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Antimicrobial activity of Vitamin-D in human macrophages isolated from older adult women peripheral blood

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Abstract
Vitamin D deficiency is becoming a public health concern all over the world including India and its deficiency is associated with several adverse health outcomes. Many studies suggest that among all age groups geriatric population are more susceptible to Vitamin D deficiency and also more prone against infections than other age groups. A vitamin D replete state appears to benefit most infections. In present study the association of vitamin D status with antimicrobial activity of cultured macrophages isolated within an exclusively rural elderly women population cohort were assessed, since they are at an increased risk of both insufficient and deficient vitamin D status. We found that *in vitro* supplementation of vitamin D increases antimicrobial activity in monocytes derived macrophages (MDM), which also influences their current Vitamin D status.

Keywords: Vitamin-D, peripheral blood mononuclear cells, macrophages, iNOS & SOD activity, geriatric women, infections

1. Introduction
Evidence exists that vitamin D has a potential antimicrobial activity and its deficiency has deleterious effects on general well-being and longevity. Vitamin D may reduce the risk of infection through multiple mechanisms; Vitamin D boosts innate immunity by modulating production of anti-microbial peptides (AMPs) and cytokine response [1]. Moreover, Vitamin D helps in boosting the activity of monocytes and macrophages thereby contributing to a potent systemic anti-microbial effect [2] A vitamin D replete state appears to benefit most infections. Antibiotics remain an expensive option and misuse of these agents results in significant antibiotic resistance and contributes to escalating health care costs. Vitamin D constitutes an inexpensive prophylactic option and possibly therapeutic product either by itself or as a synergistic agent to traditional antimicrobial agents [1] Vitamin-D deficiency is becoming a public health concern all over the world including India and its deficiency is associated with several adverse health outcomes [3]. Many studies suggest that among all age groups geriatric population (>60 years) are more susceptible to Vitamin D deficiency [4]. They are also comparatively more prone against infections than other age groups. Geriatric population is in the rise all over World (including India). India's elderly population has already crossed 100 million mark during 2011 [5]. The crucial role of $1,25(OH)_2D_3$ in the immune system was confirmed by other evidences. First, the intracrine induction of antimicrobial activity by $1,25(OH)_2D_3$ is a pivotal function of the monocyte/macrophage response to infection. Second, sub-optimal vitamin D status is a common peculiarity of many populations throughout the world, with the possible support of monocyte/macrophage metabolism of $25(OH)D_3$ and subsequent synthesis and action of $1,25(OH)_2D_3$ [6].
Inducible Nitric Oxide Synthase (iNOS) is known to play a significant role in host defense against a number of microbial infections. Intracellular microbial killing is often associated with the expression of iNOS and Nitric Oxide (NO) [7]. The ability of $1,25(OH)_2D_3$ to regulate iNOS activity in innate immune cells is already been proved, from previous studies it has been shown that $1,25(OH)_2D_3$ enhances human monocyte anti-mycobacterial activity by enhancing the synthesis of reactive oxygen intermediates (ROI) and reactive nitrogen

intermediates (RNI) in *M. tuberculosis* infected macrophages as well as THP1 cells via NADPH oxidase system and is regulated by phosphatidyl inositol 3-kinase (PI 3-K) signaling pathways [8, 9].

Another antimicrobial parameter the super oxide dismutase (SOD) activity is used to identify the bactericidal effect of polymorphonuclear leucocytes, depending on their superoxide generative capacity [10, 11], previous studies also proved that this enzyme activity is also regulated by 1,25(OH)2D3 in human monocytes andTHP1 cells [8, 9].

These findings and demographic situations have provided a new opportunity to investigate the following objectives, which is to examine the association of vitamin D status with antimicrobial activity of cultured macrophages isolated within an exclusively elderly population cohort, since they are at an increased risk of both insufficient and deficient vitamin D status. Another objective of this study is to find out the antimicrobial activity of cultured macrophages after *in vitro* supple-mentation of Vitamin D.

2. Methodology

2.1 Study Subjects

The experiment was conducted with the human subjects' understanding and consent, as well as a statement that the responsible Ethical Committee has approved the experiments. 65post –menopausal geriatric women (mean age 62.5±4.23 years); Elderly women with abnormal c-reactive protein level, chronic renal, hepatic, cardiac, acute critical illness were excluded from the study.

2.2 Isolation and culture of Human Macrophages

Peripheral blood mononuclear cells(PBMC) were isolated from heparinised blood (4ml) of healthy older adult women volunteers by density gradient centrifugation with Ficoll-Paque (HIMEDIA).The cells were washed twice in phosphate-buffered saline (PBS) and were resuspended in medium RPMI 1640 (HIMEDIA), supplemented with 10% Fetal Calf Serum and Macrophage Cell Stimulating Factor (MCSF) also added at2ng/ml concentration. Finally, cells were added to adherent 6 well plates at a density of ≈ 2 $\times 10^6$ cells per well. After incubation for 48 hours, at 37 ºC and 5% CO_2 environment, the non-adherent cells were removed by repeated vigorous washings.Selected cell culture were then supplemented with $1,25(OH)_2D_3$ at a dose of 10^{-8}M for 72 hours. After completion of seven days the cells were isolated (>10^6 cells per well) and incubated with certain bacteria *(E. coli*) for 120 minutes. The infection were given in two set, SET-1= ratio of 1: 4 and SET-2= ratio of 1:400 *E. coli* cells. After which iNOS activity and SOD activity were assayed. [12, 13].

2.3 Assessment of Serum Vitamin-D level

Biochemical estimations were carried out using standard ELISA method [14] Serum 25(OH) D level of 20.0 - <30.0 ng/ml was classified as vitamin D insufficiency (VDI), levels >30.0 ng/ml and < 20 ng/ml were classified as normal and vitamin D deficiency (VDD), respectively [4, 14].

2.4 Bacteria Killing Assay

Human MDMs (2×10^6 cells) were suspended at 1:4 (for SET-1) and 1:400 (for SET-2) ratio with *E. coli* in a final volume of 1ml of Phosphate buffer solution. This suspension was then incubated with gentle rocking, at 37 ^{0}C.Aliquots of the suspension were plated at 0 minute, and 120 minute of incubation [15]. The Agar plates were then incubated at 37 ^{0}C in incubator, and bacterial colonies were counted the next day. Results are expressed as bacterial killing =100 – (N/N_0*100) where N= is the number of colonies counted at each time point and N_0 =is the number of colonies counted at time zero [15].

2.5 Estimation of inducible Nitric Oxide Synthase (iNOS) and Superoxide Dismutase (SOD) activity

Macrophage cell lysate from individual subjects were assayed for iNOS activity and Super Oxide Dismutase activity (SOD) using standardized protocol at 420µM [12, 13].

2.6 Statistical analysis

Data are expressed as mean ±SD. Within a given group, different treatment conditions were compared using a Paired Student's t test, where $P<0.05$ considered to be statistically significant. Test of significance of correlation coefficient were also performed.

3. Result and Discussion

The subjects consisted of 65 geriatric women (mean 62.5±4.23 year), including 37 women having 10.03 ±4.46 ng/ml serum $25(OH)D_3$ level, considered as Deficient Group, 9 women having 26.52±2.49 ng/ml serum $25(OH)D_3$ level, considered as Insufficient Group, and 19 women having 53.37±23.65 ng/ml serum $25(OH)D_3$ level, considered as Normal Group. For each cases monocytes derived macrophages were cultured and infected with different strength of *E. coli* cultures (SET-1 and SET-2) and incubated for 120 consecutive minutes. Assessments of inducible Nitric Oxide Synthase activity, Super oxide dismutase activity and at the same time reduction in *E. coli* colony counts were analyzed.

iNOS is an essential enzyme in protective immunity against different bacterial infections [16, 17]. Nitric Oxide can inhibit both microbial DNA replications and cellular respirations [16]. In the presence of oxygen, nitric oxide can be further catalyzed into nitrogen dioxide (NO_2), nitrogen trioxide (N_2O_3), nitrate (NO^-_3) and other reactive nitrogen species. The expression of iNOS could be differentially regulated by different microbial products. In addition to live bacteria, bacterial components such as endotoxin, lipoproteins or exotoxins could also effectively stimulate macrophages to express iNOS activity [17].

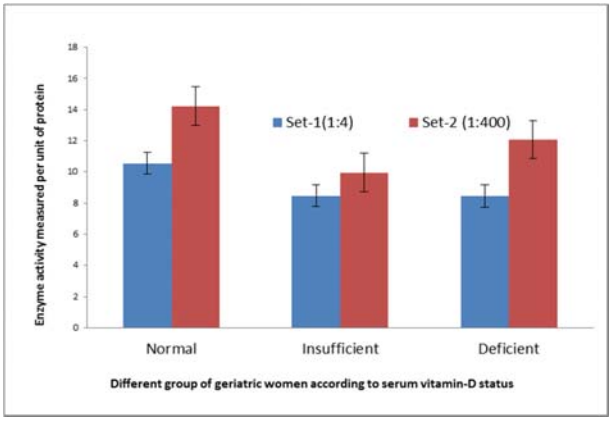

Fig 1: Inducible Nitric Oxide Synthase Assay among different group of geriatric women according to serum vitamin-D status

Observing iNOS activity among above mentioned three groups (e.g.; Normal, Deficient, and Insufficient group) has shown interesting interpretation (Fig.1); where iNOS activity

increases with increase in serum 25(OH)D level (r = 0.214, $p<0.05$),which means person with low vitamin-D level(i.e., Deficient group) also have low iNOS activity (8.45±4.54 for SET-1 infection and 12.07±8.44 for SET-2 infection).Again person having normal 25(OH)D level have shown high iNOS activity (10.55±4.05 for SET-1 infection and 14.23±5.67 for SET-2 infection).

The above mentioned data suggested that to obtain maximum immune response related to iNOS activity optimum serum level of vitamin-D may be desirable among human monocytes/macrophages.

Again after *in vitro* vitamin D supplementation among these selected target groups have shown another interesting interpretation (Fig. 2A, 2B, 2C).Macrophages cultured from PBMC of Normal Serum Vitamin-D consisting women shown no marked increase in iNOS (Fig.2A) activity (14.50±5.67 for SET-1 infection and 14.35±6.85 for SET-2 infection) when supplemented with $1,25(OH)_2D_3$ at a dose of 10^{-8}M for 72 hours. It indicate that as their serum consists of optimum vitamin D level, excess *in vitro* Vitamin D supplementations has no influence on its iNOS activity.

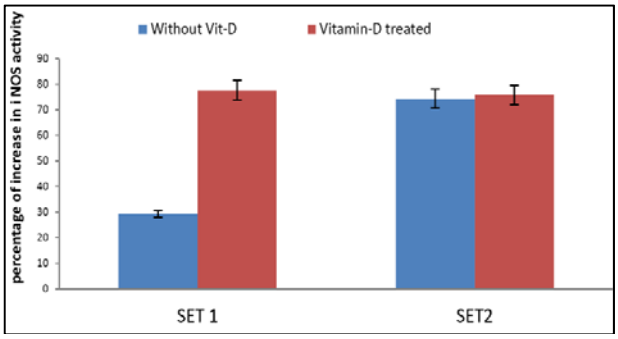

Fig 2A: Inducible Nitric Oxide Synthase Assay among Normal older women

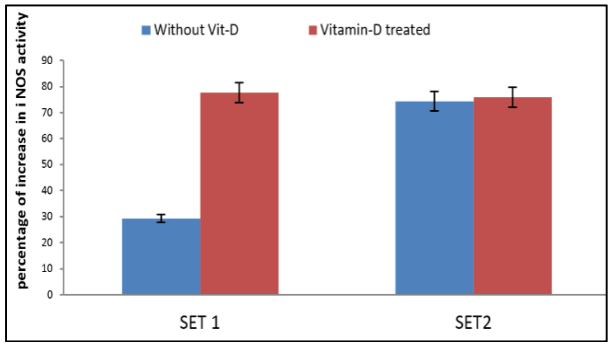

Fig 2B: Inducible Nitric Oxide Synthase Assay among Deficient older women

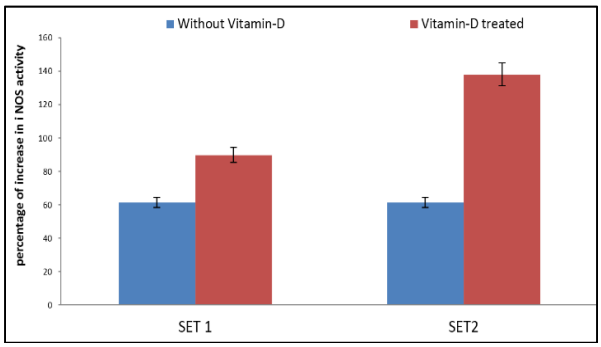

Fig 2C: Inducible Nitric Oxide Synthase Assay among Insufficient older women

Macrophages cultured from PBMC of Deficient Group of women shown marked increase in iNOS (Fig.2B) activity from its untreated one (10.11±6.52 for SET-1 infection and 14.93±5.13 for SET-2 infection) when supplemented with $1,25(OH)_2D_3$ at a dose of 10^{-8}M for 72 hours and it is significant at 5% level (t=2.58,p=0.0011 for SET-1 infection and t=3.80 p=0.003 for SET-2 infection). As their serum consists of low vitamin D level, therefore without sufficient vitamin D these iNOS activity was not its maximum level when exposed to infections, which indicates its poor immunological response against infections, but after supplementation of vitamin D at a certain standardized dose help these macrophages to show its maximum iNOS activity against infections. Again for Insufficient Group (Fig. 2C) of women, like Deficient Group similar activities were observed (7.16±3.202 for SET-1 infection and12.48±6.02 for SET-2 infection). These data clearly suggest normal function of macrophages from aged people in presence of $1,25(OH)_2D_3$.

Molecular oxygen is an essential element of life, yet as a result of incomplete reduction of oxygen to water, reactive oxygen species (ROS) are generated in all aerobes. Most ROS are generated as superoxide anions), ($O^{-}_2$and are rapidly dismutated either non-enzymatically or enzymatically by the action of superoxide dismutase (SOD) to hydrogen peroxide and oxygen [18]. Pathogen-derived generation of O^{-}_2 by NADPH oxidase in mammalian neutrophils is a common feature [10]. In fact, the bactericidal effect of polymorphonuclear leucocytes depends on their superoxide generative capacity [11, 19], and biosynthesis of SOD is mainly controlled by increased intracellular fluxes of O^{-}_2 in numerous micro-organisms [17] as well as in higher organisms [18, 19].

Observing SOD activity among above mentioned three groups (e.g.; Normal, Deficient, and Insufficient group) has shown in Fig.3; The SOD activity for Normal Group is 8.36 ±3.95 for SET-1 infection and 10.52±5.72 for SET-2 infection and that for insufficient group (8.60 ±4.56 for SET-1 infection and 12.70±4.72 for SET-2 infection) have shown increased activity after infection, whereas the SOD activity for Deficient Group is 9.71 ±4.31 for SET-1 infection and 10.81±5.07 for SET-2 infection.

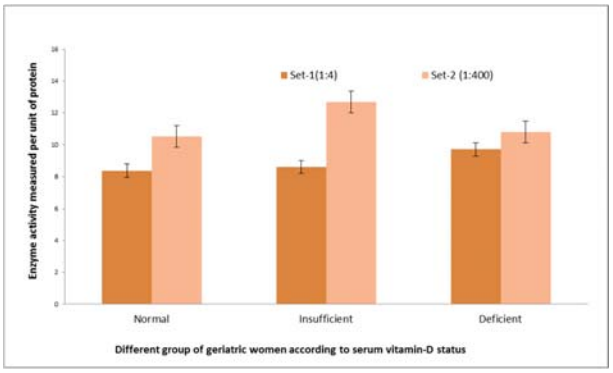

Fig 3: Super Oxide Dismutase Assay among different group of geriatric women according to their serum vitamin-D status

In vitro vitamin-D supplementation among these selected target groups have shown another significant observations (Fig. 4A, 4B, 4C). Macrophages cultured from PBMC of Normal Serum Vitamin D consisting women shown increase in SOD (Fig.4A) activity but it is insignificant at 5% level, (15.41±5.46 for SET-1 infection and 10.99±6.04 for SET-2 infection) when supplemented with $1,25(OH)_2 D_3$ at a dose of 10^{-8}M for 72 hours.

Fig 4A: Super Oxide Dismutase Assay among Normal older women

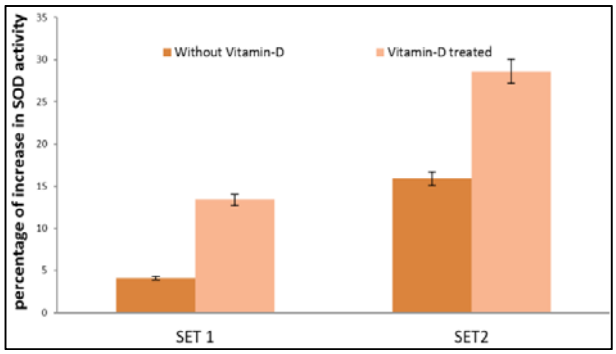

Fig 4B: Super Oxide Dismutase Assay among Deficient older women

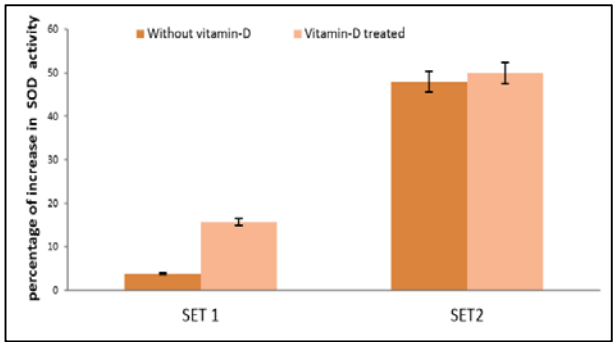

Fig 4C: Super Oxide Dismutase Assay among Insufficient older women

Macrophages cultured from PBMC of Deficient Group of women also shown increase in SOD (Fig.4B) activity from its normal one (10.58±6.62 for SET-1 infection and12.0±7.04 for SET-2 infection) when supplemented with $1,25(OH)_2D_3$ at a dose of 10 $^{-8}$M for 72 hours, but all are insignificant at 5% level.

Bacteria killing assay was performed where among Normal group the percentage of colony forming unit (CFU) reduction is higher than the other two group, again deficient group has comparatively less CFU reduction. Vitamin D plays crucial role in bactericidal activity of macrophages, which is already been proved by many previous works [8, 9, 15, 20, 21]. In present study it is also observed that macrophages cultured from normal serum vitamin D consisting people has more bactericidal capacity than other two groups, again deficient group has comparatively less bactericidal capacity due to inadequate serum vitamin D level.

After *in vitro* supplementation of $1,25(OH)_2D_3$ among Normal group, (Fig. 6A & 6B) no significant increase in CFU reduction has observed which indicates its maximum bactericidal capacity has reached without excess vitamin D supplementation. In case of Deficient group and Insufficient group the percentage of CFU reduction has increased but they are statistically insignificant at 5% level. Study with more sample may give us one clear picture regarding this.

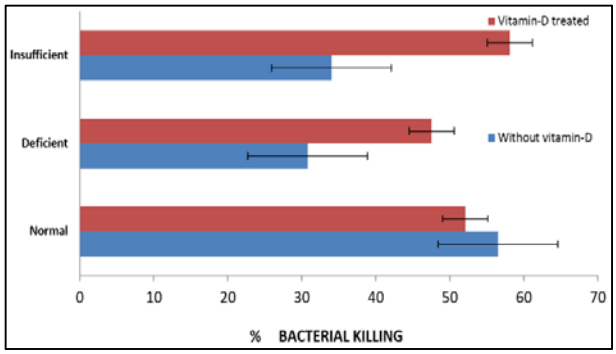

Fig 6A: Bacteria Killing Assay for SET-1 Infection after *in vitro* Vitamin-D supplementation

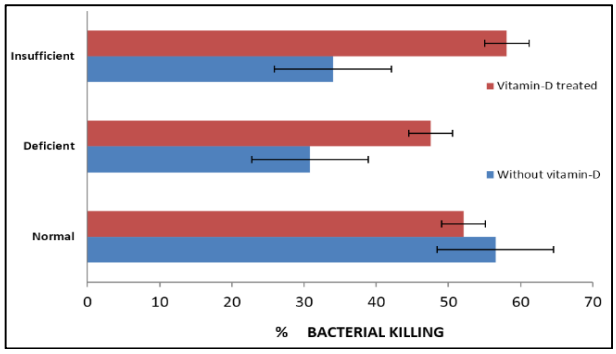

Fig 6B: Bacteria Killing Assay for SET-2 Infection after *in vitro* Vitamin-D supplementation

4. Conclusion

In summary, our results support the conclusion that Vitamin D is an important mediator of antimicrobial effects in normal human MDMs, (Monocytes Derived Macrophages). Additionally Vitamin D may have crucial effect on regulation of iNOS activity, SOD activity, Bactericidal activity and further stimulates the macrophages to act better against the invading pathogens. Many previous studies and this present study showing a host deficient in 25(OH) D could suffer impaired innate immune responses from the barrier and innate immune effector cells resulting in increased susceptibility to infection. Mostly among geriatric people improper Vitamin D status is one such important specific nutritional deficiency that directly affects their immunological status other than bone metabolism. These present findings support epidemiological reports and case studies associating the importance of Vitamin D with an increase in opportunistic infections, susceptibility to human immunodeficiency, especially for geriatric people. Further work needs to be carried out to reveal the detailed mechanism underlying this outcome. These preliminary observations can now help to explain some of the speculations about the role of Vitamin D in different infections. Consequently, consideration might be given to clinical trials of inexpensive vitamin D supplementation at appropriate doses to enhance innate immunity to microbial infections.

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