Vitamin C supplementation improve the sputum conversion culture rate in pulmonary tuberculosis treatment while rifampicin susceptible

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Abstract. The failure of first-line tuberculosis treatment greatly affects multiple drug-resistant tuberculosis. In vitro study of vitamin C induces the death of *M. tuberculosis* bacteria and accelerates healing of tuberculosis, so the multiple drug-resistant tuberculosis can be avoided. This research aimed to identify the effect of vitamin C as a supportive treatment on the sputum conversion rate. The randomizedand double group with a parallel design by matching pair method was used to collect samples. The first group was treated with standard tuberculosis treatment, and the other was given vitamin C supplementation. Vitamin C plasma level analyzation was performed before and after two months of treatment. Sputum conversion was evaluated every week for eight weeks. The comparison of vitamin C plasma level in pre and post-treatment group was significant (p=0.03) but not in the other group. There was no significant difference in vitamin C plasma level between two groups (p=0.21). The proportion of sputum conversion rate in both group in the first week was 0% vs. 9.6% (p=0.83) and the last week of study was 83.9% vs. 100% (p=0.02). In conclusion, vitamin C supplementation has effects in improving the healing process of tuberculosis patients as indicated by higher in sputum conversion rate.

1. Introduction

In this world, 1.5 million people died because of tuberculosis (TB) diseases and in Indonesia alone, the incidence of TB is about one million while 100.000 died from the diseases. Treatment failure among TB patients caused TB with multidrug-resistant[1]. The efforts made by the World Health Organization (WHO) for TB disease control, are with Directly Observed Treatment Short (DOTS) strategies. The DOTS strategies by applying two months as intensive period will greatly affect the rate of cure for TB disease in the later treatment period[2]. It is important to improve sputum conversion rate to ensure healing process. The feasible option to consider in helping to accelerate the process of sputum conversion in the intensive period of treatment is supplementation of vitamin C that accompanies the current DOTS treatment.

Vitamin C is a molecule that serves as a reducing and antioxidant compounds and as regulatory in the transduction of redox reaction signal from toxin to control the inflammatory response[3].The combination of vitamin C with some antibiotics is able to suppress the growth of bacteria *Bacillus cereus*[4], *Pseudomonas auruginosa*[5], *Staphylococcus aureus* and *Escherichia coli*[6]. An in vitro study

showed that vitamin C could serve as a bactericide in *M. tuberculosis* bacteria[7]. The bactericidal process by vitamin C to *M. tuberculosis* bacteria occurs through the combination of Haber-Weiss reaction and the Fenton reaction which produces oxidative stress[8].

The aim of the study was to prove the effect of oral supplementation of vitamin C as a supportive treatment and its effect on the sputum conversion culture rate. If this hypothesis was proven true, then the supplementation of vitamin C can be added to the tuberculosis treatment regimen. It is done so that the bacterial sputum culture conversion can be accelerated and the possibility of treatment failure can be reduced.

2. Methods

This study used a cohort prospective, randomized and double group parallel design. Sputum culture conversion in each group was followed every week for eight weeks. The measurement of vitamin C plasma levels was performed before and after two months of treatment. Acid-fast bacilli (AFB) sputum culture examination was done at Multi-Drug Resistances (MDR) TB Laboratory of H. Adam Malik Hospital Center (Lowenstein-Jensen solid medium). The vitamin C plasma levels were measured by using the spectrophotometric method at the Integrated Laboratory of Medicine Faculty of Sumatera Utara University (EnzyChrome^{TM} ascorbic acid assay kit EASC-100 by BioAssay Systems).

The subjects of the study were pulmonary tuberculosis patients while susceptible Rifampicin who were determined by the Molecular Fast Test tool (GeneXpertDx System), aged 17 to 65 years, HIV-seronegative, non-diabetic patients, not taking immunosuppressive drugs and were not pregnant and lactating.

Tuberculosis patients were selected through inclusion/exclusion criteria and randomized. They were then divided into two groups.

The first group was treated with standard tuberculosis treatment and supplementation of vitamin C (vitamin C type Ester 500 mg/day). The second group was treated with only standard tuberculosis treatment (control group). The independent variable was vitamin C plasma levels, and the dependent variable was the sputum culture conversion rate which was performed every week for eight weeks. To see the difference in vitamin C plasma levels before and after two months of treatment, and to see the difference in conversion rates between the control group and the treatment group, we used the t-test with 95% confidence level.

3. Results

During the study, sixty-two tuberculosis patients were diagnosed, among the participants in both group, there were 26 males (83.9%) and 5 females (16.1%). The control group means age was 36.39±13.98, and the treatment group was 35.03±11.76 years. All patients completed the study.

Table 1. Characteristics of study samples.

Characteristics	Control Group	Treatment group	p value
Sex			
- Male	26 (83.9%)	26 (83.9%)	
-Female	5 (16.1%)	5 (16.1%)	1.00[a]
Total	31 100%)	31 (100%)	
Age (years)	36.39 ± 13.98	35.03 ± 11.76	0.66[a]
Result of Genexpert system			
-Very low:	5 (16.1%)	4 (12.9%)	
- Low:	8 (25.8%)	7 (22.6%)	
- Medium:	9 (29.0%)	10 (32.3%)	0.57[b]
- High:	9 (29.0%)	10 (32.3%)	
Total	31 (100%)	31 (100%)	
Mean vitamin C plasma level pre-treatment (μM/L)	228.36 ± 121.3	201.19 ± 183.1	0.41[b]

[a]Wilcoxon Signed Rank Test
[b]paired t-test

There was no marked difference in the sex, mean of age, gene expert results and mean of vitamin c plasma level pre treatment of the two groups (p>0.05).

The mean of vitamin C plasma level in both group before and after treatment are shown in Table 2.

Table 2. Mean vitamin C plasma level.

Mean vitamin C plasma level	Control group (μM/L)	Treatment Group (μM/L)	p value
Before treatment	228.35	201.19	0.41[a]
Two months after treatment	200.29	273.61	0.21[b]
p Value	0.13[a]	0.03[b]	

[a]paired t-test
[b]Wilcoxon Signed Rank Test

In control group, the mean vitamin c plasma level before and after two months of treatment was not significant (p>0.05). In the treatment group, the mean vitamin c plasma level before and after two months of treatment was significant (p<0.05). However, the comparison between two groups was not significant (p>0.05).

The rate of sputum culture conversion at the first week was 0% in control group and 9.6% in the treatment group (p>0.05). They were not statistically significant differences in the first week, however, were significantly different in the second week. 16.1% patients in the treatment group had a negative sputum culture whereas, in control group, there was no sputum conversion. The differencein sputum culture conversion rates in both groups is shown in Table 3.

Table 3. Comparison of sputum culture conversion rate.

Culture time	The number of cultures converted		P value
	Control group	Treatment group	
Week I	0	3 (9.6%)	0.83
Week II	0	5 (16.1%)	0.003
Week III	2 (6.5%)	14 (45.2%)	0.000
Week IV	6 (19.4%)	21 (67.7%)	0.000
Week V	15 (49.5%)	25 (80.6%)	0.023
Week VI	17 (54.8%)	29 (93.5%)	0.001
Week VII	20 (64.5%)	30 (96.8%)	0.002
Week VIII	26 (83.9%)	31 (100%)	0.023

Continuity of sputum culture conversion in treatment group goes on to end of the study. In control group, the sputum culture conversion started at the third week,and at the end of the study, sputum culture conversion was 83.9%. So, there were 16.1% subjects who had not experienced sputum culture conversion. Different t-test analysis indicated sputum culture conversion rate in the treatment group was quicker (two weeks; p=0.03).

4. Discussion

The sample characteristics of both groups showed no significant differences due to the use of the sampling method which equated the variables that might be the confounding between the two groups. Statistical data showed that both group each consisted of 26 men (83.9%) and 5 women (16.1%) with a ratio of 2.4. The mean age in the control group was 36.39±13.98 and in the treatment group was 35.03±11.76 years (p>0.05).

The rates of tuberculosis disease transmission based on gene expert tool results in the control group were 29.0% each high and medium levels, 25.8% at low and 16.1% very low levels. In the treatment group were 32.3% each at the high and Medium level, 22.6% at Low level and 12.9% at very low (p>0.05).

The results of measurement of vitamin C levels before and after treatment in the control group showed no significant differences, despite a decline but still in high level. Normal levels of vitamin C

in the plasma are 70-80 μM/L[9]; this suggests that the mean of vitamin C plasma level in control group are above the average vitamin C plasma levels. This fact can be understood because the vitamin C plasma level in humans are very dependent on adequate intake of food sources consumed because the human body cannot synthesize vitamin C itself[10].

In the treatment group, the mean vitamin C plasma level before and after two months of treatment showed a significant increase. The mean vitamin C plasma levels in the treatment group were higher when compared with the results of the study conducted by Padayatty *et al.* that reported vitamin C supplementation of 1.25 grams through oral yields a concentration of vitamin C in plasma of 134.8±20.6 μM/L[11]. This occurs because the supplementation given is a vitamin C type ester which is slower in the absorption of the body and lasts longer in the body[12]. In the treatment group, inaddition in obtaining a source of vitamin C from food also received supplementation continuously so that the maximum levels of vitamin C in the blood can be maintained. The ability of cells to store ascorbic acid is up to 1500μM/L[9].

In the control group, subjects who experienced a negative sputum culture conversion started the third week of 6.2% and the ended study sputum culture conversion was 83.9%. Our study was similar to the research conducted by Lee *et al.* that showed the sputum culture conversion rate was 90.1%[13]. Findings by Kayigamba *et al.* which published the proportion of TB treatment healing after two months of treatment was 82%[14]. Slowing of AFB culture conversion rates at two months of treatment in the control group was due to many extrinsic factors, drug tolerance[15] and may without any support that speeds up the death of bacteria.

The percentage of sputum culture conversion in the treatment group started since the first week was 9.6% while sputum culture conversion at the end of the study was 100%. The increase of sputum culture conversion rate and sputum culture negative rate in the treatment group was because of the maximal role of tuberculosis drug because the subjects chosen were still rifampicin susceptible. The bacterial cell death by rifampicin is increasingly accelerated by the mechanism of action of vitamin C. The presence of activities termed “the combo attack” are collaborations of first-line tuberculosis drugs and the role of vitamin C through the Fenton reaction within the bacterial cell causes the death of the bacterial cell getting larger.

The mechanism of action of rifampicin will result in superoxide formation (O_2^-) which will damage Fe and Sulfide bonds, and Fe ion will trigger a Fenton reaction[16]. It will also be used by vitamin C to produce a will larger Fenton reaction. Treatment with vitamin C supplementation will form a long chain free fatty acid 2-hydroxylation containing a hydroxyl radical. *M. tuberculosis* bacteria do not produce this fatty acid because it is toxic to *Mycobacterium* compared to the same saturated fatty acids[17]. The Fenton reaction induced by vitamin C begins with the reduction of Ferri ion into a highly reactive Ferro ion so that when reacting with oxygen it produces superoxide. This superoxide will react with hydrogen ions to produce hydrogen peroxide compounds. When hydrogen peroxide reacts with Ferro ions, it oxidizes back into Ferric ions and hydroxyl radical and cause fatty acid oxidation and DNA damage[18].

The requirement for the Fenton reaction of induced by vitamin C to produce oxidative stress is the concentration of high vitamin C in *M. tuberculosis* bacterial cells, and this is closely related to the mechanism of insertion of vitamin C into bacterial cells. Availability and role of Fe ion in *M. tuberculosis* bacterial cells is an important component that plays a role in the occurrence of Fenton reactions

In vitro concentration of vitamin C, less than 4mMol/L will only inhibit the growth of *M. tuberculosis* bacteria[7]. At 32mMol/L, *M. tuberculosis* bacteria culture will be sterile after three weeks[19]. 10mMol/L vitamin C concentration affects the transcription process in *M. tuberculosis* culture[20]. There is a variation in the concentration of vitamin C to be able to be antibacterial, but high concentrations seem to be an absolute requirement. Achievement of vitamin C concentration of at least 4mMol/L in lung cells and macrophage cells is a difficult thing to achieve. The absorption and distribution of vitamin C are saturable and dose-dependent, so there is an effort to maintain vitamin C levels during a low intake but limit the highest levels of serum at high intake. With an average

concentration of vitamin C levels in the control group of only 273.61 μM/L (≈0,27mM/L) (See Table 2) the ability of vitamin C independently to function as an antibacterial would not have been possible.

The mechanism of nutrient absorption in *M. tuberculosis* bacteria is highly dependent on the permeability of the cell wall. Porin is a non-specific protein channel located on the outer membrane of bacteria that allows the entry of hydrophilic solutes[21]. Vitamin C is hydrophilic molecules, so the greatest suspicion of how vitamin C enters the cells of *M. tuberculosis* bacteria is through the protein transport (porin). There is an alleged overlapping function between vitamin C absorption and nutrients so that the process of entering vitamin C into *M. tuberculosis* bacteria cells competes with other hydrophilic nutrients. This is the reason that vitamin C in vivo can only serve as bacteriostatic because of the inadequate attainment of vitamin C concentration as well as the alleged absorption of vitamin C by *M. tuberculosis* bacteria that competes with the absorption of nutrients.

The role of Fe ion in Fenton's reaction is also vital. Vitamin C will utilize the Fe ion present in *M. tuberculosis* bacteria is also very important because Fe ions are highly needed by *M. tuberculosis* bacteria to survive primarily in macrophages, while macrophages also require Fe ions for the continuity of the process of eliminating the *M. tuberculosis* bacteria has been tagged[22]. Macrophage obtains Fe ions from Heme and non-Heme iron sources. Fe ions from the heme iron source will form aRv0203-Fe protein bond. These proteins attach to the mmpL3 and mmpL11 receptors in the bacterial membrane, and by activating the MhuD protein will degrade the Fe-protein bond in the cytoplasm to obtain Fe[23]. Non-Heme iron sources (lactoferrin and ferritin) bind to receptors in macrophages. The existing ion ions are acquired by *M. tuberculosis* bacteria by activating Carboxymicobactin/ mycobactin proteins and bringing Fe ions into bacterial cells through the IrtAB transporter[24]. Fe Ions are stored by bacteria in the form of bacterioferritin and will be used when necessary or in conditions of iron deficiency bacteria. The availability of sufficient iron is a potential material that can be exploited for the occurrence of Fenton reactions either induced by Rifampicin drugs or by vitamin C.

Although the achievement of vitamin C concentration was less than optimal, vitamin C was able to increase the percentage of sputum culture conversion in two months of intensive treatment rather than the group without vitamin C as anti-TB standard. The effect of vitamin C cannot stand alone, in this case, the influence of vitamin C can only serve as a bacteriostatic to the rate of growth and not as a bactericide, however, vitamin C has a very helpful role as the main work of TB drug in eliminating *M. tuberculosis.*

5. Conclusion

Vitamin C supplementation has effects in improving healing process in tuberculosis patients as indicated by higher in sputum conversion rate.

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