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EVALUATION OF NITRITES IN MONONUCLEAR CELLS IN GOATS EXPOSED TO BCG VACCINE

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Abstract: Tuberculosis (TB) is considered a re-emerging disease and the methods for its diagnosis have low sensitivity, due to the fact that it is a slow-growing bacterium. It is caused by species of the genus Mycobacterium and is a very important public health problem. The objective was to evaluate nitrites in mononuclear cell culture supernatants in goats exposed to the administration of the M. bovis BCG vaccine. BCG vaccination of goats. Ficoll-Hypaque method. Trypan blue dye exclusion test. Detection and quantification of NO_2- by the ELISA method. In the first treatment (control) the concentration of NO_2^- fluctuated between 7.86 and 19.82 µM/l, in the second treatment (BCG vaccine for M. bovis) they were between 13.76 and 84.81 µM/l, observing a greater response three days after starting treatment. In the group to which the BCG vaccine was applied, there was a high production of NO by the activated macrophages, since they have a powerful antimicrobial mechanism. Therefore, it is feasible to evaluate nitrites, to determine latent tuberculosis infection.
Keywords: Tuberculosis, Nitrate reductase, BCG vaccine, *Mycobacterium tuberculosis.*

INTRODUCTION

Tuberculosis (TB) is a communicable disease that has been known since ancient times, it has also been considered the most frequent cause of deaths (Jurado et al., 2015), it is considered a health, economic and social problem of great magnitude to worldwide (Campanico et al 2018). TB is an infectious disease that is among the top 10 causes of death worldwide, and the leading bacterial cause of death (Simoes et al 2020). In 2019, an estimated 10 million people developed TB and 1.5 million people died as a result of the disease (Appetecchia et al 2020; World Health Organization 2020). Currently, the WHO (2023) reports that, in the world, every day, about 4,400 people lose their lives due to TB and about 30,000 people suffer from this preventable and curable disease.

Global efforts to combat TB have saved an estimated 74 million lives since 2000. However, the COVID-19 pandemic and socioeconomic inequalities have reversed years of progress in the fight against TB and have further increased the burden on those affected, especially the most vulnerable. On March 24, 1882, the German doctor Robert Koch discovered the causative agent of this entity Mycobacterium tuberculosis (M. tuberculosis), it is one of the most studied and feared diseases in the history of humanity, of infectious, curable, preventable and currently a re-emerging disease. According to the WHO, it is estimated that a third of the world population has latent TB infection (LTBI), of which 10% will have TB disease, having the capacity to infect 10 to 15 people in a year. (WHO, 2017). 30% of the people who carry the microbacterium are unaware of the TB problem (SSA, 2023), so it is necessary to strengthen the detection capacity of this infectious disease with the active search for cases by health personnel in the community through Through the performance of molecular tests as far as possible, it is necessary to ensure the study of all contacts for each case of TB, as well as adherence to the treatment of the affected person and carry out close follow-up until cure is achieved. The SSA in 2016 reported 64,096 new TB cases nationwide in Mexico, of which Durango has 841 and Coahuila 1,774 cases (SSA, 2016). In 2022, more than 28,000 TB cases are registered in Mexico (SSA, 2023). The only licensed TB vaccine, Bacillus Calmette-Guerin (BCG), is an attenuated M. bovis strain developed through a simple technique by subjecting a virulent M. bovis strain to serial culture in vitro for 13 years, which has been used since 1921 as a preventive measure (Dietrich, Mollenkopf et al. 2002;

Aguilo, Alvarez-Arguedas et al. 2015 and Gu, Chen et al. 2016). One of the immunological indicators that are being used to make the diagnosis of LTBI is the production of nitric oxide (NO), interferon-gamma (IFN-γ) and interleukins (IL) (Waters, Palmer et al. 2002), in At this stage the disease remains inactive. In this regard, it has been observed that the level of exhaled NO increases in TB patients (Liu, Wang et al. 2010). However, an elevated expression level of NOS2 has been immunohistochemically detected in macrophages obtained by alveolar lung lavage from individuals with active pulmonary TB (TBP) (Harjani, Yap et al. 2016). In fact, the inhalation route of drug administration serves as the primary choice for powder or aerosol inhalers, as they provide preferential drug accumulation at the target site as well as being non-invasive and surpassing gastrointestinal drug degradation. Therefore, they can also restrict drug exposure to healthy cells (Nabi et al 2020). In this regard, PAHO (2020) published the unified World Health Organization guidelines on the treatment of drug-resistant TB. First-line drugs are rifampicin (RIF), isoniazid (INH), pyrazinamide, ethambutol, and streptomycin. The second-line group is made up of prothionamide, cycloserine, capreomycin, clofazimine, and rifamycins, and the third group includes ciprofloxacin, levofloxacin, ofloxacin, moxifloxacin, gatifloxacin, amoxicillin/clavulanic acid, and macrolides, among others. The problem with TB is that when the bacteria is activated, it spreads rapidly through saliva, that is, a person who is sick with M. tuberculosis, when talking or coughing, emits aerosols (droplets of saliva) with which can spread the disease to other people who are close to it. The elevated production of nitric oxide (NO) by activated macrophages is a potent antimicrobial mechanism. Experimentally, reactive nitrogen intermediates (RNI) play a protective role in persistent acute and chronic tuberculosis infection. Due to the ability of Mycobacterium to reduce nitrates (NO3-) to nitrites (NO2-) by the action of the enzyme nitrate reductase, a fact that leads to the possibility of evaluating the NO2- activity through culture supernatants of goat mononuclear cells after administration of the M. bovis BCG vaccine. In clinical practice, the nitrate reductase test is useful in the early detection of M. tuberculosis resistance for patient management and infection control, therefore. The objective of the present investigation was to evaluate the activity of nitrites in culture supernatants of peripheral blood mononuclear cells (PBMC) in goats exposed to the administration of the M. bovis BCG vaccine.

MATERIAL AND METHODS

Twelve Sannen breed goats were selected, with an approximate age of 24 months, free of TB, brucellosis and clinically healthy (Figure 1). The animals used in the study were kept in pens isolated from the rest of the cattle. The marking was carried out and each animal was weighed. Of the 12 animals assigned to the study, 6 goats were control and 6 animals to apply the M. bovis BCG vaccine. Sampling was performed by jugular venipuncture using 4 ml Vacutainer® tubes with EDTA. Six goats (control) were inoculated with 0.1 ml of saline solution (Pasteur Merieux, Lyon France).

Another six goats were immunized with the M. bovis BCG vaccine used by the Ministry of Health. 0.1 ml of solution was applied with an intradermal insulin syringe. Samples were taken at the time prior to inoculation (zero days), and subsequently at 3, 7, 14, 21 and 27 days after inoculating the animals, and the RNI were evaluated. (NO_2^-) in PBMC in µmoles/l (Feng, Bean et al. 1999).

Figure 1. Saanen breed goats.

OBTAINING MONONUCLEAR CELLS FROM WHOLE BLOOD BY THE FICOLL-HYPAQUE METHOD

Peripheral blood was collected in Vacutainer® tubes with EDTA and processed in a period of no more than four hours at room temperature. Subsequently, the whole blood was centrifuged at 1500 rpm for 15 min, the plasma was collected and placed in a 15 ml conical tube, the layer of mononuclear cells or cell suspension (SC) present between the plasma and the globular package, subsequently the SC was resuspended in RPMI supplemented with 10% fetal bovine serum (FBS) and 27 mM sodium bicarbonate bringing it to 6 ml, then it was transferred to another conical tube with 3 ml of Ficoll-Hypaque in a slow manner through the walls. of the tube, it was centrifuged again at 1,500 rpm for 15 min (remove the centrifuge brake), 4 layers were obtained, Ficoll, mononuclear, Ficoll, and polymorphonuclear with erythrocytes, respectively (Figure 2).

Subsequently, the mononuclear cell-rich ring was recovered by aspiration with a pipette and washed twice in sterile PBS bringing it up to 10 ml to wash away any remaining Ficoll, keeping on ice and centrifuging at 1500 rpm for 5 min.

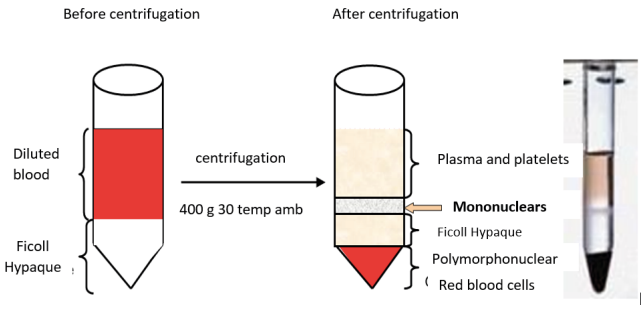

Figure 2. Obtaining peripheral blood mononuclear cells by Ficoll-Hypaque.

DETERMINATION OF CELL VIABILITY USING THE TRYPAN BLUE DYE EXCLUSION TEST

Cell suspensions were resuspended in complete RPMI with SFB. Cell viability was determined using the trypan blue dye exclusion test (Huttunen, Pelkonen et al. 2004). A dilution was made with 100 µl of SC: 90 µl of PBS plus 10 µl of SC (1:10) were added to an eppendorf tube, 10 µl of this suspension was taken and transferred to another eppendorf tube with 80 µl of PBS plus 10 µl of trypan blue (1:100) and immediately afterwards mixed and 10 µl taken to add to a hematocytometer as shown in Figure 3, to count mononuclear cells under a microscope with the weak (10X) dry objective (Gallily, Aalseth et al. 1970). Lymphocytes from four quadrants (1, 3, 7, 9) were read and cells adjusted to 1.32 X 106. Non-viable cells stain dark blue, viable cells exclude dye, remaining unstained (Berger and Edelson 1976).

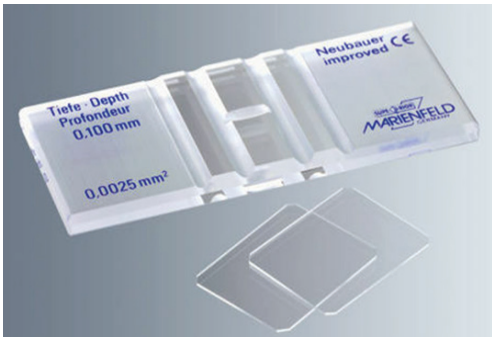

Figure 3. Neubauer Chamber (Brand) or Hematocytometer.

DETECTION AND QUANTIFICATION OF NITRITES (NO2-) BY THE ELISA METHOD

The Griess test is a chemical test that detects the presence of organic nitrites. The Griess diazotization reaction, on which the Griess reagent is based, was first described in 1858 by Peter Griess. Nitrite is detected and analyzed by the formation of a pinkish-red color upon treatment of a sample containing NO2- with Griess's reagent.

When sulfanilic acid is added, the nitrites form a diazonium salt. When α-naphthylamine is added, a pink color develops. Typical commercial Griess reagent contains 0.2% naphthylenediamine dihydrochloride, and 2% sulfanilamide in 5% phosphoric acid.

Sodium nitrite stock solutions ($NaNO_2^-$) (SIGMA®) in 100 mM of water were stored at 4°C, the solution of NO_3^- reductase (Boehringer Mannheim®, Lewes, United Kingdom) at 2.5 U/ml and a mixture of NADPH (SIGMA®) at 1.67 mg/ml plus flavin adenine dinucleotide (FAD) (Boehringer Mannheim®, Lewes, United Kingdom) at 0.05 mg /ml in water were stored at -20°C.

The assay was performed in 96-well flat bottom microplates. 100 µl of mononuclear cell culture was transferred to 96-well plates, adding 50 µl to each, one for the measurement of

NO_2^-. Water (20 ml per well) was added to the microplate of the NO_2^- and the enzyme mixture (20 ml per well). All microplates were incubated for 30 minutes at room temperature. Griess's reagent (100 ml per well) was added and after 5 minutes at room temperature, the optical densities (OD) were read at 620 nm (reference) in all the microplates (Jeevan, McFarland et al. 2006), the absorbance was measured in a ELISA reader (Organon Tecknika®, Microwell system), (**Figure 4**).

Figure 4. ELISA reader (Organon Tecknika®, Microwell system)

NO2- concentrations were calculated directly from the standard curve of sodium nitrite (Rhoades and Orme 1997) in PBS solution in a concentration range of 0 to 100 mM. The tests were carried out in duplicate, obtaining the mean of these.

STATISTIC ANALYSIS

For the statistical analysis of the concentration values obtained from the control and treatment with the BCG vaccine for determination of NO_2^- of the samples, a statistical treatment was carried out for quantitative variables, the Student's T test was used as a parametric test to assess if there is a significant difference at $p<0.05$ between two groups of data, the analysis was carried out with the EXCEL 2010 program.

RESULTS

A total of 12 Sannen goats were included, with an average weight of 53.85 kg (Table 1), with an approximate age of 24 months, free of TB, brucellosis and clinically healthy.

Animal number	Weight (kg)
8	47.62
5	58.96
13	56.69
14	52.16
2	54.43
26	49.89
39	61.23
36	52.16
21	56.69
34	49.89
20	58.96
3	47.62
Total	646.3
Average	53.8583333

Table 1. Average weight of goats.

The viability of the cells fluctuated between the ranges of 80 and 85%, in some cases it was possible to obtain a viability of up to 95%, which indicated that the work being carried out was of very good quality.

NITRITE LEVELS (NO2-)

The optical density of the mononuclear cell cultures was obtained, a standard curve of $NaNO_2^-$ (**Table 2**) to calculate the existing concentrations in each sample of NO2-. For the standard solution, 0.5 g of $NaNO_2$ pure, to dissolve in a liter of water, free of nitrites. Dilute 10 ml of this solution to one liter with distilled water (1 ml = 0.005 mg of $NaNO_2$).

Sodium nitrite solution 100 µmol/mL	0	**0.1**	0.2	0.5	1
Distilled water (nitrite free) - distilled	3	2.9	2.9	2.5	2
Griess test	2	2	2	2	2
µmol nitrito/mL	0	**10**	20	50	100
DO	0.059	**0.117**	0.162	0.333	0.576

Table 2. Sodium nitrite standard curve

Mix thoroughly and after 20 min read in the UV-visible spectrophotometer at l= 520 nm. Adjust zero with blank. Plot a curve plotting concentration (mg NaNO2) versus absorbance.

100 µmol – 1 mL

x µmol – 0.1 mL x= **10 µmol nitrito/mL**

10 µmol – **0.117**

x µmol – 0.162 x= **13.8461538 µmol/mL** (Control, first sampling. Day zero)

The behavior of the different treatments during the sampling period, although it was variable, an increase of NO_2^- **(Table 3).**

Treatment	Maximum concentration	Minimum concentration
Saline solution (Control)	7.86324786 mmol/mL	19.8290598 mmol/mL
BCG vaccine of *M. bovis.*	12.6495726 mmol/mL	94.5299145 mmol/mL

Table 3. Concentrations of NO_2-

With a significance level of 0.05 ($p < 0.05$), a significant difference was obtained in the effect of the variation of the optical densities, as well as the concentration in µmol/l of NO_2- during the sampling periods of the 27 days.

Since the interaction type of treatment to goats in the sampling periods is significant, the presentation of results will only be subject to this variation factor, since it more fully describes the biological behavior of the study. With a significance level of 0.05 (p <

0.05), a significant difference was obtained in the effect of DO variation, as well as in the concentration in μmol/l of NO_2- during the 27-day sampling periods.

DISCUSSION

The ability of M. tuberculosis to survive and reproduce within macrophages, even in the presence of adverse factors such as NO, essential for the development of the infection. The identification of the mechanisms used by mycobacteria to evade nitrosant damage is an important objective as it could help develop strategies to prevent TB (Espinosa-Cueto et al., 2015). In a previous investigation, the ranges of NO2- and NO3- concentrations in plasma samples from healthy individuals were observed to be 1.3-13 μM/l (mean 4.2 μM/l) and 4.0-45.3 μM/l (average of 19.7 μM/l), respectively (Moshage, Kok et al. 1995) and despite the fact that the present experiment was carried out in goats, as a comparison we show the average value of NO2- levels of control animals during the sampling of 27 days was 7.86324786 and 19.8290598 mM/l (average 14.54629628 μM/l), in the second treatment (BCG vaccine of *M. bovis*) between 12.6495726 and 94.5299145 mM/l (media de 33.09164306mM/l).

This value coincides with the average range reported by other researchers in the determination of NIR, which was 25.1 ± 3.59 μM of NO2-/NO3- in cultures carried out for 1, 3 and 7 days of macrophages derived from peripheral blood monocytes in patients with leprosy. (Khare, Bhutani et al. 1997). This fact is evidence that the induction of the immune system through PBMC is achieved with the stimulation of a live, dead or attenuated microorganism. Waters, Palmer et al. (2003) published that the NO_2^- s are an oxidation product of NO, and the amount of NO2- produced by cells in culture is indicative of the amount of NO produced. Similar results were obtained from a study carried out to determine the accumulation of NO2- after four or more days of infection (Rhoades and Orme 1997). Previous studies reported a decrease in NO2-/NO3- levels with treatment, which was correlated with the reduction in bacillary load (Rada, Ulrich et al. 1997).

These findings agree with the results obtained in this study when observing that in the control animals production of NO_2^-/NO_3^- during the duration of the study. In addition to this assertion, there are reports on research carried out on macrophage cultures in patients with Hansen's disease, which demonstrated that macrophages from patients with leprosy and TB were capable of producing NO in the absence of stimulation. The former produced high levels of NO after stimulation. 24 hours of culture and subsequently a gradual decrease was observed as the culture time increased, in the controls very low levels were observed in the release of NO_2^- (Khare, Bhutani *et al.* 1997).

In other research carried out in this regard, it was deduced that NO inhibits the development of many bacteria and parasites in vitro, due to the production of NIR formed by the oxidation of NO (Burgner, Rockett et al. 1999). Previous studies reported high levels of NO_2^-/NO_3^- 1020 +/- 471 mM (n=22).

Concentrations of 1079 +/- 446 μM, (n=12) respectively, were observed in urine samples from healthy Ethiopian patients and patients with leprosy, and these presented type I reversal reactions (Schon, Gebre et al. 1999).

Waters and Palmer (2003) found that the amount of NO2- in culture supernatants is indicative of the amount of NO produced by cells in culture. The target of NIRs in mycobacterial diseases is not yet known, and understanding of their role in mycobacterial killing is essential (Rhee, Erdjument-Bromage et al. 2005).

The Ministry of Health of Peru (2011)

determined that the nitrate-reductase (Griess) method is useful for the rapid detection of susceptibility to INH and RIF, in a validation study of the sensitivity and specificity of the Griess method carried out at the Mycobacteria Laboratory found a sensitivity and specificity of 99.1% and 100% for INH and 93.5% and 100% for RIF.

With the present investigation, it is deduced that the production of NO is associated with the biochemical nature of the antigen, for which reason it is proposed to carry out this same assay using certain mycobacterial components such as phenolic glycolipid.

It would be interesting, in another work, to increase the number of samples, with more specific studies of nitrite expression and activity, which does not exist in resting macrophages, but can be induced in response to LPS in combination with IFN-g, since the enzyme iNOS catalyzes the conversion of arginine to citrulline, releasing NO.

CONCLUSION

The elevated NO production by activated macrophages is a potent free radical-based antimicrobial mechanism. NIRs play a protective role in acute and chronic latent TB infection. The present investigation allowed the evaluation of the activity of the enzyme nitrate reductase determining NO_2- in PBMC culture supernatants from goats exposed to the M. bovis BCG vaccine, measuring the activity of the NO_2-, which allows a precise evaluation of the latency state of Mycobacterium as a fundamental element of public health to control TB. In the face of primary infection, a high production of NO by activated macrophages occurs, even in patients with active TB it is observed that the level of exhaled NO increases, as well as the expression of the enzyme nitrate reductase in macrophages obtained by lung lavage. alveolar.

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