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Effect of the analogue of L-carnitine, *β*-hydroxyphosphocarnitine on the metabolism of *Nocardia brasiliensis*

Efecto del análogo de L-carnitina, la *β*-hidroxifosfocarnitina sobre el metabolismo de *Nocardia brasiliensis*

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Abstract

Objectives: To determine whether β-HFC, in addition to its immunomodulatory effect as a therapeutic alternative in the combat of the mycetoma has an effect on the biochemical activity of *N. brasiliensis*. **Methodology:** determination of bacterial growth using N. brasilensis in the presence of β-HFC and biochemical analysis of its metabolism. **Contribution:** The immunomodulatory function of β-HFC in the treatment of chronic infections has recently been studied but it is not known whether it also has any antimicrobial effect, so in this paper the direct effect of β-HFC in *N. brasiliensis* will be elucidated in part.

Inmunomodulator, β-HFC, *Nocardia brasiliensis*

Resumen

Objetivos: Determinar si la β-HFC, además de su efecto inmunomodulador como alternativa terapéutica en el combate del micetoma tiene efecto en la actividad bioqímica de *N. brasiliensis*. **Metodología:** Se determinó el crecimiento bacteriano utilizando de *N. brasilensis* en presencia de β-HFC y análisis bioquímico de su metabolismo. **Contribution:** Recientemente se estudia la función inmunomoduladora de la β-HFC en el tratamiento de infecciones crónicas pero no se sabe si también tiene algun efecto antimicrobiano, por lo que en este trabajo se dilucidará en parte el efecto directo de β-HFC en *N. brasiliensis*.

Inmunomodulador, β-HFC, *Nocardia brasiliensis*

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Introduction

L-carnitine is a quaternary amine that in eukaryotes acts as a shuttle of long-chain fatty acids through the inner membrane of the mitochondria so that beta oxidation is carried out in the mitochondrial matrix (1), in bacteria, Carnitine is transported to the cytosol by the ABC system, or by the biotin / choline / carnitine transporter, in Gram positive and negative it is used by aerobic and anaerobic routes, as the final electron acceptor or as the sole source of carbon and nitrogen, to be catabolized to trimethylamine and malic semialdehyde, the latter will enter the cycle of tricarboxylic acids (2).

Beta-hydroxyphysphocarnitine (β-HFC), is an analogue of L-carnitine, which in addition to participating in lipid metabolism, lowers blood levels of glucose, cholesterol and increases circulating T lymphocytes (3), therefore β-HFC It is a good candidate to be an immunomodulator in infectious processes. Such as the mycetoma, chronic infection of the skin and underlying tissues with a tendency to affect bone, is characterized by a relatively painless increase in volume and fistulas through which pus and grains constituted by filaments are eliminated. In Mexico, the mycetoma is caused in 65% of cases by *Nocardia brasiliensis* (4), a Gram positive bacterium belonging to the phylum Actinobacteria, Cummins (5), Lechevalier (6) and Goodfellow (7), they place it Mycobacterium and Corynebacterium taxonomic group: all of them are distinguished by being aerobic, alcohol-acid variables, have branched mycelium or an aerial mycelium that tends to fragment, classification IV based on the chemotype of the wall and serology the walls of *N. brasiliensis, Mycobacterium tuberculosis and Corynebacterium diphtheriae types gravis and mitis* are closely related, since these genera cause diseases in humans and are rarely isolated from soils rich in decaying matter even when it is the natural habitat of actinomycetes (7).

The microbiological diagnosis of *N. brasiliensis*, long and thin filaments are observed in Gram staining, or forming clusters or tangles, are positive catalase, negative oxidase, use glucose, inositol and mannitol by oxidative route, hydrolyze casein, hypoxanthine, tyrosine and urea.

In addition they have in their genome other 32 proteases (8) Nocardia as a genus is biologically active, produces biomolecules by the route of synthesis of non-ribosomal peptides (NRPS) with three domains: A of adenylation, T of thiolation and C of condensation, domain A acts on an L-α-amino acid catalyzing the adenylation of the carboxyl end, in an ATP-dependent manner in a reversible classic ATP / Ppi reaction, (9). On the other hand, *in silico* studies of the lipid metabolism of said bacterium, it includes 15 acyl-CoA synthetases, 6 long-chain acyl CoA synthetases, 12 enoyl-CoA hydrostases / isomerases, 12 acetyl CoA acetyltransferases and a beta oxidation complex FadA / FadB (8) *N. cyriacigeorgica* for example, hydrolyses to petroleum-derived n-alkanes using an oxidative route from which alcohols, acid aldehydes and CoA esters are obtained with the subsequent beta oxidation (10), which demonstrates that these bacteria have developed strategies to produce surfactants or to join oils by hydrophobic bonds (11). Because the physiopathogenesis of *N. brasiliensis* depends on hydrolytic reactions that provide space and substrate in the host, it is necessary to know if the proteolysis and hydrolysis of fatty acids is susceptible to modulation by molecules that are used in immunomodulatory therapies so that The objective of this work is to determine whether, in addition to its immunomodulatory effect as a therapeutic alternative in the fight against mycetoma, β-HFC has an effect on the biochemical activity of *N. brasiliensis*, on the synthesis of biotechnological contribution molecules.

Methodology

Determination of antimicrobial activity by minimum inhibitory concentration (MIC)

To determine if β-HFC has an antibiotic effect, the minimum inhibitory concentration (MIC) and agar diffusion were evaluated, sulfamethoxazol-trimethoprim was used as a control antibiotic against *N. brasiliensis*, in both methods the concentration 62-64 μg / mL. Gram positive bacteria strains Staphylococcus aureus, Rhodococcus equi, *N. brasiliensis* FM-825, Nocardia brasiliensis HUJEG-1 ATC700358 and Escherichia coli were used as Gram negative, purity was verified according to Cowan and Steele's methodology (14), Conventional microbiology material was used, at a temperature of 25° C.

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For tests with *N. brasiliensis* and Rhodococcus equi, brain and heart infusion broth (BHI) agar, sheep blood agar, Dibico ©, for the other bacteria were used Müeller-Hinton Dibico © (MH) broth and agar was used, the Mc Farland turbidity standard $1.5x10^8$ bacteria / ml (tube 0.5) was used and for the challenges the pure sulfamethoxazole salt (Sigma®) was used and the formula Sulfamethoxazole-Trimethoprim injection (Agro-Vet® Laboratories), β-HFC was provided by Nucitec © laboratories

Preparation of work solutions

320µg of sulfamethoxazole, solubilized in 2 ml of dimethylsulfoxide (DMSO) and graduated to 5ml, the working concentration was 64 µg / ml; 320µg of β-HFC were solubilized in 5 ml of PBS with the same working concentration, both solutions were sterilized by filtration with low bonded membrane and cooled until use.

In a 24-well plate, 1000 µL were dispensed in each of them, in well A1, 1000 microliters of the antimicrobial solution was served at the concentration indicated in document M-24-A, starting from this well obtained a 1: 2 concentration, 10 double dilutions were made, well B-5 was growth control (+) and well B-6 as sterility control (-) as shown in figure 1.

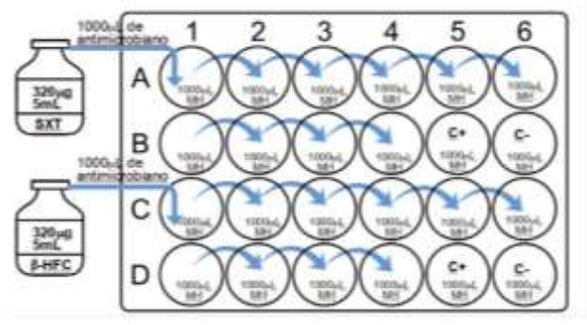

Figura 1 Preparation of strains and bacterial suspension for the MIC

In 50 mL of BHI broth, an inoculum of *N. brasiliensis* was seeded, after 7 days from the surface of the broth the growth film was taken, the bacterium was washed with PBS and disintegrated in Ten Broeck macerator until an orange suspension was obtained sui generis smell. The other bacterial strains were seeded in broth and M-H agar and manipulated according to microbiological techniques of antimicrobial susceptibility testing.

A suspension was adjusted to the turbidity standard 0.5 of the Mc Farland $1.5X10^8$ nephelometer (150,000,000 CFU / mL), hence 0.5 mL was taken and 4.5 mL was added to a PBS tube (1:10 dilution), 1.5X107 (15,000,000 CFU / ml) 4 mL of the previous suspension was added to 36 mL of MH broth (1:10 dilution) $1.5X10^6$ (1,500,000 CFU / mL)

10 microliters were served in 100 microliters of the bacterial suspension (1:10 dilution) $1.5X\ 10^5$ (150,000 CFU / mL) per well of a 96-well plate (12 wells for this work modifying the quantities but retaining the concentrations) was dispensed in each of the wells of the plate as shown in Figure 2.

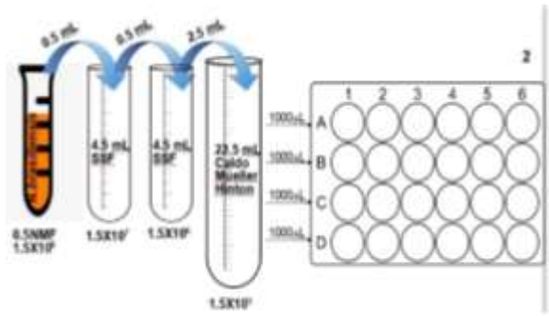

Figure 2 Microbial susceptibility testing protocol

From the suspension adjusted to 0.5 of the Mac Farland standard, a swab was taken and massively seeded in blood agar plate, commercial unidisks (Biorad) were impregnated with sulfamethoxazol-trimethoprim (STX), erythromycin 15 µg (E 15)) and dicloxacillin 1µg (DC), for the β-HFC were made well os with punch in agar and / or filter paper discs (64 µg / mL).

Determination of plate hydrolytic activity

From a solution of *N. brasiliensis* adjusted to 0.5 of the Mac Faland nephelometer in PBS plus β-HFC with a concentration of 64 mg / mL, in tween 80 agar plates, egg yolk, casein and tyrosine, prepared according At the indications of Cowan & Steele's (12) and López Martínez (13), 10 µL of the bacteria were sown in the media, in addition a batch of control group was prepared where *N. brasiliensis* was not in contact with the β-HFC. media were seeded the same day and allowed to grow at 25 ° C for 7 days.

The hydrolysis was developed with congo red or with lugol, the indicator was poured on the surface completely covering the agar, it was allowed to interact with the medium for 15 minutes, after time it was removed, the excess was rinsed with sterile SSF and dried to room temperature.

To obtain the power index, the diameter of the growth (A) and the diameter of the hydrolysis (B) were measured and B / A divided, the same experiment was performed but in broths with the substrates, plus β-HFC at a concentration of 64 μg / mL, the total volume of the bacteria plus the substrate was 2 mL, the supernatants were recovered and centrifuged to release them from the bacterium. The absorbance was read on Tecan GeniOS®, DO plate reader. 405, 540, 595 and 620.

Results

β-HFC has no bacteriostatic or bactericidal effect

The bacteria used in the test both Gram positive and negative showed no problems in their growth when incubated in the presence of β-HFC, in fact in the first well where it is more concentrated, there was greater growth (Figure 3), same as was corroborated by the technique of Miles & Misra (14), where the standard 0.5 of Mac Farland gave 240,000 CFU against 720,000 CFU of a solution of *N. brasiliensis* plus 64 mg / mL of β-HFC, in addition the STX was chosen as Control antibiotic since it is the one indicated to treat people suffering from mycetoma against *N. brasiliensis*, surprisingly, the MIC and agar diffusion tests showed that the bacteria are resistant (Figures 4 and 5a) to this antibiotic with the exception of S aureus, which was sensitive to a concentration of 0.25 mg / mL, *Rhodhococcus* equi had intermediate sensitivity (Figure 5b).

Figure 3 Bacterial growth in the presence of increasing concentrations of B-HFC

Figure 4 Bacterial growth in the presence of increasing concentrations of B-HFC

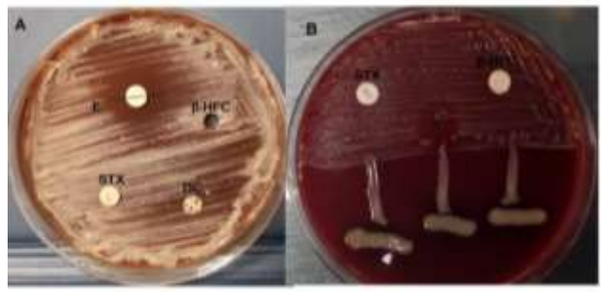

Figure 5 Babcterial growth by diffusion in agar. In the presence of B-HFC and STX. A) Nocardia brasiliensis, partial sensitivity (10 mm) to erythromycin, resistance to other antibiotics, B) Rhodococcus equi, partial sensitivity (10mm)

B-HFC increases the metabolic activity of *N. brasiliensis*

In the solid media with β-HFC the bacteria grew abundantly, and a difference was observed in the hydrolysis of the media with respect to the control, as shown in Figure 6.

Figure 6 *N. Brasiliensis* hydrolytic activity in the presence of B-HFC (left) and control (right)

The power index was obtained, the results are shown in table 1, with all the substrates used for the growth of N. brasilensis the index was greater than 1 indicating that β-HFC stimulated the metabolism of the bacteria.

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Sustrato	Tratamiento	Crecimiento (cm) (A)	Halo de hidrólisis (cm) (B)	Índice B/A
Tirosina	*N. brasiliensis*	2	2.57	1.28
	N. brasiliensis + β-HFC	2	2.7	1.35
Caseína	*N. brasiliensis*	2	2.37	1.18
	N. brasiliensis + β-HFC	2	2.47	1.23
Yema de huevo	*N. brasiliensis*	2	2.23	1.11
	N. brasiliensis + β-HFC	2	2.3	1.15
Tween 80	*N. brasiliensis*	2	5.3	1.06
	N. brasiliensis + β-HFC	2	5.37	1.1

Table 1 Power index of substrate hydrolysis by *N. Brasiliensis*

The supernatants recovered from the broth hydrolysis also showed some differences, Figure 7 shows the graphs of the quantified absorbances analyzed by an ANOVA, a significant difference was observed, which is checked in the graph of pairs where tween 80, yolk of Egg and tyrosine show significant differences between them. As shown in the graph boxes and mustaches (figure 7A) and pairs (figure 7B). Additionally, a viable account was made, and it was confirmed that β-HFC has a growth activity of the bacterium by the amount of CFU counted (Table 2)

Sustrato	Estándar UFC/ml	Control UFC/ml	β-HFC UFC/ml
Tween 80	$2.4X10^5$	$4.58X10^6$ ± (1.2)	$1.1X10^7$ ±(1.2)
Caseína	$2.4X10^5$	$9X10^4$ ± (8.4)	$9X10^5$ ± (6.7)
Tirosina	$2.4X10^5$	$5.88X10^6$ ± (2.3)	$7.46X10^6$ ± (9.0)
Yema de huevo	$2.4X10^5$	$7.8X10^5$ ± (1.2)	$1.73X10^5$ ± (4.5)

Table 2 Colony forming units (CFU) of N. *brasiliensis* + β-HFC

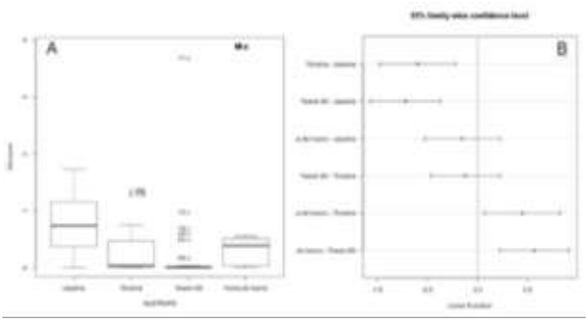

Figure 7 Quantification of the hydrolysis of N. Brasiliensis on different substrates a. Measurement of optical density. B. Peer quantification

Discussion

A bactericidal or bacteriostatic activity for L-carnitine has not been reported, it is used in weight and height control in obese people (15), and lately the immune response (16) has taken an important role in modulation, has studied its activity in tuberculosis, a chronic infectious disease of great importance worldwide, a report mentions an "antibacterial" effect of carnitine when administered orally, which resulted in an increase in the activity of CD4 + and CD8 + T lymphocytes possibly due to the increase in ATP that carnitine contributes to metabolism (17). In this work a carnitine analogue was used, and it was found that not only does it not have any antimicrobial activity, but it can also be used as a source of carbon and nitrogen (2). Because *N. brasiliensis* has a hydrolytic metabolism which causes chronic infections and remains viable for a long time, it is possible to think that it may not be appropriate to use carnitine as a therapy against it, perhaps the bacteria is able to carry Just β oxidation, according to the results obtained, substrates rich in fatty acids (tween 80 and egg yolk) and with amino acids (casein and tyrosine) were used for the proteolysis analysis of *N. brasiliensis* on the same over said compounds, Results obtained qualitatively speaking with the β-HFC analogue indicate that if the hydrolysis of the substrates increases (table 1), for the verification of significance in the difference, the analyzes were performed in visible spectrophotometry, the results show significant difference in the substrates rich in fatty acids (figure 7B), which suggests that the bacterium performs β oxidation and that it also allows mul type your number. The results obtained show that β-HFC does have an effect on the metabolism of *N. brasiliensis*, the main effect is on the increase of CFU, so carnitine favors both bacteria and T lymphocytes (2,17- 19).

Conclusions

Many questions arise with the findings, if carnitine favors both bacteria and T lymphocytes, will the bacteria and the immune system have to compete in a mycetoma infection? If the bacterium is stopped being treated as a pathogen and included as a biotechnological entity, if the administration of β-HFC favors the synthesis of biomolecules, can they be used against it to eliminate the microtome in patients suffering from it?

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Elucidate the metabolic pathway that is induced to confirm that the β-HFC effectively activates the beta oxidation of *N. brasiliensis*.

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