicative of NADPH oxidase activity. The enhanced of nitric oxide synthase (iNOS), consequently producing ROS and NO respectively; causes the inhibition of the cytochrome oxidase activity [8], whereas Mg^{++} ATPase and AcPase activities were increased (Fig. 2).

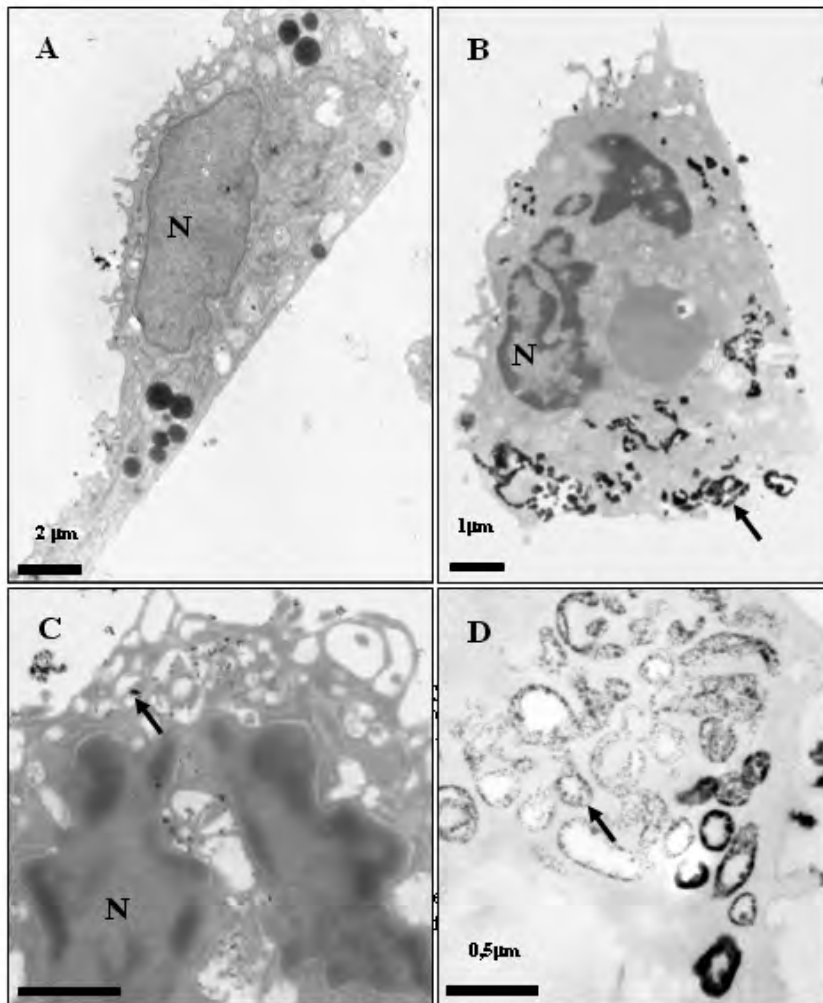


Fig. 2 - Transmission electron microscopy: A – Control macrophage routinely processed for transmission electron microscopy; B – Canova treated macrophage processed for Mg^{++} ATPase showing positive reaction for enzyme activity (arrows); C – Canova treated macrophage showing positive reaction for NADPH oxidase enzyme activity (arrows); D – Canova treated macrophage showing positive reaction for AcPase activity (arrows). N = nucleus.

Acridine orange and confocal microscopy showed the majority of Canova treated macrophages presented great amount of acid vesicles, also inside of the innumerable cytoplasmic projections (Fig. 3). CA stimulates an increase of the endosomal/lysosomal system as well as the phagocytic activity of macrophages when interacted with *Saccharomyces cerevisiae* and *Trypanosoma cruzi* epimastigotes [9].

The modulatory effects of CA were also observed both *in vivo* and *in vitro* in experimental infection by *Leishmania amazonensis* and *Paracoccidioides brasiliensis*, controlling infection progression and limiting its dissemination [10-11]. Moreover, it is neither toxic nor mutagenic [12].

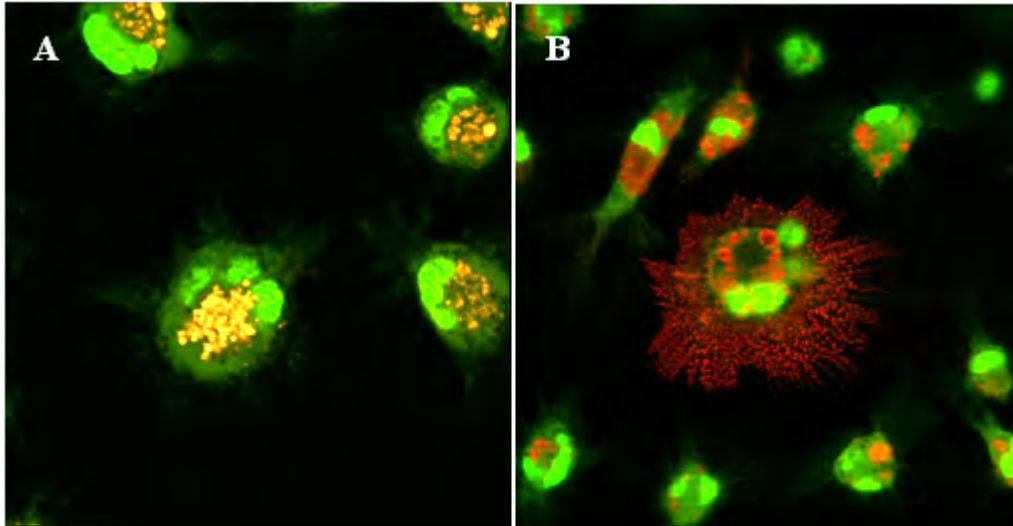


Fig. 3 - Confocal microscopy: Macrophages incubated with acridine orange; **A** – Control macrophage; **B** – Canova treated macrophage showing many acid vesicles in red and nucleus in green;

The results with CA treatment suggest an improvement in resistance against infectious and foreign agents and show an enhanced immunity favoring a specific immunological response to these microorganisms through the phagocytic pathway. The treatment induces morphological alterations of macrophages, including an increase in their spreading areas. This can be a key event for the enhancement of phagocytic ability of non-infective forms observed. To-date, several potent biological response modifiers, which are able to activate macrophages, has been extensively studied. Canova, a homeopathic medication with no side effects, provides a good alternative for macrophage activation. After these results, two master dissertations were concluded with clinical results after the treatment of HIV/AIDS patients with Canova (Figure 4). The evolution of HIV/AIDS+ patients in Botswana, Africa, was evaluated in a prospective study. Participants were assessed on site, Gabane Home Care, prior to and after the completion of one and eighteen months period of Canova treatment, using a specific quality of life in HIV/AIDS questionnaire. The data indicate that the treatment is highly effective in reducing symptomatology and improving quality of life in individuals with HIV by recovering parameters like general pain feeling, appetite, capability to do small efforts and absenteeism among others. The evolution of HIV/AIDS+ patients was also evaluated in Brazil, before and after one and six months of continuous use of Canova. Patients were assessed linked to Non Governmental Organizations (NGO) and to a public institution of health in Curitiba, Paraná, Brazil. The patients were evaluated clinically, in a laboratory, and through a specific quality of life questionnaire, form 17 from The Measurement Groups. The data indicate that the treatment is effective in reducing opportunistic diseases, increasing the number of CD4 cells and erythrocytes number, and in improving the quality of life by parameters of recovering such as decreasing general pain and depression, and increasing appetite and energy to work [13-14].

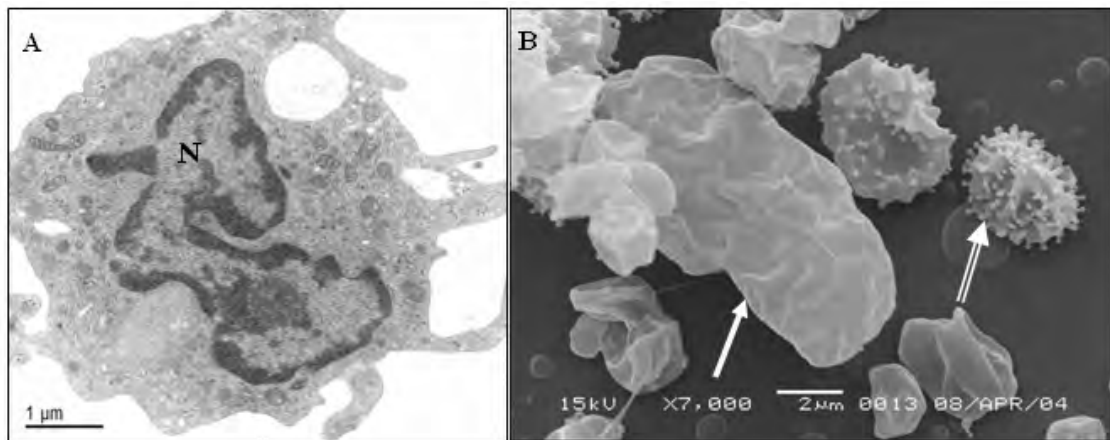


Fig. 4 - Transmission and scanning electron microscopy: blood cells from HIV/AIDS patient after 30 days of Canova treatment showing activated monocytes (**A** in TEM and **B** in SEM). N = nucleus, white arrow activated monocyte, and open arrow infected lymphocyte.

Similarly, the improvement in immune response of CA-treated mice was demonstrated in studies with Sarcoma 180. A reduction in sarcoma size was observed and a significant infiltration of lymphoid cells, granulation tissue and fibrosis occurred, surrounding the tumor. All animals from the treated group survived, and in 30% of them a total regression of the tumor was shown. The treatment with CA increased total numbers of leukocytes. Among lymphocytes, T CD4, B and NK cells increased [15]. These results suggested a direct or indirect action of the CA on hematopoiesis. So the bone marrow cells were treated and processed for light, transmission and scanning electron, and confocal microscopy, and also for flow cytometry. All microscopy techniques showed that monocytic lineage (CD11b) and stromal cells (adherent cells) were activated by treatment (Fig 5 and 6). Canova also increased cell clusters over adherent cells, suggesting areas of proliferation and differentiation [16-17].

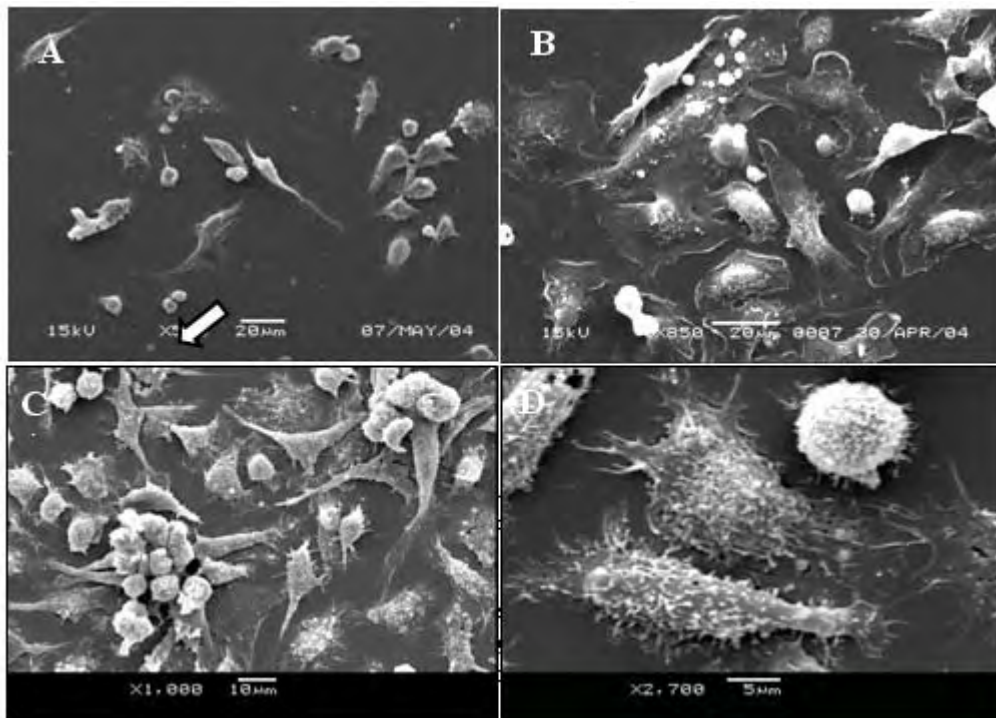


Fig. 5 - Scanning electron microscopy: A – bone marrow adherent control cells; B, C and D – bone marrow Canova treated adherent cells showing an activated morphology; cell niche over adherent monolayer suggesting cellular proliferation (arrow).

CA medication successfully activated mononuclear phagocytes, represented by monocytes, tissue macrophages, dendritic cells (DCs), microglia and osteoclasts, cells that maintain tissue homeostasis and provide a first line of defense against invading pathogens.

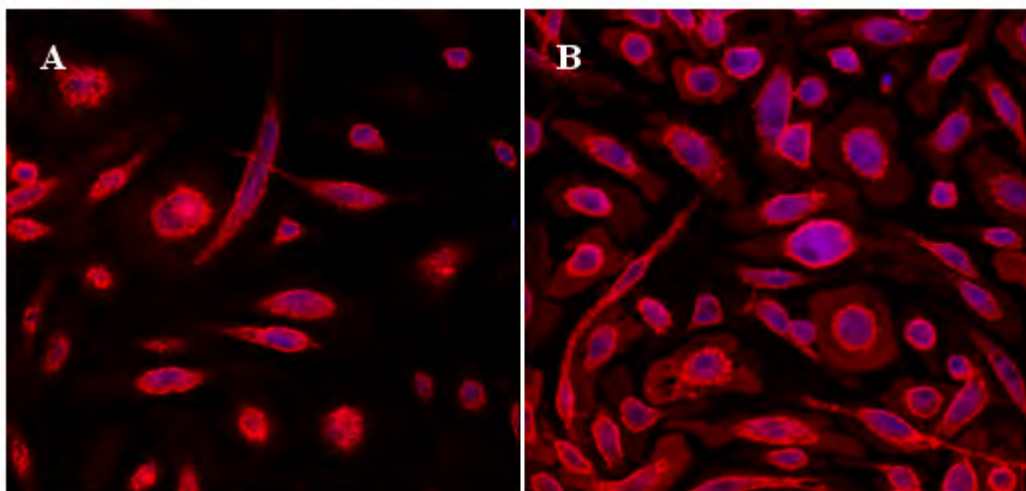


Fig. 6 - Confocal microscopy: Bone marrow adherent cells labeled with Cd11b (in red) for monocytes/macrophages lineage; DAPI for nucleus in blue; A – Control cells; B – Canova treated cells showing more adhered with activated morphology and positive labeled for CD11b.

4. Conclusion

Our results point to the need of a careful examination of the interplay between homeopathic medicaments and macrophages in the treatment of a malignancy. At this knowledge is extended, the ability to selectively influence the activation state of macrophage hopefully will allow us to manipulate these treatments in the malignant diseases. This will be an important area for future research, in particular the functional relationship between immune cells production of cytokines and the response to homeopathic treatment. One of the ultimate goals of biological research is the constant improvement of methods to obtain responses to our unsolved questions. It seems to be clear that the reliable information about the morphology and organization of the cell constituents may solve some of them. We have demonstrated that the samples analysis using several microscopy techniques allied to cell culture is an efficient manner to study the mechanisms of action of medicines.

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