Anti-bacterial Properties of Melatonin against *Mycobacterium Tuberculosis in Vitro*

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Abstract

57 isolates of *Mycobacterium tuberculosis* and *Mycobacterium bovis* were identified; they were isolated from different clinical sources which included sputum, bronchial wash, abscess, pleural fluid, gastric fluid, eye fluid, and CSF, also urine and ear swab. This investigation was carried out on 198 patient attended National Reference Laboratory for T.B during September 2009. Also the study declared that the ratio of separation of this bacterium from male was (67.6%) and it's higher than the ratio of separation this bacterium from females which was (32.3%). The susceptibility of *Mycobacterium tuberculosis* to melatonin was evaluated. Many concentrations of melatonin were prepared to investigate it as antibacterial drug against multidrug resistant *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Suspension bacteria (10^{-1}, 10^{-3} and 10^{-5}) were cultured on Lowenstein-Jensen media (LJ) contains melatonin, while control media without this drug. Six isolates were chosen according to their susceptibility patterns; they were resisting to Rifampicin, Streptomycin, Isonicotinic –hydrazide and sensitive to Ethambutol. In conclusion, these *in vitro* studies clearly demonstrate anti-bacterial effects of melatonin. Among possible mechanisms, it is concluded that melatonin showed antibacterial effects against multidrug resistant T.B by reducing intracellular substrates. Identifying the mode of action could be of great help in developing and researching new anti-bacterial drugs.

***Key words: Antibacterial, Melatonin, Mycobacterium tuberculosis*.**

الخلاصة

تم تشخيص 57 عزلة موجبة من العصيات الدرنية الفطرية والعصيات الدرنية البقرية من المصادر السريرية المختلفة والتي تضمنت القشع ، الغسل القصبي، الخراج، سائل الجنب والمعدة والعين والذخاعي كذلك الادرار ومسحة الأذن. نفذت هذه الدراسة على 198 مريض راجع المختبر الوطني المرجعي للتدرن خلال شهر ايلول 2009. أوضحت الدراسة ان نسبة اصابة الذكور(67.6%) هي اعلى من نسبة اصابة الأناث (32.3%) تم تقييم حساسية العصيات التدرنية لعقار الميلاتونين. تم استخدام عدة تراكيز من الميلاتونين كمضاد للبكتريا ضد عصيات التدرن *M. Tuberculosis* و *M. bovis* ذات المقاومة المتعددة للمضادات. زرعت عدة تخافيف للعالق البكتيري (10^{-1},10^{-3},10^{-5}) على وسط (LJ) والذي يحتوي على عقار الميلاتونين بينما وسط السيطرة لايحوي هذا العقار. اختيرت ستة عزلات بالأعتماد على الأنماط التحسسية حيث كانت هذه العزلات مقاومة للريفامبسين، الستربتوميسين، ايزونيكوتنك هايدرازايدINH، وحساسة للايثامبيتول. نستنتج من هذه الدراسة التاثيرالتثبيطي للميلاتونين على البكتريا مختبرياوالميكانيكية المحتملة بواسطة تأثيره على داخل الخلية للبكتريا وبذلك يفتح الآفاق لمضاد بكتيري جديد.

Introduction

Although tuberculosis (TB) is a preventable and treatable disease, it causes 3 million deaths annually. The current situation of TB is unique, mainly due to two aspects: the association between TB and human immunodeficiency virus (HIV) infection, and the global spread of virulent strains resistant to key antituberculous drugs. There is a direct association between HIV infection and the reactivation of latent TB or the progression of TB following newly acquired infection [(1)]. Melatonin originally identified as an effector for circadian rhythms, is now known to be a hormone involved in a vast range of homeostasis maintenance activities, for example seasonal timing, sexual development, the antioxidant defense system and immune response [(2)]. Melatonin is synthesized from tryptophan within the serotonin pathway mainly in the pineal gland, and in a number of extrapineal organs such as retina, lens, bone marrow, intestine, skin and so on. To date, three mammalian melatonin receptors, G-protein coupled receptors MT1 and MT2, and a quinone reductase family receptor, MT3, have been identified [(3)]. Melatonin is a highly studied endogenous molecule. This indolamine has a variety of beneficial effects within cells and organisms, including cell cycle regulation [(4)]. Although there are a plethora of studies on melatonin, only a few of them relate to it in vitro antimicrobial activities [(5–7)] on to its effects in infectious diseases [(8-10)].

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New in vitro antimicrobial studies using melatonin suggested that it has limited antimicrobial properties [11, 12] while one study found that melatonin inhibited *Candida albicans* at a concentration of 300 µg/ml [12].In contrast to the in vitro studies, virtually all in vivo studies performed with melatonin in infectious disease models documented it as a successful therapy [9, 13]. Melatonin is a highly versatile molecule. One example is its ability to limit the growth of a variety of tumor types. One of several proposed mechanisms to explain melatonin's inhibitory actions on cancer growth is its ability to curtail the uptake of growth factors which promote cell proliferation [14]. Linoleic acid (LA), an essential omega-6 polyunsaturated fatty acid is a growth factor for a number of tumor types. Via an action on the cell membrane, melatonin prevents the uptake of this fatty acid by cancer cells which reduces the activation of genes that promote cell proliferation [15]. Similar actions of melatonin on the bacterial wall thus may restrict the survivability of bacteria. Additionally, melatonin has a high metal binding capacity. Melatonin binds iron (III), copper and zinc thereby reducing their cytoplasmic availability. Bacteria are strongly dependent on free metals, in particular, free iron for growth [16]. Clearly, there are several potential mechanisms that may explain the possible antibacterial efficacy of melatonin. In the current study we tested the antimicrobial effects of melatonin against resistant strains of *Mycobacterium tuberculosis*.

Materials and Methods

Samples

A total of 198 clinical samples from National Reference Laboratory for T.B, were included in this study. They were collected during November 2009. Identification of these isolates as *Mycobacterium tuberculosis* and *Mycobacterium bovis* were based on biochemical properties [17]. The most samples were sputum; they are transfer to the laboratory in sterile container. Three samples from each patient were taken to diagnose tuberculosis. Susceptibility test was done to 13 isolates from 57 positive samples to tuberculosis according to routine work of national reference laboratory for tuberculosis. All samples treat with sodium hydroxide and hydrochloric acid to remove all microorganisms and epithelial cells (Petroffs method). Zeihl-Neelsen stain was done for all specimens.

Culture the Specimens

The specimens was cultured on Lowenstein-Jensen media (LJ) pouring in screw –capped tubes in final volume 6ml, these tubes put in oven at 80-85 C for 45 min. The media left for 24 hr to be insure that there is no contamination. The treated specimens will culturing by using Pasture pipette, (0.4-0.2) ml from inoculme of specimen must be transferred to LJ media and let the culture for 72 hr at 37 C in incubators(in slope position), then for 50 days at 37 C (in vertical position).

Susceptibility Test

Susceptibility test was done by using the proportional method[18] , four antibiotic solutions were used; Rifampicin 40 µg/ml, Streptomycin 4 µg/ml, Ethambutol 2 µg/ml, Isonicotinic hydrazide 0.2 µg/ml. *Mycobacterium tuberculosis* suspension was prepared in many dilutions 10^{-1} -10^{-5}, the primary dilution adjusted with MacFerland solution(3×10^{8}) CFU/ml. 100 µl from 10^{-1}, $10^{-3}$and 10^{-5} dilution was cultured on media (LJ) contain antibiotics and without antibiotics as control. After 6-8 weeks the result must be read, if the growth on media contains antibiotics more than control media this means resistant bacteria. While the growth consider sensitive when the growth less than control media, for this test we chose 4 isolates from *Mycobacterium tuberculosis* and 2 isolates from *Mycobacterium bovis*, according to their typing (catalase, nitrase test, niacin test and colony form).

Melatonin drug

In this study, many concentration of melatonin dissolved in 96% ethanol (0.4, 0.2, 0.1, 0.05) mg/ml were prepared to investigate it as antibacterial drug against multi-drug resistant *Mycobacterium tuberculosis* and *Mycobacterium bovis*.Suspension bacteria (10^{-1}, 10^{-3} and 10^{-5}) were cultured on LJ media contains melatonin, while control media without this drug. Six isolates were chosen according to their susceptibility patterns, they were resist to Rifampicin, Streptomycin, Isonicotinic –hydrazide and sensitive to Ethambutol [19].

Results

Table -1 showed us the number of *Mycobacterium tuberculosis* isolated from many sources. It was found that 57 samples were positive samples. Table-2 shows number and percentage of infection with *M. tuberculosis* and *M. bovis* according to the sex, it was found that 134(67.6%) of patients were males and 64(32.3%) were females with present of statistical significant. Table-3 shows the percentage of infected with *M. tuberculosis* and *M.bovis* according to the age. The higher percentage rate (46.2%) was found in the group of (30-39 year) while the lowest

percentage rate (6.7%) was found in the group (70≥ year) with present of statistical differences. Table-3 shows the number and percentage of infection with *Mycobacterium SPP*. According to the sex, it was found that 134 (67.6%) of patients were males and 64(32.3%) were females with present of statistical significant differences. Table-4 shows the effect of many concentration of melatonin on *M. tuberculosis* and *M.bovis* isolates. It was found that *M. tuberculosis* and *M.bovis* sensitized to the concentrations 0.05, 0.1, 0.2 and 0.4mg/ml. Table-5 shows the susceptibility patterns of many dilution of *M. tuberculosis* and *M. bovis* to different concentration of melatonin. It was found that dilution of bacteria 10^{-1}, 10^{-3} and 10^{-5} were sensitized to all concentrations of melatonin that were used. Figure -1 shows the growth of *M. tuberculosis* and *M bovis*. Resistant to Rifampicin, streptomycin and INH and were sensitize to Ethambutol.and Melatonin.

Table 1 : Number of *M.tuberculosis* and *M.bovis* isolated from many sources

Isolates source	Number of Examined Samples	Number Of Positive Sample	
		No.	(%)
Sputum	145	55	96.5
Bronchial wash	19	1	1.8
Abscess	5	1	1.8
Pleural Fluid	13	-	-
Gastric Fluid	3	-	-
Eye Fluid	2	-	-
Knee Fluid	1	-	-
C.S.F	1	-	-
Ear Swabs	1	-	-
Urine	8	-	-
Total	198	57	

Table 2 : Number and percentage of infection with *M. tuberculosis* and *M. bovis* according to the sex

Sex of Patients	Examined samples		Number of positive M. tuberculosis isolates		Number of positive M. bovis isolates	
	No.	%	No.	%	No.	%
Male	134	67.7	23	65.7	14	63.6
female	64	32.3	12	34.3	8	36.4
Total	198		35		22	

Table 3 : Percentage of infected with *M. tuberculosis* and *M.bovis* according to age.

Age Groups	Number of Examined Samples	Number of Positive Sample	
		No.	(%)
10< year	6	0	0
10-19 year	16	6	*37.5 %
20-29 year	36	14	38.9 %
30-39 year	39	18	46.2 %
40-49 year	29	6	37.5 %
50-59 year	41	8	19.5 %
60-69 year	16	4	25 %
70≥ year	15	1	**6.7 %
Total	198	57	

** Statistically significant difference

Table 4 : Effect of many concentration of melatonin on *Mycobacterium SPP*. Isolates

Code of isolates	Melatonin concentration (mg/ml)				
	(0.4)	(0.2)	(0.1)	(0.05)	Control
	S	S	S	S	R
L_2	S	S	S	S	R
L_3	S	S	S	S	R
L_4	S	S	S	S	R
L_5^-	S	S	S	S	R
L_6^*	S	S	S	S	R

L =isolate, S =Sensitive, R=Resist, *= *M. bovis*

Table 5 : Susceptibility patterns of many dilutions M.tuberculosis and M.bovis to many concentration of Melatonin

Dilution of Bacteria	Melatonin concentration mg/ml				
	(0.4)	(0.2)	(0.1)	(0.05)	Control
3×10^7 CFU/ml (10^{-1})	S	S	S	S	R
3×10^5 CFU/ml (10^{-3})	S	S	S	S	R
3×10^3 CFU/ml (10^{-5})	S	S	S	S	R

Figure 1 : Growth of M.tuberculosis resists to Rifampicin,Streptomycin, and INH and was sensitized to Ethambutol and Melatonin on a Lowesten agar slant (from the left to the right).

Discussion

The results of the present study indicate that melatonin has in vitro antimicrobial activity against strains of antibiotic-resistant mycobacterium tuberculosis. As melatonin is weakly soluble in water, investigators generally use ethanol to dissolve the indolamine. The antibacterial effects of melatonin could be a result of the metal binding capacity of the indolamine. Normally, tissue fluids contain unsaturated iron-binding proteins including transferrin in plasma and lymph and lactoferrin in other secretions such as milk or mucous [20, 21]. These proteins ensure that the concentration of free iron in these fluids is virtually zero. This is essential for the normal bactericidal and bacteriostatic effects of plasma and extracellular fluids. If iron becomes freely available, the antibacterial effects of these fluids are lost. This can lead to rapid extracellular bacterial growth and a major increase in bacterial virulance. Pathogenic bacteria have also ways of extracting essential iron from the low iron environment in vivo via siderophores such as enterochelin, which can remove iron from unsaturated transferrin or lactoferrin [22]. An intriguing aspect of this issue is that, because iron is absolutely essential for bacteria, it could make the development of bacterial resistance very difficult for any organism deprived of iron. Of the concern being expressed over the increasing resistance to antibiotics now being encountered, particularly within hospitals, studies on the development of novel drugs against both iron transport and/or intrabacterial free iron availability should be undertaken. Melatonin reportedly has a high metal binding capacity including iron. Limson et al. [23] observed that melatonin and its precursors exhibited the ability to bind metals in situ. Gulcin et al. [24] also noted that melatonin is an effective metal chelating agent. This feature of melatonin has been typically thought to be related to the antioxitant properties of the indole by making transition metals unavailable for the Fenton reaction. However, in case of bacterial growth, an agent which binds free iron in the cytoplasm has great importance. As melatonin easily passes all biological barriers including bacterial cell wall, it may bind free iron in the cytoplasm and restrict bacterial growth via this mechanism. In the present study, melatonin was tested against resistant mycobacterium tuberculosis. In gram-negative bacteria, the cell envelope is composed largely of protein glycopeptide, lipopolysaccharide, and also, substantial amounts of lipid [25]. Melatonin has been shown to limit the uptake of LA and total fatty acids by human breast cancer cells. This feature may also work against an extremely fast dividing prokaryote. Konar et al. [7] reported that melatonin, at the concentration of 1000 µg/ml, significantly reduced the lipid level of Sacchoramyces cerevisae. Furthermore in the same study, melatonin, at concentration of 300 µg /ml, was shown to be one of the most effective agents in reducing lipid levels of Candida albicans. In organisms, melatonin can be administered via any of several routes, e.g. orally, sublingually, etc., and it is available either as an over-the-counter supplement or as a prescription drug (depending on the country). The molecule has a long shelf-life and is inexpensive relative to conventional drugs used to treat the variety of bacterial infections.Melatonin is also very safe with few side effects and a very wide safety margin. The current in vitro studies should be expanded to investigate the efficacy of melatonin as an in vivo antibiotic. It has been previously shown that melatonin is beneficial in newborn humans suffering from septicemia [26]. Those findings coupled with the data reported here suggest further investigations of the role of melatonin in reducing bacterial growth.

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