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Larrea divaricata Cav (Jarilla): Production of Superoxide Anion, Hydrogen Peroxide and Expression of Zymosan Receptors

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Larrea divaricata is a plant widely used in folk medicine in Argentina. This work aimed to study the mechanisms of decoction activity on the release of oxygen reactive species. Decoction increased the binding of zymosan-FITC and superoxide production. Cadmium decreased the superoxide production as well as malonate and barbital. Decoction decreased the release of hydrogen peroxide. Decoction increased the reduction of MTT but not when malonate and barbital were included. Together, decoction increased the expression of dectin-1 leading to increased superoxide production. It is possible that decoction increases the activity of peroxidase, and decreases the Cu, Zn-superoxide dismutase.

Keywords *Larrea divaricata*, Decoction, Superoxide, Hydrogen Peroxide.

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INTRODUCTION

Phagocytic cells have long been recognized as fundamental components of the immune system of most organisms.[1] In carrying out their protective task, phagocytes engulf and destroy infective organisms or degraded host cells without damaging themselves or other cells.[2] Phagocytic cells such as polymorphonuclear leukocytes and macrophages (Mϕs), respond to a variety of membrane stimulants with the production and extracellular release of a number of reactive oxygen and nitrogen species (ROS and RNS), respectively.[3,4] This coordinated sequence of biochemical reaction, known as "oxidative burst," is initiated by an increase in oxygen uptake followed by the one-electron reduction of oxygen to superoxide anion (O_2^-) using NADPH or NADH as the electron donor and catalyzed by a NAD(P)H oxidase.[5]

Other ROS are produced from the superoxide anion, such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and singlet oxygen. The main source of ROS in animal cells is the mitochondria, and *in vitro* studies indicate that superoxide anion is the primary ROS produced as a result of the single electron reduction of oxygen.[6–8] There is growing evidence that they can serve as specific signaling molecules.[9] Within the mitochondria, the main sites of superoxide production have been localized to the electron transport chain. During electron transport, electron leaks, primarily at complexes I and III, can pass single electrons to oxygen and give rise to O_2^-.[10] The damaging potential of superoxide is muted by superoxide dismutases (SODs), which catalyze its dismutation to oxygen plus hydrogen peroxide.[11]

The cytosol was found to contain a Cu, Zn-SOD and the mitochondrial matrix a Mn-SOD. At that time Cu, Zn-SOD was also noted in the intermembrane space of mitochondria, and in nuclei.[12,13] The mice who lacked MnSOD die within a few days of birth,[14] while those lacking the cytosolic isoform, Cu, ZnSOD, survive.[15] Mitochondria from various aerobic organisms have been recognized as effective sources of H_2O_2.[16,17] Hydrogen peroxide diffuses rapidly through membrane[18] and cross to cytosol.[19]

Dectin-1 is a β-glucan receptor, which is involved in the binding and phagocytosis of opsonized zymosan and *C. albicans*[20–22] by Mϕs. Dectin-1 activates the generation of O_2^- through the membrane-bound enzyme NADPH-oxidase.[23,24] Besides, it has been shown that the coordinate signaling through both dectin-1 and TLR-2 activates NF-κB.[22]

Larrea divaricata Cav. (Zygophyllaceae) is widely used in folk medicine in Argentine. The following claims have been made for this plant: healing sores and wounds, rheumatism, inflammation of respiratory and intestinal tract, gastric disturbance, venereal diseases, tonic, corrective, antiseptic, stimulating expectorant, emetic,[25] arthritis, cancer,[26] tuberculosis,[25–27] common cold,[27] and rubefascient.[28] The aim of this study was to evaluate *in vivo* the

possible mechanisms by which decoction of *L. divaricata* is able to increase the superoxide anion production[29] on murine Mϕs.

MATERIALS AND METHODS

Preparation of Plant Extracts

Leaves and tender branches of *L divaricata* were collected in San Luis, Argentina. The plant was identified in the herbarium of the National University of San Luis (voucher number: UNSL # 467). Leaves and tender branches were dried in a stove at 45°C during 5 days and reduced to fine dust.[30] From this material we prepared: Decoction (D): 5 g of dust plus 100 ml of distilled water heated to boiling temperature and left in for 20 min. Percentage yield: 21.01 % (w/w).

The extract was filtered and centrifuged at 3.500 rpm for 15 min. Supernatants were filter-sterilized through a 0.22 μm filter, lyophilized, aliquoted and stored at –20°C until use.[30] Before assays, all drugs and extracts used were subjected to the *Limulus* test for the detection of LPS contamination. The LPS was either low (0.01 EU/ml) or not detectable.

Stimulation of Mϕs *in vivo* with Decoction

Male and female Rockland mice of 20 g were used. Animals were housed and cared for at the Animal Resource Facilities, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, in accordance with institutional guidelines. Groups of four mice were injected i.p. twice in a 48 h period with 0.2 ml of D (0.5 mg/kg). As control group, animals received 0.2 ml of PBS. [29] Peritoneal cells were harvested from each mouse 48 h after the second injection, as described below.

Cell Preparation

Peritoneal cells (PC) were harvested by sterile lavage with 20 ml HBSS (Sigma Aldrich, St. Louis, MO, USA) supplemented with 20 μg/ml gentamicin (Sigma Aldrich, St. Louis, MO, USA) and heparin (Sigma Aldrich, St. Louis, MO, USA) (50 U/ml) as previously[31] and adjusted to 1×10^6 cell/ml. Mϕs were purified from PC by adherence onto 96 well flat bottomed tissue culture plates in DMED (Sigma Aldrich, St. Louis, MO, USA) with 20 μg/ml gentamicin and 5% heat-inactivated fetal calf serum (FCS) (Gibco, Rockville, MD). Non-adherent cells were removed after 2 h at 37°C and complete medium was added with 0.1 mg/ml of D. In our hands, the adherent Mϕ monolayers showed > 90% of purity according to morphologic analysis or non-specific esterase staining. All cells used in this work were obtained according to this procedure, named as treated cells o treated Mϕs.

DETECTION OF ZYMOSAN RECEPTORS

Zymosan (Sigma Aldrich, St. Louis, MO, USA) (1.44 mg/ml) was labeled by incubation at 25°C during 30 min in 0.1 mg/ml FITC (Sigma Aldrich, St. Louis, MO, USA). All particles were washed twice in HBSS containing 20 mM HEPES (Sigma Aldrich, St. Louis, MO, USA) and 0.25% BSA (Sigma Aldrich, St. Louis, MO, USA) (HBSA) and then diluted to 1.44 mg/ml in HBSA. Cultured cells were washed with PBS and fixed with cold methanol (Quantum, New York, USA). Fixed cells were incubated with 1 ml of zymosan-FITC (Z-FITC) for 30 min at 37ºC. Cells were examined with a fluorescent microscope with a 100 × objective (Axiovert 100. Zeiss, Germany) as previously.[32]

Production of Superoxide

Opsonized zymosan (OPZ) was used as particulate stimulus. Zymosan A (Sigma Aldrich, St. Louis, MO, USA) was opsonized with normal mice serum (1:3) for 30 min at 37°C, then washed three times and suspended at 1 mg/ml. For these assays we used the protocol described by Schoff [33] and O_2^- anion was evaluated by the reduction of NBT (Sigma Aldrich, St. Louis, MO, USA) to formazan. Mϕs obtained from mice treated with D (0.5 mg/kg) were incubated during 1 h at 37°C with 15 µl of anti–Dectin-1 mAb, 2A11 (kindly provided by Gordon Brown, University of Oxford, Oxford, England), 1mM malonate (Sigma Aldrich, St. Louis, MO, USA) and 1 mM barbital (Sigma Aldrich, St. Louis, MO, USA).

Treated cells also were incubated with 1mM cadmium chloride (Sigma Aldrich, St. Louis, MO, USA) and 2 mM H_2O_2 (Tetrahedron, Buenos Aires, Argentine) for 30 min at 37° C. After incubation, 300 µl of NBT (0.1%) and OPZ (1 mg/ml) were added and left for 30 min. The reaction was stopped with 1N HCl (Tetrahedron, Buenos Aires, Argentina). Controls included untreated Mϕs (control Mϕs) cultured with or without OPZ. Formazan was extracted with dioxane (Dorwill, Buenos Aires, Argentina) and the absorbance was measured in a microplate reader at 525 nm (Microplate Reader Benchmark. Bio-Rad, Hercules, CA, USA).

Release of Hydrogen Peroxide (H_2O_2)

Briefly, the determination of the production of H_2O_2 induced by D was carried out by using treated Mϕs incubated during 1 h with 2 mM of sodium azide (peroxidase inhibitor). After incubation, cells were washed and a solution of phenol red containing 140 mM NaCl, 10 mM potassium phosphate pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 0.01 mg/ml type II horseradish peroxidase (Sigma) was added. After 1 h incubation the reaction was stopped with 10 ml of 4 N NaOH and absorbance was measured at 650 nm in a microplate reader (Microplate Reader Benchmark. Bio-Rad, Hercules, CA, USA). Zymosan (250 µg/well) was used as a positive control. The results were expressed as

nanomoles of H_2O_2 /10^6 cells, from a standard curve established in each test, constituted of known molar concentrations of H_2O_2 in buffered phenol red.[34]

MTT Reduction

Reduction of this salt 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was assayed as previously described[35] Briefly, the treated cells with D (0.5 mg/kg) or treated cells with D (0.5 mg/kg) plus 1mM malonate and 1mM barbital were incubated with 100 µl of DMEM and 10 µl of 5 mg/ml MTT (Sigma Aldrich, St. Louis, MO, USA) during 4 h. The purple formazan formed was solubilized by the addition of acidic isopropanol. The absorbance was measured using a microplate reader (Microplate Reader Benchmark. Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm. Untreated cells were used as control of viability (100%) and results were expressed as % of viability relative to control.

Statistical Analysis

Differences between group means were assessed using one way ANOVA followed by comparisons with Tukey´s test. A $p \leq 0.05$ was considered statistically significant.

RESULTS

Assessment of Zymosan Receptors

The intensive fluorescence observed in Figure 1 shows that D increased clearly the binding of Z-FITC on Mϕs compared with control cells (Mϕs without the treatment with D). The strong spotted fluorescence on Mϕs suggested that an important increment in the number of Z receptor molecules occurred.

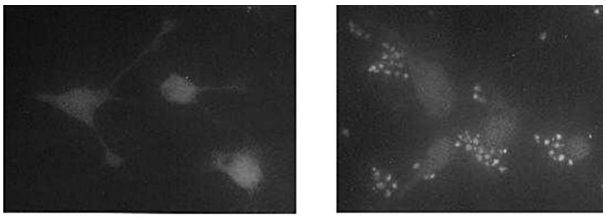

Figure 1: Expression of Z receptors after *L. divaricata* Cav treatment. Cells of untreated (Control) or treated mice (D) were incubated in the presence Z-FITC (1.44 mg/ml). After extensive washing cells were evaluated with an Axiovert 100 fluorescent microscope. Magnification: X 100. Results are a representative of three separate experiments.

Superoxide Production

Besides, we assayed the effect of D on the release of O_2^-. Figure 2A shows that D increased the production of this specie ($p < 0.0098$). These results correlate with those obtained elsewhere.[29] The production of O_2^- decreased in Mϕs treated with D, OPZ and the monoclonal antibody 2A11 respect to Mϕs without mAb ($p < 0.0037$). We also demonstrated that the production of O_2^- was not modified, when mAb 2A11 was added to Mϕs treated with D. Figure 2B shows that O_2^- production in Mϕs treated with D was not affected by cadmium. The incubation of treated Mϕs with malonate and barbital decreased the superoxide anion production ($p < 0.008$). Cadmium decreased the release of O_2^- in Mϕs

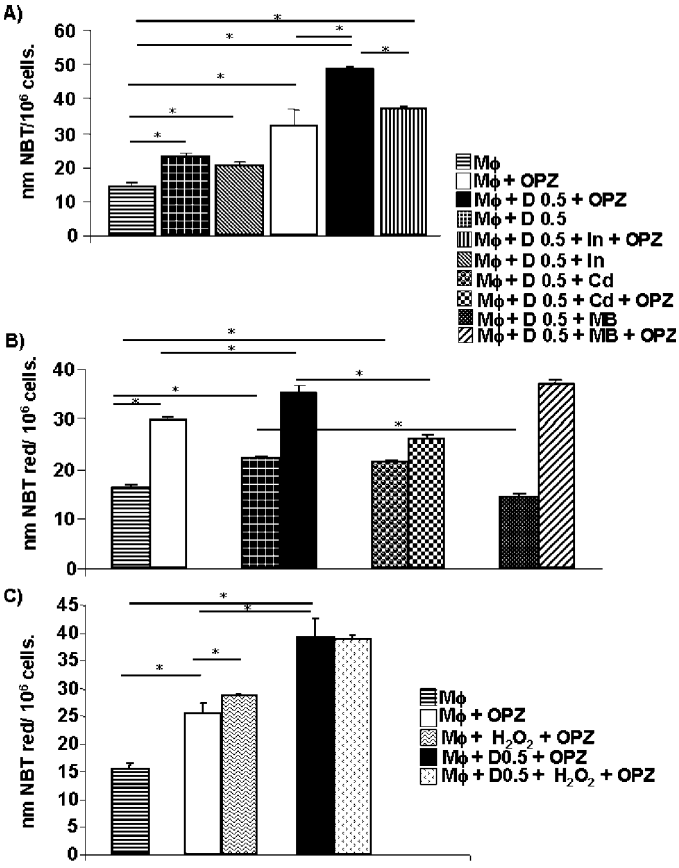

Figure 2: Effect of *L. divaricata* Cav decoction on the superoxide production by peritoneal Mϕs. Mϕs obtained from D treated mice were incubated for 1 h at 37°C with anti–Dectin-1 mAb (In) (**A**), 1mM malonate and 1 mM barbital (MB), 1mM cadmium chloride (Cd) (**B**) and 2 mM H_2O_2 (**C**) for 30 min at 37°C. As controls, cells of untreated mice cultured with or without OPZ were used. The production of superoxide anion was assessed by the reduction of nitro blue tetrazolium (NBT) to the insoluble formazan. Experiments were performed by triplicate and one representative is shown. *$p \leq 0.05$.

treated with D and OPZ ($p < 0.01$). As shown in Figure 2C, the superoxide anion production was not modified by H_2O_2 when Mϕs were treated with D.

Determination of Hydrogen Peroxide (H_2O_2)

The results obtained when we assessed the effect of D on the release of H_2O_2 i*n vivo* are shown in Figure 3B: the extract *per se* decreased the levels of H_2O_2 in D treated cells ($p < 0.0045$). When sodium azide was added to D treated cells, the levels of H_2O_2 increased significantly ($p < 0.0042$).

MTT Reduction

Figure 3A shows that the reduction of MTT was increased in Mϕs of mice treated with D ($p < 0.0006$). Reduction of MTT decreased when malonate and barbital were added but the levels of reduced MTT were even significantly greater that those observed in control Mϕs ($p < 0.009$).

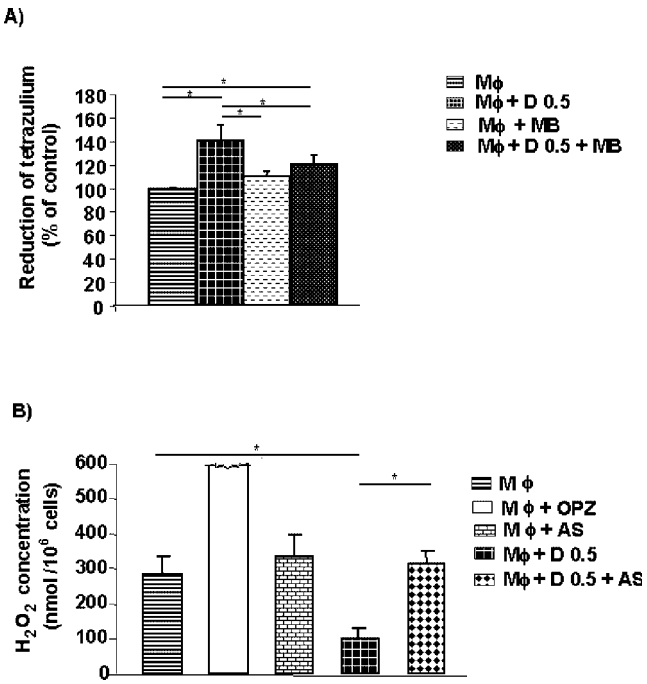

Figure 3: Effect of *L. divaricata* Cav on MTT reduction and H_2O_2 production by peritoneal Mϕs. **A**) Cells obtained from mice treated with D were incubated with or without 1mM malonate and 1mM barbital. After incubation, 10 µl of MTT (5 mg/ml) were added. The formazan formed was solubilized and the absorbance was measured at 570 nm. As control, untreated cells were used (100% of viability). Results are expressed as % of viability with respect to the control. **B**) Mϕs obtained from mice treated with D were incubated with 2 mM of sodium azide (AS) and washed. A solution of phenol red was added. After 1 h incubation the reaction was interrupted by the addition of 4 N NaOH and absorbance was measured at 650 nm. Zymosan was used as standard. The results are expressed in nmol of H_2O_2 /10^6 cells.

DISCUSSION AND CONCLUSIONS

The past decade has witnessed a tremendous resurgence in the interest and use of medicinal plants. In North America, for instance, plant medicinal usage increased from just about 3% of the population in 1991 to over 37% in 1998.[36] The use of medicinal plants is also a common practice in Argentina, although in most cases the active compounds of the plants are not yet tested.[37]

The results from the present study show that the *in vivo* treatment of mice peritoneal Mϕs with D of *L. divaricata* Cav. induces an increase of Z receptors. Dectin-1 is a small type II transmembrane receptor containing one lectin-like carbohydrate recognition domain, which recognizes β 1,3- and/or β 1,6-linked glucans and intact yeasts,[38] and an immunereceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail.[39]

In addition to these exogenous ligands, the receptor can also recognize an endogenous ligand on T cells.[39] The receptor is expressed at high levels on Mϕs and neutrophils, and to a lesser extent on dendritic cells and a subpopulation of T cells.[40] The human homologue was found to be structurally and functionally similar to the murine receptor.[41] Dectin-1 is the major receptor for unopsonized and opsonized zymosan on Mϕs.[20] These data suggest that dectin-1 could be the Z receptor increased in our experiments. Previously we demonstrated that D increases the production of O_2^-.[29] In this study, we confirmed these results and we showed that the production of O_2^- decreased in Mϕs treated with D and after incubated with opsonized zymosan and mAb 2A11. This antibody blocks dectin-1.[42]

The amount of O_2^- in cytoplasm of Mϕs stimulated with OPZ is due to the membrane-bound enzyme NADPH-oxidase.[43] Dectin-1 induces NADPH oxidase activation.[24] Our results suggest that dectin-1 is involved in the O_2^- increase after treatment with D and the production of O_2^- was not modified when mAb 2A11 was added to treated Mϕs. These results suggest that it exist a dectin-1 non-dependent way by which D stimulate the O_2^- production.

The electron-transfer chain of mitochondria is a well-documented source of cytosolic O_2^-.[44] Superoxide anion is produced from both Complexes I and III of the electron transport chain. Complex I-dependent superoxide is exclusively released into the matrix and no detectable levels escape from intact mitochondria. Complex III shows direct extra mitochondrial release of superoxide anion.[45] Superoxide crosses to cytoplasm through outer membrane voltage-dependent anion channels.[44] It is known that O_2^- can penetrate phagosoma membranes in a protonated form ($HO_2^{\cdot}$). This uncharged specie should comprise 0.2% of the superoxide population at pH 7.5. [46] The O_2^- production in Mϕs treated with D was not affected by cadmium (NAD(P)H oxidase inhibitor) at the used concentration (1mM).[47]

Malonate and barbital inhibit the mitochondria Complex II and I, respectively and reduce ROS production.[48–51] The incubation of treated Mϕs with

malonate and barbital decreased the levels of superoxide. These results suggest that the mitochondria could be involved in the increased O_2^- production in treated Mϕs. Cadmium decreased the release of O_2^- in Mϕs treated with D and OPZ; these effects confirm the results obtained with mAb 2A11.

MTT is a membrane-permeable tetrazolium salt[52] which is reduced to formazan by superoxide anion[53] in living cells. It is known that MTT is reduced in the mitochondria and also in intracellular vesicles identified as endosomes and lysosomes.[54] Our results show that reduction of MTT was increased in murine Mϕs treated with D. Reduction of MTT decreased when malonate and barbital were added but the levels of reduced MTT were significantly greater that observed in untreated Mϕs. These results confirm again that there are different ways of superoxide production by Mϕs when these cells were treated with D. Our results show that the superoxide production in D-treated Mϕs was not modified by H_2O_2 which is a Cu,Zn SOD inhibitor.[55] These results suggest that Cu,Zn SOD could be inhibited by D. Besides, we found that the production of H_2O_2 decreased when cells were treated with D. Sodium azide (peroxidase inhibitor) increased the production of H_2O_2. We can suppose that after treatment with D the activity of peroxidase is improved.

Bhattacharya et al. (2001), using *Withania somnifera* glycowithanolides, showed that this plant normalizes the augmented SOD activity and enhances the activity of peroxidase in a chronic footshock stress in rats.[56] Both a crude extract and the P3 fraction (polysaccharides fraction) of *Asparagus racemosus* protect against radiation-induced loss of protein thiols and inactivation of SOD in membrane damage induced by the free radicals generated during γ-radiation in rats.[57] Anesini et al. (2004) showed that an aqueous extract of *L. divaricata* increased the peroxidase activity in female rat submandibular glands.[58] We can suggest that D of *L. divaricata* increases expression of dectin-1, as well as the activity of peroxidase and decreases the activity of Cu, Zn superoxide dismutase. Besides, superoxide production induced by D could be derived from NADPH oxidase and mitochondrial ways.

The effects of *L. divaricata* on different enzymes could be due to several active compounds present in D such as flavonoids (aglycones, glycosides), saponins, phenolic acids, ligans.[25,59] To the best of our knowledge, this is the first report addressing the effects of *L. divaricata* compounds on Mϕs. The results obtained will help to understand the equilibrium between pro-inflammatory and anti-oxidant activities of *L. divaricata* that could be relevant for the treatment of diseases where ROS are altered.

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