



# International Journal of Medical Research & Health Sciences

www.ijmrhs.com

Volume 4 Issue 2

Coden: IJMRHS

Copyright @2015

ISSN: 2319-5886

Received: 31<sup>st</sup> Jan 2015Revised: 10<sup>th</sup> Feb 2015Accepted: 31<sup>st</sup> Mar 2015

Research article

## 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

Talaulikar Nikita.S <sup>1</sup>, \* Dsouza Delia.B <sup>2</sup> Rodrigues Savio <sup>3</sup>, Kulkarni MS <sup>2</sup>

<sup>1</sup> II MBBS student, Goa Medical College, Bambolim, Goa, India

<sup>2</sup> Department of Preventive and Social Medicine, Goa Medical College, Bambolim, Goa, India

<sup>3</sup> Department of Microbiology, Goa Medical College, Bambolim, Goa, India, 403202.

\*Corresponding author email: deliadsouza@rediffmail.com

### 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-Jensen 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 <sup>[1]</sup>. 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"

[1]. 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'. Vilchère 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.<sup>[11]</sup> Sensitivity and resistance pattern was interpreted as per the

Revised National TB Control Programme Training Manual Guidelines<sup>[11]</sup>.

## RESULTS

**Table 1: Results of the Drug susceptibility testing**

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

**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 Vilchère C et al<sup>[9]</sup> 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 an 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<sup>[12]</sup> and Munyaka AW<sup>[13]</sup> 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.<sup>[7-10]</sup>

## CONCLUSION

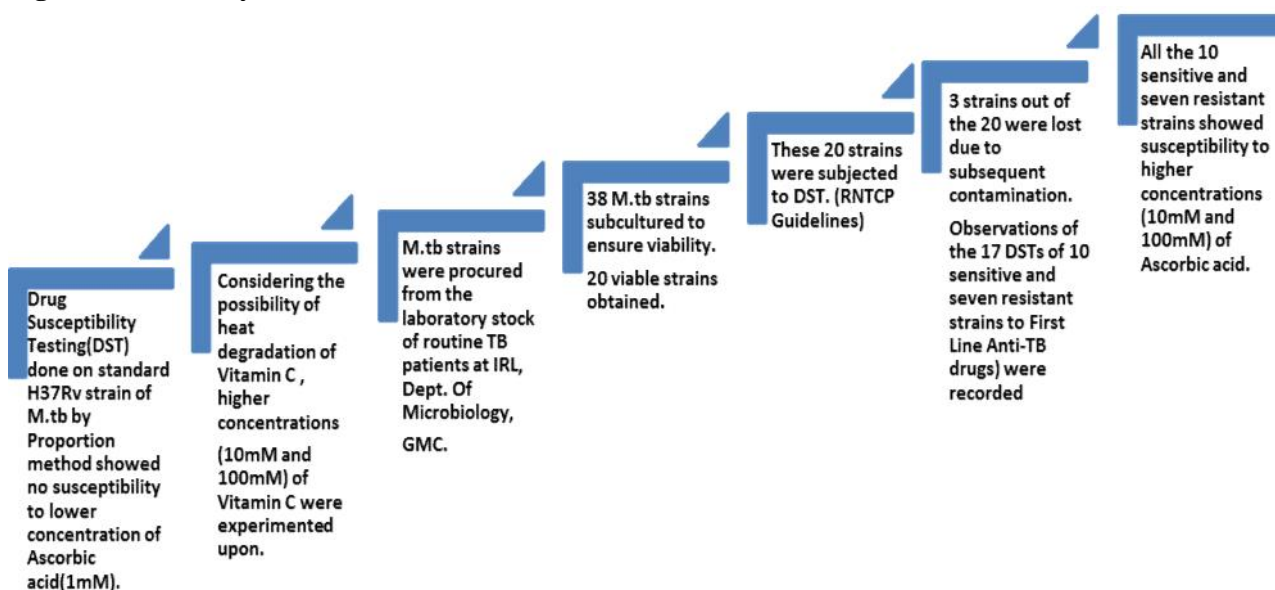
The objectives with which we started the pilot study were largely met. With regards to the first objective the authors found that all 17(100%) of the laboratory isolates of Mycobacterium tuberculosis which were sensitive (10 strains) as well as resistant (seven strains) to first line anti –TB drugs used 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.

Figure 1: Summary



## ACKNOWLEDGEMENT

The authors would like to thank the Indian Council of Medical Research for the research grant awarded to the first author (undergraduate student from II MBBS) to conduct this experimental pilot study through it's Short Term Studentship program. The authors express their sincere thanks to Ms Puja A. Parulekar (Senior Laboratory Technician at IRL, Goa Medical College and Dr Cigy C Borges,

Microbiologist at IRL, Goa Medical College for the immense guidance, technical support and cooperation, received while conducting this pilot experiment in the Microbiology laboratory of Goa Medical College without whom it would not be possible to conduct such a study. We also thank the Institutional Ethics Committee for approving this pilot research study.

**Conflict of interest: NIL**

## REFERENCES

1. World Health Organisation (WHO). Global Tuberculosis Report 2013. Geneva: WHO; 2013.
2. World Health Organisation (WHO). Global Tuberculosis Report 2014. Geneva: WHO; 2014.
3. World Health Organisation (WHO). Global Tuberculosis Report 2012. Geneva: WHO; 2012.
4. Colijn C, Cohen T, Ganesh A, Murray M. Spontaneous Emergence of Multiple Drug Resistance in Tuberculosis before and during Therapy. PLoS ONE.2011; 6(3): e18327. Doi: 10.1371/journal.pone.0018327.
5. Selkon JB.. The emergence of isoniazid-resistant cultures in patients with pulmonary tuberculosis during treatment with isoniazid alone or isoniazid plus PAS. Bull. World Health Organ. 1964; 31: 273–94.
6. Klenner FR. Massive doses of vitamin C and the virus diseases. South Med Surg. 1951 Apr; CIII (4): 101-7.
7. McConkey M, Smith DT. The Relation of Vitamin C Deficiency to Intestinal Tuberculosis in the Guinea Pig. J. Exp. Med. 1933; 58: 503–512.
8. Taneja NK, Dhingra S, Mittal A, Naresh M, Tyagi JS. Mycobacterium tuberculosis transcriptional adaptation, growth arrest and dormancy phenotype development is triggered by vitamin C. PLoS One. 2010; 5: e10860.
9. Vilchèze C, Hartman T, Weinrick B, Jacobs W R. Mycobacterium tuberculosis is extraordinarily sensitive to killing by a vitamin C-induced Fenton reaction. Nat Commun.2013; 4: 1881.
10. Narwadiya SC, Sahare KN, Tumane PM, Dhumne UL, Meshram VG. In vitro antituberculosis effect of vitamin C contents of medicinal plants. Asian J. Exp. Biol. Sci. 2011; 2: 151–54.
11. Directorate General of Health Services, Ministry of Health and Family Welfare, Central TB Division. Revised National TB Control Programme Training Manual for Mycobacterium tuberculosis Culture and Drug Susceptibility Testing. New Delhi: 2009: 1-76.
12. Alvarado JD, Palacios VN. Effect of temperature on the aerobic degradation of vitamin C in citric fruit juices. Arch Latinoam Nutr. 1989 Dec; 39(4): 601-12.
13. Munyaka AW, Makule EE, Oey I, Van Loey A, Hendrickx M. Thermal stability of L-ascorbic acid and ascorbic acid oxidase in broccoli (*Brassica oleracea* var. *italica*). J Food Sci. 2010 May; 75(4): C336-40. . doi: 10.1111/j.1750-3841.2010.01573.x