A Trial of Improving Antituberculous Regimens in Mice Model of Tuberculosis

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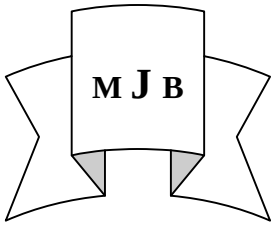

Abstract

Dormancy is a mycobacterial property of persistence in a viable nonreplicating form. It stands for a real community health threat because of its contribution to drug resistance and recurrence of tuberculosis. In order to arrange for a trial of solving this problem, the basic mechanisms by which mycobacterium become dormant were considered in order to partially reverse these factors. Factors that induce partial reactivation from dormancy include: presence of oxygen free radicals and L-alanine nutrient supply. For evaluating antituberculous regimens that include the test agents added to the conventional rifampicin plus INH, both in vitro (MIC) and in vivo (mice) models were used. Two fold serial dilution of modified Lwenstein-Jensen medium have showed equipotent inhibitory effect of metranidazole, L-alanine and metronidazole plus ginsenoside when each added to rifampicin plus INH but those were significantly more potent than rifampicin plus INH only regimen; $P< 0.05$ at intial concentrations of 4 microgram/ ml for each except ginsenoside 8 microgram/ ml. In vivo assessment include dividing 36 mice into 6 groups 5 of them were induced with tuberculosis by inoculation of subcutaneous aspect of left abdominal side with 5000 CFU of *M. tuberculosis* and monitored for temperature, nodule diameter and intramacrophage mycobacteria count in histopathological analysis along 4 weeks of treatment course. Regimen that included metronidazole plus ginsenoside added to rifampicin plus INH had the more potent antituberculous activity in comparism with other regimens in regard to prevention of increase in body temperature and decreasing nodule diameter from 7 mm to 2 mm whereas nodular intramacrophage bacilli were just 4 bacilli in comparison with untreated mean 8 3. Other regimens came second in potency and were significant in comparism with rifampicin plus INH only regimen. From the overall results, there was a significant augmentation of antituberculous potency upon adding some dormancy reversal metronidazole and L-alanine that could be a promising future regimen for eradicating dormant persistant tuberculosis.

محاولة استئصال التدرن ببعض الانظمة العلاجية المضادة للسبات التدرني في النموذج المحدث في الفئران

الخلاصة

ظاهرة السبات في المايكوبكتريا هي خاصية البقاء بشكل حي غير متكاثر. وهي تمثل التهديد الحقيقي لصحة المجتمع لمساهمتها في ظهور المقاومة الدوائية والانتكاسات في التدرن. وكمحاولة لحل تلك المشكلة يتعين الاخذ بنظر الاعتبار الالية التي يحدث بها السبات الماكوبكتيري لغرض عكسه بشكل جزئي.

حيث ان من العوامل التي تحفز التنشيط العكسي الجزئي للسبات هو وجود الجذور الاوكسجينية الحرة وحامض الالنين .

ولاجل تقييم التاثير المضاد للتدرن للانظمة الاختبارية المضافة الى العلاج التقليدي الريفامبيسين واي ان اج تم عمل نموذج في الزجاج وهو التركيز التثبيطي الادنى ونموذج الكائن الحي الذي يشمل نموذج التدرن المحدث في الفئران. تم تخفيف وسط لوفنستاين جنسن بالضعف المتوالي الحاوي على المترونيدازول لوحده او مع الجنسينوسايد وكذلك الالنين عندما يضاف كل منها

الى الريفامبيسين والاي ان اج حيث اظهرت الانظمة الاختبارية تاثيرات مضادة للتدرن بصورة متساوية بالمقارنة مع نظام الريفامبيسين والاي ان اج عند معامل الثقة 95% وبتراكيز اولية تبلغ 4 مايكروغرام\مل لكل منها عدا الجنسينوسايد 8 مايكروغرام\مل.

يتضمن التقييم في نموذج الفار تقسيم 36 فارة الى 6 مجاميع 5 منها تم احداث التدرن فيها باعطاء 5000 وحدة جرثومية مولدة تحت الجلد للجانب الايسر للبطن، ثم تمت مراقبة درجة حرارة الجسم وقطر عقدة التدرن على مدى 4 اسابيع من العلاج وعدد الجراثيم التدرنية داخل الماكروفيج في التشخيص النسيجي بعد اتمام العلاج وقتل الفئران.

لقد اظهر النظام الحاوي على المترونيدازول والجنسينوسايد المضاف الى الرفامبيسين والاي ان اج التاثير الاكثر فاعلية من الانظمة الاخرى في منع ارتفاع درجة الحرارة وتخفيض قطر العقدة الجلدية من 7 ملم الى 2 ملم وتخفيض عدد المايكوبكتريا في الماكروفيج الى 8 عصيات بالمقارنة ب 83 في المجموعة التي لم تعالج. اما فاعلية المجاميع الاخرى فقد اتت بالدرجة الثانية ولكن تاثيرها ايضا معتدا بالمقارنة مع نظام الريفامبيسين والاي ان اج عند معامل الثقة 95%. ومن مجموع النتائج فان هنالك اثرا علاجيا واعدا للتدرن من تقييم وتطبيق هذه الانظمة

العاكسة جزئيا لحالة السبات التدرني.

Introduction

Many microorganisms exert a considerable threat for community due to their variable virulence factors. One of the most obstacling virulence factors is their abstinence from taking antimicrobials owing to their cell signal directed metabolic inactivitation, a phenomenon called dormant state [1]. The most common pathogenic microorganisms that reveal dormancy are Mycobacteria, Salmonellae, Brucellae and some viral infections. Dormancy is a major contributor of 1- drug resistance by these microorganisms[2] 2- mandation of prolonged antimicrobial course 3- reactivation of the same disease after cessation of acute phase [3] 4- necessitation of multiple drugs regimens to avoid resistance. Variable molecular mechanisms can mediate microbial dormancy , these include 1- presence of toxic environmental factors like immune cytokines secreted by innate cells; macrophages, dentritic cells, NK and phagocytes examples of these cytokines are IL1, IL12, CC, Igs, INF lysosomal proteases and phospholipases in addition to those secreted by T and B lyphocytes and free radicals[4] 2- Host tissue PH 3- Lack of vital requirements for microbial survival [5] like hypoxia [6] or low CO2 tension whether as a disease condition like fibrosed, granulomatous or caseous tuberclous center or tissue specific relative low CO2 tension that explains a diminished incidence of development of tuberculosis in lower aerated organs like kidneys and higher incidence in right upper lung.

Another important vital factor for mycobacteria growth and replication are the nutrients and energy sources including amino acids, cofactors, sugar and carboxylic acids [5].

A trial of partial reactivation of mycobacteria by provision of oxygen free radicals could theoretically overcome drug abstinence behavior and hence improves outcome of antituberculous regimens [7], however free radicals could be scavenged by different electron scavengers and reducing agent as antistress factors that can regulate phases of partial reactivation of these

microorganisms. Animal model of *M. tuberculosis* is very important for surveillance and assessment of new strategies of treating this community threatening disease. From the most commonly used animals are mice which are considered to be a reliable model of monitoring experimental tuberculosis [8,9].

Materials and Methods

The used materials

Test agents

1- Metronidazole vial (500 mg; Kimadia. Jordan)
2- L- alanine powder (8 mg; Chemika-Fulka; Germany)
3- Gensinoside tablet (200 mg; Gensing; Canada)

Antituberculous agents

1- Rifampicin cap (300 mg; Ajanta; India)
2- INH tablet (100 mg; Ajant; India)

Staining with Ziehl-Neelsen stain.

Culture media

1- Brain-Heart nutrient medium as transport medium
2- Lowenstein-Jensen medium

Racks for MIC of 2 fold dilution of blank solvents with modified Lowenstein-Jensen medium [10].

Instrument

1- light microscope 2- Sensitive thermotransducer 3- physiograph
4- Dissecting set 5- incubator.

In vitro methods of assessing antimycobacterial potency.

Mycobacterium tuberculosis was requested from the Institute of Respiratory Diseases where it had been identified and tested for rifampicin and INH susceptibility. It had been transported in a brain-heart medium to be cultured and incubated in Lowenstein-Jensen medium for cultivation and preparing of a unique subcutaneous inoculum of 5000 CFU to be injected subcutaneously into the mice.

Mice Model of Tuberculosis

Thirty-six mice aging 2-3 months with 25-30 grams average body weights of both sexes were given a standard oxoid diet and water ad libitum. They were bred in standard breeding cages under a specially care and monitoring. They were divided into 6 groups:

Healthy group; N = 6 were injected 0.1 ml of transport media (lacking M. tuberculosis) subcutaneously at the left side of the abdomen as a healthy control group. The same procedure done for the following groups except for the content of injection and treatment.

Tuberculosis induced groups include:

Induced untreated; N = 6 injected with 0.1 ml of normal saline solution contains (5000 CFU) Mycobacterium tuberculosis subcutaneously at left lower abdominal aspect. These mice were given 0.5 ml distilled water orally daily for 4 weeks as a controlled untreated

Control treated group N = 6 were given 1mg rifampicin + 1mg INH in 0.5 ml distilled water orally daily for 4 weeks as a controlled treated group

Test regimen 1 group N = 6 were given 1mg rifampicin + 1mg INH + 2mg metronidazole in 0.5 ml distilled water orally for 4 weeks.

Test regimen 2 group N = 6 were given 1mg rifampicin + 1mg INH + 2mg metronidazole in addition to 2mg gensinoside in 0.5 ml distilled water orally for 4 weeks.

Test regimen 3 group N = 6 were given 1mg rifampicin + 1mg INH in addition to 2mg L-alanine powder in

0.5 ml distilled water orally for 4 weeks.

For all groups, sublingual temperature was weekly measured with a sensitive thermotransducer in addition to repeated measuring of the lesion diameter weekly for 4 weeks after which period the autopsy histopathologic examination of the induced tuberculous nodule was done with assessing the severity of the lesion after treatment.

Results

Table 1 Growth of *M. tuberculosis* in modified Lowenstein-Jensen medium as an indicator of MIC of different antituberculous regimens.

Types of anti-tuberculous regimens	Minimal antimycobacterial inhibitory conc. MIC of serial 2 fold dilutions for the initial 4,4,4,4 and 8 blank concentrations in micrograms/ml for rifampicin, INH, metronidazole, gensinoside and L-alanine respectively				Test of significance In comparison to Rifampicin+INH
	0	**1**	**2**	**3**	
Blank solvant	+	+	+	+	-
Rifampicin + INH	-	+	+	+	-
Rifampicin + INH + Metronidazole	-	-	-	+	Significant
Rifampicin + INH + L-alanine	-	-	-	+	Significant
Rifampicin + INH +Metrodidazole + Gensinoside	-	-	-	+	significant

Combining metronidazole 4 micrograms/ml plus 4microgram/ml gensinoside with 4 microgram/ml rifampicin and 4 microgram/ml INH had the same mycobacterium inhibitory potency to the regimens that include adding either 4 microgram/ ml metronidazole or 8 microgram/ml L-alanine to rifampicin plus INH in the 2 fold diluted modified Lowenstein-Jensen medium at the 3rd dilution (0.5 microgram/ml for each) in comparison with rifampicin plus INH only treatment :MIC at 1st dilution (2 microgram rifampicin and 2 microgram INH)

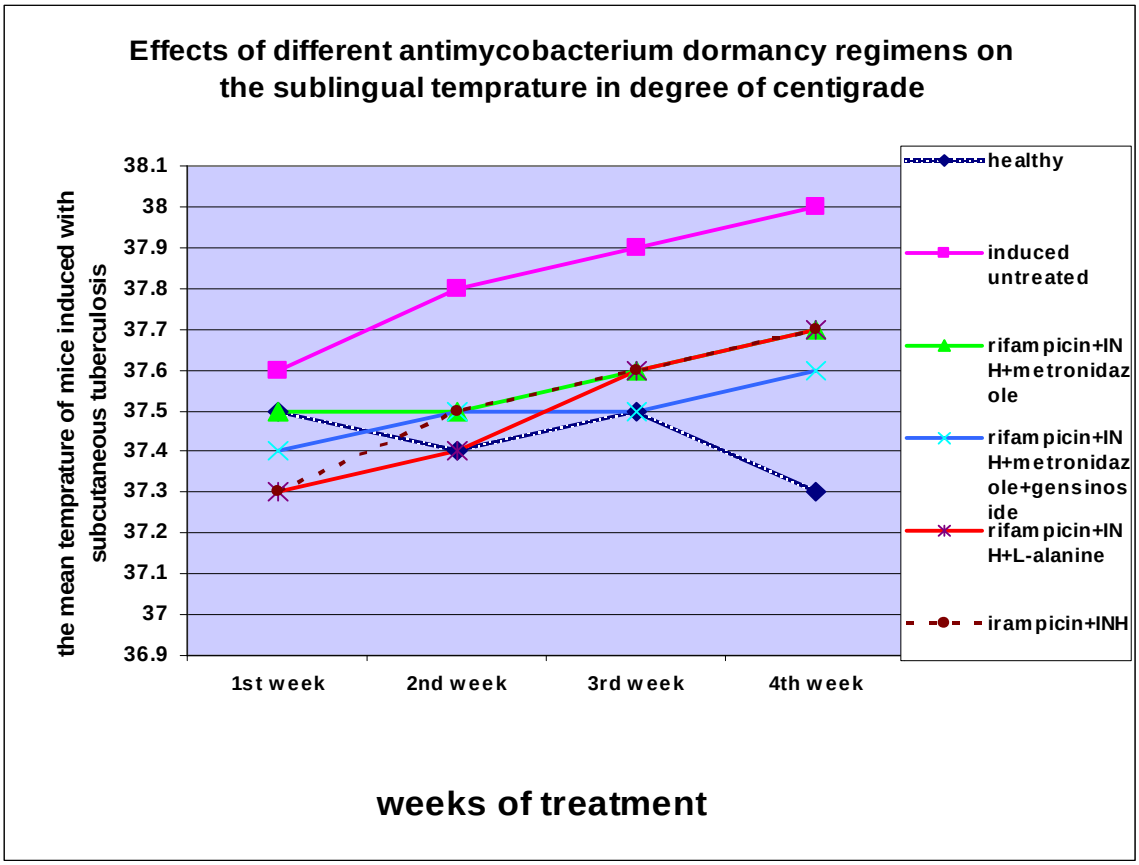

Figure 1 The effect of different antituberculous regimens in reducing mice temperature after induction with subcutaneous *M. tuberculosis*.

There were no significant changes between different regimens, however, it was obvious that antituberulous drugs prevent progressive rise in temperature noticed with the untreated group, measured as a cumulative correlation coefficient R at $P<0.05$.

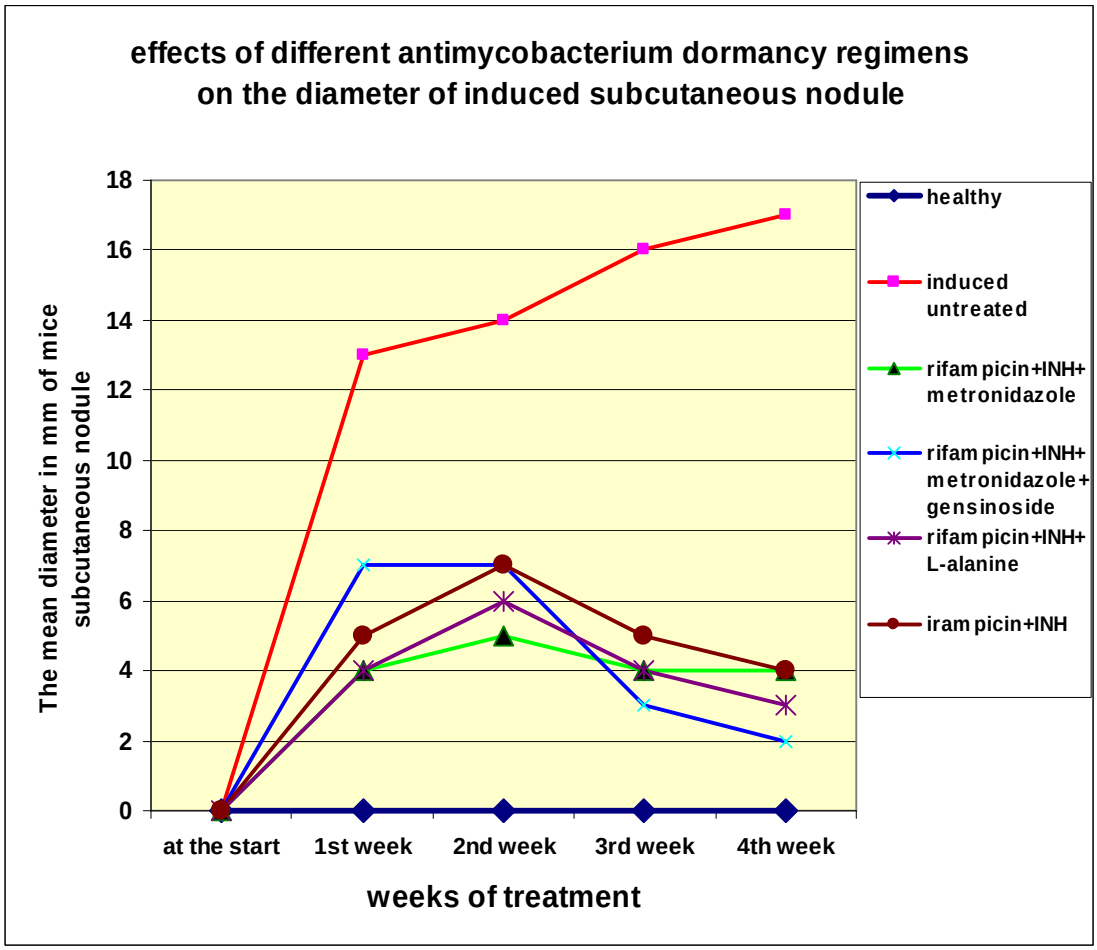

Figure 2 The mean diameter of the induced subcutaneous tuberculous nodule as a cumulative response to different antimycobacterial regimens.

The most potent improving activity in the nodule size was obtained by combining both metronidazole and ginsenoside with rifampicin plus INH: r = 0.988 at P< 0.05. This combination caused a reduction of nodular size from 7 mm to 2 mm at the end of the treatment course whereas a clear progressive increase in size if untreated.

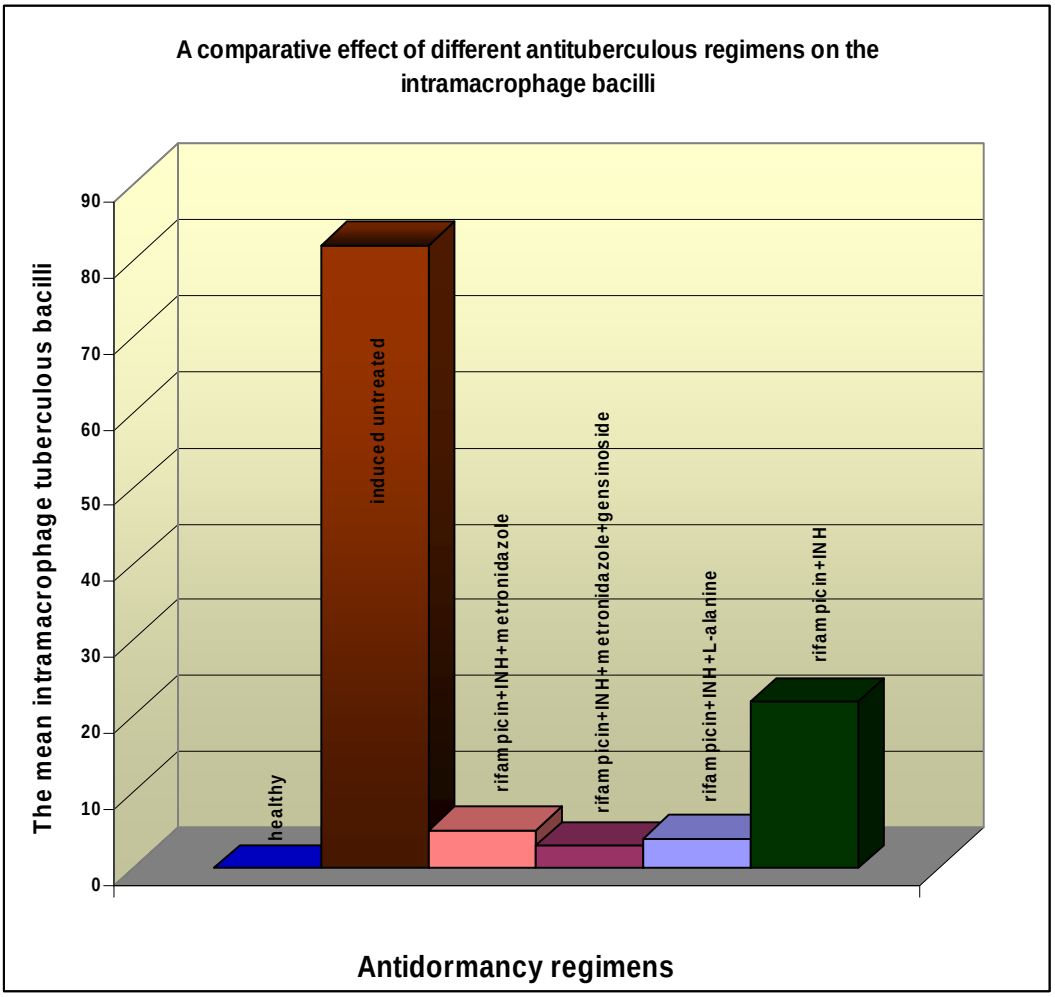

Figure 3 The mean intramacrophage mycobacterial bacilli in response to different antituberculous regimens after autopsy histopathological sectioning and examination of the tuberculous nodule macrophages when treatment course been completed.

Adding metronidazole or L-alanine to rifampicin plus INH caused significant decrease in intramacrophage bacilli in comparison to rifampicin plus INH only; $P< 0.05$ after the end of treatment course.

Discussion

Dormancy is a dangerous bacterial response that despite avoidance of serious acute proliferation, microorganisms like mycobacteria remain viable but inactive for years or even decades which endangers human life upon reactivation by any reason for example immune compromisation, malnutrition, immune deficiency and iatrogenic immunosuppression [11]. However, dormancy process makes reactivated M.tuberculosis even more virulent due to abstinence from antimicrobial intake by these bacteria giving them a further chance of mutation and escape from antibacterial activity [12]. This property necessitates a prolonged treatment course with multiple

antimicrobial regimens that means more toxicity and less patient compliance. On the other hand, *M. tuberculosis* usually resides in a thick inflammatory caseous or granulomatous center that cause diminished drug distribution kinetics for tuberculous center which will further predispose for mycobacterial resistance [13]. A close monitoring of *M. tuberculosis* pathogenesis gives a clue regarding the mechanisms by which dormancy could occur. Different environmental factors such as immune system elements including phagocytes, lymphocytes and their secretions in addition to mycobacterial surrounding PH, O2 tension, free radicals and nutrients are the triggering factors for [3,4] enhancing signal directed dormant state. A partial reversing of one or more of these factors will theoretically overcome antimicrobial abstinence by these microorganisms.

In this current study, a trial of provision of oxygen free radicals with metronidazole was assessed; on the other hand, addition of M. tuberculosis nutrients factors such as L-alanine amino acid was also monitored. These groups were controlled by standard treatment with INH and rifampicin with and without gensinoside supplement group as an electron scavenger factor [14] in form of alternating administration with oxygen free radicals inducer metronidazole.

Tuberculosis model included an induction of subcutaneous isolated, identified and cultivated *M. tuberculosis* to the left lateral aspect of abdomen of the mice with constitutional clinical signs and histopathological follow up of the lesion.

In vitro assessment of inhibitory potency for the test regimens with MIC; table 1. revealed that combining metronidazole 4 micrograms/ml plus 4microgram/ml gensinoside with 4microgram/ml rifampicin and 4 microgram/ml INH had the same mycobacterium inhibitory potency to the regimens that include adding either 4 microgram/ ml metronidazole or 8 microgram/ml L-alanine to rifampicin plus INH in the 2 fold diluted modified Lowenstein-Jensen medium at the 3rd dilution (0.5 microgram/ml for each) in comparison with rifampicin plus INH only treatment :MIC at 1st dilution (2 microgram rifampicin and 2 microgram INH). That means there was no influence by the gensinoside as a direct antimycobacterial agent although gensinoside intensified the anti-tuberculous activity in the mice model. So, in vivo activity of gensinoside may include modulation of mice immune response against tuberculosis. One study regarding assessment of MIC values of rifampicin and INH revealed mycobacterial inhibitoy concentrations that approximate findings of this study: at 1 micrograms/ml for each [15].

In regard to L-alanine, antimycobacterial potentiation is attributed according to in vivo findings that alanine induces resuscitation of the dormant M. tuberculosis owing to activation of bacterial cell wall enzymes including alanine dehydrogenase. This process could render the bacteria more susceptible to INH inhibitory effects [16, 17].

Concerning the constitutional

evaluation of the sublingual temperature in figure 1, all antituberculous regimens showed the same antipyretic effect with nonsignificant variations in between groupd although it was expected that group contains gensinoside will cause obvious and more potent reduction of mice temperature due to its scavenger activity [14], however, this similarity may be explained by the rapid and potent cidal activity of the applied regimens as compared with the control at $P<0.05$.

The cumulative tuberculous nodule reducing effect was also evaluated; figure 2. It was highly significant upon combining metronidazole and ginsenoside with rifampicin and INH regimen which had reduced nodule diameter from 7 mm +/- 2 mm at ist week to 2 mm +/- 1mm at the end of treatment that was even more potent than other regimens, $R = 0.988$, $P< 0.05$. Similar findings were obtained upon estimating the mean intramacrophage tubercle bacilli as an indicator for the killing activity of both macrophages and drugs; figure 3 so that adding metronidazole alone or with ginsenoside to rifampicin plus INH or adding L-alanine to rifampicin plus INH had approximately the same activity, however their potency was significantly more than rifampicin plus INH only regimen at $P< 0.05$. Many studies have assessed the intramacrophage mycobacteria as a reliable parameter to assess antituberculous activity
for test drugs like clarithromycin and gatifloxacin [18].

Metronidazole will exert oxygen free radicals toxic to maycobacterial bacilli, an effect which is under trial for arranging for eradication of tuberculosis [19]. Whereas potentiation of antimycobacterial activity by adding alanine could be attributed to ability of alanine to enhance a partial reactivation of mycobacterial cell wall synthesis a step of triggering reactivation of the dormant bacteria by inducing the enzyme alanine
dehydrogenase [20].

Conclusion

There was a promising tuberculosis eradicating activity obtained from adding factors that can partially reverse the dormant state like metronidazole and L-alanine.

Recommendation

Further assessment of antidormant regimens in human trial is to be carried out in order to make a schedule of antituberculosis that includes complete eradication of tuberculosis.

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