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Research Article

Complementary Medicine in Tuberculosis

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ABSTRACT

Vitamin A and Vitamin D play an important role in immunity. The purpose of this study is to analyze whether vitamin D3 and retinyl palmitate could induce the effectiveness of 2nd line Anti-Tuberculosis (TB) drugs. We majorly focus on cell death process. C3HeB/FeJ mice were infected with Multidrug-Resistant (MDR) strain *Mycobacterium tuberculosis* and grouped randomly. The 1st group was euthanized to evaluate TB germs grown in the lungs. 2nd group is received no therapy. The 3rd group was given 2nd line anti-TB drugs. The 4th was given retinyl palmitate along with 2nd line drugs. 5th group was given 2nd line drugs with D3. 6th group was given a combination of 2nd line drugs, retinyl palmitate and D3. Immunohistochemistry demonstrated the quantitative measurement of nuclear receptor expression of Vitamin D (VDR) and Vitamin A (Retinoic Acid Receptor Gamma 2) (RARγ2), apoptosis Caspase-3 (*CASP3*) marker, autophagy markers Cathelin-Related Antimicrobial Peptide (CRAMP) and Microtubule-Associated Protein 1 Light Chain 3B (*MAP1LC3B*), marker of necrosis namely, Receptor Interacting threonine Kinase 3 (*RIPK3*) and interstitial collagenase Matrix Metalloproteinase 1 (*MMP1*). TB germs in lung were counted in Colony Forming Units (CFU). Partial Least Square Structural Equation Modeling (PLS-SEM) with SmartPLS 3.2.6 software was used to analyze structural model within variable. Vitamin D3 plays an important role in increasing autophagy of infected cells and *MMP1*. Both vitamin D3 and retinyl palmitate played a role in increasing Casp3 expression and reducing CFU. The combination of D3 and retinyl palmitate reduced cell necrosis which was characterized by a decrease in *RIPK3*. Our study proves that the combination of D3 and retinyl palmitate supplementation on the 2nd line anti-TB drugs reduces cell necrosis directly.

Keywords: Cell death process, D3, Retinyl palmitate, MDR-TB, Caspase-3

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INTRODUCTION

The efficacy of 2nd Anti-Tuberculosis (TB) drugs is very low. The success rate of Multidrug-Resistant Tuberculosis (MDR-TB) therapy in Indonesia is 51% (WHO, 2016). Literature shows that many of TB patients are having deficiencies of vitamin A and D (Srinivasan A, *et al.*, 2013). The biological effects of the two vitamins are closely related. Vitamin D Receptors (VDR) can form heterodimers with Retinoid X Receptor (RXR) or Retinoic Acid Receptor (RAR) (Schräder M, *et al.*, 1994; Koszewski NJ, *et al.*, 2010; Syal K, *et al.*, 2015). The heterodimer binds to the Vitamin D Response Element (VDRE) and initiates the transcription of the target gene. More than 60 genes have VDRE sequence. These genes are classified into 6 bioresponses-

- Bone metabolism
- Mineral homeostasis
- Detoxification
- Cell cycle (proliferation, differentiation, migration and cell death)
- Immune system
- Amino acids, fats and carbohydrates metabolism (Chengprapakorn W, 2016).

This study aims to analyze how the combination of D3 and retinyl palmitate could improve the effectiveness of the 2nd line anti-TB drugs. Analysis was done by observing the infected cells death pathway i.e., necrosis and apoptosis, or autophagy. Necrosis is a form of death that can release bacteria to infect new cells. Apoptosis is a double-edged knife that kills host cells and bacteria that infect it, while autophagy facilitates bacterial death through the formation of autophagolisosomes. Increased effectiveness of therapy was evidenced by a decrease in the necrosis, and increase in the apoptotic and autophagy pathway. The necrosis pathway was observed with Receptor Interacting Protein Kinase 3 (*RIPK3*) marker, apoptosis was observed with Caspase3 marker (*CASP3*), autophagy was measured with Cathelicidin Related Antimicrobial Peptide (CRAMP) and Light Chain 3B (LC3B) markers. The supplementation goal decreased MTR-TB Colony Forming Units (CFU), and reducing collagenase Matrix Metalloproteinase-1 (*MMP-1*).

MATERIAL AND METHODS

Animals and experimental procedures

Animal care and use were approved by the Institutional Animal Care and Use Committee of Faculty of Veterinary Medicine, Airlangga University, Indonesia (393-KE). 48 C3HeB/FeJ mice aged 5-8 weeks were infected with MDR strain *Mycobacterium tuberculosis* (100 µl) through intratracheal instillation (10^5 CFU/ml) and divided randomly into 6 groups of 8 animals. The 1st Group (G1) was euthanized 2 weeks after infection to see if pulmonary TB had occurred. 2nd Group (G2) was considered as control group which did not receive any therapy. 3rd Group (G3) was treated with the 2nd line anti-TB drugs as recommended by the Ministry of Health of the Republic of Indonesia after 2 weeks of infection. Similarly 4th Group (G4) was given retinyl palmitate and the 2nd line drugs. 5th Group (G5) was given 2nd line drugs and D3 while 6th Group (G6) was given the 2nd line drugs, retinyl palmitate and D3.

Kanamycin (Sigma K1876) was injected in 150 mg/kg Body Weight (BW) once daily on 5 days each week. Pyrazinamide (Sigma P7136) 150 mg/kg BW, levofloxacin (Sigma 28266) 200 mg/kg BW, ethionamide (Sigma E6005-5G) 50 mg/kg BW, cycloserine (Sigma 30020-1G) 300 mg/kg BW, retinyl palmitate (Sigma-Aldrich R1512) 16 IU/g BW and vitamin D3 (Dvion Drops, Merck) 1.25 IU/g BW were given through esophageal cannula 20 gauge once daily on 7 days, each week. After 6 months of therapy, the left lung was processed for immunohistochemistry, and the right lung was used to count bacterial viability.

Primary antibodies used include-anti-RAR2 (ab188000, abcam), anti-VDR antibodies (ab3508, abcam), anti-cathelicidin antibody

(ab64892 abcam), anti-LC3B antibody (ab63817, abcam), anti-*MMP1* antibody (ab137332, abcam), anti-*CASP3* (P17) (PA1961-1, BosterBio), and anti-*RIPK3* (PA2242, BosterBio). Five visual fields were analyzed by 2 independent investigators using the Olympus BX51 light microscope 400X magnification. The brown precipitate indicated the presence of the target antigen.

Statistical analysis

Data was processed with Statistical Package of Social Sciences (SPSS) version 20. Confidence intervals are calculated at a confidence level of 95%. The structural model between variables was analyzed by Partial Least Square Structural Equation Modeling (PLS-SEM) SmartPLS 3.2.6.

RESULTS AND DISCUSSION

Association of nuclear receptor expression

VDR and RARγ2 form heterodimers to start transcription, then it might be interesting to analyze whether VDR expression can be increased by administration of retinyl palmitate or otherwise the expression of RARγ2 can be increased by administration of D3. The post-Hoc Tukey HSD test concluded that the administration of retinyl palmitate 16 units/g BW of mice per day did not increase VDR expression (p=0.993). Vice versa, the Tukey HSD test concluded that the administration of D3 1.25 IU/g BW of mice per day did not increase the expression of RARγ2 (p=0.991). Each pathway is specifically activated by each ligand (*Figures 1* and *2*).

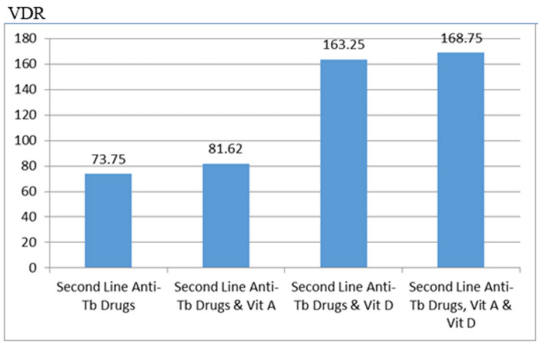

Figure 1 Supplementation of retinyl palmitate on 2nd line anti-TB did not increase VDR expression

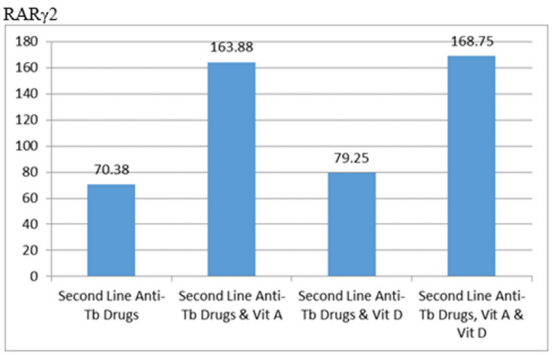

Figure 2: Supplementation of D3 on 2nd line anti-TB did not increase RARγ2 expression

Role of vitamin D3 in autophagy and decreasing MMP1

The Tukey HSD test concluded that D3 is absolutely essential in cathelicidin production (p=0.000), whereas retinyl palmitate did not increase CRAMP (p=0.860) (*Figure 3*). Autophagy was also analyzed by measuring LC3B-insoluble autophagosome membrane. The Mann-Whitney test concluded that cholecalciferol had a role in increasing autophagy membrane formation (p=0.001), retinyl palmitate did not increase LC3B (p=0.834) (*Figures 4* and *5*).

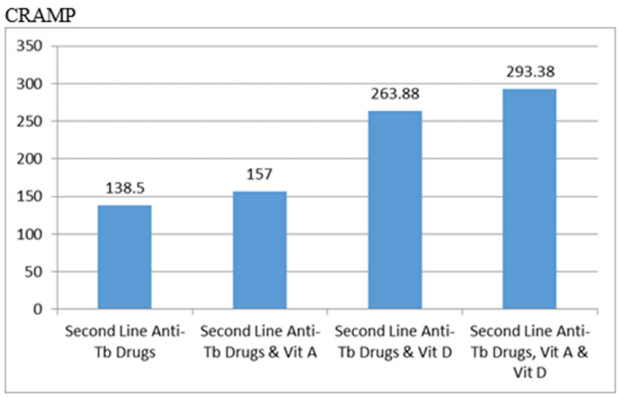

Figure 3: Vitamin D3 supplementation increasing cathelicidin concentration

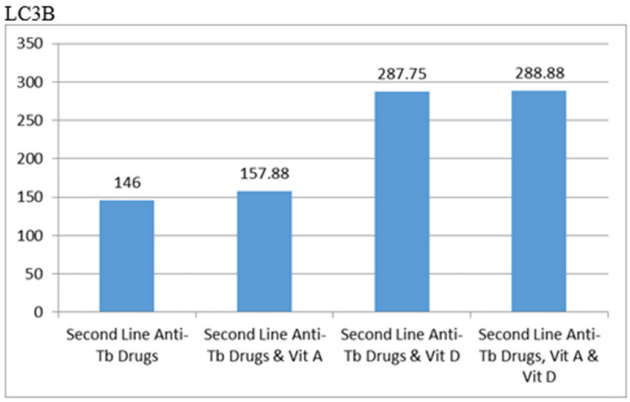

Figure 4: Role of vitamin D3 in the formation of autophagy membrane

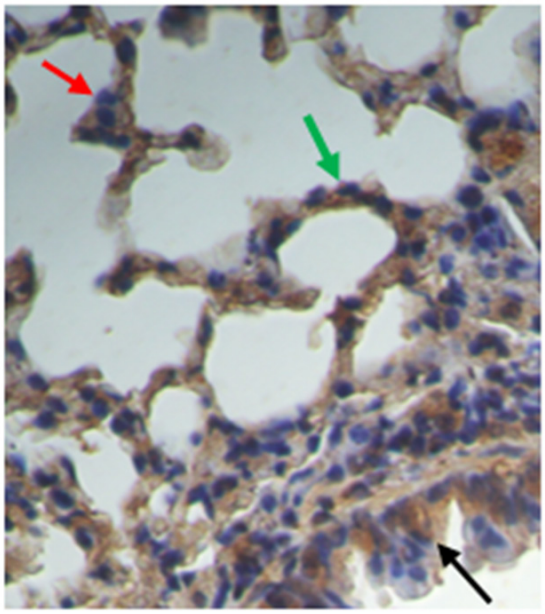

Figure 5: LC3B immunoreactivity is expressed in bronchiolar epithelium (↖), alveolar epithelium (↑) and macrophage (↘)

Some studies use pulmonary collagenase enzymes as markers of the effectiveness of therapy. *MMP1* will decrease along with the success of therapy (Ugarte-Gil CA, *et al.*, 2013). This study concluded that D3 supplementation significantly reduced *MMP1* compared to 2nd line anti-TB therapy alone (*Figures 6* and *7*).

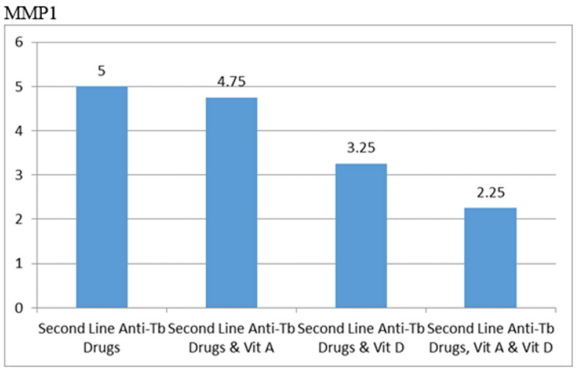

Figure 6: Supplementation of D3 on the 2nd line anti-TB significantly decreased *MMP1* compared to 2nd line anti-TB therapy alone

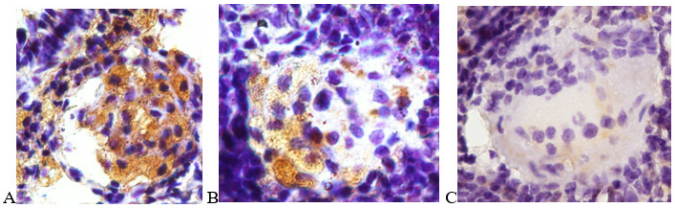

Figure 7: Tubercles in lung mice (immunohistochemistry *MMP1*, 1000X). A: Without therapy, (B): With 2nd line anti-TB therapy, (C): 2nd line anti-TB drugs and D3 supplementation

Role of vitamin D3 and retinyl palmitate in apoptosis induction and bacterial suppression

Apoptosis is the mechanism of infected cells to eliminate bacteria. The Mann-Whitney test concluded that both D3 and retinyl palmitate contribute to increase *CASP3* (*Figure 8*) (p=0.035 and p=0.027). This is supported by the calculation of bacterial viability in lungs, both D3 and retinyl palmitate play a role in reducing CFU (p=0.000 and p=0.000) (*Figure 9*).

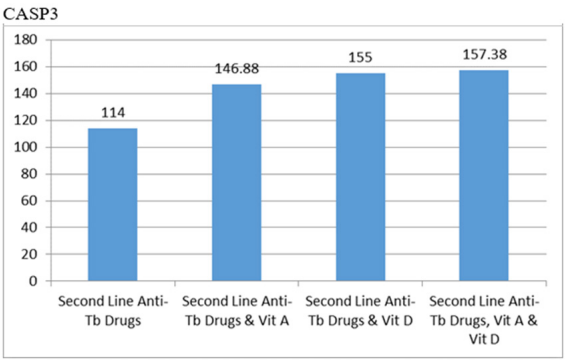

Figure 8: Both D3 and retinyl palmitate play a role in increasing apoptosis

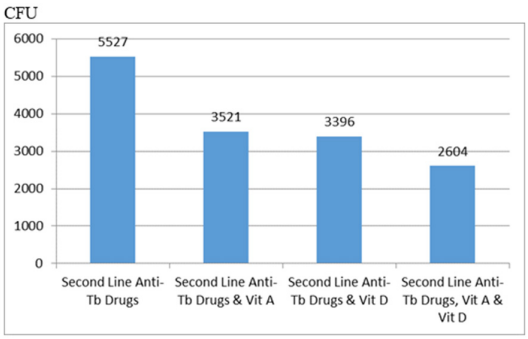

Figure 9: Both D3 and retinyl palmitate play a role in decreasing bacterial viability

Association of vitamin D3 in combination with retinyl palmitate in necrotic cell death

In previous study we showed that vitamin D3 reduce necrosis indirectly through increasing apoptosis or autophagy (Wahyunitisari MR, *et al.*, 2017). This time, PLS-SEM proves that the combination of D3 and retinyl palmitate directly decreases *RIPK3* (*Figures 10* and *11*).

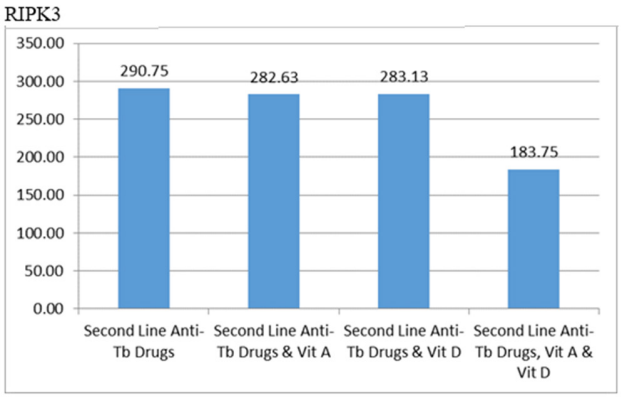

Figure 10: The combination of D3 and retinyl palmitate significantly decreases necrotic cell

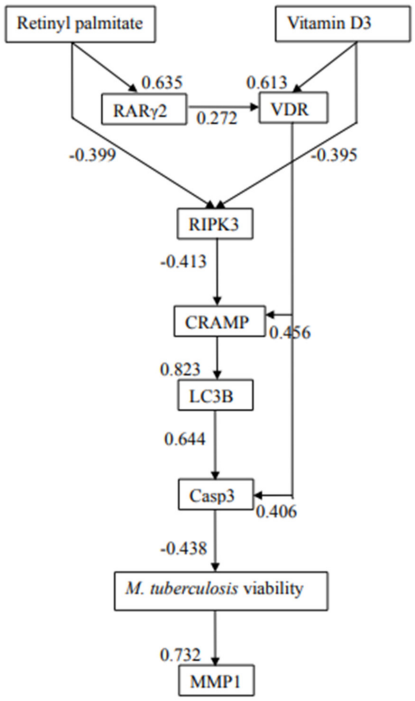

Figure 11: PLS-SEM analysis showed that the combination of D3 and retinyl palmitate directly decreased necrosis cell
Note: Numbers denote Beta (β)-path coefficient, the amount of contribution of the independent variables affects the dependent variables. The t-test value >1.96 indicates a meaningful relationship

TB and malnutrition have complex relationships. Although vitamin D can be produced by epithelial cells, lymphocytes, and antigen presenting cells, supplementation of vitamin D for active TB is still needed because of the high requirement (Wang Q, *et al.*, 2018). Vitamin D nuclear receptors were first discovered in intestine (Brumbaugh PF, *et al.*, 1974), then began to be found in other tissues including lung epithelial cells (Colston K, *et al.*, 1982), and alveolar lymphocyte (Biyoudi-Vouenze R, *et al.*, 1991).

Although this study proved that VDR and RARγ2 were only induced by each ligand, PLS-SEM analysis proved that in TB pathogenesis, RARγ2

had an effect on VDR activities with path coefficient 0.272 (*Figure 11*). SEM not only evaluates the measurement but also asses the structural model. Structural model has been able to estimate the relationships among latent variable and model disturbances. VDR is a chromosomal protein detected in macrophages, activated lymphocytes, bronchioles epithelium and alveolar epithelium (*Figure 12*). RAR is proven to be able to bind to DNA enhancer sequence VDRE (Schüle R, *et al.*, 1990). RAR can form heterodimers with VDR (Schräder M, *et al.*, 1993). In lung tissue, RARγ2 immunoreactivity is detected in the bronchiolar epithelium, endothelial, and alveolar epithelial (*Figure 13*).

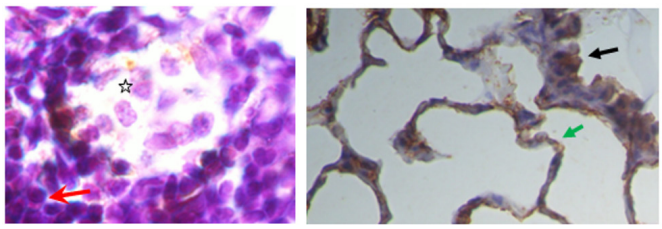

Figure 12: VDR expression is found in macrophages (☆), activated lymphocytes (), bronchiolar epithelium (←) and alveolar epithelium () (VDR immunohistochemistry) (left: 1000X and right: 400X)

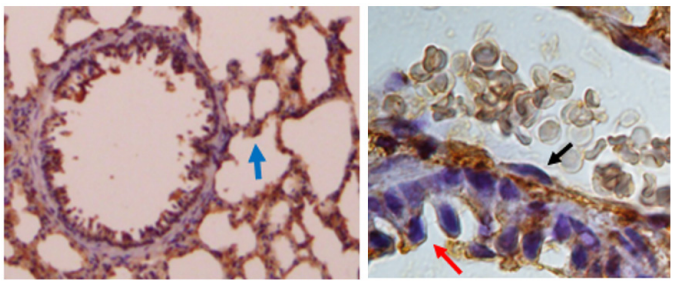

Figure 13: RARγ2 immunoreactivity is detected in the bronchiolar epithelium (), endothelial (), and alveolar epithelium () (Immunohistochemistry RARγ2, left 100X, right 1000X)

Autophagy is self-eating process. The two autophagy markers analyzed in this study were LC3B autophagy membrane precursor and antimicrobial CRAMP. CRAMP is expressed in bronchiolar epithelium, macrophage and alveolar epithelium cells (*Figure 14*). This study concluded that vitamin D3 plays an important role in autophagy, which is consistent with the literature. Vitamin D3 induces antimicrobial CRAMP through VDR (Liu PT, *et al.*, 2009; Dhawan P, *et al.*, 2015). Supplementation of retinyl palmitate does not improve CRAMP. The anti-mycobacterial activity of vitamin A was concluded through autophagosome acidification (Rajawat Y, *et al.*, 2011; Wheelwright M, *et al.*, 2014).

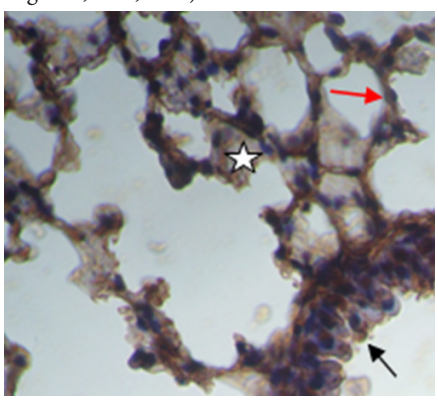

Figure 14: CRAMP is expressed in bronchiolar epithelium (), macrophage () and alveolar epithelium cells () (Immunohistochemistry CRAMP, 400X)

Vitamin D3 increases the expression of LC3B, this complete the data that vitamin D also increases the expression of *ATG16L1* (Sun J, 2016), *ATG5* and Beclin-1 (Yuk JM, *et al.*, 2009; Campbell GR and Spector SA, 2011). Vitamin D supplementation is useful for immunocompromised patients, such as TB with diabetes mellitus (Kota SK, *et al.*, 2011; Lopez-Lopez N, *et al.*, 2014), and TB/HIV co-infection (Campbell GR and Spector SA, 2012). Vitamin D does not inhibit Th1 cell differentiation (Rode AK, *et al.*, 2017). There is no literature that links RARγ with autophagy membrane formation. Vitamin A nuclear receptor that associated with LC3 is RARα (He W, *et al.*, 2014).

As a general rule, host inflammatory mediators have been associated with tissue destruction. This study concluded that D3-VDR reduced lung damage through decreasing *MMP1*, this completes data from previous studies that concluded vitamin D3 reduced *MMP7* and *MMP9* expression and increased *TIMP1* (Anand SP and Selvaraj P, 2009). Vitamin D3 is alveolar type-II cell growth factor (Edelson JD, *et al.*, 1994).

Vitamin D3 reduces TB immunopathology (Gemelli C, *et al.*, 2013; Dauletbaev N, *et al.*, 2015; Reeme AE, *et al.*, 2016), and prevents pulmonary fibrosis (Ramirez AM , *et al.*, 2010; Li F, *et al.*, 2015). PLS-SEM analysis shows retinyl palmitate 16 IU/g BW decreases *MMP1* expression through intermediate variables (*Figure 11*). The literature shows that RARγ agonist work depending on its concentration (Kimura K, *et al.*, 2017). It is necessary to find the right concentration of supplementation so that retinyl palmitate can decreases *MMP1* expression directly.

Apoptosis is a form of programmed cell death without causing an inflammatory reaction. Apoptosis is known to be an innate anti-mycobacterial mechanism in the presence of efferocytosis. According to the literature, this study proves that the retinyl palmitate-RARγ pathway induces *M. tuberculosis* infected cell apoptosis (Szondy Z, *et al.*, 1997). It has also been noted that vitamin A increases apoptosis receptor Tumor Necrosis Factor Receptor Superfamily member 6 (*TNFRSF6*) and apoptosis regulator gene Reticulon 3 (*RTN3*) (Balmer JE and Blomhoff R, 2002). The D3-VDR pathway has also been shown to increase *M. tuberculosis* infected cell apoptosis. In addition to the genomic pathway, D3 also increases apoptosis by increasing intracellular Ca^{2+} concentrations that lead to mitochondrial membrane potential change and cytochrome C release (Mehto S, *et al.*, 2015).

Vitamin D3 is significantly associated with decreased viability of *M. tuberculosis* (Bloom BR and Modlin RL, 2016). In addition to direct antimycobacterial action (Greenstein RJ, *et al.*, 2012), vitamin D reduces bacterial viability through increased macrophage activity (Abe E, *et al.*, 1984), hepcidin expression inhibition which results in decreased intracellular iron concentrations. *M. tuberculosis* requires iron for its growth (Szymczak I and Pawliczak R, 2016). Vitamin A inhibits *M. tuberculosis* growth directly (Greenstein RJ, *et al.*, 2012; Crowle AJ and Ross EJ, 1989), and also activating lymphocyte T and macrophages (Yamada H, *et al.*, 2007).

Necrosis is a form of premature death due to excessive oxidative stress. Necrosis and necroptosis have the same subcellular process, only the onset of necrosis is faster (Berghe TV, *et al.*, 2010). Necrotic death of infection cells is a major contributor to lung injury. *RIPK3* as a marker of necrosis, is known to play a role in NF-κB activation and inflammasome which are closely related to oxidative stress (Moriwaki K and Chan FM, 2017; Ramya D, *et al.*, 2012; Wang Z, *et al.*, 2016; Bohn T, 2017; Teixeira TM, *et al.*, 2017). PLS-SEM analysis concluded that the combination of D3 and retinyl palmitate supplementation decreased *RIPK3* expression directly.

CONCLUSION

This is supported by a literature stating that vitamin A and vitamin D increase the factors that contribute to an endogenous antioxidant activity such as Nuclear Factor like 2 (NRF2), Superoxide Dismutase 2 (SOD2), Glutathione Peroxidase (GPX), Nicotinamide adenine dinucleotide phos-

phate hydrogen Quinone Oxidoreductase 11 (NQO1), and Heme Oxygenase 1 (HO1). If vitamin D supplementation alone decreases cell necrosis through intermediate variables, this study proves that a combination of vitamin A and vitamin D supplementation can reduce cell necrosis directly. Proper supplementation can improve the effectiveness of TB therapy.

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