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N-acetyl-L-cysteine reduces *Leishmania amazonensis*-induced inflammation in BALB/c mice

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Abstract

Background: Leishmaniasis is a emergent disease characterized by different clinical manifestations in both humans and dogs. Predominant clinical features of cutaneous leishmaniasis are ulcerative painless skin lesions. Several data reported that pain is associated with human and dog leishmaniasis, out with areas of painless ulcerative lesions per se. Actually, current medications used for leishmaniasis management are characterized by several side effects and, in addition, some cases of the disease are refractory to the treatment. On this background it is mandatory the identification of new and safe candidates for designing less toxic and low-cost remedies. Therefore, the search for new leishmanicidal compounds is indispensable.

Methods: In the present paper we investigated the effect of orally N-acetyl-L-cysteine (NAC) supplementation at dose of 200 mg/Kg for 10 weeks, in subcutaneous *Leishmania* (*L. amazonensis*) infected BALB/c mice. And evaluating the effect of NAC on inflammatory response such as TNF- α , IL-6, IL-1 β levels, and on thermal and mechanical hyperalgesia.

Results: In the present paper we showed how NAC supplementation affected parameters of oxidative stress (GSH, MDA, SOD), inflammation such as cytokines levels (IL-1 β , IL-6, TNF α) and mast cell activation and consequently on induced pain, during leishmaniasis in BALB/c mice.

Conclusions: The findings of our study provided the scientific data demonstrating that *L. amazonensis* infection induces inflammation and pain in BALB/c mice that are reversed by administration of NAC.

Keywords: Leishmaniasis, Inflammation, Mice, Pain

Background

Leishmaniasis, a severe public health problem worldwide, is proved by other than 20 various protozoan species of the genus *Leishmania* [1]. This disease represents one of the most frequently occurring painful and inflammatory conditions in both humans and animals [2, 3]. These parasites are classified as digenetic organisms because they live one phase of their lifecycle in an insect host from the genus *Lutzomyia* in the New World or *Phlebotomus* in the Old

World and the other stage inside a mammalian host. A wide umbrella of clinical manifestations characterized leishmania infections and are recognized by distinctive symptoms principally related to infections and diverse *Leishmania* species [4]. The different types of the disease have been categorized in tegumentary, also named cutaneous leishmaniasis, and visceral leishmaniasis. About 200,000–400,000 new visceral leishmaniasis cases and 700,000–1,200,000 new cutaneous leishmaniasis cases happen each year worldwide [5]. The transmission of parasites occur by phlebotomine sand flies which become infected during blood meal on mammalian hosts. Though dogs represent the most know domestic reservoirs of specific *Leishmania* parasites such as *L. infantum* the role of *Didelphis* spp. as reservoirs have been proposed and its synanthropic facility

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could simplify the link between wild and peridomestic environments [6, 7]. Literature data showed that various rodent species have been classified as probable pools of *L. amazonensis*, *L. braziliensis* and *L. infantum*, screening competence to maintain these parasites [8]. Because of this, leishmaniasis is classified as a re-emergent and new infection with geographical extension due to human migration, urbanization, human-driven environmental alterations and co-infection with other illnesses [9]. The important expansion of this disease has pointed the development of new foci of transmission and reactivation in controlled sets [10, 11]. In the spectrum of Leishmania parasites, the *L. amazonensis* belongs to the *L. mexicana* complex and produces localized cutaneous and diffuse cutaneous leishmaniasis [5]. This particular parasite frequently capture the host's innate immune response to start an infection, in particular in macrophages. The infections caused by *L. amazonensis* are featured by the suppression of the natural early response, as reported by inhibition of macrophage production of pro-inflammatory molecules. It was demonstrated that in the initial stage of infection by *L. amazonensis*, many inflammatory cytokines are downregulated when matched with *L. major* infected mice [12]. Others reports demonstrated that the suppression of pro-inflammatory cytokines such as IL-12, IL-17 and IL-6 in macrophages infected with *L. amazonensis* and preserved with lipopolysaccharide (LPS) as compared with infection by *L. major* [13]. Clinical manifestation of the disease comprise a widespread range of expressions, among these skin ulcers at the set of the infection or distribution in visceral organs followed by leucopenia, anemia, weakness and fever [14]. Actually there is no efficient vaccine against leishmaniasis and also chemotherapy comprises restricted plethora of drugs. The pharmacological treatment employed to counteract the effects of infections showed several difficulties such as high toxicity and cost, chronic administration, inconsistency of efficiency and parasite resistance [15]. For these reasons the elaboration of new current and also reasonable drugs is essential. In the pathophysiology of leishmania a prominent role is coated by both inflammation and oxidative stress. The relationship between inflammation and the production of reactive species is well known and characterized as a fundamental component of oxidative stress, the oxidative stress often represents an additional component to the tissue damage induced by leishmaniasis. Reactive oxygen species (ROS) are generated in the mitochondria reported as respiratory chain products [16] and are implicated in several biological processes, such as hormonal biosynthesis [17], cellular signalling [18] and destruction of intracellular pathogens [19]. Moreover, ROS are also classified as relevant effector agents against intracellular pathogens, induced by Toll-like receptors or IFN- γ [20, 21]. Data obtained from investigations carried out in murine model of leishmaniasis proved that nitric oxide (NO) induction is one of the key

effector mechanisms of macrophages for abolition of Leishmania parasites [22]. Researches data suggests that the susceptibility of a selected strain of mice such as BALB/c mice to *L. amazonensis* as well recognized to *L. major* infection appears to be dependent of Th2 cytokines like IL-4 and IL-10 [23]. Currently, the most frequently used drug for dogs are systemic antimonials [24]. Nevertheless, because of the high toxicity related with this group and the recent emergence of drug resistant strains, alternative therapeutic options to be considered. Oxidative stress is considered to be an important etiologic factor in leishmaniasis and the antioxidant NAC has been used with success as an adjunct in human and experimental leishmaniasis diseases [25]; formulations of NAC have utilized, both in the human [24] and veterinary fields [26]. NAC is classified as an antioxidant, both directly as a GSH substitute and indirectly as a precursor for GSH [27]. Therefore, research is now focused on the identification of more effective and safe tools, as part of an ideal multimodal management of leishmaniasis and its complications in veterinary patients. On this basis, we investigated the possible effects of NAC supplementation on the course of *L. amazonensis* infection in BALB/c mice to better clarify both pathogenesis and development of leishmaniasis. In particular we focused our research on the inflammatory process and consequently algescic sensitization associated with leishmaniasis disease. We used a multimodal approach evaluating the inflammatory response and the involvement of important immune cells such as mast cell in pain sensitization, and also the antioxidant activity of NAC. Our study confirms and suggest an important role for NAC as a useful strategy or coadjuvant in the treatment of this multi symptomatic and complex disease.

Results

NAC decreases paw edema, histological damage and neutrophilic infiltration in *L. amazonensis*-infected animals

The inflammatory response in the paw skin revealed a significant difference in infected animals when matched to the treated-infected animals. Oral administration of NAC (200 mg/kg) significantly reduced the development of paw edema beginning, namely from the second week (Fig. 1a). Skin from sham mice appeared normal (Fig. 1b). In contrast, a evident increase of inflammatory cells was evident after *L. amazonensis* infection into the right hind paw (Fig. 1c). Orally NAC administration (200 mg/kg) significantly reduced this histological alteration, as well as inflammatory cell infiltration (Fig. 1d).

Histological injury progression was also linked with neutrophil infiltration revealed by an increase in MPO activity (Fig. 1e).

The infection with *L. amazonensis* stimulated significant increase in MPO compared with control, whereas

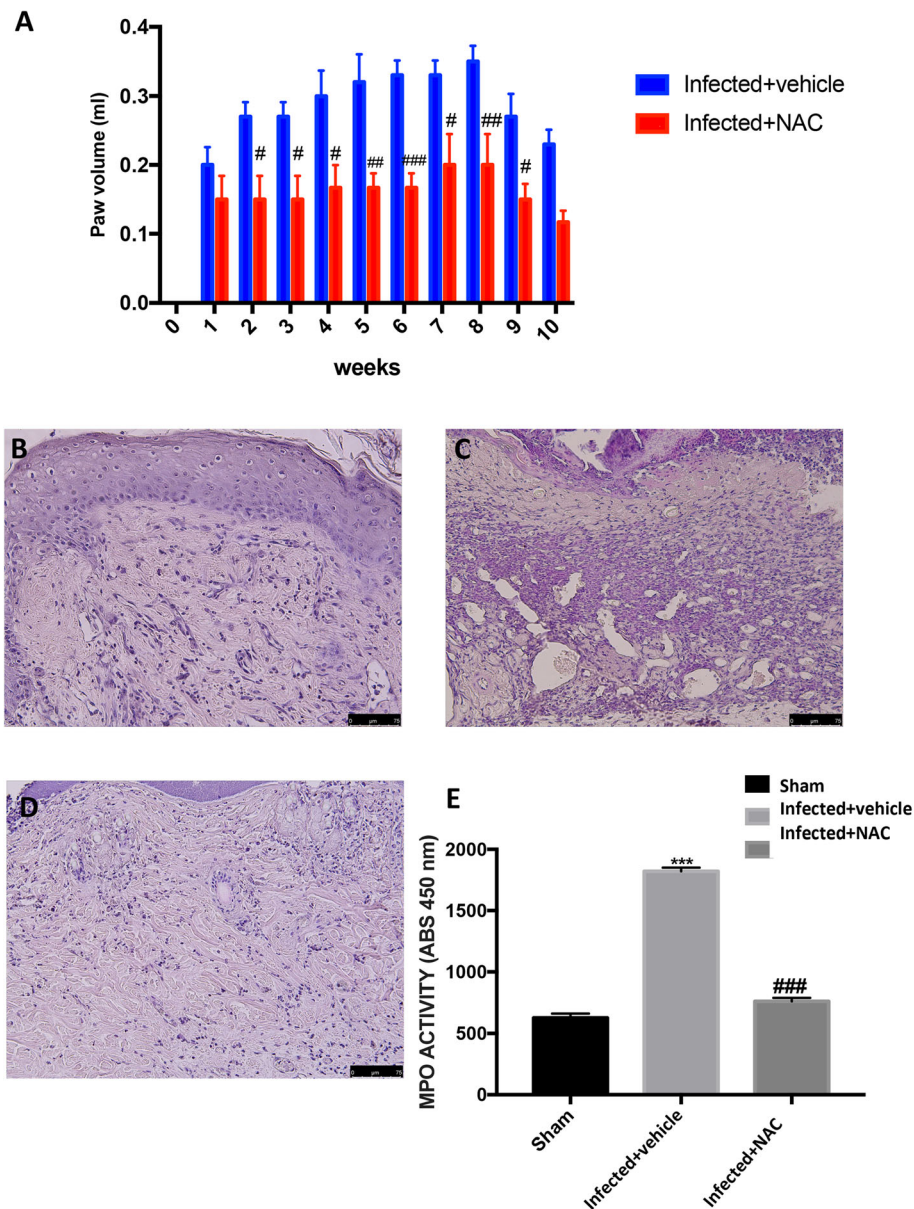


Fig. 1 Effect of oral NAC on *L. amazonensis* infected mice on paw edema histological damage and neutrophil infiltration in paw skin. Paw edema (A) was assessed at the time points indicated after subcutaneous injection of *L. amazonensis* into the mice hind paw. NAC administration (200 mg/kg) produced significant improvements in this scores. Histological analysis was achieved by hematoxylin/eosin staining in sham; (B), *L. amazonensis* subcutaneously infected mice into the hind paw (C), and *L. amazonensis* subcutaneously infected mice + NAC (200 mg/kg) (D); moreover myeloperoxidase (MPO) activity in paw skin was evaluated (E). Figures are representative of at least three independent experiments for all animals from each group. Values are means ± SEM of five animals for each group. ###*P* < 0.001 vs. *L. amazonensis* infected mice; ****P* < 0.001 vs. sham

treatment with NAC (200 mg/kg) notably reduced MPO activity when compared to vehicle (Fig. 1e).

Effect of NAC on chronic mechanical and thermal hyperalgesia in *L. amazonensis* infected animals

Subsequently we evaluated the effect of NAC on mechanical and thermal hyperalgesia. Oral administration of NAC (200 mg/kg) significantly reduced the development of mechanical

and thermal hyperalgesia starting from the second week (Fig. 2b, c).

Effect of NAC on mast cell infiltration and nociceptive marker in *L. amazonensis* infected mice

Level expression of the nerve sensitizer NGF (Fig. 3a,a') was increased in the *L. amazonensis* infected animals matched to sham animals. Administration of NAC (200

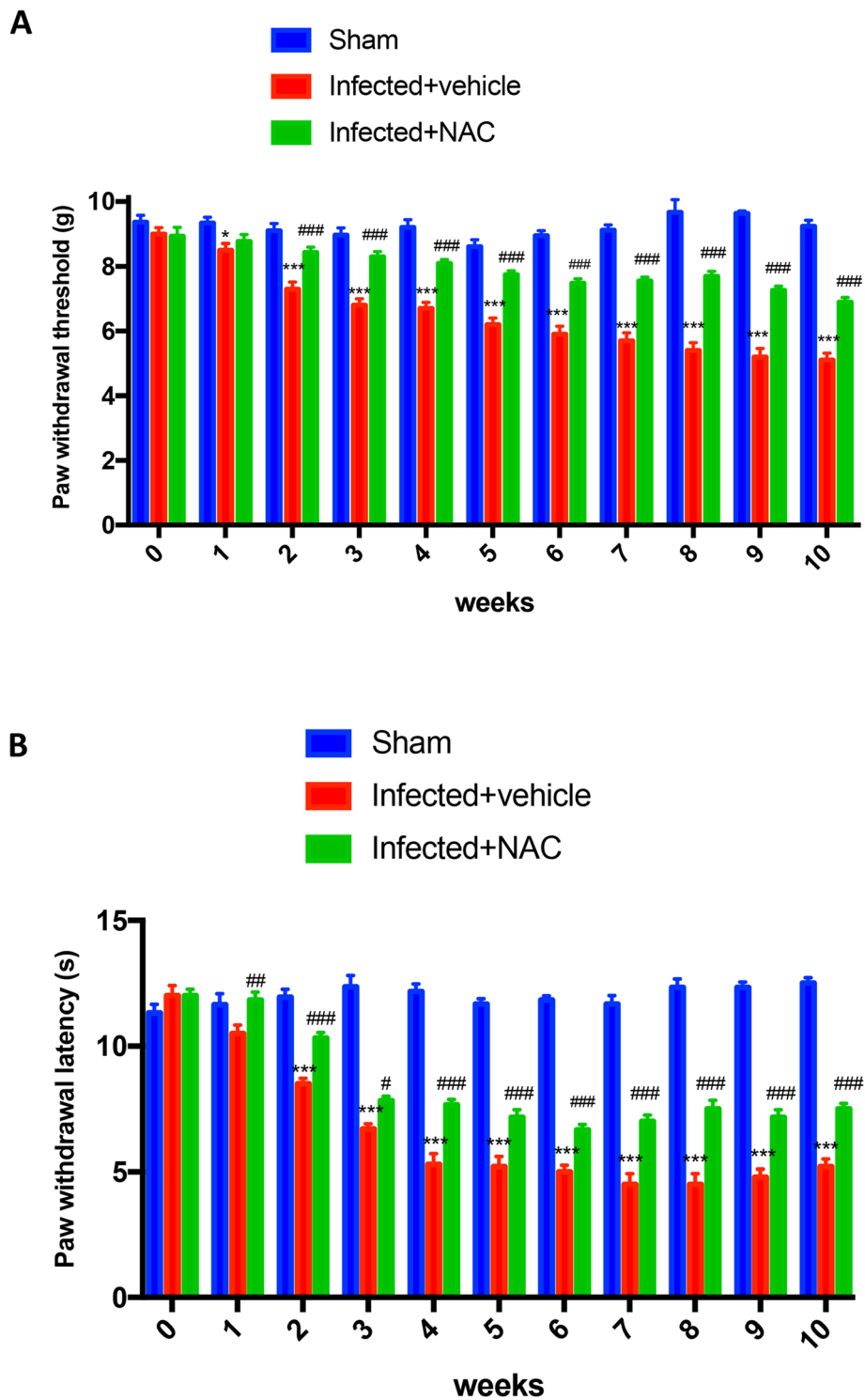


Fig. 2 Effect of oral NAC administration on *L. amazonensis* infected mice on mechanical and thermal hyperalgesia. Mechanical (A) and thermal hyperalgesia (B) were assessed at the time points indicated after subcutaneous infection of *L. amazonensis* into the mice hind paw. NAC administration (20 mg/kg) produced significant improvements in both scores. Values are means \pm SEM [#]*P* < 0.05, ^{##}*P* < 0.01, and ^{###}*P* < 0.001 vs. *L. amazonensis* infected mice

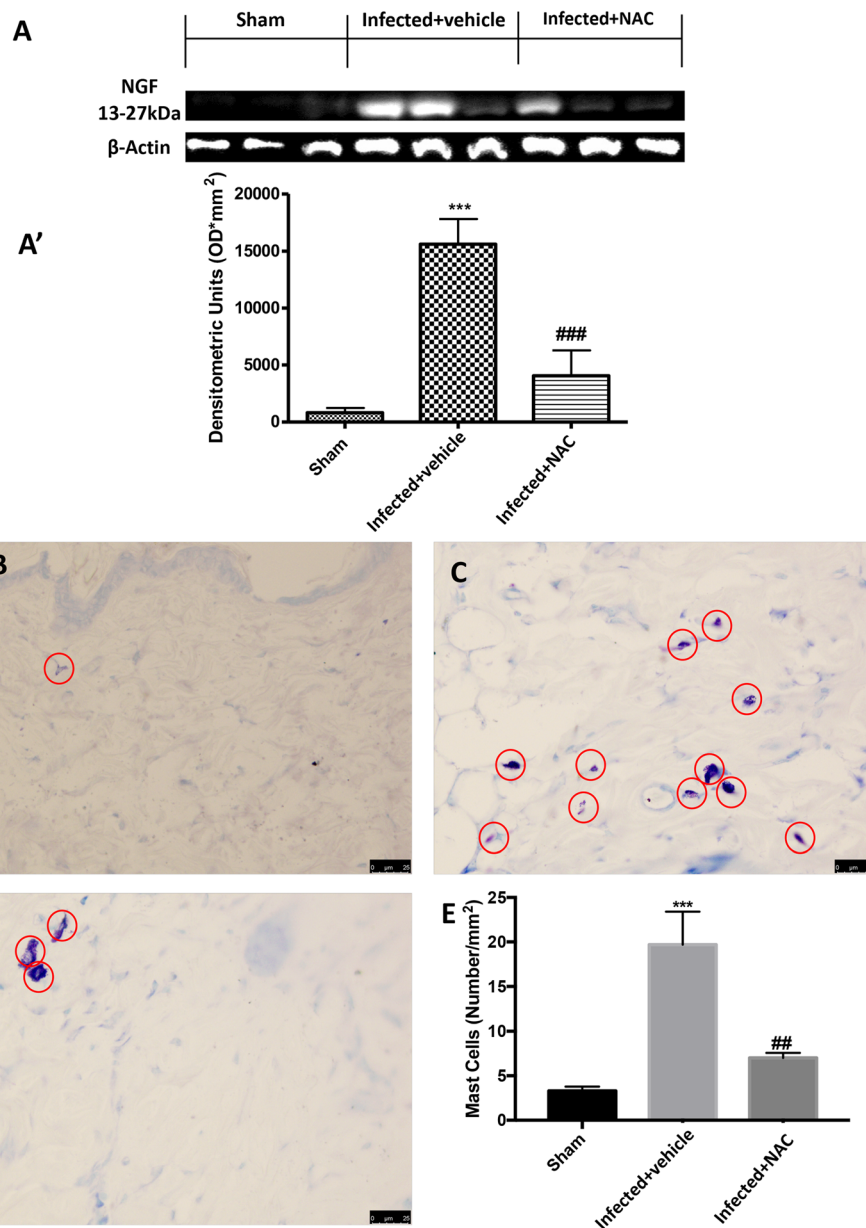


Fig. 3 Effect of oral NAC administration on *L. amazonensis* infected mice on NGF and mast cell infiltration in paw skin. Representative western blot showing the effect of NAC (200 mg/kg) on NGF (**A**, **A'**). Levels of NGF presented in the densitometric analyses of protein bands were normalized for β -actin. The number of mast cell was increased in *L. amazonensis* infected mice (**C**) compared to the sham animals (**B**). Administration of NAC (200 mg/kg) (**D**) reduced mast cells infiltration in paw skin; the mast cells score was done by independent observer (**E**). Data are representative of at least three independent experiments and are expressed as mean \pm SEM from $N=5$ mice/group. A representative blot is shown and densitometric analysis is reported. All figures are representative of at least three independent experiments for all animals from each group. Values are means \pm SEM [#] $P < 0.05$, ^{##} $P < 0.01$, and ^{###} $P < 0.001$ vs. *L. amazonensis* infected mice

mg/kg) significantly counteracted such increase (Fig. 3a, a'). *L. amazonensis* infected mice paw skin, stained with toluidine blue, showed a strong infiltration of mast cells (Fig. 3c,e), as compared to sham mice (Fig. 3b,e). Orally NAC administration (200 mg/kg) significantly reduced mast cell infiltration (Fig. 3d,e).

NAC reduces cytokine release in *L. amazonensis* infected animals

The decrease of mice paw edema, mechanical and thermal hyperalgesia after NAC administration (200 mg/kg) was related with a significant reduction in paw skin content of pro-inflammatory and pro-nociceptive cytokines

such as TNF- α (Fig. 4a), IL-6 (Fig. 4b) and IL-1 β (Fig. 4c), as matched to the *L. amazonensis*-infected mice.

Effect of NAC on oxidative stress markers alteration in *L. amazonensis* infected animals

To evaluate the antioxidant effect of NAC, we investigated some markers of oxidative stress, such as glutathione (GSH), malondialdehyde (MDA) level and superoxide dismutase (SOD) activity, as show in Fig. 5a in infected + vehicle mice we observed a significant depletion of GSH levels, that have been restored by NAC treatment. As illustrated in Fig. 5b, the result showed that the level of MDA was decreased significantly in tissue collected from mice treated with NAC compared to *L. amazonensis* + vehicle infected animals. Furthermore, we observed that the activities of SOD Fig.5c, were significantly increased in tissue taken from NAC group treated compared to infected + vehicle mice group.

Discussion

In the present research we have verified whether and how orally NAC act an anti-inflammatory antioxidant and consequently an analgesic effect in subcutaneous *L. amazonensis*-infected BALB/c mice, in an attempt of confirming the impact of this treatment in both pathogenesis and development of leishmaniasis disease. *L. amazonensis* is the contributory cause of cutaneous and cutaneous diffuse leishmaniasis; in both forms of infection chronic lesions are disseminated through the skin and for this reason are classified as disabling disease with difficult management. Moreover, several patients refer pain independently of the region of the body infected [28]. The inflammatory process related with infection is also related with pain condition. This symptom is due to the development of immune response including inflammatory mediators formation, leading to nociception sensitization of primary and second-order neurons implicated in nociceptive impulse transmission. Nevertheless, these processes cause central sensitization and therefore the sensation of pain perceived as hyperalgesia [29, 30]. The inflammatory process includes, among others, a severe involvement of cytokines participating in the determination of pain. Literature data with *Leishmania* parasites encourage the development of chronic inflammatory lesions associated with cytokines release that provoke tissue damage and pain [31, 32]. It was demonstrated that chronic *L. amazonensis* infection in BALB/c mice displays induction of inflammatory response in particular in the site of infection; this inflammatory condition is also associated with hyperalgesia, parasite migration and injury in secondary organs like liver and spleen [33, 34]. Moreover, a nociceptive behavior was seen in both rats and mice infected with *L. major* and *L. amazonensis*, despite absence of pain was reported in patients with cutaneous lesions [33, 35]. In the field of inflammatory process, cytokines such as IL-1 β ,

TNF- α and IL-6 have a role as hyperalgesic endogenous molecules and analgesic targets [36]. *Leishmania* parasite resides within macrophages and may cause inhibition of mitochondrial respiratory activity, also inactivates peroxidases, increases susceptibility to oxidant damage, inhibits glycolysis, S-nitrosylation, ADP-ribosylation, nitrosine tyrosine of proteins, disrupts the Fe-S clusters, zinc fingers or heme groups and causes peroxidation of membrane lipids [37]. Still many of the pathogenetic mechanisms of leishmaniasis are not clear, as well as the interactions with the immune system and the host resistance mechanisms. An important role is attributed to macrophages, in fact the recruitment and the activation of macrophages can influence the outcome of *Leishmania* infection, the activated macrophages are also responsible for the production of high reactive species qualities of nitrogen and oxygen during oxidative burst, even if some species are resistant to this oxidative stress [38]. This type of parasite resistance probably developed as a defense mechanism against the host's response, moreover, this type of resistance of the parasite stimulates a continuous and intense inflammatory response by the host. Therefore the inflammatory response and the continuous production of oxidizing agents during this phase contributes to tissue injury and therefore to disease progression [39]. It is widely recognized that oxidative stress and oxidative damage to biomolecule are involved in the progression of many diseases, especially in association with pro inflammatory conditions. This has been seen not only in infectious diseases but in a whole series of pathologies of various nature such as cardiovascular conditions, several types of cancer, neurodegenerative diseases and diabetes, where the major regulator of the pathological process is the activation at the molecular level of a pro-inflammatory condition [39, 40]. The current therapies for leishmaniasis are all characterized by important side effects as well as by toxicity if used for long periods [41, 42]. In light of the relationship between activation of pro-inflammatory pathways and production of reactive species, oxidative stress is an additional factor that contributes to tissue damage caused by leishmaniasis, and therefore to be taken into consideration as a pharmacological target. Our data showed a structural abnormalities and neurotrophic infiltration in skin of *L. amazonensis* infected mice and that in terms of paw edema, thermal and mechanical hyperalgesia, neutrophilic infiltration and skin damage. NAC also counteracted the *L. amazonensis* -induced output of pro-inflammatory and pro-nociceptive mediators in paw skin. It is well known that mast cells play an important part both in physiological and pathological situations, since they are able to release different proinflammatory mediators and thus increase inflammation of leukocytes in inflammatory states. It is known that the combination of oxidative stress and inflammatory activation is commonly associated to several degenerative processes triggered by parasitosis. It was also seen as TNF- α promotes

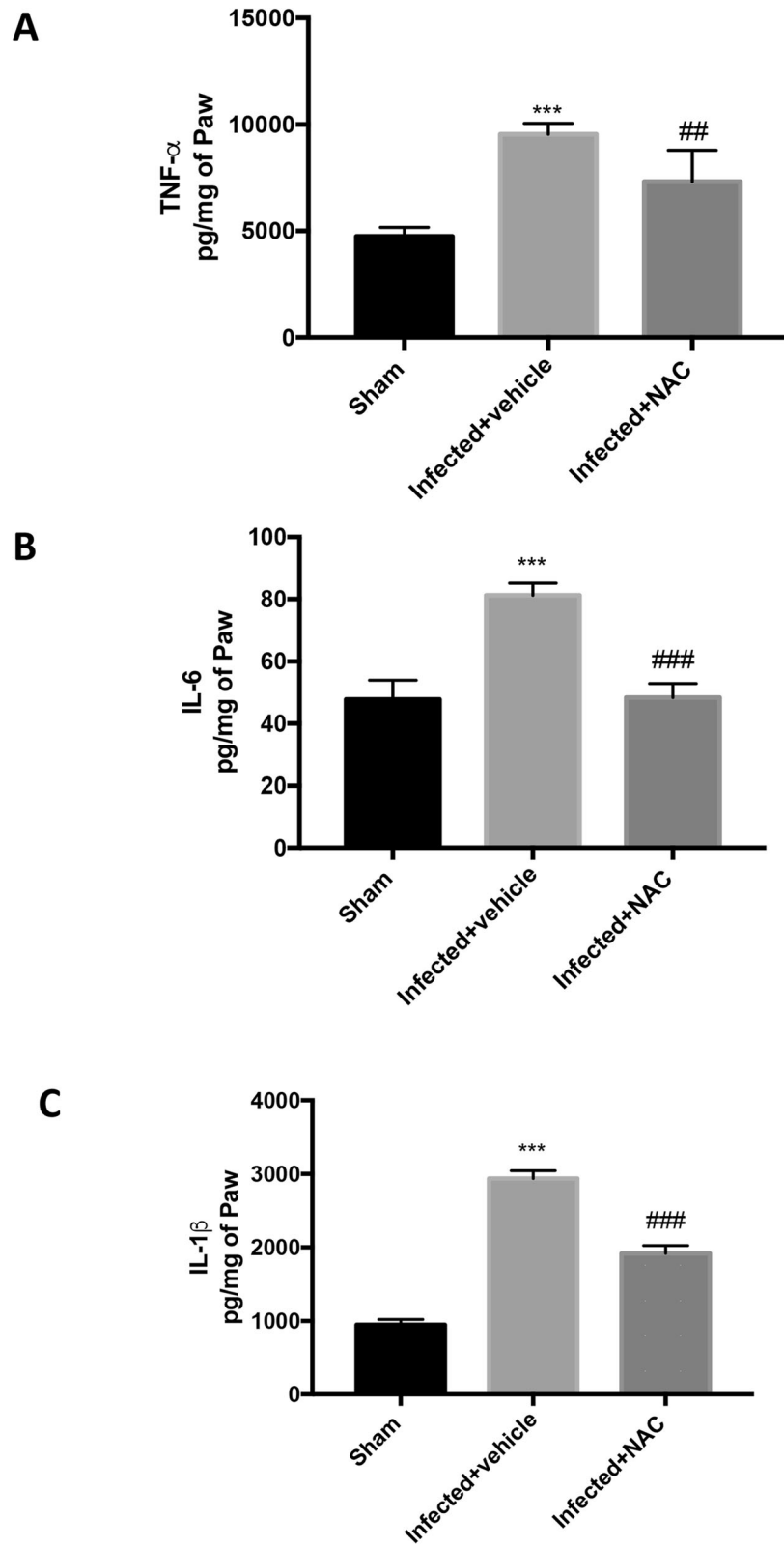


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Fig. 4 Effect of oral NAC administration on *L. amazonensis* infected mice on cytokine expression. A significant increase of TNF- α (A), IL-6 (B), and IL-1 β (C) levels was detected in paw skin after subcutaneous infection of *L. amazonensis* into the mice paw skin. Orally NAC administration (20 mg/kg) significantly reduced levels of all analyzed cytokines. Values are means \pm SEM of five animals for each group. ### $P < 0.001$ vs. *L. amazonensis* infected mice; *** $P < 0.001$ vs. sham

the induction of IL-6 and IL-1 β ; high levels of these pro-inflammatory cytokines can cause systemic inflammation [43]. Glutathione (GSH) and the other proteins containing thiol groups are the main endogenous components responsible for protection from oxidative stress, in fact they have the ability to reduce the excess of electrophilic species that would otherwise cause damage to biomolecules. GSH is primarily responsible for the detoxifying action of liver, by both xenobiotics and endogenous metabolites, which are thus eliminated by being conjugated with GSH. In response to oxidative stress, GSH and other proteins containing thiol groups are up regulated, due to their action capable of to scavenge superoxide and hydroxyl radicals [44] [45]. The protective effects of NAC have been suggested to be due to scavenging reactive oxygen intermediates thought a stimulation of glutathione synthesis [46] [45], which modulates the immune response [47].

Conclusions

In conclusion, the present findings demonstrate that *L. amazonensis* infection induces inflammation, pain and oxidative stress in BALB/c mice. In particular, in this study we have shown that BALB/c mice subjected to infection with *L. amazonensis* exhibit an increase in paw edema associated with histological damage and neutrophilic infiltration.

Chronic treatment with NAC decreased inflammation, pain and the levels of the principal pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α . Based on our results, a clinical use of NAC as an adjuvant to current therapies against leishmaniasis would seem to be of great help in the management of this complex pathology, which could help to prevent the development of disorders resulting from the association between inflammation, pain and oxidative stress. These preclinical findings contribute to address significant questions related to biological events observed in human leishmaniasis and may represent great advances in the complex understanding about the evolution of lesions in cutaneous leishmaniasis. Although, the limitation of pre-clinical models on the transferability of efficacy in veterinary medicine, the present findings shed new light on some of the inflammatory and nociceptive pathways and molecules targeted by NAC. To date there is an increasing interest in the search for new drugs for the treatment of leishmaniasis, which can also have a different mechanisms of action from those that are the currently available drugs. Collectively the data presented in our study suggest an important role of NAC in the management of pain and

especially of the inflammatory process in leishmaniasis, therefore NAC could be a great resource for the management of these pathologies in veterinary medicine.

Methods

Animals

Male BALB/c mice (6–8 weeks old; 25–30 g) were purchased from Envigo (Milan, Italy) and were housed in a controlled location and delivered with typical rodent water and food. Mice were acclimatized to their environment for 1 week. Animal care was in accord with Italian (DM 116192) and European Economic Community regulations (OJ of EC L 358/1 12/18/1986) for the protection of experimental animals. Animals study was conducted according to ARRIVE guidelines.

Parasites and mice infections

L. amazonensis (WHOM/R/75/Josefa) was cultivated in vitro in Schneider Insect Medium (Sigma-Aldrich) added with 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin. Stationary promastigote forms were transferred to fresh medium at a density of 10^7 parasites/ml, at 26 °C. BALB/c mice were subcutaneously infected in the footpad with 2×10^5 stationary phase promastigotes of *L. amazonensis*, as previously described [48].

Experimental groups and treatment

Animals were randomly assigned into the following groups:

Sham (non-infected animals): mice ($N = 10$) were exposed to same surgical procedures excluding for the infection with parasite.

Infected + vehicle: mice ($N = 10$) were subcutaneously infected in the footpad with 2×10^5 stationary phase promastigotes of *L. amazonensis*.

Infected + NAC: mice ($N = 10$) were administered 2 days before the infection with NAC 200 mg/kg once a day during all the infection period (10 weeks) by oral gavage as previously seen [48].

At the end of experiment, animals were terminally anesthetized with overdose of isoflurane (5.0% isoflurane (Baxter International) in air, until saturation) for further analysis.

The minimum number of animals for each technique was calculated with G*Power software statistical test. This test provides an efficient method to determine the

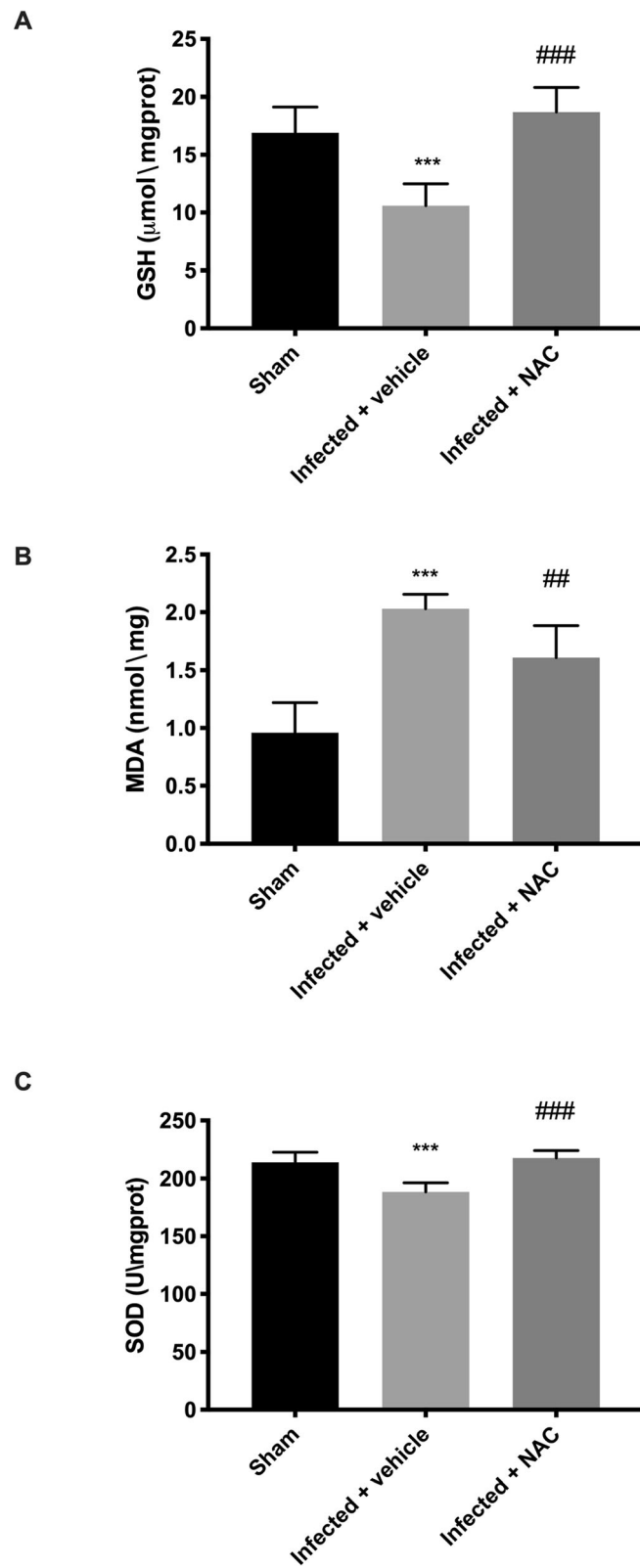


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Fig. 5 Effect of NAC on oxidative stress markers alteration in *L. amazonensis* infected animals. As show in Fig. 5A in infected + vehicle mice we observed a significant depletion of GSH levels, that have been restored by NAC treatment. The result in Fig. 5B, showed that the level of MDA was decreased significantly in tissue collected from mice treated with NAC compared to *L. amazonensis* + vehicle infected animals. Furthermore, we observed that the activities of SOD Fig.5C, were significantly increased in tissue taken from NAC group treated compared to infected + vehicle mice group. Values are means \pm SEM of five animals for each group. $^{***}P < 0.01$, $^{###}P < 0.001$ vs. *L. amazonensis* infected mice; $^{***}P < 0.001$ vs. sham

sample size necessary to carry out the experiment before the experiment itself is actually conducted.

Paw edema

Volume of paw edema was calculated by quantitating the variation in the paw volume using a plethysmometry (model 7140; Ugo Basile).

Histological analysis and myeloperoxidase (MPO) activity

Sections of 7 μ m-thick were stained with haematoxylin and eosin and then observed by light microscopy coupled to an Imaging system (Leica DM2000, Milan, Italy) The MPO activity, an indicator of neutrophil infiltration, was made and calculated as previously described [49].

Thermal hyperalgesia

The responses to thermal hyperalgesia was calculated by the Hargreaves' Method using a Basile Plantar Test [50] (Ugo Basile; Comerio, Italy) with a cut-off latency of 20 s employed to prevent tissue injury. The withdrawal latency time of irritated paws was calculated with an electronic clock circuit and thermocouple. Foot withdrawal latencies were acquired before infection (baseline) and after every week for all experimental time to calculate the analgesic effect of NAC treatment. A significant ($P < 0.05$) decrease in paw-withdrawal latency over time is described as thermal hyperalgesia.

Mechanical Hyperalgesia

Mechanical allodynia was measured by Electronic von Frey test (BIO-EVF4, Bioseb, France). The withdrawal threshold was expressed as the force, in grams, at which the mouse withdrew its paw. Withdrawal was determined 3 times, and the reported value is the mean of the 3 different measures.

Western blot analysis

Western blot analysis on cytosolic fraction of the paw skin was made as previously described [51]. The filters were probed with anti-nerve growth factor (NGF) (1:1000) (Santa Cruz Biotechnology Cat# sc-549, RRID:AB_632012) and β -actin (1:500) (Santa Cruz Biotechnology Cat# sc-69,879, RRID:AB_1119529) for the standardization. Signals were detected with enhanced chemiluminescence (ECL) detection system reagent and the relative expression of the protein bands was quantified

by densitometry with BIORAD ChemiDoc™ XRS + software. A representative picture of blot signals was imported to analysis software (Image Quant TL, v2003).

Staining of mast cells (MCs)

The identification of MCs was evaluated in the palm of paw sections as previously described by Petrosino et colleagues [52]. Briefly, sections were detected with toluidine blue and the mast cells were marked purple. Metachromatically stained MCs were calculated by counting five high-power fields (100 \times) per section using Leica DM2000 (Milan, Italy) microscope.

Determination of cytokine levels in paw skin

TNF- α , IL-1 β and IL-6 released in the paw skin were measured by ELISA (R&D systems, Minneapolis, MN) as described previously by Salvemini et al. [53], and the results expressed as pg per paw normalized to the volume recovered from each paw.

Assay of oxidative stress

Assay of oxidative stress was performed as previously described [54–56]. MDA level, SOD activity, GSH level were spectrophotometrically analyzed according to the manufacturer instructions.

Statistical analysis

All values are expressed as mean \pm standard error of the mean (S.E.M.) of N observations. Each experiment was performed in triplicate on three different occasions. All analysis was executed in a blinded manner. The significance level was determined by one-way analysis of variance (ANOVA). GraphPad Software Prism 7 (La Jolla, CA) was used for all statistical analyses.

Abbreviations

ECL: Enhanced chemiluminescence; GSH: Glutathione; L. amazonensis: *Leishmania amazonensis*; LPS: Lipopolysaccharide; MCs: Mast Cells; NAC: N-acetyl-L-cysteine; NGF: Nerve growth factor; S.E.M.: Standard error of the mean

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Authors' contributions

RC conceived of the study and drafted the manuscript. EG, RS, DI and MC carried out the experiments and revised the manuscript. RDP performed the statistical analysis. DB and SC designed the study and critically revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and analyses during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The University of Messina Review Board for the care of animals authorized the study. Animal care was in accordance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M.116192) as well as with EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Consent for publication

Not applicable.

Competing interests

None of the authors have any conflicts of interest to declare. In order to ensure transparency we declare that Dr. Enrico Gugliandolo is a member of the editorial board of this journal.

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