Parasitology

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Vitamin D increases killing of intracellular *Leishmania amazonensis in vitro* independently of macrophage oxidative mechanisms

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Research Article

Cite this article: Machado PdeA, Escrivani DO, Gomes DCO, Rossi-Bergmann B, Chaves SP, Coimbra ES, de Matos Guedes HL (2020). Vitamin D increases killing of intracellular *Leishmania amazonensis in vitro* independently of macrophage oxidative mechanisms. *Parasitology* **147**, 1792–1800. https://doi.org/10.1017/S0031182020001791

Received: 11 May 2020
Revised: 11 September 2020
Accepted: 11 September 2020
First published online: 22 September 2020

Key words:
Leishmania amazonensis; macrophages; NO; ROS; Vitamin D

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Abstract

Vitamin D has been reported to activate macrophage microbicidal mechanisms by inducing the production of antimicrobial peptides and nitric oxide (NO), but conversely has been shown to contribute to a greater susceptibility to *Leishmania amazonensis* infection in mice. Thus, this study aimed to evaluate the role of vitamin D during intracellular infection with *L. amazonensis* by examining its effect on macrophage oxidative mechanisms and parasite survival *in vitro*. Vitamins D_2 and D_3 significantly inhibited promastigote and amastigote growth *in vitro*. Vitamin D_3 was not able to induce NO and reactive oxygen species (ROS) production in uninfected macrophages or macrophages infected with *L. amazonensis*. In addition, vitamin D_3 in combination with interferon (IFN)-γ did not enhance amastigote killing and in fact, significantly reduced NO and ROS production when compared with the effect of IFN-γ alone. In this study, we demonstrated that vitamin D directly reduces parasite growth in infected macrophages (approximately 50–60% at 50 μM) but this effect is independent of the activation of macrophage oxidative mechanisms. These findings will contribute to a better understanding of the role of vitamin D in cutaneous leishmaniasis.

Introduction

Leishmaniasis is a protozoonosis affecting around 700 000 to 1 million individuals annually and causing 26 000–65 000 annual deaths (World Health Organization, 2019). During their life cycle, these parasites alternate between two main morphological forms: (1) amastigotes, which are intracellular and mainly found in macrophages, and (2) promastigotes, which are present in the digestive tract of the insect vector (Hoare and Wallace, 1966; Anversa *et al.*, 2018). Pentavalent antimonials are used as the first-line drugs in the treatment of cutaneous leishmaniasis, but they are not recommended by the Food and Drug Administration (FDA), due to their toxicity and the clinical failure associated to parasite resistance (Aronson and Joya, 2019). For the treatment of visceral leishmaniasis, liposomal amphotericin B is recommended as the first-line antileishmanial drug, but this therapy is expensive and in East Africa and Brazil the efficacy of this drug is lower than expected, meaning high doses are required (van Griensven and Diro, 2019). However, in Brazil and in most parts of the world the treatment of choice for all clinical forms of leishmaniasis remains to be the pentavalent antimonials (Anversa *et al.*, 2018; Maxfield and Crane, 2020).

Leishmania infection mainly occurs in poor communities, where diets are often inadequate. Furthermore, in *Leishmania*-infected individuals, malnutrition is more pronounced (Custodio *et al.*, 2016). It has already been demonstrated that pre-existing malnutrition can interfere with cell-mediated immune responses against *L. infantum*, altering T cell migration and suppressing the control of parasite, which contributes to the pathophysiology of visceral leishmaniasis in malnourished individuals (Losada-Barragán *et al.*, 2017).

Vitamin D is the precursor of the steroid hormone calcitriol (Rodriguez-Cortes *et al.*, 2017). The major source of vitamin D for humans comes from exposure to sunlight (Holick, 2008), in which vitamin D is synthesized in the skin after exposure to UVB (Prietl *et al.*, 2013). However, vitamin D can also be obtained orally through diet in the two main forms, vitamins D_2 (ergocalciferol) and D_3 (cholecalciferol). This vitamin reaches the liver, where it is converted into 25-hydroxyvitamin D [25(OH)D]. This pre-hormone then undergoes hydrolysis by the enzyme 1-α hydroxylasein in the kidneys and is converted to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], the hormone also known as calcitriol. Calcitriol is the most biologically

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active vitamin D metabolite and increases calcium absorption, which is essential for bone health. In addition, vitamin D or 25 (OH)D can also reach other organs, such as the breast, colon, skin, brain, ovary and prostate where it is converted into 1,25 $(OH)_2D$. In these organs, cells express the vitamin D receptor, through which 1,25$(OH)_2D$ can regulate cell growth (Hollis and Wagner, 2013). Furthermore, the interaction between vitamin D and its receptor can regulate various cellular processes, such as intracellular signalling cascades, cytokine secretion, response to stress, cell communication and cell differentiation (Heikkinen *et al.*, 2011).

There are reports describing the antimicrobial (García-Barragán *et al.*, 2018; Huang *et al.*, 2019) and antiparasitic activities of vitamin D (Yamamoto *et al.*, 2017, 2019). Moreover, studies have shown that vitamin D is able to modulate immune responses (Waters *et al.*, 2001; García-Barragán *et al.*, 2018). The nuclear vitamin D receptor and vitamin D_3-metabolizing enzymes are present in many cells of the immune system, such as dendritic cells, monocytes, macrophages, T cells and B cells, and this fact strongly suggests an important role of vitamin D as a modulator of immune responses (Vanherwegen *et al.*, 2017). It has been reported that this vitamin is able to improve the phagocytic capacity of human monocytes (Xu *et al.*, 1993). Calcitriol can also alter the function and morphology of dendritic cells to induce tolerogenic properties in these cells. This is characterized by a decrease in the expression of MHC class II and co-stimulatory molecules (CD40, CD80 and CD86) in dendritic cells, which leads to reduced antigen presentation (Prietl *et al.*, 2013). Vitamin D can also control cells of the adaptive immune system. Direct effects of calcitriol on B cells have already been demonstrated, promoting the inhibition of memory cells and plasma cells generation and the induction of apoptosis in immunoglobulin-producing B cells (Chen *et al.*, 2007; Prietl *et al.*, 2013). In addition, calcitriol is able to suppress the proliferation and differentiation of T helper (Th) cells and modulate the cytokine production by these cells (Lemire *et al.*, 1985), specifically by inhibiting the secretion of proinflammatory Th1 cytokines, such as interferon (IFN)-γ and tumour necrosis factor (TNF)-α, and stimulating the production of anti-inflammatory Th2 cytokines, including IL-4 and IL-5 (Prietl *et al.*, 2013). Studies have already shown that vitamin D induces nitric oxide (NO) production, contributing to the effect of this vitamin against *Mycobacterium* (Waters *et al.*, 2001; García-Barragán *et al.*, 2018).

With regards to *Leishmania* parasites, the data reported on the effect of vitamin D are contradictory. It was verified that the treatment with vitamin D reduced lesion size of mice infected with *L. mexicana*, without affecting the parasite load (Ramos-Martínez *et al.*, 2013). In accordance with this, Rodriguez-Cortes and colleagues have shown in a cohort study that dogs naturally infected with *Leishmania* and exhibiting symptoms of the disease presented lower vitamin D levels in the serum compared with asymptomatic or non-infected animals (Rodriguez-Cortes *et al.*, 2017). In contrast, *Leishmania amazonensis*-infected mice receiving a vitamin D-deficient diet control lesion development better than those receiving a regular diet, which was shown to be due to the involvement of Th1 cells in both C57BL/6 and BALB/c mice (Bezerra *et al.*, 2019). Furthermore, during *L. major* infection, C57BL/6 mice deficient for the vitamin D receptor (VDR knockout) developed significantly smaller lesions, with a reduced inflammatory response and healing time 3 weeks faster than wild-type (WT) C57BL/6 mice. However, these differences were not observed between VDR knockout in the BALB/c background and the BALB/c WT mice, which indicates the importance of the host background (Whitcomb *et al.*, 2012). Due to these conflicting reports with *in vivo* models, it is necessary to investigate and understand the role of vitamin D at the cellular level using a *Leishmania*-macrophage *in vitro* model infection. For *L. major*, *in vitro* treatment with vitamin D on infected macrophages did not reduce parasite load and also inhibited the anti-microbial response promoted by IFN-γ; however, the effect on *L. amazonensis*-infected macrophages, that present a different type of parasitophorous vacuole, is still unknown.

The control of *Leishmania* infection depends on the mounting of a Th1 immune response, with production of IFN-γ and TNF-α, which activate microbicidal mechanisms in infected macrophages, such as NO and reactive oxygen species (ROS) production (Kaye and Scott, 2011; Santos and Brodskyn, 2014). These free radicals cause damage to lipids, proteins and DNA, causing parasite death (Kima, 2014).

In this study, we determined the *in vitro* antileishmanial effect of vitamin D (D_2, D_3 and calcitriol), and investigated the role of vitamin D during *in vitro* infection with the intracellular protozoan *L. amazonensis*, focusing on the microbicidal responses of the infected macrophages.

Materials and methods

Compounds

Vitamins D_2, D_3 and calcitriol were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and diluted in ethanol. Amphotericin B, obtained from Cristália (Itapira, São Paulo, Brazil), was used as a positive control in antileishmanial tests.

Parasite culture

Wild-type (WT) *Leishmania amazonensis* [IFLA/BR/67/PH8] (*L. amazonensis*–WT) and *L. amazonensis* [MHOM/BR/75/Josefa] transfected with green fluorescent protein (GFP) (*L. amazonensis*–GFP) were cultured in Warren's medium, brain-heart infusion (HiMedia, Mumbai, Maharashtra, India) plus haemin and folic acid – both obtained from Sigma-Aldrich, supplemented with 10% foetal bovine serum (FBS) (Cultilab, Campinas, São Paulo, Brazil) (v/v) and 0.1% penicillin/streptomycin solution (Sigma-Aldrich) (v/v), at 25 °C in a BOD incubator.

Mice

BALB/c mice were obtained from the Central Animal Facility of UFRJ. The procedures using animals were performed according to protocols approved by the Ethical Committee for Animal Handling (CEUA 080/2018) from UFRJ.

Anti-parasite assays

Anti-promastigote test

Leishmania amazonensis–WT promastigotes, in the logarithmic phase of growth, were transferred to 96-well culture plates (3×10^6 cells mL^{-1}) and exposed to different concentrations of vitamins D for 48 h at 25 °C. The cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and readings were taken at 570 nm. The IC_{50} values were calculated using GraFit software. Three independent experiments in triplicate were performed. Amphotericin B was used as the reference drug. MTT was obtained from Sigma-Aldrich.

Anti-amastigote test

Peritoneal macrophages of BALB/c mice (2×10^6 cells mL^{-1}) were adhered in 24-well culture plates for 24 h at 37 °C under a 5% CO_2 atmosphere. Cells were infected with *L. amazonensis*–GFP promastigotes (20:1 ratio) or *L. amazonensis*–WT (10:1 ratio) for 4

https://doi.org/10.1017/S0031182020001791 Published online by Cambridge University Press

h at 33 °C. After infection, the non-adhered promastigotes were washed and the cells were maintained in RPMI medium (Cultilab) supplemented with 10% FBS (v/v) and 0.5% penicillin/streptomycin (v/v) for 24 h. The vitamins were added to the cultures, at different concentrations for 48 h at 33 °C. For *L. amazonensis*–GFP, the parasite load was determined by measuring the fluorescence intensity of live amastigotes using a spectrofluorometer (485/528 nm). For *L. amazonensis*–WT, slides were stained with Giemsa and the number of amastigotes/200 macrophages was counted on an optical microscope, with subsequent determination of the percentage of infected macrophages and the number of amastigotes per macrophage. In each case (WT and GFP) three independent experiments in triplicate were performed. Infected macrophages that not received treatment were used as a control (incubated with culture medium containing FBS and antibiotic) and amphotericin B was used as the reference drug.

To test the effect of vitamin D_3 in combination with IFN-γ, vitamin D_3 (40 μM) and IFN-γ (1 ng mL^{-1}) were used together in the same infection protocol using *L. amazonensis*–GFP. In these experiments a treatment pre-infection and a treatment post-infection were performed. In the latter, the protocol used was the same as described above. In the former, the attached macrophages were treated with vitamin D, IFN-γ or both for 24 h, the cells were washed and infected with *L. amazonensis*–GFP promastigotes (20:1 ratio) for 4 h at 33 °C. The non-adhered promastigotes were washed and the cells were maintained in RPMI medium supplemented with 10% FBS (v/v) and 0.5% penicillin/streptomycin (v/v) for 48 h. The parasite load was determined by measuring the fluorescence intensity of live amastigotes using a spectrofluorometer (485/528 nm). Infected macrophages that not received treatment were used as a control (incubated with culture medium containing FBS and antibiotic). Three independent experiments in triplicate were performed. IFN-γ was obtained from the supernatant of L1210 cells cultivated in RPMI medium supplemented with 10% FBS (v/v), 1% non-essential amino acids (v/v) and 0.5% penicillin/streptomycin solution (v/v), at 37 °C with 5% CO_2.

Cytotoxicity assay

To evaluate the toxicity of the vitamins on mammalian cells, peritoneal macrophages from BALB/c mice (2×10^6 macrophages mL^{-1}) were adhered in 96-well culture plates for 24 h at 37 °C in 5% CO_2. The vitamins were then added to the culture in different concentrations for 48 h at 37 °C and 5% CO_2. Cell viability was assessed by the MTT method. CC_{50} values were calculated using GraFit software. Three independent experiments in triplicate were performed and 2% Triton X-100 (Dinâmica, Diadema, São Paulo, Brasil) was used as a positive control.

Reactive oxygen species assay

Peritoneal macrophages from BALB/c mice (1×10^6 cells mL^{-1}) were plated in 96-well black microplates with a clear flat bottom. For the treatment post-infection, these cells were infected with *L. amazonensis*–WT promastigotes (10:1 ratio) for 4 h at 33 °C, non-adherent promastigotes were washed and after 24 h, the cells were treated with vitamin D_3 (40 μM), IFN-γ (1 ng mL^{-1}) or both for 48 h. For the treatment pre-infection, the macrophages were treated with vitamin D_3 (40 μM), IFN-γ (1 ng mL^{-1}) or both for 24 h, washed and infected with *L. amazonensis*–WT promastigotes (10:1 ratio) for 4 h at 33 °C, non-adherent promastigotes were washed and were incubated for 48 h. The ROS levels also were analysed in uninfected macrophages that had been treated for 48 h with vitamin D_3 (40 μM), IFN-γ (1 ng mL^{-1}) or both added at the same time as the post- and pre-treated infected macrophages. In all cases, the plates were washed with phosphate-buffered saline and 20 μM H_2DCFDA (Invitrogen, Carlsbad, California, USA), an oxidative probe, was added for 30 min. ROS production was evaluated using a spectrofluorometer (485/528 nm). *Leishmania amazonensis*-infected macrophages stimulated with H_2O_2 (4 mM) for 30 min were used as a positive control. Four independent experiments in triplicate were performed.

Table 1. Effect of vitamins D against peritoneal macrophages, promastigotes of *Leishmania amazonensis* and SI

	Peritoneal macrophages – CC_{50} (μM)[a]	*L. amazonensis* promastigotes – IC_{50} (μM)[b]	SI[c]
Vitamin D_2	61.95 ± 1.49	36.47 ± 2.88	1.70
Vitamin D_3	59.97 ± 1.07	39.64 ± 1.88	1.51
Amphotericin B[d]	–	0.12 ± 0.01	–

[a]Cytotoxic concentration of 50% of macrophages (CC_{50}).
[b]Inhibitory concentration of 50% of growth parasites (IC_{50}). Values are the mean ± S.D.
[c]SI, calculated by: CC_{50} against macrophages/IC_{50} against parasite.
[d]Amphotericin B was used as reference drug.

Nitrite assay

Nitrite production was determined in the supernatant of the cultures (uninfected and infected macrophages with *L. amazonensis*) from treatment pre- and post-infection with vitamin D_3 (40 μM), IFN-γ (1 ng mL^{-1}) or both, by the Griess method (Green *et al.*, 1982). Nitrite concentration was estimated using a sodium nitrite standard curve. *Leishmania amazonensis*-infected macrophages stimulated with 1 ng mL^{-1} IFN-γ were used as a positive control. Four independent experiments in triplicate were performed.

Statistical analysis

All the graphs presented in this paper show a set of data taken from three or four independent experiments, performed in triplicate.

Comparison between groups was made using one-way analysis of variance (ANOVA) followed by Dunnett post-test and the significance was considered when $P < 0.05$.

Results

Anti-Leishmania amazonensis *activity of vitamin D*

It has been previously reported that vitamin D can regulate innate and adaptive immune mechanisms (Ramos-Martínez *et al.*, 2015), such as through the modulation of macrophage effector functions (Gough *et al.*, 2017). As macrophages are the main host cell for the *Leishmania* parasites (Carlsen *et al.*, 2015), the role of vitamin D during infection of macrophages by *L. amazonensis* was investigated.

Initially, we evaluated separately the toxicity of vitamins D_2 and D_3 against host macrophages and *L. amazonensis* promastigotes. Vitamins D_2 and D_3 presented some toxicity against peritoneal macrophages, with CC_{50} values of 61.95 and 59.97 μM, respectively (Table 1). The positive control, 2% Triton X-100, killed 93.36% of macrophages. Both of these forms of vitamin D were active against *L. amazonensis* promastigotes, with IC_{50} values of 36.47 and 39.64 μM, for vitamins D_2 and D_3 respectively (Table 1). Amphotericin B was used as a positive control and showed an IC_{50} of 0.12 μM (Table 1 and Fig. S1A). The selectivity index (SI = CC_{50} of macrophages/IC_{50} against *L. amazonensis* promastigotes) of vitamins D_2 and D_3 gave values of 1.70 and

https://doi.org/10.1017/S0031182020001791 Published online by Cambridge University Press

Table 2. Effect of vitamins D against intracellular amastigotes of *L. amazonensis* (WT and GFP), promastigotes of *L. amazonensis* and peritoneal macrophages at 50 μM

	% inhibition at 50 μM[a]			
	L. amazonensis amastigotes			
	WT[b]	GFP[c]	*L. amazonensis* promastigotes – WT[b]	Peritoneal macrophages
Vitamin D_2	60.80 ± 4.67***	51.16 ± 2.02***	89.35 ± 9.81***	13.68 ± 7.72[ns]
Vitamin D_3	66.89 ± 2.3***	54.23 ± 6.44***	64.19 ± 4.31***	18.64 ± 11.97[ns]

[a]Percentage of inhibition compared to untreated control ± standard error of the mean (S.E.M.).
[b]*L. amazonensis* – WT = not transfected.
[c]*L. amazonensis* – GFP = transfected with green fluorescence protein (GFP).
***$P < 0.0001$ and [ns]not significant ($P = 0.0803$), when compared with the percentage of inhibition of control group, that is 0%.

1.51, respectively (Table 1), which indicates that these vitamins are more toxic to the parasite in the promastigote form than to the macrophage.

Next, the effect of vitamins D_2 and D_3 on intracellular *L. amazonensis* amastigotes was evaluated. To avoid toxicity to the macrophages, the highest concentration of the vitamins tested was 50 μM. First, the anti-amastigote effect was determined by fluorimetry using *L. amazonensis*–GFP, which showed a reduction of the amastigote viability of 51.16 and 54.23% at 50 μM for vitamins D_2 and D_3, respectively, after 48 h of incubation (Table 2). The anti-amastigote effect was also assessed using the *L. amazonensis*–WT by manually counting the intracellular parasites, and the reduction of the amastigote viability at 50 μM was 60.80 and 66.89% for vitamins D_2 and D_3, respectively (Table 2). Still regarding *L. amazonensis*–WT, vitamins D_2 and D_3 significantly reduced the percentage of infected macrophages at concentrations of 50, 25 and 12.5 μM – $P < 0.05$ (Fig. 1A and C and Fig. S2). Furthermore, vitamin D_2 significantly decreased the number of amastigotes per macrophage in all concentrations tested – 50 μM to 6.25 μM – $P < 0.0001$ (Fig. 1B and Fig. S2), while vitamin D_3 caused this reduction at 50 to 12.5 μM – $P < 0.0001$ (Fig. 1D and Fig. S2). Amphotericin B was used as a positive control and inhibited about 65% of the amastigote growth at a concentration of 0.5 μM, with an IC_{50} of 0.28 μM (Fig. S1B).

Overall, Table 2 shows that at 50 μM the vitamins had low toxicity on peritoneal macrophages: 13.68 and 18.64% of cell death after treatment with for vitamins D_2 and D_3, respectively, when compared to untreated control, and these values showed no statistical difference in relation to the untreated control ($P = 0.0803$). However, these vitamins significantly inhibited ($P < 0.0001$) the growth of *L. amazonensis* promastigotes (89.35 and 64.19% for vitamins D_2 and D_3, respectively, compared to untreated control) and intracellular amastigotes (both vitamins inhibited about 50% of the GFP parasites and 60% of the WT parasites, when compared to untreated control).

The calcitriol, the most biologically active vitamin D metabolite, was not toxic to macrophages up to the highest concentration tested (5 μM) (Fig. S3A) and did not show an antileishmanial effect up to 5 μM (Fig. S3B and S3C).

Combination of vitamin D_3 and IFN-γ did not alter parasite load

It has previously been demonstrated that vitamin D_3 and IFN-γ in combination can increase the activity of human monocytes against *Mycobacterium tuberculosis* reducing the proliferation of this microorganism (Rook *et al.*, 1986). Based on this, the combination of vitamin D_3 and IFN-γ during *L. amazonensis* infection *in vitro* was examined. Infected macrophages were treated during 48 h with 40 μM vitamin D_3 together with 1 ng mL^{-1} IFN-γ but this did not enhance the amastigote killing compared to the vitamin D_3 alone (Fig. 2A). IFN-γ (1 ng mL^{-1}) inhibited about 15% of amastigote growth (Fig. 2A), when compared to untreated control. Pre-infection treatment with vitamin D_3 did not cause a significant inhibition of amastigote growth (only about 5%) and IFN-γ (1 ng mL^{-1}) inhibited about 10% of amastigote growth (Fig. 2B). Vitamin D_3 in combination with IFN-γ (pre-infection treatment) did not alter the effect of vitamin D_3 used separately (Fig. 2B). This combination nullified the antileishmanial effect of IFN-γ (Fig. 2B), when used separately.

Vitamin D reduced microbicidal mechanisms in macrophages

Vitamin D_3 has been reported to increase NO production from peripheral blood mononuclear cells (PBMCs) in *Mycobacterium bovis*-infected cattle (Waters *et al.*, 2001). Therefore, we investigated the effect of vitamin D_3 in the production of NO and ROS by murine macrophages uninfected and infected with *L. amazonensis*.

The NO levels produced by *L. amazonensis*-infected macrophages and uninfected macrophages after treatment with vitamin D_3 (40 μM) were comparable to the untreated control (Fig. 3A). IFN-γ (1 ng mL^{-1}) significantly stimulated the NO production by both infected and uninfected macrophages (Fig. 3A). However, the combination of vitamin D_3 and IFN-γ deactivated the NO production induced by the IFN-γ (Fig. 3A). Similar results were observed for the pre-infection treatment with vitamin D_3 and vitamin D_3 with IFN-γ (Fig. 3B). Pre-infection treatment with vitamin D_3 (40 μM) maintained NO levels comparable to the untreated control; IFN-γ (1 ng mL^{-1}) significantly stimulated the NO production by both infected and uninfected macrophages; while the combination of 40 μM vitamin D_3 and 1 ng mL^{-1} IFN-γ maintained NO levels compared to vitamin D_3 alone or to untreated control. Therefore, this pre-infection combination also was able to deactivate the NO production induced by IFN-γ used separately (Fig. 3B).

Vitamin D_3 at 40 μM reduced ROS production from uninfected and *L. amazonensis*-infected macrophages (Fig. 3C). IFN-γ (1 ng mL^{-1}) and H_2O_2 (4 mM) stimulated significantly the ROS production by both infected and uninfected macrophages (Fig. 3C). The combination of vitamin D_3 and IFN-γ deactivated the ROS production induced by IFN-γ used alone (Fig. 3C), which was observed for both uninfected and infected macrophages, similarly to that which occurred for the NO production. Pre-infection treatment with vitamin D_3 maintained the ROS levels comparable to the untreated control, and the combination of vitamin D_3 and IFN-γ deactivated the ROS production induced by IFN-γ used separately in macrophages infected with *L. amazonensis* (Fig. 3D).

These results suggest that vitamin D_3 is able to modulate effector functions of macrophages, leading to a decrease in ROS levels produced by uninfected macrophages and macrophages infected with *L. amazonensis*. In addition, combination of vitamin

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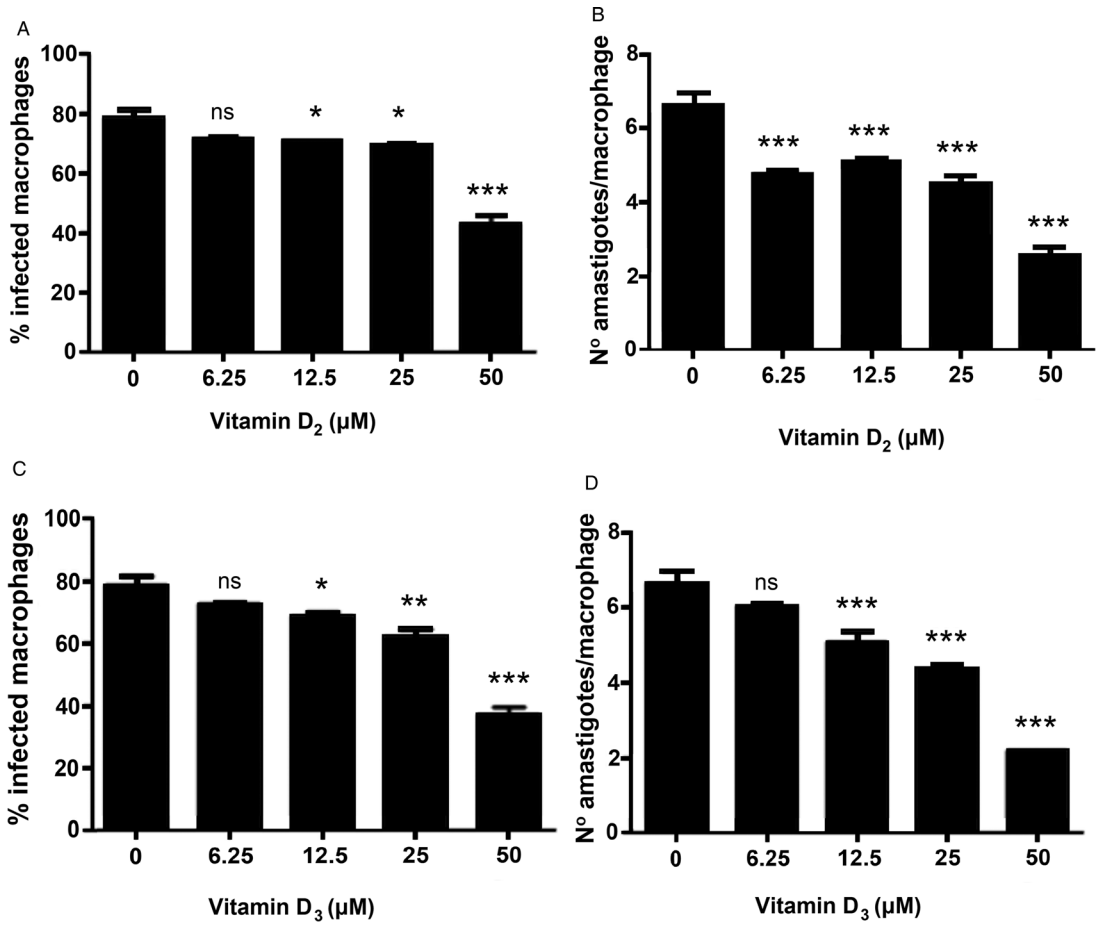

Fig. 1. Effect of vitamins D_2 and D_3 against intracellular amastigotes of *Leishmania amazonensis*. Peritoneal macrophages infected with *L. amazonensis* amastigotes were treated with vitamin D_2 or D_3 for 48 h and the parasitic load was determined by counting the intracellular parasites after staining with Giemsa. Three independent experiments in triplicate were performed. (A and C) Percentage of infected macrophages with *L. amazonensis* amastigotes after treatment with vitamin D_2 (A) and D_3 (C). (B and D) The number of amastigotes per macrophage after treatment with vitamin D_2 (B) and D_3 (D). Graphs were prepared using the GraphPad Prism software, in which one-way ANOVA was used to realize the statistical analysis and Dunnett post-test was applied to compare the groups (50, 25, 12.5 and 6.25) with 0 μM (untreated control): $P < 0.001$ (***); $P < 0.01$ (**); $P < 0.05$ (*) and ns (not significant, $P > 0.05$).

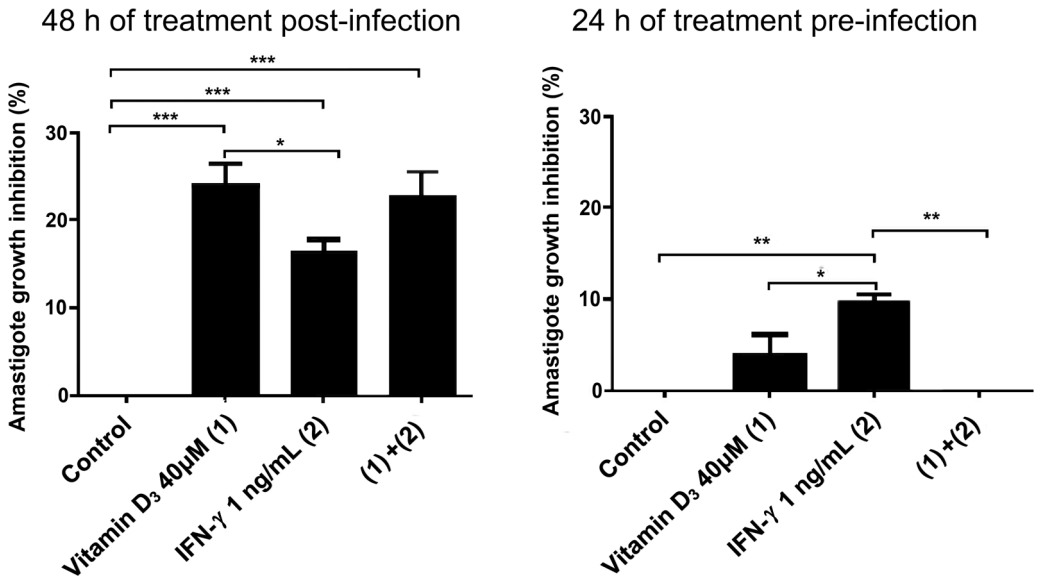

Fig. 2. Percentage of growth inhibition of *L. amazonensis* amastigotes after 24 h for treatment pre-infection and 48 h for treatment post-infection with vitamin D_3 (40 μM) – (1), IFN-γ (1 ng mL^{-1}) – (2) and vitamin D_3 (40 μM) + IFN-γ (1 ng mL^{-1}) – (1) + (2). Each value was calculated based on the control, which caused 0% of growth inhibition. The parasite load was determined by measuring the fluorescence intensity of live amastigotes (*L. amazonensis*–GFP) using a spectrofluorometer (485/528 nm). Three independent experiments in triplicate were performed. Graphs were prepared using GraphPad Prism software, in which one-way ANOVA was used to realize the statistical analysis and Dunnett post-test was applied to compare the groups indicated in the figure: $P < 0.001$ (***); $P < 0.01$ (**); $P < 0.05$ (*) and ns (not significant, $P > 0.05$). Control = macrophages that were infected with *L. amazonensis*, but did not receive the treatment.

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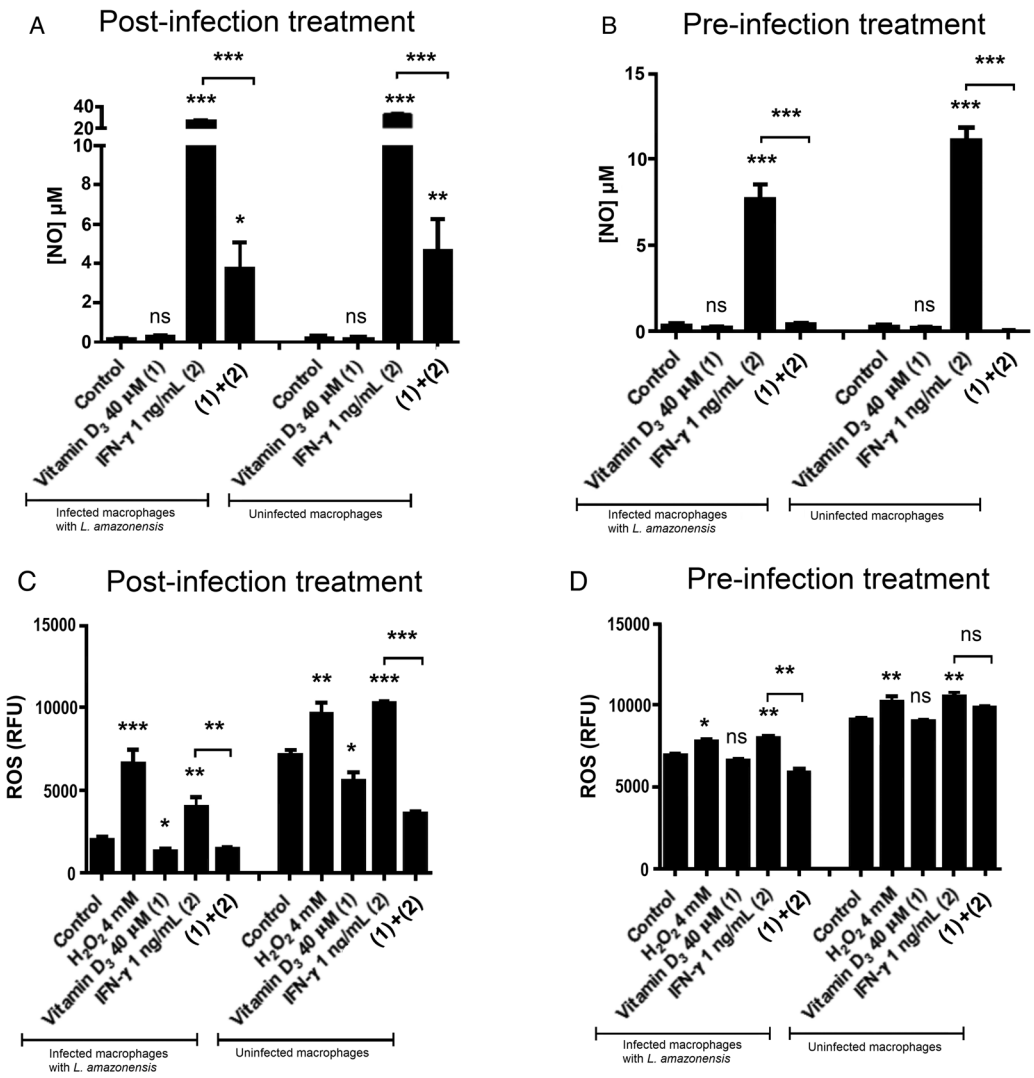

Fig. 3. Effect of post-infection treatment (48 h) and pre-infection treatment (24 h) with vitamin D_3 (40 μM) – (1), IFN-γ (1 ng mL^{-1}) – (2) and vitamin D_3 (40 μM) + IFN-γ (1 ng mL^{-1}) – (1) + (2) on NO (A and B) and ROS (C and D) production by uninfected macrophages and macrophages infected with *L. amazonensis* amastigotes. (A and B) The NO levels were evaluated by the Griess method and the NO concentration is represented in μM. The treatment with IFN-γ 1 ng mL^{-1} was used as a positive control. (C and D) The ROS levels were evaluated by fluorimetry after labelling with H_2DCFDA and the ROS amount is expressed in relative fluorescence units (RFU). Uninfected and infected macrophages with *L. amazonensis* treated with H_2O_2 (4 mM) were used as a positive control. Four independent experiments in triplicate were performed. Graphs were prepared using GraphPad Prism software, in which one-way ANOVA was used to realize the statistical analysis and Dunnett post-test was applied to compare all the groups with control and these analyses are represented above each column: $P < 0.001$ (***); $P < 0.01$ (**); $P < 0.05$ (*) and ns (not significant, $P > 0.05$). The uninfected groups only are compared with the uninfected control and the infected groups only are compared with the infected control. The IFN-γ 1 ng mL^{-1} treatment – (2) was also compared with (vitamin D_3 + IFN-γ) treatment – (1) + (2) and these analyses are indicated above the line between these groups: $P < 0.001$ (***); $P < 0.01$ (**) and ns (not significant, $P > 0.05$). In the experiments using only uninfected macrophages, the control is macrophages untreated with vitamin D_3 or IFN-γ or both. In experiments with infected macrophages the control is macrophages that were infected with *L. amazonensis*, but did not receive the treatment.

D_3 and IFN-γ deactivates macrophage microbicidal mechanisms induced by IFN-γ, such as the NO and ROS production, by both uninfected and infected macrophages.

Discussion

Vitamin D interferes with the regulation of the immune response (Ramos-Martínez *et al.*, 2015), with several immune targets such as dendritic cells, monocytes, macrophages, T cells and B cells (Hart *et al.*, 2011), which are important for the treatment of infectious diseases (Ramos-Martínez *et al.*, 2015). Interestingly, vitamin D_3, also known as cholecalciferol, has been shown to be effective against different strains of *Mycobacterium* spp., like *M. avium* subspecies *paratuberculosis*, *M. avium* subspecies *avium* and *M. tuberculosis* complex, inhibiting bacterial growth (Greenstein *et al.*, 2012). It was also shown by Kim and colleagues that vitamin D contributes to the antimicrobial activity of macrophages against *M. leprae*, with the bacterial burden decreasing significantly with an increasing concentration of vitamin D (Kim *et al.*, 2018). Against *Leishmania* parasites, another intracellular pathogen like *Mycobacterium*, the role of vitamin D is still under debate, as some studies show a positive role while others a negative. For example, Rodriguez-Cortes and colleagues have shown that symptomatic dogs naturally infected with *Leishmania* present lower vitamin D levels compared with asymptomatic or non-infected dogs (Rodriguez-Cortes *et al.*, 2017). In agreement with this study, *L. mexicana*-infected mice treated with vitamin D exhibit a reduction in lesion size (Ramos-Martínez *et al.*, 2013). In contrast, *L. amazonensis*-infected mice that received a vitamin D-deficient diet controlled the lesion development better than mice that received a regular diet (Bezerra *et al.*, 2019). In addition, C57BL/6 mice deficient for the VDR and infected with *L. major* developed significantly smaller lesions and had a reduced inflammatory process when compared to C57BL/6 WT mice (Whitcomb *et al.*,

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2012). Therefore, it is necessary to use a simpler model to investigate the role of vitamin D, for this, the direct effect on macrophages during *Leishmania* infection can be evaluated.

In this study, an *in vitro* model of cutaneous leishmaniasis (infection by *L. amazonensis*) was used to assess the role of vitamin D. Vitamin D (D_2 and D_3) was slightly effective against *L. amazonensis*. Vitamin D is produced in the skin (Prietl *et al.*, 2013), therefore it is in close proximity to the *L. amazonensis* parasite, which resides within cells, primarily macrophages, in the host skin (Anversa *et al.*, 2018). In addition, the chemical structure of vitamin D reveals that it has lipophilic properties, which could favour the penetration of this compound into the host macrophage. These two facts tend to favour the effect of vitamin D against this intracellular parasite, as we observed. However, it is important to highlight that the *in vitro* concentrations used in this study would not be achieved in plasma in an *in vivo* model, since the plasma concentrations of vitamin D are: 50 nmol L^{-1} (=0.02 μg mL^{-1} or 0.05 μM), according to the Institute of Medicine (IOM) and 75 nmol L^{-1} (=0.03 μg mL^{-1} or 0.08 μM), according to the Endocrine Society (ES) (Bendik *et al.*, 2014). In addition, it is worth noting that the concentration of vitamin D_3 in an inflammatory site appears to be greater than in the blood because activated monocytes and T lymphocytes, cells found in inflammatory infiltrates, metabolize vitamin D_3 to the active form (calcitriol) (Xu *et al.*, 1993).

IFN-γ, a potent macrophage-activating cytokine, can induce an antimicrobial response in human macrophages cultured in vitamin D-sufficient sera, which does not occur with cells cultured in sera containing low amounts of vitamin D (Fabri *et al.*, 2011). Rook and colleagues also showed that the combination of vitamin D_3 and IFN-γ increases the activity of human monocytes against *M. tuberculosis* (Rook *et al.*, 1986). Previous study by Qi and colleagues observed an increase in the replication of the *L. amazonensis* within infected macrophages after treatment with IFN-γ (post-infection treatment) (Qi *et al.*, 2004). In this study, IFN-γ poorly inhibited the *L. amazonensis* amastigote growth and the combination of vitamin D_3 and IFN-γ was not favourable to the leishmanicidal effect, as it caused a similar effect in the growth inhibition percentage as the vitamin D_3 alone. Helming and colleagues also showed that vitamin D suppressed the listericidal activity of bone marrow-derived macrophages stimulated by IFN-γ, due to an inhibition of oxidative burst (Helming *et al.*, 2005). It was shown by Henard and colleagues that the overexpression of a tryparedoxin peroxidase in *L. amazonensis* increases the parasite resistance to toxicity mediated by peroxynitrite produced by activated macrophages (Henard *et al.*, 2014). In a previous study conducted by Ehrchen and colleagues, the treatment of *L. major*-infected macrophages with vitamin D led to the conclusion that although there was no reduction in the parasitic load within the macrophages, this vitamin caused a reduction in the microbicidal properties of macrophages induced by IFN-γ, which appeared to be dependent on signalling by VDR (Ehrchen *et al.*, 2007).

Interestingly, it has also been shown that vitamin D supplementation increases NO production from PBMCs of *M. bovis*-infected cattle, which contributes to bacterial death (Waters *et al.*, 2001). Whitcomb and colleagues determined that, when pre-incubated with vitamin D prior to infection with *L. major*, NO production from dendritic cells of VDR knockout C57BL/6 mice was increased compared to dendritic cells from WT C57BL/6 mice. Nevertheless, no differences were observed in NO production by the macrophages from VDR knockout or WT C57BL/6 mice after pre-stimulation with vitamin D followed by infection with *L. major* (Whitcomb *et al.*, 2012). In this study, we show that vitamin D (D_3) was able to modulate effector mechanisms of uninfected and *L. amazonensis*-infected macrophages, decreasing the ROS levels produced by these cells. However, the combination of vitamin D_3 and IFN-γ inhibited the microbicidal mechanisms, the NO and ROS production, in both uninfected and *L. amazonensis*-infected macrophages induced by IFN-γ, whereby the levels were lower than the treatment with IFN-γ alone.

Bacchetta and colleagues reported that vitamin D supplementation increases the production of antimicrobial peptides, such as cathelicidins, from peritoneal macrophages of patients on peritoneal dialysis (Bacchetta *et al.*, 2014). Vanherwegen and colleagues also reported that vitamin D_3 can induce the production of cathelicidins (Vanherwegen *et al.*, 2017), and this could be the mechanism involved in the antileishmanial effect of vitamin D against *L. amazonensis* amastigotes within macrophages, since it appears that NO and ROS production are not related to this process. It has already been reported that vitamin D may induce the production of antimicrobial peptides, such as LL-37, a member of the cathelicidin family (Kościuczuk *et al.*, 2012). It has also been reported that vitamin D induces LL-37 expression, contributing to the host immune response against *M. tuberculosis* (Jo, 2010). Rivas-Santiago and colleagues (2008) stated that vitamin D stimulated the production of LL-37 in macrophages by sunlight through the skin. Because of this, sunbathing is recommended for patients with tuberculosis, in order to favour the production of LL-37 and consequently the kill of *M. tuberculosis* (Rivas-Santiago *et al.*, 2008). In addition, the LL-37 peptide has been shown to have antileishmanial activity against promastigotes and amastigotes of *L. donovani* and *L. major* (Marr *et al.*, 2016). It also has been shown that cathelicidins interact with membranes of pathogens causing permeation and disintegration of these cell membranes (Kościuczuk *et al.*, 2012).

It has already been reported that vitamin D decreases the production of IL-12 and TNF-α from monocytes and macrophages, and IFN-γ from $CD4^+$ T cells. In addition, vitamin D is able to decrease the differentiation of Th1 cells and increase the differentiation of regulatory T cells (Hart *et al.*, 2011). All these effects promoted by vitamin D in the immune system are unfavourable to the development of an effective immune response against *Leishmania* spp., which thus favours the parasite growth and the consequent disease progression in the host. Our previous data have demonstrated that in the absence of vitamin D in the diet, there is a decrease in the lesion size of infected mice due to an increase of Th1 cells (Bezerra *et al.*, 2019). However, in this paper, we have investigated the vitamin D role at the host cell level, and we suggest that vitamin D (D_2 and D_3) can directly cause a slight increase in the control of the parasite load. During *in vivo* experiments, a combination of effects may happen, it is important to point out that despite the direct effect on the macrophage, during a Th1 response with production of IFN-γ, vitamin D could inhibit the NO and ROS response, thus increasing susceptibility. These results provide important information to better understand the complexity of the role of vitamin D directly on macrophages during *in vitro* infection by the protozoan parasite *L. amazonensis*.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182020001791.

Acknowledgements. The authors are grateful to Programa Jovem Cientista do Nosso Estado; Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Agency for Support and Evaluation of Graduate Education (CAPES).

Financial support. This study was funded by Programa Jovem Cientista do Nosso Estado (FAPERJ – E-26/203.215/2015); Productivity Fellowships from CNPq (304712/2016-7), FAPERJ (E-26/202-422/2017) and the Agency for Support and Evaluation of Graduate Education (CAPES) Finance code 001.

Conflict of interest. The authors declare that they do not have any conflict of interests.

Ethical standards. The procedures using animals were performed according to protocols approved by the Ethical Committee for Animal Handling (CEUA 080/2018) from UFRJ.

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