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Determine Suitability of Isoxyl, A Mycolic Acid Inhibitor for Intermittent Chemotherapy of Tuberculosis

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A B S T R A C T

Intermittent chemotherapy is attractive. If drugs can be given less frequently than once a day, fully supervised treatment is much easier. In managing patients with tuberculosis (TB), administration of drugs at intermittent intervals would reduce cost and possibly toxicity of drugs, as well as enhance adherence through greater feasibility of directly observed therapy. There is an urgent need to develop new effective antitubercular compounds, compounds that increase the permeability of the mycobacterial cell wall by inhibiting the synthesis of cell wall components and enhance the activity of conventional drugs as a result of increased penetration of these latter agents to susceptible internal targets. As drug development is a long and expensive process, it becomes predominant to reexamine drugs that were formerly deemed effective against TB and increase the permeability of the Mycobacterial cell wall. One such drug is Isoxyl (ISO). ISO is an old drug, used for the clinical treatment of TB in 1960's. Hence there was a thought to recheck its efficacy against *Mycobacterium tuberculosis* strains as it is an old drug which have proven its efficacy. Present study was conducted with the objective, to determine suitability of ISO for intermittent chemotherapy of TB. In vitro Pulsed exposure study of ISO against Test culture and Standard strain of *M. tuberculosis* H37 Rv after growth of test organisms in log phase was carried out. Viable counts were carried out before addition of drug, immediately after washing and at intervals thereafter. Sensitivity tests were set up on sterile LJM slants. The effect of exposure was estimated by noting the delay before the organisms began to grow. Pulsed exposure study of ISO in infected macrophages was also done. The rate of growth in the cultures after exposure to ISO was similar to the usual growth rate. ISO was not bactericidal during exposure and growth began immediately after the drug was removed by washing. There was significant difference, after applying un-paired t-test, in the bacterial count observed by exposure in INH 1 mcg/ml (24 hrs exposure) and ISO 10 mcg/ml (24 hrs exposure) and 10 mcg/ml (96 hrs exposure) concentrations. Same observations made in pulse exposure study of ISO in macrophage cell line. Summarizing, the assessment of suitability for intermittent chemotherapy, it seems likely that ISO would be least satisfactory. Some caution must be expressed about extrapolating the results of in vitro experiments and to the treatment of human TB. There is no adequate substitute for clinical studies of intermittent treatment of pulmonary tuberculosis with ISO.

Introduction

Intermittent chemotherapy is attractive. If drugs can be given less frequently than once a day, fully supervised treatment is much easier. Indeed in most cases this is the only way that supervised administration can be achieved. The suitability of a drug for intermittent doses appears to depend on the length of time that bacterial multiplication is inhibited after exposure to it and not merely on its bactericidal activity. If growth is not suppressed between doses there may, in some circumstances, be rapid emergence of resistance (Dickinson and Mitchison, 1966). The significant Postantibiotic effect (PAE) of a single drug or a combination of drugs may allow wider dosing intervals without the loss of therapeutic efficacy. PAE refers to the continued suppression of bacterial growth following limited exposure of organisms to an antimicrobial agent (Vogelman and Craig, 1985). In managing patients with tuberculosis (TB), administration of drugs at intermittent intervals would reduce cost and possibly toxicity of drugs, as well as enhance adherence through greater feasibility of directly observed therapy. Earlier work, a few decades ago on pulsed exposure of Rifampicin (RF) and Isoniazide (INH) for 6 to 96 h provided some therapeutic hints on such issues (Dickinson and Mitchison, 1970).

There is evidence that the growth of tubercle bacilli is inhibited soon after the start of chemotherapy with INH or Streptomycin (SM) and that no further multiplication occurs during treatment. Inhibition of growth was thought to occur even if the doses were spaced at intervals of one-three days. However, if bacterial multiplication was not suppressed in the intervals between doses, conditions for the rapid emergence of resistance and the subsequent failure of treatment would exist. Thus the suitability of

a drug for intermittent doses appears to depend partly upon its bactericidal activity, but principally upon the length of time that bacterial multiplication is inhibited after exposure to the drug.

According to World Health Organization (WHO) global TB report 2015, TB now ranks alongside HIV as a leading cause of death, killing 1.5 million people worldwide in year 2014. With a high rate of relapse indicates that, the current oral therapy although effective but fails to completely eliminate the infection from lungs and reinfects when the immunity of person weakens. Also patient's noncompliance is a very big challenge and to a very large extent is responsible for relapse. India top the list of highest TB burdened country worldwide and has alone accounted for 23 % cases of TB reported in year 2014.

Developing new anti tubercular drugs is an expensive exercise and TB is not a disease of rich nations. Some development projects are underway, but more are needed. TB still remains a neglected disease in relation to drug development (Chopra *et al*, 2008). The needs, challenges and recent advances towards development of novel chemical molecules against TB have been reviewed recently. There are 8 new or repurposed anti-TB drugs are in advanced phases of clinical development. 42 countries have started using Bedaquiline to treat patients as part of efforts to expand access to treatment for MDR-TB. Also, Healing rates were significantly better in patients who additionally took Delamanid (recently approved by EMA for MDR-TB) along with first line ATDs.

There is an urgent need to develop new effective anti tubercular compounds, compounds that increase the permeability of the Mycobacterial cell wall by inhibiting the

synthesis of cell wall components and enhance the activity of conventional drugs as a result of increased penetration of these latter agents to susceptible internal targets (David *et al.*, 1988).

This enhancement of antimicrobial activity theoretically affords the use of lower concentration of antibiotics associated with toxicity (Matlola *et al.*, 2001). As drug development is a long and expensive process, it becomes predominant to reexamine drugs that were formerly deemed effective against TB and increase the permeability of the Mycobacterial cell wall. One such drug is Isoxyl (ISO). ISO is an old drug, used for the clinical treatment of TB in 1960's (Winder *et al.*, 1971). Urbancik (1970) and Tischer (1966) demonstrated modest therapeutic efficacy of ISO monotherapy in cases of untreated pulmonary TB of various degree of difficulty (Tischer, 1966; Urbancik, 1970).

Scmid (1970) concluded that INH and ISO were more effective than monotherapy with either drug (Schmid, 1970). The NCDDG group led by DR Patrick Brennan recently evaluated this drug and found it to be effective against MDR strains of *Mycobacterium tuberculosis* (MTB). ISO, a thiourea (thiocarlide, 4, 4 – diisoamyloxythiocarbanilide) demonstrated potent activity against standard strain of MTB (Phetsuksiri *et al.*, 1999). It had been noted that it strongly inhibited mycolic acid synthesis in *Mycobacterium bovis* (Winder, 1971).

Hence there was a thought to recheck its efficacy against MTB strains as it is an old drug which have proven its efficacy. Present study was conducted with the objective, to determine suitability of ISO for intermittent chemotherapy of TB.

Material and Methods (Mor *et al.*, 1995)

In vitro Pulsed exposure study of ISO

Test culture and Standard strain of *M. tuberculosis* H37Rv was grown in sterile Dubos broth with glucose and albumin supplement with 0.05% Tween 80 (Hi Media laboratories Pvt Ltd) in conical flasks in triplicate. After growth of test organisms in log phase, containing 10^5 organisms/ml, INH (Lupin pharmaceuticals) and ISO were added in final concentration of $1\mu\text{/ml}$ and $10\mu\text{/ml}$ respectively. It was incubated for 24 and 96 hours.

After each incubation drug was removed by filtration through sterile preassembled filter units (Millipore) with membrane filter having porosity $0.45\ \mu$, size 47 mm (Sartorius). Drug was further removed by washing with fresh, warm, drug free Dubos broth with glucose and albumin supplement (Hi Media laboratories Pvt Ltd). Membrane filter was inoculated in Dubos broth with glucose and albumin supplement with 0.05% tween 80. Viable counts were carried out before addition of drug, immediately after washing and at intervals thereafter.

Sensitivity tests were set up on sterile LJM slants. The effect of exposure was estimated by noting the delay before the organisms began to grow.

Pulsed exposure study of ISO in infected macrophages (Mor *et al.*, 1995)

Twenty-Four well tissue culture plates (Nunc) were seeded with 10^6 macrophage cells/ml/well and incubated at 37°C in 5% CO_2 atmosphere. Cells used were J774A. 1 Macrophage cell line procured from N.C.C.S., Pune.

After 4 hours, the macrophage monolayer were washed with sterile Hanks balanced salt solution (Haffkine Bio-pharmaceuticals Co. Ltd) to remove non-adherent cells.

Macrophage monolayer were infected with 1.0 ml of test culture containing 10^6 bacteria per ml. After 3 hours of incubation non-phagocytosed organisms were removed by washing the wells three times with warm sterile Hanks balanced salt solution. The cells were replaced with fresh DMEM (Hi Media laboratories Pvt Ltd) with 5% FBS containing different concentrations of ISO

Drug concentrations (mcg/ml) tested as follows: (1) INH: 1.0 (2) ISO: 5.0 and 1.25

Study was carried out in triplicate. Culture medium controls and cell control was included in the study. The tissue culture plates were incubated at 37° C in 5% CO₂ atmosphere for 4 hours. Infected macrophages were exposed to the drugs for 24 hours. After 4 hours incubation drug containing DMEM was removed and replaced with drug free DMEM. The monolayer were incubated for 2 weeks further. The samples of macrophage cells for determining the number of CFU were taken on days 0, 1,3,5,7, 9, 11 and 13. Macrophage cells were lysed with 1ml of sterile water for injection containing 0.25% Sodium dodecyl sulfate (Qualigen). Three, 10 fold dilutions

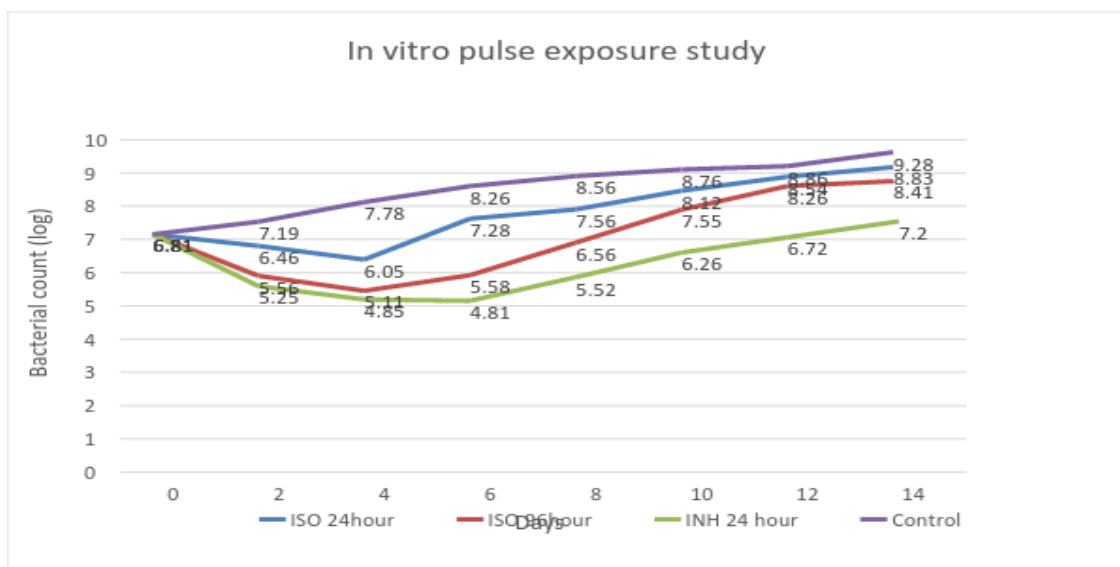
of lysate were made and 0.1 ml of each dilution was plated on sterile LJM slants in duplicate. LJM slants were incubated at 37° C for 21 days. Number of viable bacteria in each well was scored by counting the number of colonies resulting from each dilution on LJM.

As a control, cells in 3 control wells were lysed immediately after initial infection to determine the number of bacteria phagocytosed and to assess the extent of growth over time. Numbers of CFUs indicate the long-term effect of short exposure to the drugs.

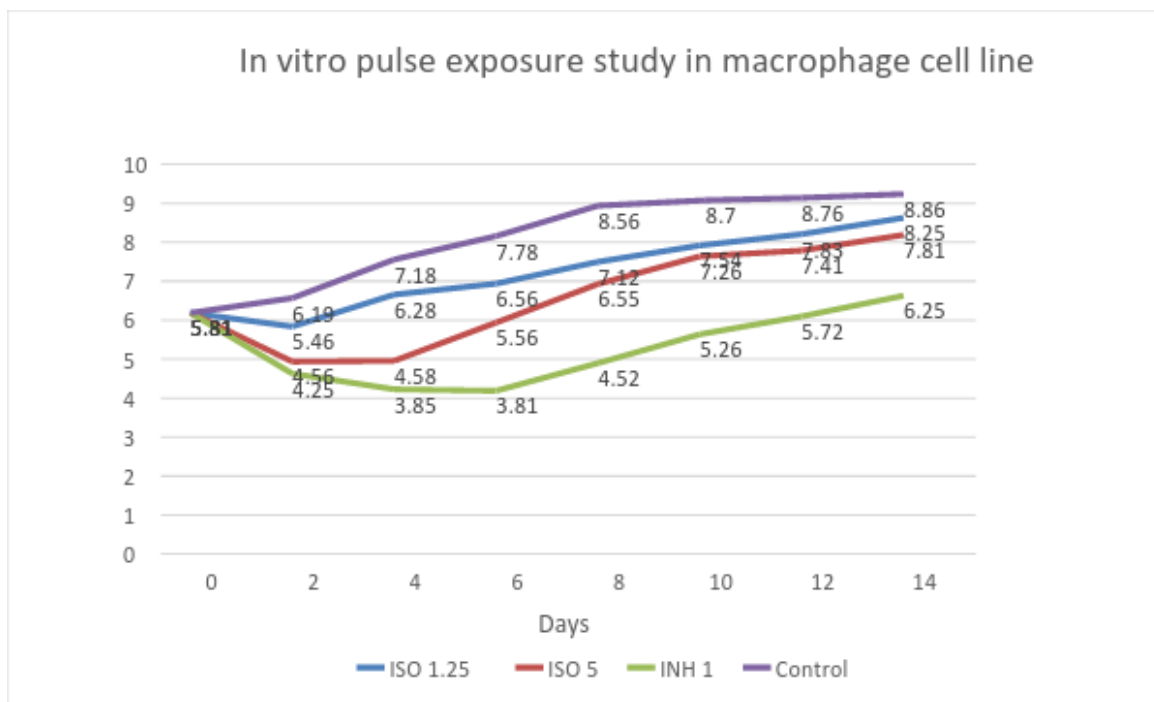
Results and Discussion

ISO was found to be less bactericidal than INH. Lag period after the pulse of ISO appeared to be nil in vitro. Slow recovery of test culture started immediately after a pulse exposure to ISO with the growth rate only returning to normal immediately after 24 and 96 hours of pulse in vitro. No definite bactericidal activity was shown by ISO in vitro, even after exposure for 96 hours.

Pulse exposure study in vitro



Pulse exposure study in macrophage cell line



Returning to normal growth rate took immediately after exposure of 1.5 mcg/ml and 5 mcg/ml of ISO for 24 hours in cell line. Initial reduction in bacterial count in first 2 days in 5 mcg/ml of ISO exposure was similar to INH exposure. But multiplication of test organisms began immediately after removal of the drugs.

Inhibition of the growth of *Staphylococci* following temporary exposure to penicillin was first noted by Bigger (1944) and further studied by Parker and Marsh (1946). Egle and Musselman (1949) exposed *Streptococci* and *Staphylococci*, inactivated penicillin with penicillinase and then did serial viable counts on the culture. As the duration of the exposure to penicillin was increased the delay, before growth began, reached a maximum of about three hours, irrespective of the concentration of penicillin used. The authors recognized the importance of the

phenomenon in explaining the efficacy of intermittent treatment with penicillin. Hurwitz *et al.*, (1964) found a delay in growth of *Mycobacterium fortuitum* following exposure to streptomycin.

In traditional chemotherapy drugs are administered in small doses at frequent intervals in order to maintain a bacteriostatic concentration in the blood for a period as long as possible. During investigations at the Tuberculosis Chemotherapy Centre, madras 1960-1963, it was found that when large doses of INH were given at less frequent intervals than usual, there was an increase in efficacy without corresponding rise in toxicity. This led to consideration that other drugs might also be more effective if given in a similar manner. Increased spacing out of drug doses makes it possible to administer fully supervised treatment and also permit developing countries to use their limited

resources to greater advantage (Dickinson and Mitchison, 1966).

The suitability of a drug for intermittent chemotherapy depends on its ability to inhibit bacterial multiplication between doses when bacteriostatic concentration is no longer present, and to a lesser extent, on its bactericidal activity. Oral chemotherapy for pulmonary tuberculosis is traditionally administered in several doses during the day with the aim of maintaining a continuous bacteriostatic drug concentration in the serum. Streptomycin is, however, given as a single daily injection, even though serum concentrations fall rapidly during the following 6-10 hours. The first definite evidence that improved results might be obtained by intermittent chemotherapy was obtained from a study in which patients fared better when treated with INH alone given in a single daily dose of 8.7 mg/kg, than in two doses of 4.4 mg/kg, in the day (Tuberculosis Chemotherapy Centre, 1960)

In these patients the response to treatment was related to the peak concentration of INH attained in the serum, but not to the period in each day during which a bacteriostatic concentration was maintained. The incidence of peripheral neuritis was higher in slow inactivators of INH than in rapid inactivators, suggesting that the risk of toxicity was related not only to the peak serum concentrations but also to the length of time that the free drug was present in the serum (Tuberculosis Chemotherapy Centre, 1960) (Tuberculosis Chemotherapy Centre, 1963) Thus the spacing out of the drug doses at intervals of about one day might be obtained without an increase in the toxicity risk. Application of this principle might also improve results of treatment with such second-line drugs as Ethionamide and Cycloserine. The spacing of doses at intervals greater than one day makes it

easier to administer the drug under full supervision, and so should improve the regularity of drug-taking. Encouraging results have already been obtained in clinical practices with a regimen of high doses INH and SM given together twice weekly (Tuberculosis Chemotherapy Centre, 1964)

In present study we have evaluated suitability of ISO for intermittent chemotherapy of TB in vitro and in cell line.

When patients with TB are given their first few drug doses, many of the tubercle bacilli in their lesions are actively growing. The response of these bacilli is mirrored in the experiment in which a log phase culture of *M. tuberculosis* was exposed to a pulse of ISO and viable counts done during and after the pulse. ISO was found to be less bactericidal than INH and lag period after the pulse of ISO appeared to be nil in vitro. Slow recovery of test culture started immediately after a pulse exposure to ISO with the growth rate only returning to normal immediately. Returning to normal growth rate took immediately after 24 and 96 hours of pulse in vitro. No definite bactericidal activity was shown by ISO in vitro, even after exposure for 96 hours. The multiplication of the test organisms began immediately after removal of the drugs. The rate of growth in the cultures after exposure to ISO was similar to the usual growth rate. ISO was not bactericidal during exposure and growth began immediately after the drug was removed by washing. There was significant difference, after applying unpaired t-test, in the bacterial count observed by exposure in INH 1 mcg/ml (24 hrs exposure) and ISO 10 mcg/ml (24 hrs exposure) and 10 mcg/ml (96 hrs exposure) concentrations

Same observations made in pulse exposure study of ISO in macrophage cell line.

Returning to normal growth rate took immediately after exposure of 1.5 mcg/ml and 5 mcg/ml of ISO for 24 hours in cell line. Initial reduction in bacterial count in first 2 days in 5 mcg/ ml of ISO exposure was similar to INH exposure. But multiplication of test organisms began immediately after removal of the drugs. The rate of growth in the cultures after exposure to ISO was similar to the usual growth rate. ISO was not bactericidal at concentration of 1.5mcg/ml, while there was little bactericidal activity with 5 mcg/ml concentration during exposure and growth began immediately after the drug was removed by washing.

There was significant difference after applying un-paired t-test, in the bacterial count observed by exposure in INH 1 mcg/ml and ISO 1 mcg/ ml and 5 mcg/ml concentrations. Dickinson *et al* (1966) reported similar findings in vitro. They reported mean delay of growth as zero day periods after exposure of test culture to 24 and 96 hours. While mean change in viable counts after exposure for 24 and 96 hours was zero. These findings suggest that if ISO were to be used intermittently in treatment, there would only be a delay in the growth of an organism.

Furthermore, alternating periods of inhibition and growth would promote the rapid emergence of drug resistance. Thus, if patients were treated intermittently with a double drug combination such as INH and ISO, frequent failures to respond would be expected, due to the growth of INH resistant mutants, which would also soon acquire ISO resistance. Summarizing, the assessment of suitability for intermittent chemotherapy, it seems likely that ISO would be least satisfactory. Some caution must be expressed about extrapolating the results of in vitro experiments and to the treatment of

human TB. There is no adequate substitute for clinical studies of intermittent treatment of pulmonary tuberculosis with ISO.

Conclusion

Summarizing, the assessment of suitability for intermittent chemotherapy, it seems likely that ISO would be least satisfactory. Some caution must be expressed about extrapolating the results of in vitro experiments and to the treatment of human TB. There is no adequate substitute for clinical studies of intermittent treatment of pulmonary tuberculosis with ISO.

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