

Reactive Nitrogen Intermediates Production by Macrophages of *Mycobacterium bovis*-Infected Goats and Supplemented with Dihydroxyvitamin D3 *in vivo*

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Abstract: Problem statement: Tuberculosis (TB) remains one of the world's major health problems. To evaluate *in vivo* indices of cellular sensitization, antigen-induced Reactive Nitrogen Intermediates (RNI) responses by blood mononuclear cells from *Mycobacterium bovis* BCG-infected goats supplemented with 1, 25 dihydroxyvitamin D3 [1, 25-(OH)₂D3]. **Approach:** An experimental, longitudinal and comparative study was planned. Twelve animals of goat cattle 20-to 24-month-old *sannen* selected themselves. Five samplings were made, previous to the inoculation (zero d), 3, 7, 14 and 21 d after applying the treatments. The mononuclear cells by the Ficoll-hypaque method were obtained. The RNI, nitrites and nitrates (NO₂⁻ and NO₃⁻) were quantified by Enzyme-Linked Immunosorbent Assay (ELISA). **Results:** The treatment with the 1, 25(OH)₂D3 stimulated the NO₃⁻ synthesis indicating, that by itself it is a good modulator of the micobacterial replication and in the treatment with *M. bovis*-BCG vaccine increased as a result to the treatment with 1,25(OH)₂D3. The exhibition to *M. bovis*-BCG vaccine with the treatment with 1, 25(OH)₂D3 was able to increase answer NO₃⁻ in exposed animals. **Conclusion:** The 1, 25(OH)₂D3 stimulated *in vivo* the production of RNI in goats exposed to *M. bovis* BCG vaccine.

Key words: *M. bovis*-BCG vaccine, 1,25 dihydroxyvitamin D3, nitrites, nitrates, goats

INTRODUCTION

Tuberculosis (TB) remains one of the world's major health problems and the leading cause of death from a single infectious agent called *Mycobacterium tuberculosis*, the primary causative agent^[1]. TB is a zoonotic disease that often becomes chronic with high rate of recurrence. The World Health Organization has declared TB a global emergency, the first disease so designated *M. tuberculosis* infects a third of the world-wide population to the year, in spite of the efforts of

public health to control their dissemination^[2,3], the majority of such infections remain clinically latent^[4], the pathogenicity include its ability to resist the harsh environment of the host macrophage and to persist within immunocompetent hosts^[5]. The entry and survival of *Mycobacterium* in macrophages are central to the pathogenesis of TB. In addition, the ability of the macrophage to resist the growth of the microorganisms is dependent on the activation state of the macrophage. One essential component of tubercular host defense includes Nitric Oxide (NO). Waters, Palmer *et al.*^[12]

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determined that there are two essential components of tubercular host defense include NO and Tumor Necrosis Factor alpha (TNF- α). Stimulation of inducible NO Synthase (iNOS) in macrophages and subsequent generation of RNI are potent mechanisms for mycobacterial killing^[6,7]. Interestingly, it has been associated with risk of dissemination and mortality the absence of iNOS^[8]. Furthermore, it has been used, NO inhaled like treatment for TB^[9]. Therefore, iNOS synthesis is essential for maintaining stationary-level infection^[10], has been investigated iNOS presence in biopsies exhibiting dermal nerves from patients with untreated leprosy^[11]. NO is readily produced by *Mycobacterium*-induced Peripheral Blood Mononuclear Cells (PBMC) from *M. bovis*-infected cattle. NO responses play an important role in organism and host defense. NO metabolism of the host is markedly altered in all infections. In recent reports, NO concentrations were found to be increased in culture supernatants and this is gaining wide acceptance for use in TB diagnosis^[12]. Evaluation of NO response may prove useful for diagnosis of bovine TB, nitrite (NO₂⁻) is the stable oxidation product of NO and the amount of NO₂⁻ within culture supernatants is indicative of the amount of NO produced by cells in culture^[12]. The 1, 25-(OH)₂D₃ is a powerful stimulus for the production of NO by macrophages, which are indicative of the defense of the host against the TB^[13]. Has been considered that the active principle of vitamin D, the 1, 25 Dihydroxyvitamin D₃ [1,25-(OH)₂D₃], it has turned to be a powerful regulator of the immune response. Additionally, it has been observed that 1,25-(OH)₂D₃ suppresses to the growth of *Mycobacterium* in the macrophages through a dependent mechanism of NO^[14]. An important point is that 25-OH-D₃ is turned to 1,25-(OH)₂D₃ within the macrophage and the proportion of conversion increases within the alveolar macrophages and in followed human monocytes of the stimulation with IFN- γ ^[15,16], it is probable that the 1,25-(OH)₂D₃ triggers a dependent mechanism of NO that increases the destruction of *Mycobacterium*^[17,18]. The *M. bovis* Bacillus Calmette-Guerin (BCG) vaccine, an attenuated strain of *M. bovis*, was developed for control of human TB more than 70 years ago and is still the only TB vaccine available. The protection against TB requires the induction of Th1 immune response, but studies with new-born animal have shown that they preferentially develop Th2-Type responses following immunization and are deficient in Th1 responses^[19]. As a result, the bacterial transcription changes of the Th1-mediated immune response are likely induced, directly or indirectly, by NO generated by infected macrophages^[20]. BCG vaccination at birth induced a

high level of immunity and that the sensitization of very young animals to antigens of environmental mycobacteria by 6 weeks of age did not affect the effectiveness of BCG^[19]. Characteristic changes in RNI metabolism, are an integral part of the cellular immune assays. Weatherby *et al.*^[21], have confirmed that macrophages are among the first cells in innate resistance to intracellular microbial pathogens, they have been determining that the cytokines gamma-Interferon (IFN- γ), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and TNF- α activate macrophages to resist the growth of intracellular pathogens during the improvement in the production of antimicrobials molecules, including the RNI and the Reactive Oxygen Intermediates (ROI)^[8,21], proinflammatory cytokines such as interleukin (IL-12) play critical roles in the induction of host resistance to *Mycobacterium*^[22]. In addition, the activated macrophages can turn the L-arginina to NO₂⁻ in the presence of the iNOS enzyme, with the development of a cytotoxic activity against tumor cells^[23] and in front of bacterial infections^[24]. The RNI are protective agents in infectious processes as VIH, *Helicobacter pilori*, *M. tuberculosis*, malaria and infections of respiratory tract and urinary, also have been observed in a great diversity of autoimmune diseases and chronic inflammatory diseases^[25]. Additionally, as an end product in the reaction of iNOS has been determined the presence of nitrotyrosine in lung and tissues several, finding very similar levels^[26]. Nevertheless, the targets of RNI in *Mycobacterium* are unknown^[27]. Different studies have raised that the exhibition to the *M. bovis* BCG vaccine alters the specific immune response, it causing an increase of the immune response mediated by Th1 cells and an stimulation of the immune response Th2. In addition, Flynn *et al.*^[28] demonstrated that *Mycobacterium* reactivation occurs if the production of Reactive Nitrogen Intermediates (RNI) is inhibited in a murine model of latency^[28]. However, little is known about the role of these intermediates during latent infections. Different studies have been raised that the exhibition to the *M. bovis* BCG vaccine alters the specific immune response, it causing an increase of the immune response mediated by Th1 cells and a stimulation of the immune response Th2. In addition, Flynn *et al.*^[28] demonstrated that *Mycobacterium* reactivation occurs if the production of Reactive Nitrogen Intermediates (RNI) is inhibited in a murine model of latency^[28]. However, little is known about the role of these intermediates during latent infections. It has been observed which in infections of *M. bovis* the 1, 25-(OH)₂D₃ accelerates the specific production of and RNI^[29]. But, it is necessary to consider the development

of new investigations to clarify these discoveries. The aim of the present study was to quantify RNI production by macrophages from *M. bovis*-BCG infected goats supplemented with 1, 25-(OH)₂D₃.

MATERIALS AND METHODS

Animals and immunizations: Twelve goats were 20-to 24-month-old *sannen*. They were maintained with a diet supplemented with grain; they were supplied with water ad libitum. Four treatments were carried out, control, supplemented with *M. bovis* BCG vaccine (Pasteur Merieux®, Lyon France), 1, 25 (OH)₂D₃ and *M. bovis* BCG vaccine plus 1, 25 (OH)₂D₃. Three goats were immunized by the intramuscular injection of 0.1 mL volumes of saline solution, three goats with 0.1 mL volumes of *M. bovis* BCG vaccine, three goats with 0.25 µg de 1,25-(OH)₂D₃ (CALCITRIOL -alfa 25-hihidroxicolicalcitrol GELDEX-GELPHARMA®) and three with *M. bovis* BCG vaccine plus 1,25(OH)₂D₃. Whole blood was collected from the jugular vein of antigen-primed goats. Samples of peripheral blood were collected in Vacutainer tubes (BD Vacutainer™ K₃ EDTA; Becton Dickinson, Franklin Lakes, NJ).

Peripheral Blood Mononuclear Cells (PBMC): Whole blood samples were collected and blood mononuclear cells were obtained immediately before challenge [day 0] and 3, 7, 14 and 21 days postchallenge. Whole blood was centrifuged at 3000×g for 15 min, after was separated leucocytes layer in a 15 mL screw cap polypropylene centrifuge tube (Corning® 430790). Mixtures containing goat blood were overlaid on Ficoll-Hypaque (Sigma® H8889 d = 1.077 g mL⁻¹) at a ratio of 6 mL of calcium and magnesium free Phosphate Buffered Saline (PBS), it is designed to maintain a physiological pH in an open system, to 3 mL of Ficoll in a 15 mL polypropylene centrifuge tube. The resulting gradient was centrifuged at 1500×g for 15 min. Caprine PBMC were then collected, washed one time in PBS solution and suspended in RPMI 1640 medium supplemented^[12].

Cell viability: Viability of the samples was measured by trypan blue (derived from toluidine, that is, any of several isomeric bases, C₁₄H₁₆N₂, derived from toluene) dye exclusion method where the viable (unstained) cells were counted in a hemocytometer under a light microscope. Samples of at least three independent experiments were analyzed in duplicate^[30]. The reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not interact

with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable. Trypan Blue Staining of Cells was Place 0.5 mL of a suitable cell suspension (dilute cells in complete medium without serum to an approximate concentration of 1×10⁵-2×10⁵ cells mL⁻¹) in a screw cap test tube, add 10 µL of 0.4% Trypan Blue Stain. Mix thoroughly; allow standing 5 min at 15-30°C (room temperature). Fill a hemocytometer as for cell counting and under a microscope, observe if non-viable are stained and viable cells excluded the stain.

Cell culture: The wells of 24-well round-bottom microtiter plates (Corning Costar®24-well T-C Treated microplates, Individually Wrapped No. 3526) were seeded with 2×10⁵ PBMC in a total volume of 200 µL per well. The goat macrophage cell was obtained from a cell culture. The cells were cultured at 37°C in 5% carbon dioxide atmosphere in RPMI 1640 medium supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 units of penicillin mL⁻¹, 0.1 mg of streptomycin mL⁻¹, 1% nonessential amino acids (Sigma), 2% essential amino acids (MP Biomedicals®), 50 µM 2-mercaptoethanol (Sigma) and 10% (vol/vol) heat-inactivated fetal bovine serum^[31]. The macrophages (5×10⁵ cells mL⁻¹) were dispensed to six-well plates, 2 mL⁻¹ well. The cells were allowed to adhere for 48 h, was removed the supernatant from adherent cells and were resuspended in cool PBS by scraping, the viability of the cells was assessed and the cell suspension was obtained in a microcentrifuge tube (Eppendorf Axygen®), centrifuged (5 min, 1,500 rpm) to separate the cells from the PBS. The cells were suspended in an adequate cryoprotection it was constituted by 90% of fetal bovine serum and 10% of Di-Methyl-Sulfoxide (DMS) and later were stored at -70°C for the subsequent analyses. Sterilize DMSO was by filtration through a 0.2 µm cellulose membrane and was stored in small quantities (2 mL).

Measurement of Reactive Nitrogen Intermediates (RNI): The RNI concentrations were obtained prior and after vaccination and challenge. RNI were measured spectrophotometrically as the stable metabolite NO₂⁻ according to the Griess method (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid)^[32]. NO₂⁻ and NO₃⁻ optical densities were measurement with ELISA microplate reader (Organon Tecknika, Microwell system). Stock solutions of sodium nitrite and sodium nitrate (Baker) at 100 Mm in phosphates buffer were stored at 4°C. The solution of nitrate reductase (Sigma) at 2.5 U mL⁻¹ and a mixture

of NADPH (Sigma) at 1, 67 mg mL⁻¹ plus flavin adenine dinucleotide-FAD (Sigma) at 0.05 mg mL⁻¹ in sterile desionized water were stored at -20°C. Prior to use, 1 volume of nitrate reductase was mixed 3 volumes of NADPH-FAD (enzyme mixture). The production of nitrite was quantified by comparing the results with absorbance of standard solutions of sodium nitrite. Samples of at least four independent experiments were analyzed in duplicate. The assay was performed in nonsterile 8-well plates. The samples (cell culture medium) were divided between two plates, one for measuring NO₂⁻ and the other for measuring NO₃⁻ in each one 50 µL of sample were added plus 20 µL of water for the measurement of NO₂⁻ and 20 µL of enzyme mixture for the measurement of NO₃⁻ after were incubated for 30 min at room temperature. Griess reagent (100 ml per well) was added and left for 5 min at room temperature and then the Optical Densities (ODs) in all plates were read at 620 nm (reference) and 540 nm (test)^[32]. Nitrite concentrations were calculated directly from the nitrite standard curve. To determine nitrate concentration, OD nitrite was subtracted from OD nitrate before comparison with the nitrate standard curve. Medium alone was used to calculate the assay background level and this was subtracted from all data^[14]. In order to determine whether there was any association between nitrites or nitrates responses and protection against experimental infection with *M. bovis*, these RNI were measured at regular intervals during the vaccine trial.

Statistical method: A design at random with repeat measures in the periods was used completely^[33]. The procedure that it was used was PROC MIXED (SAS, 1999). The used statistical model was the following one:

$$Y_{ijkl} = \mu + \zeta_i + C\zeta_{j(i)} + P_k + \zeta P_{ik} + \beta(x_i - x_{..}) + \epsilon_{ijkl}$$

Concentrations of nitrite were determined by comparison with a standard of sodium nitrite. All samples were assayed in duplicate (version 8.0; SAS Institute Inc., Cary, North, Carolina).

RESULTS

Differences were observed in the nitrites and nitrates responses (Fig. 1 and 2). Control animals NO₂⁻ concentrations were between 7.9-18.64 µM⁻¹, the supplemented treatment with *M. bovis* BCG vaccine were 10.84-17.06 µM⁻¹, the next treatment 1,25(OH)₂D3 were 13.93-25.38 µM⁻¹ and the last treatment *M. bovis*

BCG vaccine plus 1,25(OH)₂D3 were 12.64-8.29 µM⁻¹. Control animals NO₃⁻ concentrations were between 26.06-39.23 µM⁻¹, the supplemented treatment with *M. bovis* BCG vaccine were 17.77-64.27 µM⁻¹,

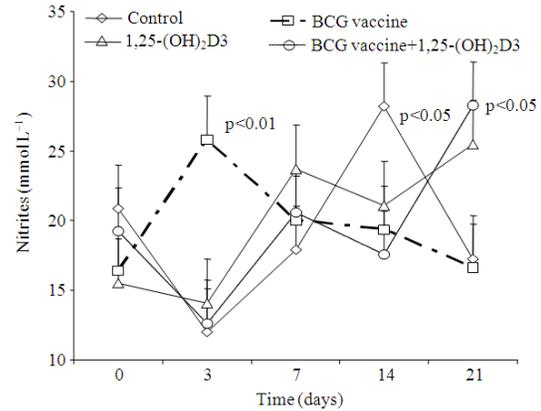


Fig. 1: Average of minimum squares and standard errors of the concentration of nitrites in cells culture (macrophages) when applying different treatments increased the answer of the immune system in goats. The measurement of nitrites was realized in 48 h cells culture. The measurements (n = 12) utilized in this analysis were from five points (prechallenge [day 0] and 3, 7, 14 and 21 days postchallenge

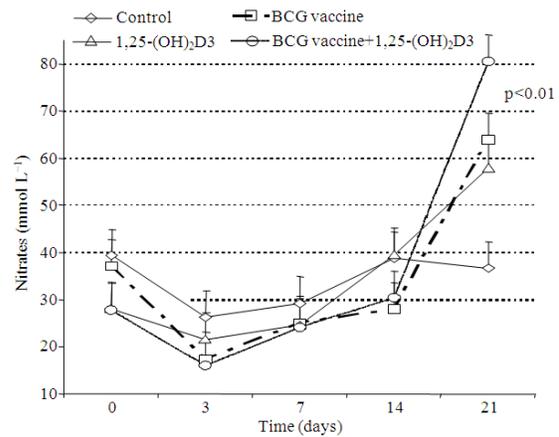


Fig. 2: Average of minimum squares and standard errors of the concentration of nitrates in cells culture (macrophages) when applying different treatments increased the answer of the immune system in goats. The measurement of nitrates was realized in 48 h cells culture. The measurements (n = 12) utilized in this analysis were from five points (prechallenge [day 0] and 3, 7, 14 and 21 days postchallenge

The next treatment 1,25(OH)₂D₃ were 20.94-57.35 μM^{-1} and the last treatment *M. bovis* BCG vaccine plus 1,25(OH)₂D₃ were 16.41-80.94 μM^{-1} . The two groups vaccinated with BCG produced significantly higher mean peripheral blood NO₂⁻ and NO₃⁻ responses to BCG and BCG plus 1,25(OH)₂D₃ than those for the nonvaccinated group (p<0.05). The group vaccinated with BCG plus 1,25(OH)₂D₃ produced significantly higher mean NO₃⁻ response than those for the BCG only group (p< 0.05). There were no significant differences between the mean NO₂⁻. The concentrations differences between NO₂⁻ and NO₃⁻ were considered significant at (p<0.01). However, the treatments effect was no significant (p>0.9). The treatment with the 1,25(OH)₂D₃ stimulated the NO₃⁻ synthesis indicating, that by itself it is a good modulator of the micobacterial replication and in the treatment with *M. bovis*-BCG vaccine increased as a result to the treatment with 1,25(OH)₂D₃. The exhibition to *M. bovis*-BCG vaccine with the treatment with 1,25(OH)₂D₃ was able to increase answer NO₃⁻ in exposed animals.

DISCUSSION

Vitamin D is known for its beneficial effects in diseases with strong Th1 responses, perhaps by altering Th1/Th2 balance *in vivo*^[34]. The monocytes^[35] and the dendritic cells^[36] express the receptors constitutively of the 1,25(OH)₂D₃. However, has been observed that 1,25(OH)₂D₃ treatment inhibited chemokine-induced migration of T cells^[37]. In this study, it was observed that NO₃⁻ concentration was significantly higher in animals with *M. bovis*-BCG plus 1,25(OH)₂D₃ compared with NO₂⁻ concentration. In addition, an increase in the NO₃⁻ production was observed with the challenge to the alone 1,25(OH)₂D₃. Indicating with that it is a good regulate of the micobacterial replication. In addition to this asseveration, has been determined that the active metabolite of the vitamin D is the 1,25(OH)₂D₃, that is a powerful regulator of the immune response^[38]. Interestingly, in a previous study was determined that it is possible that RNI and ROI can inhibit the bactericidal replication (bacteriostatic) but not eradicate the bacterium (bactericidal)^[39]. This agrees with studies^[40] that demonstrated that the reactivation with *M. tuberculosis* happens if the RNI production is inhibited. The NO is a found unstable molecule in the biological systems and quickly is turned NO₂⁻ and NO₃⁻. However, the amount of NO₂⁻ within culture supernatants is indicative of the amount of NO produced by cells in culture^[12]. In this study, the supplemented animals with *M. bovis* BCG vaccine were 10.84-17.06 μM^{-1} . In addition, other studies have

demonstrated that between 34 and 241 nmol of NO₂⁻ in culture supernatants of human peripheral blood monocytes infected with *M. tuberculosis*^[41] are generated. Has been considered that while most virulent strains of *Mycobacterium* are more susceptible to RNI^[32]. The NO in the macrophages acts like a powerful microbicidal to destroy to the ingested microorganisms. We obtained a constant elevation of the concentration of NO₃⁻ to the course of the period of the study arriving at the 21 day to a concentration of 80.94 μM^{-1} . There are evidences of endogenous NO₃⁻ production, especially in answer to inflammatory stimuli where the NO₃⁻ production *in vitro* could be induced by the macrophages in the presence of lipopolysaccharide^[42]. Therefore, it is deduced that the production of RNI in macrophages is essential for the defense of the host, mainly to exert bactericidal actions. In addition, it was demonstrated that the stimulation of iNOS in macrophages and the subsequent generation of RNI are powerful mechanisms to kill the *Mycobacterium*^[42]. Was reported that the first time that *Mycobacterium* induces mRNA for iNOS, iNOS protein, NO and peroxinitrite in human monocyte/macrophage cultures^[31]. Moreover, Waters *et al.*^[12], have investigated 49 cattle with no history of TB and infected with *M. bovis*, they reported that NO concentration was found to be significantly higher in control group than those of healthy animals and they concluded that NO concentrations were altered probably by the effects of some immunocytokines as host defense elements of organism during infection. However, in culture supernatants exists IFN- γ that increased macrophage NO₂⁻ production. In addition, IFN- γ was found to be the critical mediator of NO production^[43]. Protective immunity against intracellular bacteria such as TB species depends on the interplay between various T-cell subsets and cytokines. Cell-mediated immunity to TB is likely to include the production of cytokines that activate macrophages and lymphocytes for production of anti-tuberculosis activities. In this study we determined that 1,25(OH)₂D₃ therapy during acute TB had beneficial effects both clinically and in modulating the systemic humoral and cellular immune response for increased host defense. The present study is the first to assess the effectiveness of BCG administered to goats to induce immune responses and protection against challenge with virulent *M. bovis*.

CONCLUSION

In conclusion, the 1,25(OH)₂D₃ stimulates *in vivo* the production of RNI in goats exposed to *M. bovis* BCG vaccine. Clearly, the high RNI levels observed were associated with the increased of Th1-mediated

immune response and can help to study of many antitubercular treatments. RNI diagnosis can provide new clues about the different clinical outcomes after *Mycobacterium* infection. Further studies are necessary to determine the importance of RNI in cases with tuberculosis.

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