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Canova, a Brazilian medical formulation, alters oxidative metabolism of mice macrophages

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Summary Macrophages play a significant role in the host defence mechanism. When activated they can produce reactive oxygen species (ROS) as well as related reactive nitrogen species (RNS). ROS are produced via NAD(P)H oxidase which catalyzes superoxide (O_2^-) formation. It is subsequently converted to hydrogen peroxide (H_2O_2) by either spontaneous or enzyme-mediated dismutation. Nitric oxide synthase (NOS) catalyzes nitric oxide (NO) formation. Canova (CA) is a Brazilian medication produced with homeopathic techniques, composed of *Aconitum*, *Thuya*, *Bryonia*, *Arsenicum*, *Lachesis* in distilled water containing less than 1% ethanol. Previous studies demonstrated that CA is neither toxic nor mutagenic and activates macrophages decreasing the tumor necrosis factor-α (TNFα) production. In this assay we showed that macrophages triggered with Canova increased NAD(P)H oxidase activity as well as that of iNOS, consequently producing ROS and NO respectively. Cytochrome oxidase and peroxisomes activities were inhibited by NO. As NO and O_2^- are being produced at the same time, formation of peroxynitrite ($ONOO^-$) may be occurring. A potential explanation is provided on how treatment with Canova may enhance immune functions which could be particularly important in the cytotoxic actions of macrophages. CA can be considered as a new adjuvant therapeutic approach to known therapies.

Introduction

Macrophages play a significant role in the host defence mechanism. When activated, they produce and release reactive oxygen species (ROS) in response to stimulation with various agents[1] and can inhibit the growth of a wide variety of tumor cells and micro-organisms.[2] Another important pathway stimulated involves the generation of

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nitric oxide (NO) and related reactive nitrogen species (RNS).[3]

Oxygen metabolites play an important role in the intracellular and extracellular killing of microorganisms, and may also serve as mediators of the immune system and modulators of cellular activities such as adhesion, phagocytosis and signal transduction.[4] In this metabolic pathway, oxygen is enzymatically reduced to superoxide anion (O_2^-) through a series of one-electron reductive reactions catalyzed by the NAD(P)H oxidase system.[5,3] O_2^- is subsequently converted to hydrogen peroxide (H_2O_2) by either spontaneous or enzyme-mediated dismutation via superoxide dismutase (SOD). NAD(P)H is recognized as one of the primary enzyme complexes involved in the anti-pathogen action of phagocytic cells. It is a membrane associated multisubunit enzyme.[6] In unstimulated cells, components of NAD(P)H oxidase are distributed throughout the membrane and the cytosol.[7]

NO is a diatomic mediator generated from the five-electron oxidation of L-arginine and molecular oxygen catalyzed by the nitric oxide synthase (NOS) family of enzymes.[8] Two of these are constitutively expressed in vascular endothelial cells (eNOS or type III NOS) and in neurones (nNOS or type I NOS), whereas the expression of a third isoenzyme (iNOS or type II NOS) is inducible in a variety of cells. NO production has also been detected in mitochondrial preparations and the existence of a mitochondrial NOS (mtNOS) associated with the inner mitochondrial membrane, has been postulated.[9] The constitutive forms are low-activity enzymes that generate small amounts of NO as signaling molecules. Once induced, iNOS produces large amounts of NO which accounts for its microbicidal and tumoricidal activities.[10] Unlike active NAD(P)H oxidase, NO syntase is soluble.[9]

A medical formulation called Canova (CA) is a Brazilian medication based on homeopathic techniques. It has a patented formulation sold under prescription in specialized drugstores. CA stimulates the host defence favoring an immunologic response against several pathologic states through activation of the immune system. Previous studies demonstrated that CA activates macrophages both in vivo and in vitro. In addition, Tumor Necrosis Factor-α (TNFα) production is significantly diminished.[11] CA modulatory effects were observed in experimental infection both in vivo and in vitro by *Leishmania amazonensis* controlling infection progression and limiting it dissemination.[12] Sarcoma 180-bearing mice treated with CA showed reduction in sarcoma size and a significant infiltration of lymphoid cells, granulation tissue and fibrosis surrounding the tumor. Besides T CD4, T CD8, B and NK cells increased both in normal-treated and S180-treated mice groups.[13] Clinical reports shown successful results when CA is employed in diseases where the immune system is depressed due to its modulatory capacity.[14] Moreover, it is neither toxic nor mutagenic.[15] CA is indicated in deseases were the immune system is depressed such as acquired immunodeficiency syndrome (AIDS), hepatitis and neoplasia. Thus, providing an insite into the usefullness of this medicine may contribute to knowledge of how the immune system works.

The role of ROS/RNS on bacterial killing and tissue damage has long been explored.[1] It follows that mechanisms involving ROS/RNS production may have important implications in new therapeutic approaches. We now evaluate whether the influence of CA on mouse macrophages is accompanied by alterations linked to oxygen and nitrogen metabolism.

Materials and methods

Canova (CA)

Canova is a commercial medicine that represents a new form of immunomodulatory therapy and follows Hahnemann's ancient homeopathic techniques. Canova is an aqueous, colourless and odorless solution produced and sold in Brazilian authorized 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 ethanolic extract) several dinamizations—sucussion (shaking) and dilution in distilled water—are performed. Decimal dilutions (dH) are prepared. The final commercial product, Canova, is composed of 11 dH *Aconitum napellus* (Ranunculaceae), 19 dH *Thuya occidentalis* (Cupresaceae), 18 dH *Bryonia alba* (Curcubitaceae), 19 dH *Arsenicum album* (arsenic trioxide), 18 dH *Lachesis muta* (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.

Animals

Six–8 week old male Swiss mice from the Central Animal House of the Universidade Federal do Paraná received a standard laboratory diet (Purina®) and water ad libitum. All recommendations of

the National Law (N° 6.638, November, 5th 1979) for scientific management of animals were respected. Experiments were carried out at the Laboratório de Pesquisa em Células Neoplásicas e Inflamatórias, UFPR, which has a management program for produced residues.

CA treatment

All experiments were performed at least three times in quadruplicate and with two control groups. These were mice without any treatment and mice treated with an ethanolic solution (less than 1%). The treatment groups were held according to a previous description.[11]

In vivo treatment:
Mice were treated at daily intervals for 7 days. Seven μl/g of commercial CA were injected subcutaneously.

In vitro treatment:
Three-h cultured cells were treated with CA (200 μl/ml), and after 24 h a new dose of 20 μl/ml was given without replacing the medium. Treatment was carried out for 48 h in vitro.

Canova and the ethanolic solution were vigorously shaken, a process named sucussion, immediately before treatment.

Cell culture preparation

Macrophages were washed from peritoneal cavities with 10 ml of either cold phosphate buffer solution (PBS) or Hank's buffer salt solution (HBSS), pH 7.4. Harvested peritoneal cells were counted using a Neubauer chamber. The macrophages were incubated at 37 °C under 5% CO_2 for 15 min and the non-adherent cells were removed by washing with PBS. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 μg/ml penicillin and 100 U/ml gentamicin was added to culture and handled according to the procedures required by each experiment. Accordingly, $>90\%$ of adherent cells were macrophages[11] and the preparation was not further purified.

Immunogold to iNOS detection

Cells (2×10^7 cells/group) of in vivo and in vitro treatment were fixed for 1h in freshly prepared 0.05% glutaraldehyde, 2% paraformaldehyde/PBS, and then rinsed and incubated for 30 min in 50 nM ammoniun chloride/PBS. Monoclonal antibody against mouse iNOS (Sigma) was diluted (1:100) in 0.01% Triton-X/ PBS where cells were incubated for 1 h at 25 °C. Before adding the secondary antibody, cells were rinsed five times with PBS. Incubation with Protein A—gold 10 nm (Sigma) was performed for 30 min at room temperature, in 0.01% Triton-X/ PBS. Cells were re-fixed in 2.5% glutaraldehyde, 4% paraformaldehyde/PBS for 30 min and processed to electron microscopy. Cells were post fixed in 1% OsO_4, dehydrated in acetone and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Jeol 1200 EXII transmission electron microscope at the Centro de Microscopia Eletrônica, UFPR. A GATAN image analyser was used to produce the electronmicrographs.

Ultrastructural cytochemistry to detect NAD(P)H oxidase activity

Macrophages treated in vivo and in vitro with CA were processed for detection of NAD(P)H oxidase activity (2×10^7 cells/group). This technique is based on the reaction of H_2O_2 generated by cells with cerium chloride, which results in a precipitate of cerium perhydroxide (Ce-$[OH]_2OOH$).[16] Briefly, adherent 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. The enzyme control group is used to gain some insight into the specificity of the reaction. After incubation, the cells were washed twice with buffer and fixed in a solution containing 1% glutaraldehyde, 4% paraformaldehyde and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2. The cells were gently scraped off with a rubber policeman and processed to transmission electron microscopy as described above. The cells were observed without uranyl acetate and lead citrate stain to guarantee the results.

Ultrastructural cytochemistry to detect cytochrome oxidase and catalase

The peroxisomal enzyme marker catalase and the cytochrome oxidase activity were detected based upon the oxidative polymerization of 3,3′-diaminobenzydine (DAB) to an osmiophilic reaction product (DAB precipitation).[17–19] Reaction products

indicative of catalase and cytochrome oxidase activity were observed in both in vivo and in vitro assays. Cells (2×10^7 cells/group) were rinsed at 4 °C with 0.1 M cacodylate buffer (pH 7.5) containing 5% sucrose and 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 twice with 0.1 M cacodylate buffer (pH 7.2) containing 5% sucrose, and washed twice more with 0.05 M TRIS-HCl buffer (pH 7.6) containing 5% sucrose. The reaction medium containing 5 mg 3,3′-diaminobenzidine (DAB) in 0.05 M TRIS-HCl buffer (pH 7.6) were added and the cells were incubated for 1 h at 37 °C and subsequently incubated for 10 min with 0.05 M TRIS-HCl buffer (pH 7.6). The cells were gently scraped off with a rubber policeman and processed according transmission electron microscopy protocol as described above. These cells were also observed without stain.

Micro-organisms

Trypanosoma cruzi Dm28c[20] clones epimastigotes were maintained and grown in liver infusion tryptose (LIT) medium at 28 °C. To obtain metacyclic tripomastigotes, the epimastigotes in the late exponential growth phase were harvested from LIT medium by centrifugation and incubated for 2 h in triatomine artificial urine (TAU) (190 mM NaCl, 17 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.035% $NaHCO_3$, 8 mM sodium phosphate pH 6.0). Then, parasites were transferred to cell culture bottles containing TAU3AAG (TAU supplemented with 50 mM L-glutamate, 10 mM L-proline, 2 mM L-aspartete and 10 mM D-glucose) and incubated for 3–4 days at 28 °C.[21] *L. amazonensis* (MHOM/BR/73/M2269)[22] promastigotes were maintained in Tobie and Evans biphasic medium at 24 °C. Parasites were cultivated in RPMI 1640 medium supplemented with 20% FBS for 7 days to provide multiplication and used at log phase.[23]

Macrophage-micro-organisms interaction

Macrophages were cultivated for 24 h and then allowed to interact with the intracellular parasites at a ratio of 10:1 for 2 h in medium without FBS. The parasites were washed out and the macrophages were cultivated with standard medium for 24 or 72 h to detect NO production.

Nitric oxide (NO) production

The NO generation was estimated by sampling culture supernatants for nitrite, which is a stable product of NO reaction, as described elsewhere.[24] Macrophages treated in vivo and in vitro (5×10^5 cells/well) were plated into 96-well tissue culture plates. After 48 h, aliquots of 100 μl of cell-free supernatant were mixed with an equal volume of Griess-reagent (0.5% sulfanilamide and 0.05% *N*-1-naphtyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) in 96-well tissue culture plates and incubated for 10 min at 25 °C. 50 ng/ml LPS and 26 U/ml IFN-γ were added as a positive control for NO production. Optic density of the samples was subsequently measured at 550 nm at a microplate reader (BIO-RAD). The nitrite concentration was determined by reference to a standard curve using sodium nitrite (10–80 μM) diluted in culture medium.

Superoxide anion (O_2^-) detection

Reduction of ferricytochrome c was used to measure rates of formation of O_2^- in culture supernatant as described elsewhere.[25] For measurement of O_2^-, adherent cells treated in vivo or in vitro (5×10^5 cells/well) were incubated in HBSS containing ferricytochrome c (80 μM—Sigma) in the presence or absence of 1 μg/ml phorbol miristate acetate (PMA). Since PMA is able to induce O_2^- production by macrophages[26] it was used as positive control. Absorbance was measured at 550 nm in a microplate reader (BIO-RAD) and the extinction molar coefficient $\varepsilon=2.1\times10^4\ M^{-1}\ Ca^{-1}$ was used to determine reduced ferricytochrome c. Results are expressed as nmol $O_2^-/10^6$ cells.

Hydrogen peroxide (H_2O_2) measurement

Production of H_2O_2 by macrophages after in vivo or in vitro treatment was quantified based on the horseradish peroxidase-dependent oxidation of phenol red by H_2O_2.[5] Macrophages (3.5×10^5 cells/well) were incubated in horseradish peroxidase (15 U/ml, type IV-A-Sigma) and 194 mg/ml phenol red solution dissolved in HBSS at 4 °C, briefly before the start of the experiment. 1 μg/ml of PMA was added as a positive control of H_2O_2 production.[26] The plates were incubated at 37 °C for the desired time interval (60 and 90 min) and the reaction stopped by adding 10 μl 1 M NaOH aqueous solution per well. The absorbance of cell-free culture supernatant was read at 620 nm at a microplate reader (SLT Lab Instruments 340 ATC). The H_2O_2 concentration was determined by reference to a standard curve using 1–50 nmol of H_2O_2 in a solution containg 15 U/ml peroxidase, 194 mg/ml phenol red in HBSS.

Statistical analysis

Results are expressed as the means ± standard deviation (SD). Data were submitted to analysis of variance (ANOVA) and Tukey test ($P < 0.05$) to determine the statistical significance of the inter-group comparisons. Data are representative of three independent experiments.

Results

Results from in vivo and in vitro experiments were very similar. Our results also demonstrated no statistical differences between the control group and the ethanolic aqueous solution group, these thus being referred only as the control group. Significant differences were observed in the treated group when compared to the control group. Cells from the control groups were mainly resident macrophages (Fig. 1(A)) and few activated macrophages were also present, as already described.[11] Almost all cells from the treated group were activated, as defined by morphological alterations (Fig. 1(B)).

Immunogold to iNOS detection

The signal molecule NO is synthesized on demand, after enzyme activation. The inducible NOS (iNOS) once expressed, produces NO for long periods (hours to days).[27] For a better understanding, the mechanism through Canova acts, namely the expression of iNOS from peritoneal macrophages, was analysed. There was a marked difference between the morphology of control and treated cells. The control cells were smaller, had few vesicles and membrane projection compared with the treated cells. Macrophages treated with Canova contained increased iNOS levels when compared with the control group. The enzyme was found on the cytoplasm located mainly near the nuclei (Fig. 2(A)), mitochondria (Fig. 2(B)) and vesicles (Fig. 2(C) and (D)). The control group only contained iNOS in the few activated macrophages detected.

Ultrastructural cytochemistry to detect NAD(P)H oxidase activity

NAD(P)H oxidase is an electron transport chain that uses NAD(P)H as a electron donor to reduce oxygen (O_2) to superoxide (O_2^-) and hydrogen peroxide (H_2O_2).[26] Reaction products (cerium precipitated) indicative of NAD(P)H oxidase activity were detected in both in vivo and in vitro assays. When the enzyme substrate was eliminated from the incubation medium (enzyme control group), no reaction product was observed (Fig. 3(A)) in both groups. It is clear that the generation of the reaction product is strongly dependent on the presence of exogenous NAD(P)H, there being a specificity of this reaction. Occasional deposits of reaction product were detected in the control group. When found, they were located mainly in the few activated cells inside vesicles and on the external surface of the plasma membrane (Fig. 3(B)). No other cytoplasmic organelles were reactive for H_2O_2. The appearance of treated cells was markedly different from the control cells. Deposits of reaction product were mainly found

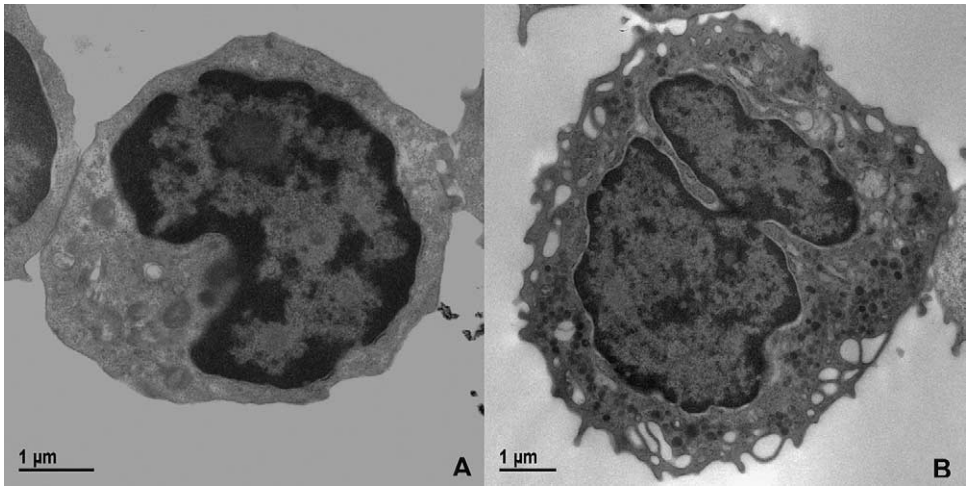

Figure 1 Electronmicrographs showing morphological characteristics of peritoneal macrophages. (A) Representative cell from control group showing typical resident morphology. (B) Activated macrophage representative of treated group. Among these cells approx. 85% had typical activated morphology. Data represent three independent experiments.

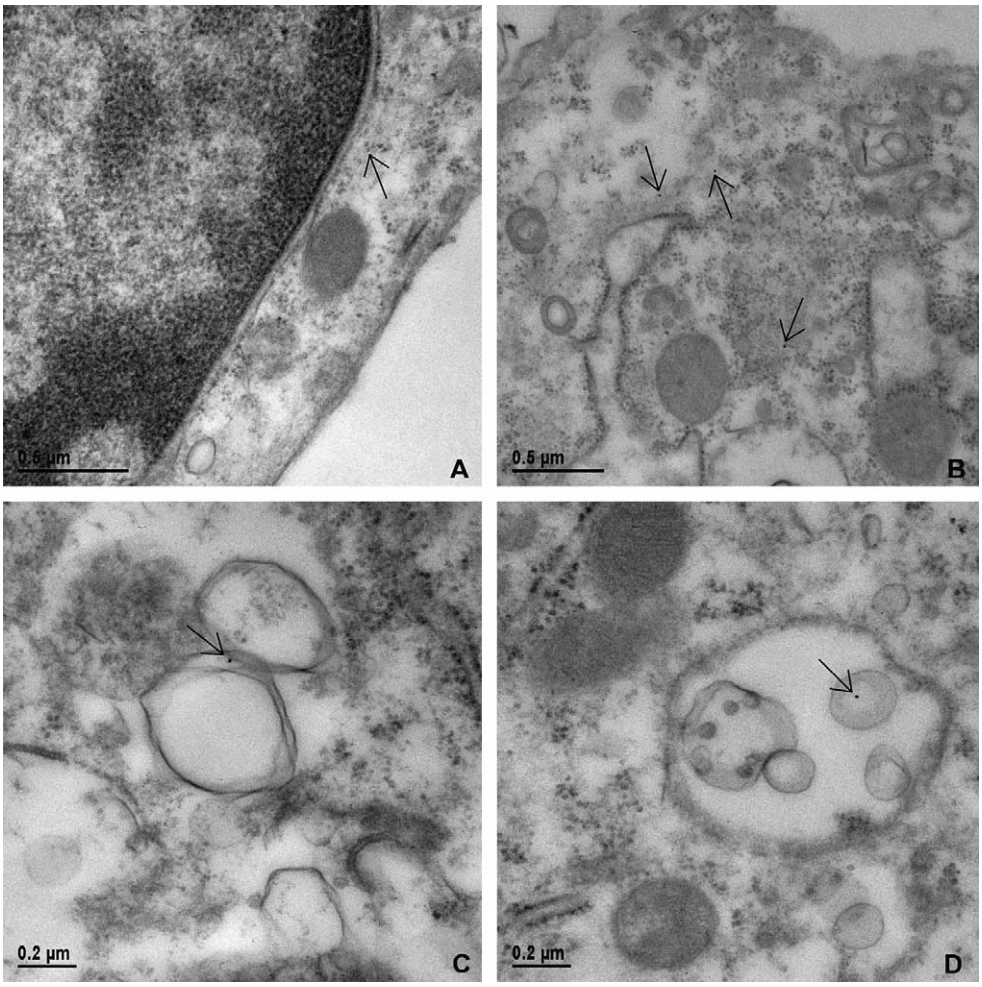

Figure 2 Immunogold to iNOS detection on treated macrophages. Canova treated cells had increased iNOS levels when compared to control group. This enzyme was located mainly near the nucleus (A), mitochondria (B), and inside vesicles (C) and (D). Arrows pointing at gold 10 nm are representative of iNOS localization. Data represent three independent experiments.

inside vesicles (Fig. 3(C) and (D)) and these were stronger than in the control. Equal amounts of electron-dense material was detected in the external surface of the plasma membrane from treated and control cells. Other cytoplasmic organelles were not found to contain H_2O_2 using this method.

Ultrastructural cytochemistry to detect cytochrome oxidase and catalase

Cytochrome oxidase catalyses the transfer of electrons from cytochrome c to oxygen forming water in the mitochondria. Catalase is involved in the peroxidative reaction of H_2O_2 inside peroxisomes. DAB donates electrons in both reactions.[18,19] Reaction products (DAB precipitated) indicative of catalase and cytochrome oxidase activity were detected in both in vivo and in vitro assays mainly with the control cells. The reaction products found in the control group were evident and distributed either inside peroxisomes or inner mitochondrial membranes (Fig. 4(A)–(C)). Generally no positive reaction was found in the treated group. However, when found, it was weak as seen in Fig. 4(D).

Nitric oxide (NO) production

NO has been shown to be part of the oxidative war chest of the immune system by virtue of its involvement in anti-tumor and anti-pathogen host response.[28] It is known to be secreted by macrophages in response to IFN-γ stimulation,[29] and LPS is recognized as a co-signal in the induction of NOS.[30] NO production was evaluated on in vivo and in vitro treatment with CA, in the presence or absence of LPS and IFN-γ. Both treatments resulted in a moderate but significant up-regulation of NO

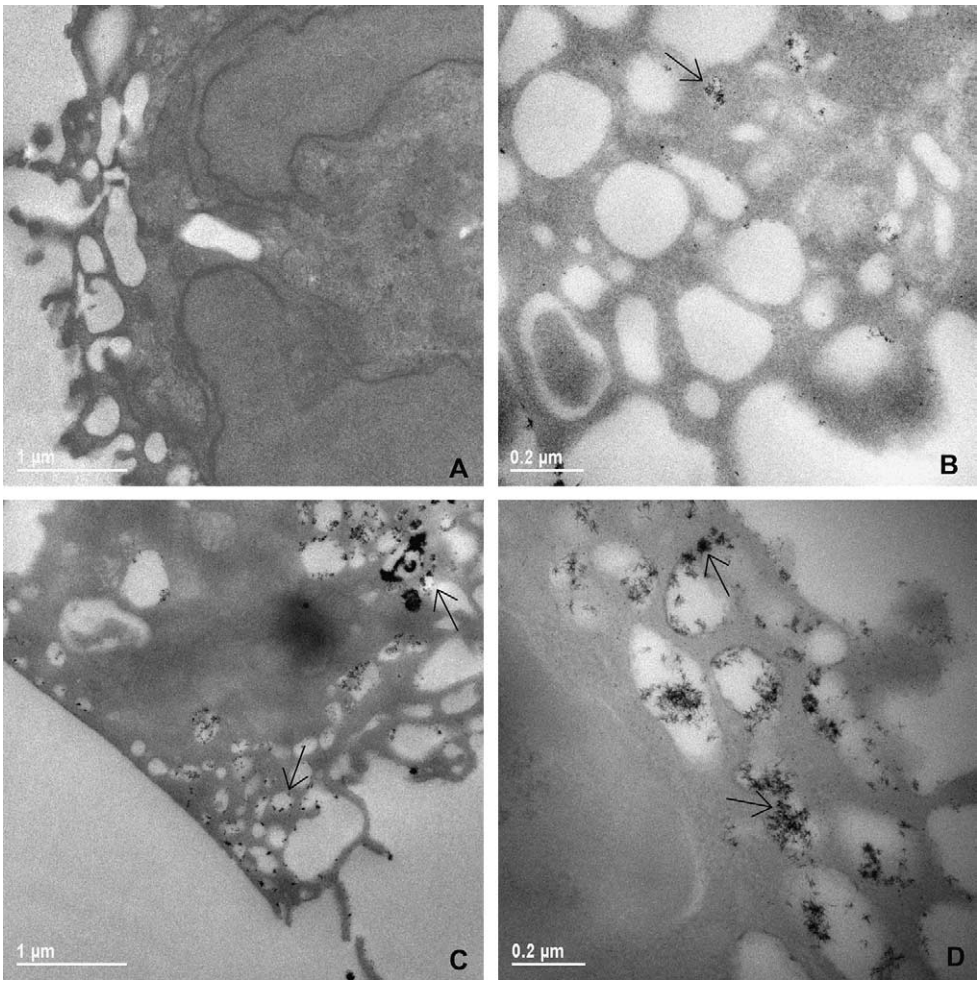

Figure 3 Electronmicrographs showing ultrastructural cytochemical localization of macrophages NAD(P)H oxidase activity. These cells were observed without stain. (A) Absence of reaction product in macrophage from enzyme control group confirming the specificity of this reaction. (B) Cell from control group showing occasional positive reaction products. Note many vesicles with no reaction products. (C) and (D) Treated cells having an intense positive reaction mainly inside vesicles. Arrows point the reaction product representative of NAD(P)H oxidase activity. Data represent three independent experiments.

production by macrophages. The NO production increased after 48 h, when treated with CA. On triggering the cells with LPS and IFN-γ, as expected, control cells increased their NO production, but macrophages from the treated group significantly increased the NO production under this condition (Fig. 5(A)).

Nitric oxide production in presence of parasites

Nitric oxide is one of the main molecules that act against intracellular parasites. NO can act directly on parasites, causing toxic and inhibitory effects on several cellular processes as growing and multiplication. The capacity to inhibiting NO production has been demonstrated to several intracellular parasites such as *Leishmania* spp.[31], *Toxoplasma gondii*[32] and fixed *T. cruzi*.[33]

NO production by macrophages of control group decreased in presence of parasites, suggesting an inhibitory process. However, this decrease was not observed in macrophages from CA treated group. Statistical tests showed that CA treatment led to a substantial enhancement of NO production by macrophages during interaction with *L. amazonensis* (46 and 32%), *T. cruzi* epimastigote (44 and 18%) and *T. cruzi* trypomastigote (32 and 5%) after 24 and 72 h, respectively (Fig. 5(B) and (C)).

Superoxide anion (O_2^-) detection

Phagocytic cells respond to a variety of membrane stimulants by the production and release of

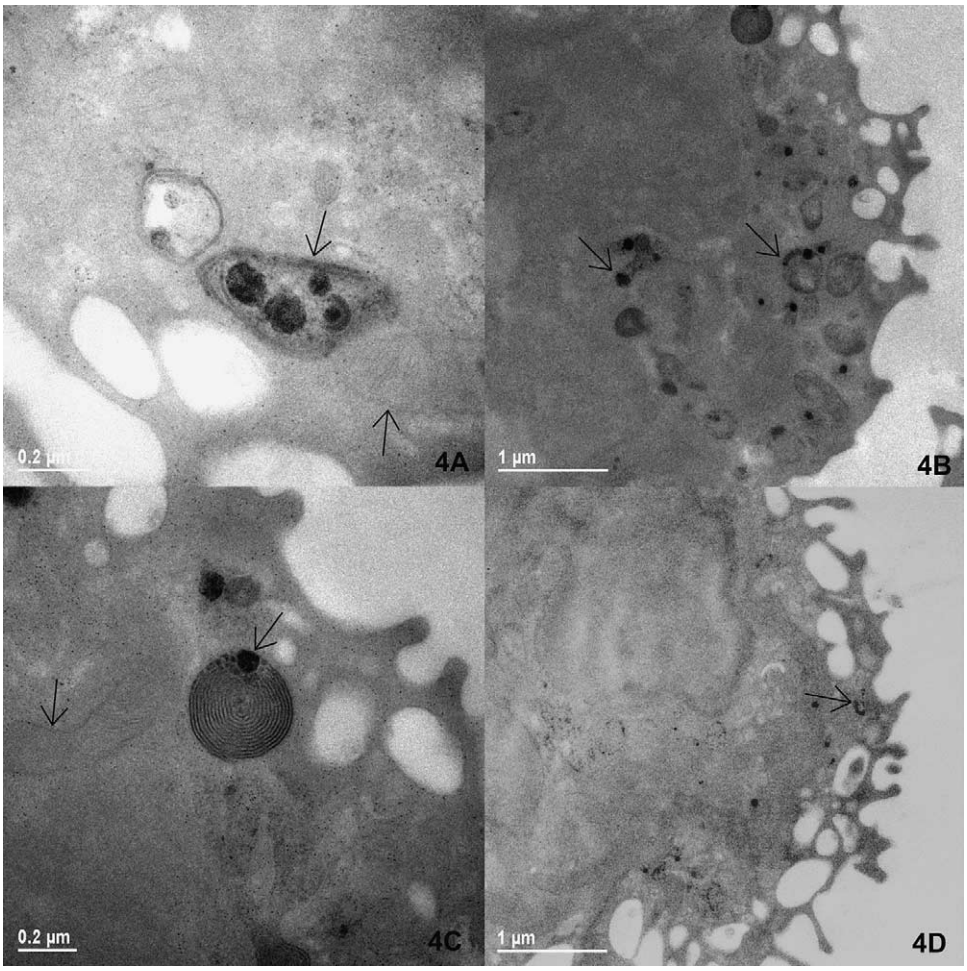

Figure 4 Ultrastructural cytochemical detection of catalase and cytochrome oxidase activity. These cells were observed without stain. (A)–(C) Showing DAB precipitated representative of catalase (peroxisomes) and cytochrome oxidase (mitochondria) activity in control cells. (D) Treated cells showing weak positive reaction products. Arrows pointing at positive reaction products. Data are representative of three independent experiments.

a number of reactive oxygen reduction products. This response is initiated by reduction of O_2 to O_2^-.[34] O_2^- detection is based on ferricytochrome c reduction. The time course for O_2^- extracellular release from control was compared to that of treated cells. At first, both groups had the same rate of O_2^- production. After 15 min, the release of from treated cells diminished considerably O_2^- (Fig. 6(A)). Under PMA stimulation, a significant decrease on O_2^- production was observed compared with the first reading (Fig. 6(B)).

Hydrogen peroxide (H_2O_2) measurement

Reactive molecules, such as H_2O_2, are produced from macrophages after various stimuli and have well-established roles in anti-microbial defence as well as in cell signaling.[30] Quantification of H_2O_2 production is based on the horseradish peroxidase-dependent oxidation of phenol red which is assayed by an increased absorbance at 620 nm[5]. The measurement of H_2O_2 on culture supernatant from treated and non-treated macrophages gave no statistically significant differences in both treatments (data not shown).

Discussion

Macrophages play an essential role in host defence against infection and tumoral cells. A large body of data indicates that macrophages must be activated in order to achieve efficiency. Recent studies have described the effects of Canova on macrophages. About 86% of treated macrophages were activated when observed in light microscopy and transmission electron microscopy.[11] The increased response capacity of activated macrophages is a result, in part, of the increase capacity of these cells to produce oxygen radicals; thus the oxidative

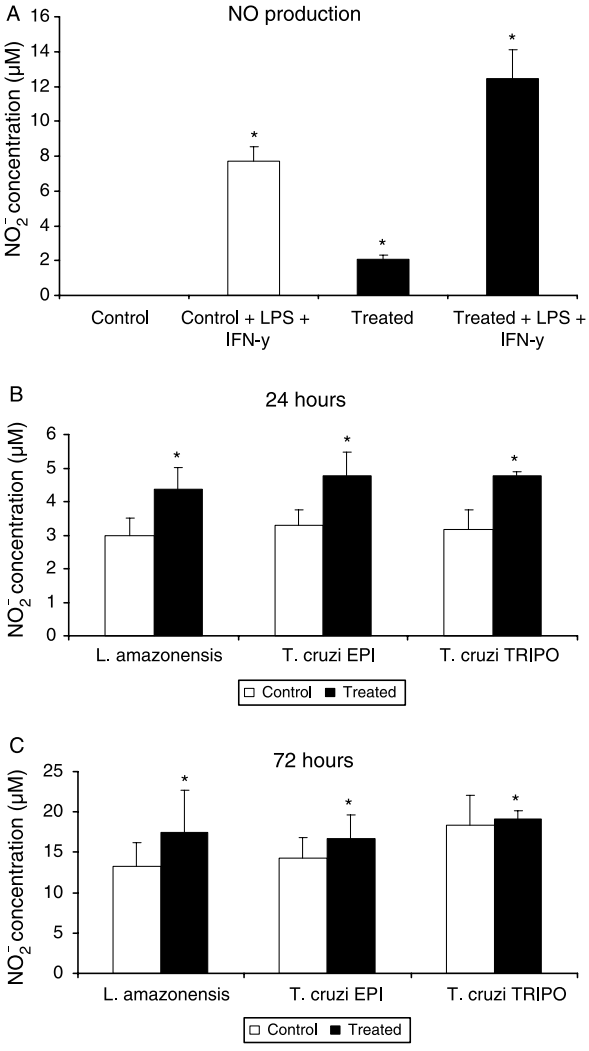

Figure 5 NO production. Macrophages were obtained either from 7-day-treated mice with 7 μl/g of MC or from non-treated animals. Nitrite (NO_2^-) concentration (μM) in supernatants was determined with Griess reagent. (A) Control versus treated group in the presence or absence of LPS and IFN-γ after 48 h. (B) and (C) Parasites were added (10:1) after 24 h of culture and allowed to interact with macrophages during 2 h. Nitrite (μM) concentration in supernatants was determined 24 (B) and 72 (C) h after interaction. Results are expressed as mean ± SD (significantly difference from the respective control group by Tukey test $^*P<0.05$). Data represent three independent experiments.

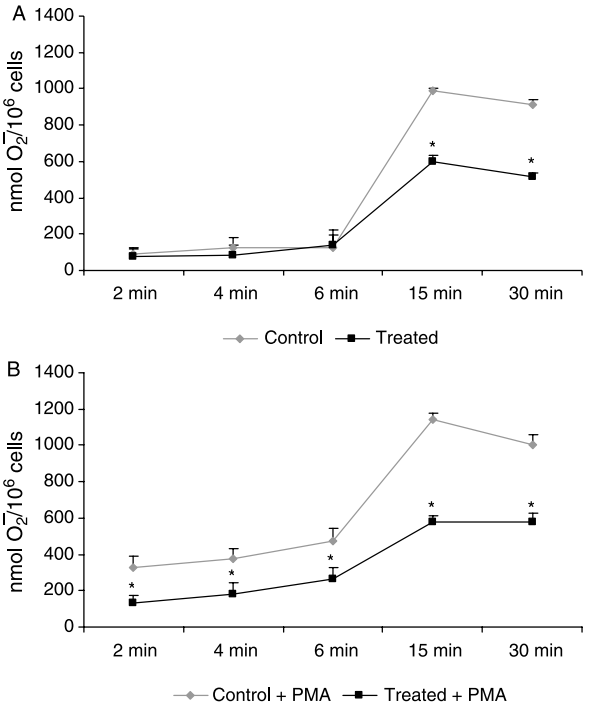

Figure 6 Time course of O_2^- extracellular release. Macrophages were obtained either from 7-day-treated mice with 7 μl/g of MC or from non-treated animals. O_2^- concentration in supernatants was determined by ferricytochrome c reduction. Results are expressed as mean ± SD (significantly different from the control group by Tukey test $^*P<0.05$). (A) Control and treated groups had the same rate of O_2^- production. After 15 min, the release of O_2^- from treated cells diminished considerably. (B) Under PMA stimulation a significant decrease on O_2^- production was observed compared with the first read value. Data represent three independent experiments.

metabolism of macrophages treated with Canova was evaluated. Results are summarize in Fig. 7.

NO is formed biologically through the oxidation of L-arginine by nitric oxide synthases. It is a cytotoxic product of activated macrophages, along with other reactive oxygen species that have been shown to be involved in numerous regulatory functions.[28,35] In vivo and in vitro assays showed that Canova acts on macrophages up-regulating nitric oxide (NO) production. These effects seem to be cumulative with LPS and IFN-γ, since NO production increased significantly under this condition (Fig. 5(A)). The in vitro administration of CA led to a substantial enhancement of NO production by macrophages after interaction with *L. amazonensis* and both forms of *T. cruzi* for 24 and 72 h (Fig. 5(B) and (C)). It seems that CA not only neutralizes the inhibitory effects of parasites but also stimulates the oxidative metabolism, as discussed above. The present study has shown that the increase in NO production is accompanied by an increase in iNOS detected. The enzyme was found on the cytoplasm located mainly near vesicles and mitochondria (Fig. 2(B)-(D)). NO and reactive oxygen species (ROS) are produced, under a variety of biological conditions and they are critical in host defence not only because they can damage pathogens and tumoral cells but also since they are immunoregulatory.[30,36]

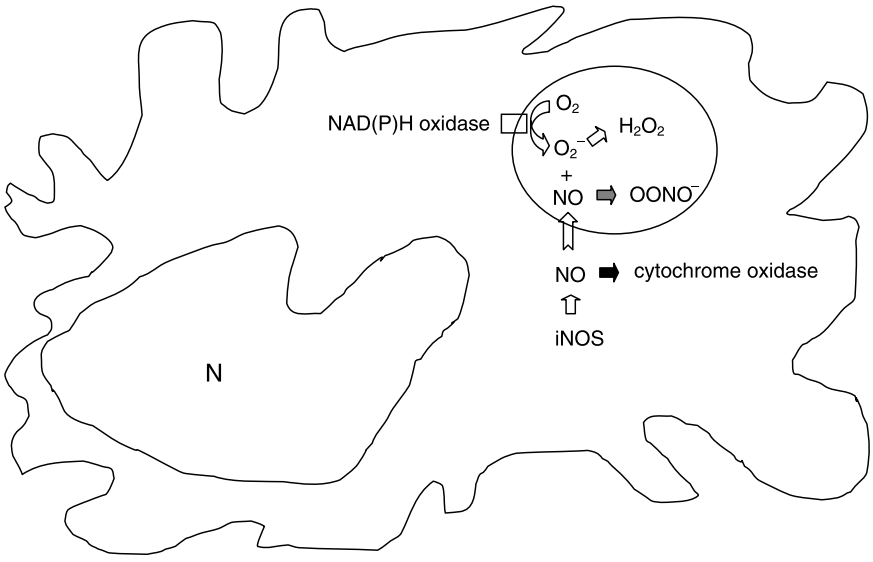

Figure 7 Activated macrophage summarizing the effects of Canova. It increased NAD(P)H oxidase in vesicles thus increasing H_2O_2 within then. Canova also increased iNOS expression increasing NO production. NO can react with O_2^- forming $ONOO^-$ besides inhibiting cytochrome oxidase. White arrow = formation; grey arrow, probable formation; black arrow, inhibition.

The enhanced capacity of activated mouse peritoneal macrophages to secrete ROS may be due to an increased affinity of their oxidase for NAD(P)H.[37] Ultrastructural cytochemical detection of NAD(P)H 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. NAD(P)H 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.[10] The control group showed electrondense products in the few activated macrophages found (Fig. 3(B)). Canova, in some way, activates this pathway because in the treated cells we observed, mainly in vesicles, stronger positive reaction products indicative of NAD(P)H oxidase activity (Fig. 3(C) and (D)). No other cytoplasmic organelles were found in both groups to contain hydrogen peroxide (H_2O_2) by this method. The external surface of the plasma membrane also presented electron-dense material, but this was apparently equally distributed in the groups. This was confirmed by measuring H_2O_2 in the culture supernatant, which showed no statistical significant differences between the groups (data not shown). Quantification of H_2O_2 on culture supernatant was based on the horseradish peroxidase-dependent oxidation of phenol red, which was assayed by increased absorbance at 620 nm. The major source of H_2O_2 in cells arises from either spontaneous conversion of superoxide anion (O_2^-) or via the action of the enzyme superoxide dismutase which catalyses the formation of H_2O_2 from O_2^-.[2,6] When O_2^- is produced and released to the outside of the cell, the most reliable method for measurement of O_2^- is the reduction of ferricytochrome c.[1] Detection of O_2^- in the culture supernatant showed no statistical differences between control and treated groups (Fig. 6(A) and (B)). These results suggest that Canova is stimulating intracellular production of ROS, thus favoring a specifically immunological response. A state of moderately increased levels of intracellular ROS is referred to as oxidative stress.[38] ROS have received increasing recognition for their role in host defence,[4] and as second messengers in the signaling pathways of macrophages. This results in a broad array of physiological responses[1] such as modulation of anti-oxidant levels, induction of new gene expression and protein modification.[38]

Cytochrome oxidase activity was determined by ultrastructural cytochemistry. We found enzyme inhibition in Canova treated cells (Fig. 4(D)). Cytochrome oxidase is an enzyme that catalyzes the transfer of electrons from its reduced substrate to molecular oxygen to form water, playing a critical role in energy metabolism.[39] It is known that NO reacts with a number of molecules, being a small gaseous free radical that binds readily to heme iron. The enzyme cytochrome oxidase contains three redox-active metal sites including

a heme iron that catalyzes electron transfer to oxygen reduction sites.[40] NO can bind to cytochrome oxidase thereby controlling cellular functions.[9] As we found an up-regulation of NO production, we can assume that NO is interacting with this enzyme. Cells producing NO as a messenger, activator, or modulator are faced with its potential toxicity. Free NO in the cell environment for a long enough periods can ultimately induce apoptosis and cell death.[41] Therefore, this interaction would be a way to attenuate NO toxicity to the host cell. NO also interacts with catalase, another heme-protein that is critical in protecting cells against H_2O_2. Catalase inhibition leads to increased levels of H_2O_2 because of reversible inhibition of H_2O_2 breakdown.[42] Treatment seems to inhibit catalase activity inside peroxisomes. Besides increased NAD(P)H oxidase activity, the elevated levels of H_2O_2 found inside vesicles can also be a consequence of this catalase inhibition by NO.

We have shown that macrophages triggered with Canova have an increase in their activity of NAD(P)H oxidase as well as that of iNOS, consequently producing ROS and NO respectively. Phagocyte anti-microbial mechanisms often work synergistically. ROS are very unstable, as they posses one or more unpaired electrons which can make them highly reactive. NO reacts very rapidly with oxygen radicals.[43] The chemical and biological interaction of NO and ROS with various biological molecules has important consequences in the mechanisms of different immunological and pathological conditions. Macrophages have the opportunity to produce O_2^- and NO in nearly equimolar amounts. As NO migrates near to the source of O_2^-, it reacts to form peroxynitrite ($ONOO^-$). Thus the primary chemistry of $ONOO^-$ would be within close proximity of the O_2^- source.[28,36] Activation of NAD(P)H oxidase leads to O_2^- increase as well as H_2O_2 inside vesicles (Fig. 3(C) and (D)). iNOS expression was seen near vesicle sites (Fig. 2(C) and (D)). As NO is small and uncharged, it can traverse the vesicle membrane[8] and we can assume that in macrophages treated with Canova $ONOO^-$ formation would be occurring within vesicles. It can be supported by the fact that O_2^- release from treated cells diminished considerably after 15 min (Fig. A and B). Reduced cytochrome c can be reoxidized by oxidants such as $ONOO^-$, diminishing apparent rates of cytochrome c reduction.[44] $ONOO^-$ is not only a free radical, but is a short-lived and far more reactive species than its precursors. Its highly reactivity with enzymes, macromolecules and lipids have been shown to influence cellular functions. Formation of $ONOO^-$ from O_2^- and NO may be useful in some situations. Phagocytes can generate $ONOO^-$ to help in killing pathogens. It can also act, in some circumstances, as an anti-oxidant defence by preventing an increase in the concentration of O_2^- and H_2O_2. $ONOO^-$ also oxidizes and nitrates a variety of biological targets and is a potential mediator of cytotoxic effects of nitric oxide.[45–47]

Canova is probably destined to control the RNS/ROS balance therefore playing an important role in the immune response. Its action leading to macrophage activation can be considered as a 'biological response modifier', as it provides an immune modulatory response directed to enhance the individual's own immunity to favor a particular immunological response.[48] The precise pathway induced by CA is still unknown, but speculations may be suggested. The NF-κB/Rel is a family of transcriptional factors that regulate the expression of numerous cellular genes and to play important roles in immune and stress responses, inflammation and apoptosis. It has been suggested that intracellular ROS levels up-regulate activity of the NF-κB/Rel family.[38] It is known that up-regulation of iNOS expression[9] and a selective repression of TNFα transcription[49] is due to NF-κB (p50 dimers). Based on our findings, the most likely mechanism that can account for the biological effects of CA on immunomodulation probably involves the activation of the NF-κB/Rel family. However it deserves further investigation

In conclusion, our findings provide a possible explanation on how treatment with Canova may enhance immune functions, which could be particularly important in the cytotoxic actions of macrophages. It can be considered as a new adjuvant therapeutic approach to known therapies.

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