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Research Article

Antimicrobial Effect of 1,25 Dihydroxy Vitamin D on *Escherichia coli* and its Association with Serum 25 Hydroxy Vitamin D Level: An Experimental Study on the Elderly Women

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Abstract

Introduction: Vitamin D plays crucial role as an antimicrobial agent, the deficiency of which has deleterious effects on the general wellbeing, especially among elderly. The incidence and severity of infectious diseases in the elderly are associated with a variety of functional, demographic and immunologic changes inside the body due to ageing.

Objective: The aim was to examine the association of vitamin D status with antimicrobial activity of cultured macrophages isolated within an exclusively elderly population cohort. In addition, the present study was also determined the antimicrobial activity (against *E. coli* infection) of cultured macrophages after *in vitro* supplementation of Vitamin D.

Materials and Methods: This experimental study was conducted among 97 randomly selected rural elderly women aged between 60 to 70 years of age, during the period of April 2014 to August 2018, at Amdanga block, North 24th parganas, West Bengal. Their vitamin D status was assessed by the estimation of serum 25(OH)D and classified into three groups viz. sufficient (40 members), insufficient (28 members) and deficient (29 members). After that the Peripheral Blood Mononuclear Cells (PBMC) were isolated and cultured from fresh blood from each and every study subject. Supplementation of 1,25 dihydroxy vitamin D[1,25$(OH)_2$D] was given selectively at a dose of 10×10^{-8} M for 72 hours in the culture media and were exposed to *Escherichia coli* and screened for their iNOS activity (inducible Nitric Oxide Sythase), SOD activity (Superoxide Dismutase) and CFU (Colony Forming Unit) reduction rate. SPSS software, version 20.0 was used to perform statistical analysis.

Results: iNOS activity and SOD activity were significantly increased in case of both sufficient and deficient group. As per the CFU reduction rate against *E. coli* infection there is no significant difference were observed according to serum 25(OH)D consisting group. After *in vitro* 1,25$(OH)_2$D supplementation,the maximum increase in CFU reduction rate was observed among Deficient group(63.57%), whereas in case of Insufficient group it was 60.11% and for Sufficient group it was 44.66%.

Conclusion: Considering bacteria killing capacity of macrophages the *in vitro* 1,25$(OH)_2$D supplementation significantly inreases the CFU reduction rateoverall. Sufficient group's macrophages always had better profile than other two groups. *In vitro* 1,25$(OH)_2$D supplementation increases iNOS and SOD activity significantly.

Keywords: *E. coli*; iNOS Activity; SOD Activity; Bacteria Killing Assay; Elderly Women

Introduction

MDR-associated infections are the current threat to any health care system, or medical community with limited therapeutic options including substantial morbidity as well as mortality with a huge economic burden [1]. Significantly higher incidence rate and multiple occurrence of infectious diseases in the elderly are associated with a variety of functional, demographic and immunologic changes inside the body due to ageing [2,3]. Recent UN report revealed that drug-resistant microbial infections kill about 700,000 people annually, and by 2050 it may increase to a crore. Reports also revealed that the AMR could force nearly 2.4 crore people into indigence by 2030, with 2-3.5% decreased gross domestic product, costing 100 trillion USD [4]. Globally India ranked first due to its per-capita usage and total consumption of antibiotics (12.9×10^9 units), thus it is cited as the global "AMR capital" [5,6]. Furthermore the global health care cost in hospitals with infections has increased in geometric proportion [7]. Although the detection of MDR-pathogens are common in community-acquired infections, furthermore the COVID-19 pandemic has shifted the focus to viral therapy. Additionally the most common incidence among elderly is that they deals with multiple medications for underlying illnesses, thus management is complicated due to age-related organ system changes, therefore antimicrobial therapy needs to be chosen keeping drug interactions and adverse events in mind [2,3,8,9]. Another concern is the imprudent use of antimicrobials in agriculture, fishery, and veterinary, making the situation worse [10]. Furthermore the wide-spread occurrence of drug-resistant strains in the environment was aggravated by pollute water-bodies, soil, and sediments [11]. Thus, immediate action on these global threats is the top priority. Vitamin D on the other hand remains one formidable antimicrobial agent [12]. At cellular level more than 200 genes are regulated by vitamin D, including cell proliferation, differentiation and apoptotic genes [13], and act as the key holder for modulating systemic inflammation, oxidative stress and mitochondrial respiratory functions. Therefore, a vitamin D replete state appears to benefit most infections [14]. At present the adjunctive treatment of vitamin D against different infection is coming in focus [15]. Whereas, Vitamin D deficiency is also a worldwide phenomenon among elderly [16-18].

Thus the objective of the present study was to examine the association of vitamin D status with antimicrobial activity of cultured macrophages isolated within an exclusively elderly population cohort. In addition, the present study was also determined the antimicrobial activity of cultured macrophages after *in vitro* supplementation of Vitamin D.

Materials and Methods

Study type and design

This was an experimental study, conducted among 97 elderly women, aged between 60-70 years (mean age 62.5±4.23 years), selected randomly from 30 villages of Amdanga block, North 24th Parganas district, West Bengal, India, within the time period of April 2014 to August 2018.

Inclusion criteria

Elderly women aged between 60-70 years, who were cooperative in nature and willing to participate were included in the study.

Exclusion criteria

Elderly women having previous history of thyroid dysfunction, on hormonal replacement therapy, amenorrhoea due to any pathological cause or surgery, on vitamin D supplementation, and physically or mentally challenged were excluded from the study. Elderly women having fever in last 20 days and having high total WBC count and high C-reactive protein level were also excluded from the study.

Data collection

At first stage the 97 elderly were selected randomly and their serum 25(OH)D level were screened accordingly and classified into three groups i.e. sufficient groups (40 members), insufficient groups (28 members) and deficient groups (29 members). Deficiency, insufficiency, and sufficiency of vitamin D were defined as ≤20, 21–29, and ≥30 ng/ml of serum 25(OH)D in the human blood, respectively [18]. In the next stage the peripheral blood mononuclear cells (PBMC) were isolated from fresh blood (4 ml) of each of the studied subjects and the collected PBMC were divided into two groups i.e. test and control; where test group received vitamin D supplementation ($1,25(OH)_2D$ was supplemented in liquid form by mixing it gently with the given culture medium) and control groups did not receive any supplementation. Finally, after the development of monocyte derived macrophages (MDMs), each test and control group were exposed to *E. coli* infection for 120 minutes.

Isolation and culture of human macrophages

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood (4ml) of each and every study subjects by density gradient centrifugation with Ficoll-Paque [19]. 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 at 2 ng/ml concentration. Finally, cells were added to adherent 6 well plates at a density of 2×10^6 cells per well. After incubation for 48 hours, at 37°C and 5% CO_2 environment, the non-adherent cells were removed by repeated vigorous washings. Selected cell culture was then supplemented with $1,25(OH)_2$ Data dose of 10×10^{-8} M for 72 hours. The dose was standardised and referred by previous reports [20-23]. After completion of seven days the cells were isolated and incubated with *E. coli* for 120 minutes. The infections were given in ratio of 1:40. After which iNOS activity and SOD (Super Oxide Dismutase) activity and bacteria killing assay were performed (Image 1).

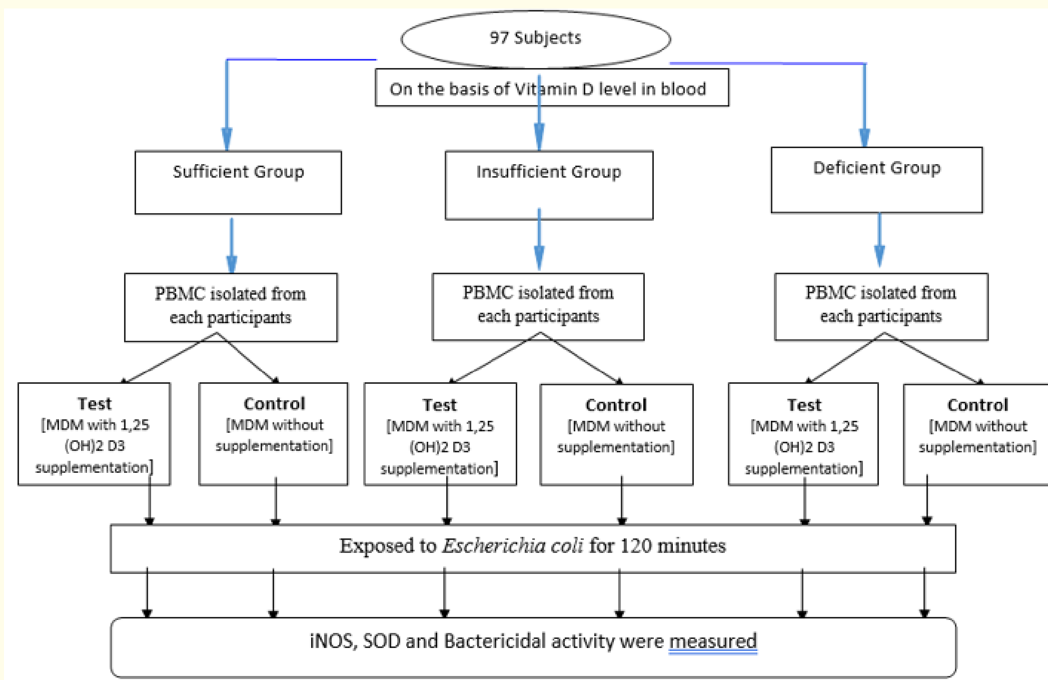

Image 1: Details work flow.

Antimicrobial activity assessment

The following assays were done following standard methods.

Inducible nitric oxide synthase (iNOS) activity assay [24]:

Macrophage cell lysate from individual subjects were assayed for iNOS enzyme using a reaction system (Arginine as substrate, potassium ferrocyanide and HEPEs buffer) and observed spectrophotometrically at 420 nm. Enzyme activity was expressed as ΔOD/mg protein per unit time

Super oxide dismutase (SOD) activity assay [25]:

Macrophage cell lysate from individual subjects were assayed for SOD enzyme using a reaction system where pyrogallol was used as substrate and observed spectrophotometrically at 420 nm. The enzyme activity was expressed as ΔOD /mg protein per unit time.

Total protein estimation [26]:

The protein contents of the samples were quantified by standard method [26]. The absorbance was measured at 660 nm.

Bacteria Killing Assay [27]

Human MDMs (2×10^6 cells) were suspended at 1:40 ratio with *E. coli* in a final volume of 1ml of 100mM Phosphate buffer, pH7.4. This suspension was then incubated with gentle rocking, at 37 ^{0} C. Centrifugation followed by washing with cold buffer twice. Cells were bursts by freeze-thaw method. Aliquots of that suspension were plated at 0 minute, and 120 minutes for incubation at 37^0 c on nutrient media. The Agar plates were then incubated at 37 ^{0} C in incubator, and bacterial colonies were counted on the next day. Results were expressed as bacterial killing = 100 – (N/N_0 multiplied by 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.

Ethics clearance

Ethics clearance was obtained from the Institutional Ethics Committee of All India Institute of Hygiene and Public Health (AIIH&PH), Kolkata. Informed written consent was obtained from each study subjects prior to the commencement of the study.

Statistical analysis

Data were put in Microsoft Excel worksheet (Microsoft, Redwoods, WA,USA) and they were checked for accuracy. Continuous data was first checked for normality distribution by Kolmogarov Smirnov Test. Significant p - value indicated skewed distribution. In presence of skewed distribution, non-parametric tests were performed. Difference between distributions of two continuous variables was determined by Kruskall Wallis test. Correlation was calculated by Spearman’s correlation coefficient (ρ, rho). Correlation was calculated by Spearman’s correlation coefficient (ρ, rho). SPSS software, version 20.0 (Statistical Package for the Social Sciences Inc, Chicago, IL, USA) was used to perform statistical analysis. p value equal to or less than 0.05 was considered as statistically significant.

Results

Association of serum 25(OH)D status with bacteria killing assay, iNOS and SOD activity

In present study in case of *E. coli* infection 42.64% Colony forming unit (CFU) reduction was observed among Sufficient group, 50.45% observed among Insufficient group and 43.45% observed among Deficient group. No significant differences (Unpaired t test, p = 0.089) were observed between these three groups.

In conditions where there is no exposure to infection or $1,25(OH)_2D$ treatment the median iNOS level was found to be more in case of deficient group, followed by sufficient group and insufficient group. After exposure to infections the median iNOS level was found to be 7.38 in deficient group,7.52 in insufficient group and 6.42 in sufficient group. Distribution of these values in 3 different group was found to be statistically significant as evident from Kruskal Wallis test (p = 0.04). Again with increase in serum 25(OH)D level, the iNOS level decreased significantly (Spearman’s rho p = 0.01). In conditions where $1,25(OH)_2D$ were treated with exposure to infection the median iNOS level was low in insufficient group 5.9 followed by increase in sufficient (9.40) group and deficient group (9.84). Although the values of iNOS level was not statistically significant in 3 different groups (p = 0.27) (Table 1).

Serum 25(OH)D status	iNOS level Median (IQR)	Kruskal-Wallis statistic(p value) Spearman’s *rho* (p value)	SOD level Median (IQR)	Kruskal-Wallis statistic (p value) Spearman’s correlation *rho* (p value)
Without infection				
Deficient group	6.14 (3.59)	4.12 (0.1269) -0.140(0.1690)	4 (4.2)	1.11(0.5741) -0.022(0.8291)
Insufficient group	3.30 (3.19)		4.79 (2)	
Sufficient groups	4.28 (3.74)		3.45(3.43)	
With Infection (*E. coli*)				
Deficient group	7.38 (8.06)	1.23 (0.539) -0.060(0.5540)	6.87 (4.71)	6.43(0.0401) -0.247(0.0174)
Insufficient group	7.52 (5.95)		4.36 (5.11)	
Sufficient group	6.42 (5.9)		3.75(4.31)	

With Infection (*E. coli*) + 1,25(OH)$_2$D treatment				
Deficient group	9.84 (8.33)	4.69 (0.0998) -0.126(0.2140)	8 (6.65)	2.60(0.2718) -0.177(0.0914)
Insufficient group	5.9 (5.62)		5.44 (6.86)	
Sufficient group	9.40(8.65)		6.5(6.38)	

Table 1: iNOS and SOD activity in *in vitro* cultured human macrophages of elderly women according to 25(OH)D level in comparison to those with or without exposure to infection (*E.coli)* (N = 97).

In conditions where there is no exposure to infection or 1,25(OH)$_2$D treatment the median SOD level was 4 for deficient group and 4.79 in case of insufficient group, 3.45 in case of sufficient group. Moreover, the difference between these three groups were insignificant (p = 0.57) as revealed from Kruskal Wallis test (Table 1).

Antimicrobial effects after *in vitro* 1,25(OH)2D supplementation

In bacteria killing assay after *in vitro* 1,25(OH)$_2$D supplementation the total reduction of CFU was significantly (p = 0.0207) high (60.64%) from without supplementation state (all three groups). This observation indicates again the important role of 1,25(OH)$_2$D in bactericidal activity of macrophages.After *in vitro* 1,25(OH)$_2$D supplementation,the maximum increase in CFU reduction rate was observed among Deficient group (63.57%), whereas in case of Insufficient group it was 60.11% and for Sufficient group it was 44.66%.

In conditions where there is no exposure to infection the iNOS level of the deficient group was 6.14, with exposure to infection (*E. coli*) it increases 7.38 and after *in vitro* 1,25(OH)$_2$D supplementation and with exposure to infection (*E. coli*) the iNOS activity level increases to 9.84, which was significant as evident from Kruskal-Wallis test (p = 0.01). Considering SOD activity among deficient group similar significant interpretation(p = 0.003) was observed (Table 1).

Where as in case of insufficient group when there is no exposure to infection the iNOS level of the insufficient group was 3.30, with exposure to infection (*E. coli*) it increased to 7.52 and after *in vitro* 1,25(OH)$_2$D supplementation and with exposure to infection (*E. coli*) the iNOS activity level increased to 5.9, which was insignificant from Kruskal-Wallis test (p = 0.08). Considering SOD activity among the insufficient group, increase in SOD activity were statistically insignificant (p = 0.75) as per Kruskal-Wallis test (Table 2).

Serum 25(OH)D status	**iNOS level**	**Statistical Test**	**SOD level**	**Statistical Test**	**25(OH)D Level**
	Median (IQR)	**Kruskal-Wallis statistic (p value)**	**Median (IQR)**	**Kruskal-Wallis statistic (p value)**	**Median (IQR)**
Deficient Group (N = 29)					
Without infection	6.14 (3.59)	21.83	4 (4.2)	16.22	5.16(2.57)
With Infection (*E. coli*)	7.38 (8.06)		6.87 (4.71)		
With Infection (*E. coli*) + 1,25(OH)$_2$D treatment	9.84 (8.33)	(0.0001)	8 (6.65)	(0.003)	
Insufficient Group (N = 28)					
Without infection	3.30 (3.19)	4.83	4.79 (2)	0.55	23.80(4.40)
With Infection (*E. coli*)	7.52 (5.95)		4.36 (5.11)		
With Infection (*E. coli*) + 1,25(OH)$_2$D treatment	5.9 (5.62)	(0.089)	5.44 (6.86)	(0.759)	

Sufficient Group (N = 40)					
Without infection	4.28 (3.74)	16.97	3.45 (3.43)	7.69	33.10(4.07)
With Infection (*E. coli*)	6.42 (5.9)		3.75 (4.31)		
With Infection (*E. coli*) + 1,25(OH)$_2$D treatment	9.40 (8.65)	(0.0002)	6.5 (6.38)	(0.02)	
Total (N = 97)					
Without infection	5.00 (4.14)	39.32	3.9 (3.43)	22.48	21.49(28.75)
With Infection (*E. coli*)	7.28 (4.75)		5.66 (4.8)		
With Infection (*E. coli*) + 1,25(OH)$_2$D treatment	9.09 (8.43)	(0.001)	6.61 (6.79)	(0.001)	

Table 2: iNOS and SOD activity in *in vitro* cultured human macrophages of elderly women according to 25(OH)D level in comparison to

Again in case of sufficient group when there was no exposure to infection, the iNOS level of the sufficient group was 4.28, with exposure to infection (*E. coli*) it increased to 6.42 and after *in vitro* 1,25(OH)$_2$D supplementation and with exposure to infection (*E. coli*) the iNOS activity level further increased to 9.40, which was significant according to Kruskal-Wallis test ($p = 0.002$). Considering SOD activity among sufficient group, again SOD activity increased significantly ($p = 0.02$) as revealed from Kruskal-Wallis test.

Discussion

In 2017, the first list of drug-resistant *priority pathogens*, appear as major threat, was published by the WHO containing 12 bacterial families, including six foremost microbes *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella*, *Serratia* and *Proteus* [28]. Thus *E. coli* remains one life threatening microorganisms specially for elderly population. Overall development of natural protection against these microbes always will be beneficial and cost effective approach for the community.

In human immune system the primary professional scavenger cells are the macrophages, capable to engulf micro-organisms, proteins and other smaller cells using several mechanisms such as Fc- receptor and complement mediated phagocytosis and endocytosis [29-30]. Vitamin D helps in promoting the activity of monocyte and macrophages, thereby contributing to a potent systemic anti-microbial effect [12-14]. Thus vitamin D deficient/ insufficient state is always beneficial to infections. Currently, several classical antibiotics are found to be ineffective against many pathogens including *E. coli*, which clarified the urgent need for new or alternative antimicrobials to prevent drug-resistance. Unfortunately, the progress in new antimicrobial research is unsteady and extremely slow, which was further delayed due to the COVID-19 pandemic.

Previous reports showed that serum 25(OH)D deficiency is associated with severity of different infection and antimicrobial activity [32-34]. In present study, considering bactericidal activity, iNOS activity and SOD activity (Table 1 and 2) also showed the similar trend where sufficient serum 25(OH)D consisting group had better result than others against *E. coli* infections.

iNOS is one essential enzyme in protective immunity against different bacterial infections [35]. Nitric oxide thus can inhibit both microbial DNA replication and cellular respiration [36]. Macrophages while activated generate massive nitric oxide, NO and superoxide radicals [37]. While the bactericidal effect of polymorphonuclear leucocytes depends on their superoxide generative capacity [35-37], and biosynthesis of SOD [37].

Reports also showed that in vitro vitamin D treatment enhanced the bacteria killing activity significantly [34,38,39]. In present study in vitro 1,25(OH)$_2$D supplementation increases iNOS and SOD activity significantly (Table 2), also significant impact was observed in CFU reduction rate among all three groups.

Present research is one basic approach towards understanding such promising molecular candidate to combat infections among elderly. It has many limitations as well. Therefore, more elaborative and specific study should be conducted to find out a novel anti-

infectious intervention, while many antibiotic classes have lost their antimicrobial efficacy and multidrug-resistance constitutes an emerging threat to global health. Present research is part of bigger project which was previously published elsewhere [17,40-41].

Limitations

The small sample size is one of the important limitations of the present study, additionally more additionally more elaborative and specific study might give clearer picture than the present one.

Conclusion

Considering bacteria killing capacity of macrophages the *in vitro* 1,25$(OH)_2$D supplementation significantly inreases the CFU reduction rate overall.Sufficient group's macrophages always had better profile than other two groups. *In vitro* 1,25$(OH)_2$D supplementation increases iNOS and SOD activity significantly.

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Conflict of Interest

Authors have no conflict of interest.

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