IN VITRO EFFECT OF VITAMIN C ON THE LABORATORY ISOLATES OF MYCOBACTERIUM TUBERCULOSIS WITH KNOWN SENSITIVITY AND RESISTANCE TO THE FIRST LINE ANTI TUBERCULAR DRUGS: AN EXPERIMENTAL PILOT STUDY

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ABSTRACT

Background and Objectives: Globally, 3.5% of new cases of Tuberculosis (TB) and 20.5% of previously treated cases are estimated to have multidrug-resistant tuberculosis, the corresponding estimates for India are 2.2%, and 15% respectively. Progress has been made in research and development of new drugs for TB over the last decade, thus fuelling the need for more innovative options. Recent in-vitro studies that claim Vitamin C to have an inhibitory effect on Mycobacterium tuberculosis could possibly prove to be a major breakthrough in Medicine. Hence this experimental study was conducted on a pilot basis with the objective of studying the in-vitro effect of the active ingredient of vitamin C on the laboratory isolates of Mycobacterium tuberculosis that were known to be sensitive and resistant to the first line anti tubercular drugs (Isoniazid, Rifampicin, Pyrazinamide and Ethambutol) and to compare the dose related response of both sensitive and resistant strains of Mycobacterium tuberculosis to varying concentrations of Vitamin C.

Materials and Methods: Using a Completely Randomized Design, a total of 17 viable Mycobacterium tuberculosis strains, 10 of which were sensitive to all first line anti-TB drugs (Isoniazid, Rifampicin, Pyrazinamide and Ethambutol) and seven strains resistant to all first line Anti-TB drugs were experimented upon. Proportion method was used to determine drug susceptibility of Mycobacterium tuberculosis to Ascorbic acid. Data is presented in a summary table. Results: With 1mM (millimole) concentration of Ascorbic acid, growth of Mycobacterium tuberculosis was observed on both drug containing as well as control media, but with higher concentration of Ascorbic acid (10 mM and 100mM), no growth was observed on Ascorbic acid containing Lowenstein Jenson media. Conclusion: Although the findings of this pilot study add to the supportive evidence of an in-vitro susceptibility of Mycobacterium tuberculosis to Vitamin C, the authors recommend that additional studies with larger sample size may be conducted to support the effectiveness of Ascorbic acid used alone or in combination with other anti-tubercular drugs to look for any drug interactions.

Keywords: Proportion method, Ascorbic acid, Mycobacterium tuberculosis

INTRODUCTION

Nearly two decades after the World Health Organization’s declaration of Tuberculosis (TB) as a global public health emergency, major progress has been made towards 2015 global targets set within the context of the Millennium Development Goals [1]. One of the five priority actions required to accelerate progress towards 2015 targets, as listed in the Global TB report, is “to ensure rapid uptake of innovations”
The increasing resistance of Mycobacterium tuberculosis (M.tb) to first line anti tubercular drugs is a cause for concern. Globally, 3.5% (95% CI: 2.2-4.7%) of new TB cases and 20.5% (95% CI: 13.6-27.5) of previously treated cases are estimated to have multidrug-resistant tuberculosis (MDR-TB), the corresponding estimates for India are 2.2(CI: 1.9-2.6), and 15(CI: 11-19) respectively [2]. Although the statistics reveal a very slow yet a hopeful decrease in the TB related mortality in India over the last few years [1-3], it is a known fact that the Tuberculosis treatment regimen has faced many dead ends with resistance developing rapidly both in vivo and in vitro [4,5], and the subsequent rise in the MDR and extensively drug-resistant tuberculosis cases has fuelled the need for a more innovative option [1]. Researchers all over the world are looking for innovative ways to treat Tuberculosis. Literature search documents the findings of Dr. Frederick R Klenner who claimed that Vitamin C fulfilled the requirements of an antibiotic due to its capacity to function as a reducing agent or the precursor of such a substance [6]. In a study by McConkey M et al [7], of the twenty-one animals which were given a tuberculous sputum feed along with a diet deficient in Vitamin C or A, C, D; seventeen developed open tuberculous ulcers, three caseous non-ulcerative lesions, and the intestinal tract of only one animal was normal at necropsy. Nine out of the ten animals, receiving supplements of Vitamin C along with tuberculous sputum feed did not develop intestinal TB. Taneja et al [8] showed that Vitamin C mimics multiple intracellular stresses and has wide-ranging regulatory effects on gene expression and physiology of M. tuberculosis which leads to growth arrest and a ‘dormant’ drug-tolerant phenotype. Vilchèze C et al [9] demonstrated ability of vitamin C to sterilize M. tuberculosis cultures. Narwadiya SC et al [10] claim Vitamin C to have similar dose related inhibitory effect on Mycobacterium tuberculosis. Given this possibility, the effect of Vitamin C on Mycobacterium tuberculosis could prove to be a major breakthrough in Medicine. This highlighted the need for an in-vitro experimentation of Vitamin C on various strains of Mycobacterium tuberculosis. Therefore this experimental pilot study was aimed at testing the effect of active ingredient of Vitamin C (Ascorbic Acid) on the sensitive as well as the resistant strains of Mycobacterium tuberculosis of routine Tuberculosis patients obtained from the laboratory stocks at Intermediate Reference Laboratory (IRL), Department of Microbiology, Goa Medical College with the following objectives: 1) To study the in vitro effect of the active ingredient of vitamin C on the laboratory isolates of Mycobacterium tuberculosis with known sensitivity and resistance to the first line Anti tubercular drugs currently used in DOTS (Directly Observed Treatment, Short Course) regimen of RNTCP (Revised National Tuberculosis Control Programme). 2) To compare the dose related response of both sensitive and resistant strains of Mycobacterium tuberculosis to varying concentrations of Vitamin C.

**METHODOLOGY**

**Type of study:** This is an experimental laboratory based study.

**Ethics approval:** The study was conducted after prior approval from the Institutional Ethics Committee. **Study design:** Completely Randomized Design was used.

**Methodology:** M. tuberculosis strains used in this study were obtained from laboratory stocks of year 2011 onwards, isolated from routine TB patients, provided by IRL at the Department of Microbiology, Goa Medical College, Goa. The isolates were first sub cultured on Lowenstein Jenson (LJ) media to ensure their viability. Thirty-eight strains of Mycobacterium tuberculosis from the laboratory stocks were first subjected to subcultures, of which 20 viable strains were further subjected to DST (Drug Susceptibility Testing). Since three strains got subsequently contaminated, a total of 17 viable strains, 10 of which were sensitive to all first line anti-TB drugs (Isoniazid, Rifampicin, Pyrazinamide and Ethambutol) and seven strains resistant to all first line Anti-TB drugs, were finally experimented upon. Drug susceptibility testing (DST) of Mycobacterium tuberculosis to Ascorbic acid (active ingredient of Vitamin C) was done using Proportion Method. [11]. Drug free/plain LJ media was used as control during the procedure for DST and the LJ media containing L-Ascorbic acid (99.7%) AR 2013 served as the drug containing media. Ascorbic acid solution of varying concentrations (1, 10 and 100 millimoles) was sterilised by Membrane filtration. The final LJ media was sterilised by Serum Inspissation. The cultured media were incubated at 37°C; the observations for
growth were made on days 28 and 42. Sensitivity and resistance pattern was interpreted as per the Revised National TB Control Programme Training Manual Guidelines.

RESULTS

Table 1: Results of the Drug susceptibility testing

<table>
<thead>
<tr>
<th>Mycobacterium tuberculosis strains</th>
<th>Ascorbic acid (1 mM) (n=17)</th>
<th>Ascorbic acid (10 mM) (n=17)</th>
<th>Ascorbic acid (100 mM) (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. growth of M.tb observed in drug containing media</td>
<td>Growth of M.tb observed in drug containing media No.</td>
<td>No growth of M.tb observed in drug containing media No.</td>
<td>Growth of M.tb observed in drug containing media No.</td>
</tr>
<tr>
<td>Strains sensitive to all four standard first line anti-TB drugs (H, R, Z, E)</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Strains resistant to all four standard first line anti-TB drugs (H, R, Z, E)</td>
<td>0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>17</td>
<td>17</td>
</tr>
</tbody>
</table>

H=Isoniazid, R=Rifampicin, Z=Pyrazinamide and E=Ethambutol

One mM concentration of Ascorbic acid, permitted growth of Mycobacterium tuberculosis strains in both drug containing as well as control media. Dose of Ascorbic acid was further increased to 10 mM and 100 mM, to study the dose-dependant response of the 10 sensitive and seven resistant M.tuberculosis strains. No growth of Mycobacterium tuberculosis strains was observed with higher concentration of Ascorbic acid (10 mM and 100mM). The LJ media with 100 mM concentration of Ascorbic acid however turned dark green in colour and the reason could not be ascertained. The observations made on day 28 and 42 remained unchanged.

DISCUSSION

In this pilot experiment, both the control (Ascorbic acid free media) as well as Ascorbic acid containing media, showed growth of Mycobacterium tuberculosis colonies at lower concentration (1mM) of Ascorbic acid. However a study done by Vilchéze C et al [9] found the minimum inhibitory concentration (MIC) of Vitamin C that prevented the growth of M. tuberculosis was one mM. This difference in the observations could be attributed to loss of biological activity of Ascorbic acid at lower concentrations subsequent to serum inspissation at 80°C for a hour on consecutive days, which happens to be the standard sterilisation procedure for preparing LJ Media.

Another possible explanation to support this could be found in studies done by Alvarado JD et al [12] and Munyaka AW [13] which state that at higher temperatures, conversion of its active ingredient, i.e. l-ascorbic acid to dehydroascorbic (DHAA) takes place. DHAA could be easily converted to other compounds that do not have the biological activity of Vitamin C. The issue of possible loss of biological activity due to heat degradation could be addressed by using a liquid media or any other selective media, which would have allowed the addition of Vitamin C after sterilisation.

In our study, we observed a dose related response (no growth of M. tb strains) with higher concentrations of Ascorbic acid. The absence of growth of Mycobacterium tuberculosis (both sensitive and resistant strains) at higher concentrations of Ascorbic acid i.e. 10mM and 100 mM, could either be due to some chemical alteration or shift of pH of LJ medium that may have possibly lead to inability of M. tuberculosis to grow in the media, or the lack of growth may also be due to the unique susceptibility
of Mycobacteria to Ascorbic acid as claimed by similar research studies conducted in the past. [7-10]

CONCLUSION

The objectives with which we started the pilot study were largely met. With regards to the first objective the authors found that all 17(100%) of the laboratory isolates of Mycobacterium tuberculosis which were sensitive (10 strains) as well as resistant (seven strains) to first line anti-TB drugs used in RNTCP, showed in-vitro susceptibility to the active ingredient of Vitamin C at higher concentrations (10mM and 100 mM). Our second objective was to compare the dose related response of both sensitive and resistant strains of Mycobacterium tuberculosis to varying concentrations of Vitamin C. We observed that at 1mM concentration, Vitamin C did not have any effect on the Mycobacterium isolates, but had effect only at higher concentrations of 10mM and 100 mM. Although the findings of this pilot study add to the supportive evidence of an in-vitro susceptibility of Mycobacterium tuberculosis to Vitamin C, the authors recommend that further studies with larger sample size may be conducted to support the effectiveness of Ascorbic acid used alone or in combination with other anti-TB drugs to look for any drug interactions. Clinical trials in humans using Vitamin C supplementation to study the in-vivo effect of Vitamin C in patients who are on DOTS regimen for treatment of Tuberculosis could also be thought of. This could revolutionize the current scenario in relation to treatment of Tuberculosis.

LIMITATIONS OF THE STUDY

1. Use of a selective liquid media would have allowed the addition of Ascorbic acid after sterilization, thus ruling out the possibility of loss of efficacy of Ascorbic acid due to degradation at higher temperatures (if any) and would probably have given us results with the lower concentration (1mM).

2. The reason for the dark-green colouration of LJ media with 100 mM concentration of Ascorbic acid could not be ascertained.

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Conflict of interest: NIL
REFERENCES