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Cathelicidin LL-37 level in presence and absence of vitamin D in cultured macrophages isolated from elderly women

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ABSTRACT

Introduction: Vitamin D deficiency and frequent infections are the two common worldwide phenomenon among elderly. Recent studies have demonstrated that vitamin D regulates the expression of specific endogenous antimicrobial peptides like cathelicidin LL-37 of macrophages and neutrophils, which is active against a broad spectrum of infectious agents. Therefore, the objective of the present study was to determine the level of cathelicidin LL-37 in macrophages of elderly women (classified according to serum 25(OH)D level) after exposure to *Vibrio cholera* infection and to find out the effect of $1,25(OH)_2D$ added *in vitro*. **Methods:** This study was conducted among 40 randomly selected rural elderly women aged between 60 to 70 years of age. Their vitamin D status was assessed by the estimation of serum 25(OH)D and classified into three groups viz. sufficient (14 members), insufficient (13 members), and deficient (13 members). Later, their peripheral blood mononuclear cells (PBMC) were isolated and cultured from fresh blood. $1,25(OH)_2D$ supplementation was given selectively at a dose of 10×10^{-8} M for 72 hours in the culture media; then exposed to infection and screened according to the objectives of this study. **Results:** Macrophages in all groups, except vitamin D deficient group, responded significantly in terms of LL-37 release during exposure to *Vibrio cholera* infection. Considering *in vitro* $1,25(OH)_2D$, supplementation responded significantly ($p<0.05$) in all three groups. **Conclusion:** Vitamin D can be used as a prophylaxis to enhance cathelicidin LL-37 release for all three groups as in the present study.

Keywords: cathelicidin LL-37 activity, elderly women, macrophages, peripheral blood mononuclear cells, vitamin D

INTRODUCTION

The world population of 60 years and above is expected to increase from 962 million to 2.1 billion in the year 2050, which will produce major difficulties in healthcare systems throughout the world (World Population Ageing, 2017). Infections and septicaemia are common among the elderly (Nasa, Juneja & Singh, 2012). Vitamin D deficiency is also a worldwide phenomenon among the elderly (Pan *et al.*, 2016). Vitamin

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D has a vital role in the body's defence mechanism towards infection by promoting the roles of macrophages and monocytes, which are important in pathogenesis (Yamshchikow *et al.*, 2009). Adjunctive treatment of vitamin D against different infections has been reported (Soeharto *et al.*, 2019).

One of the major components of vitamin D-mediated antimicrobial activity is through the production of peptides. It has already been proven that vitamin D stimulates the expression of potent antimicrobial peptides, such as cathelicidin LL-37 (Bartley *et al.*, 2010) and β defensin 2 (Bartley *et al.*, 2010). The neutrophils, monocytes, natural killer (NK) cells, and epithelial cells lining of the respiratory tract exerts these peptide synthesis with the help of vitamin D (Ginde, Mansbach & Camargo, 2009). Various research works from different parts of the world have shown that macrophages, lymphocytes, and monocytes have vitamin D receptors (VDRs) that, with 25(OH)D stimulation, increase the expression of these antimicrobial peptides (Schwalfenberg, 2011, Jeng*et al.*, 2009, Bikle, 2008).

Cathelicidin LL-37, an endogenous antimicrobial peptide, is active against a broad spectrum of infectious agents including gram negative and positive bacteria, fungi, and mycobacteria (Dürr, Sudheendra & Ramamoorthy, 2006). It is highly expressed at barrier sites including respiratory and colonic epithelium, saliva, and skin; thus provides an important first line defence mechanism for the innate immune system to respond against infectious insults (Liu *et al.*, 2006). *In vitro* $1,25(OH)_2D$ treatment of infected cultured macrophages can enhance the expression of cathelicidin LL-37 (Liu *et al.*, 2006). Stimulated macrophages cultured in vitamin D deficient sera are unable to up-regulate LL-37 and effectively kill *Myobacteriumtuberculosis* (Mtb) (Liu*et al.*, 2006). However, the addition of 25(OH)D in the media up-regulates the production of LL-37 and restores effective killing of Mtb, suggesting that vitamin D has an important role in the production of anti-microbial peptides, which is important for innate immunity (Liu *et al.*, 2006). On the other hand, despite playing such a crucial role (antimicrobial and immune benefits), this antimicrobial peptide also contributes to the host's defence through wound repair (Hiemstra *et al.*, 2007) and clearance of bacteria at various barrier sites (White, 2010).

There is a paucity of study regarding the association of vitamin D deficiency and human cathelicidin LL-37 activity against infection among macrophages isolated from adult above 60 years of age (Liu *et al.*, 2006, White, 2010, Yuk *et al.*, 2009, Martineau *et al.*, 2007). Hence, this study was planned as no such study has been conducted so far among elderly women. The objectives of this study were (i) to determine the level of cathelicidin LL-37 in macrophages of elderly women classified according to serum 25(OH)D level, and (ii) to evaluate the effect of $1,25(OH)_2D$, added *in vitro*, on cathelicidin LL-37 level in macrophages of the target population.

MATERIALS AND METHODS

Sampling design

The present study was a small part of an original research work already published (Ghosh *et al.,* 2020), where the actual sample size was 236. The sample size was calculated based on a previous prevalence of Vitamin D deficiency at 91.2% (Kota *et al.,* 2011); and using the formula $n=(Z\ (1-\infty/2))^2pq/L^2$; where L is allowable error, which was taken as 5% of p, and $Z\ (1-\infty/2)$ is the standard normal deviate at 95% confidence limit, which was 1.96. The calculated sample size came to be 145. Since multistage

random sampling was adopted, it was multiplied by 1.5 (design effect), which came to 217.5. An additional 10% was added to compensate for dropout, which was then calculated to be 239. Finally, 236 participants were included. From that 236 samples, one sub-sampling (with proper randomisation technique) was done to observe the cathelicidin LL-37 activity of cultured macrophages of the target population. Hence, the sample size was 40.

These 40 elderly women were selected randomly from those 236 samples previously screened, who were residing at 80 different villages of Amdanga block, 24 Parganas North, West Bengal, India, during April 2014 to August 2018. Mean age of the target population was 62.5±4.2 years. They were classified into three groups: vitamin D sufficient group (14 members), vitamin D insufficient group (13 members), and vitamin D deficient group (13 members) as per their serum 25(OH)D levels. Deficiency, insufficiency, and sufficiency of vitamin D were defined as ≤20, 21–29, and ≥30 ng/ml of serum 25(OH)D in human blood, respectively (National Institute of Health, 2020).

In the final stage, their peripheral blood mononuclear cells (PBMC) were isolated from fresh blood (4ml) and were screened. Elderly women having a previous history of thyroid dysfunction, on hormonal replacement therapy, amenorrhoea due to any pathological cause or surgery, on vitamin D supplementation, physically or mentally challenged, and non-cooperative in nature were excluded from the study. Elderly women having fever in the last 20 days, having high total WBC count and high C-reactive protein level were excluded from the study. Ethical clearance was obtained from the Ethical Committee of All India Institute of Hygiene and Public Health (AIIH&PH), Kolkata. Informed written consent was obtained prior to the study.

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 (Tyurina*et al.*, 2007). Isolated 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) at 2ng/ml concentration. Finally, cells were added to adherent six-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 $1,25(OH)_2D$ at a dose of 10×10^{-8} M for 72 hours. The dose was standardised and referred to previously (Dalton, Shertzer & Puga, 1999). After completion of seven days culture, isolated cells were infected with *V. cholerae* (1:40) and were kept at 37°C for 120 minutes. Uninfected cells without $1,25(OH)_2D$ supplementation, infected cells without $1,25(OH)_2D$ supplementation, and infected cells with $1,25(OH)_2D$ supplementation were prepared. For *in vitro* vitamin D supplementation, active form of vitamin D ($1,25(OH)_2D$) was used for direct acceptance of macrophages during exposure to infection and better induction for LL-37 release.

Estimation of serum 25(OH)D level and cathelicidin LL-37 level

Serum 25(OH)D and cellular LL-37 levels were measured by enzymatic immunoassay (LL-37(Human) ELISA kit., 2018; Holick, 2007). Precision of

the estimation was determined by intra-assay and inter-assay variabilities. Deficiency, insufficiency, and sufficiency of vitamin D were defined as ≤20 ng/ml, 21–29 ng/ml, and ≥30 ng/ml of serum 25(OH)D in human blood, respectively (National Institute of Health, 2020).

Statistical analysis

In LL-37 level assay, continuous data were tested for normal distribution and significant Kolmogorov-Smirnov tests were observed. Friedman analysis of variance (ANOVA) was used to compare the repeated measures of the same groups. Kruskal-Wallis test was used to compare three different groups. The Graph pad prism 7.04 and IBM SPSS Statistics version 20.0 (IBM Corp, Armonk, New York, USA) were used for statistical analysis. P-value of less than 0.05 was considered as statistically significant.

RESULTS

In the vitamin D sufficient group, cathelicidin LL-37 levels in isolated macrophages were significantly increased (p<0.05) after exposure to *V. cholerae*, which was further increased significantly (p<0.05) on *in vitro* $1,25(OH)_2D$ supplementation (Table 1).

Cathelicidin LL-37 levels in isolated macrophages of vitamin D insufficient group were increased significantly after infection (p<0.05), which were further increased significantly owing to *in vitro* $1,25(OH)_2D$ supplementation (p<0.05). On the other hand, cathelicidin LL-37 levels from macrophages of vitamin D deficient group, though not increased significantly after exposure to infection, did increase significantly after *in vitro* supplementation of $1,25(OH)_2D$ (p<0.05).

No significant differences were observed in the cathelicidin LL-37 levels in isolated macrophages of the three groups viz. vitamin D sufficient, insufficient, and deficient; without *V. cholerae* infection, with *V. cholerae* infection, and with *V. cholerae* infection accompanied by $1,25(OH)_2D$ supplementation.

DISCUSSION

Recent discovery of the immuno-modulatory role of vitamin D, specifically its induction of antimicrobial peptide gene expression, explains the 'antibiotic' effect of vitamin D that has greatly renewed interest in the ability of vitamin D to improve immune functions (Wang *et al.*, 2004). Up-regulation of antimicrobial peptide gene expression because of

Table 1. Cathelicidin LL-37 level in cultured human macrophages of elderly women according to 25(OH)D levels with or without exposure to *Vibrio cholerae* infection (n=40)

Cathelicidin LL-37 (ng/ml)	*Vitamin D deficient group Median (IQR) (ng/ml)*	*Vitamin D insufficient group Median (IQR) (ng/ml)*	*Vitamin D sufficient group Median (IQR) (ng/ml)*	*Kruskal Wallis test*
Without infection	78.22(34.71)	75.01(56.32)	67.20(45.37)	2.03
Infection	77.58(56.48)	138.94(91.39)	99.24(89.78)	4.24
Infection with $1,25(OH)_2D$ treatment	99.83(38.63)	192.00(123.25)	111.12(103.10)	5.13
Friedman ANOVA	6.61*	14.00*	17.71*	

*p<0.05

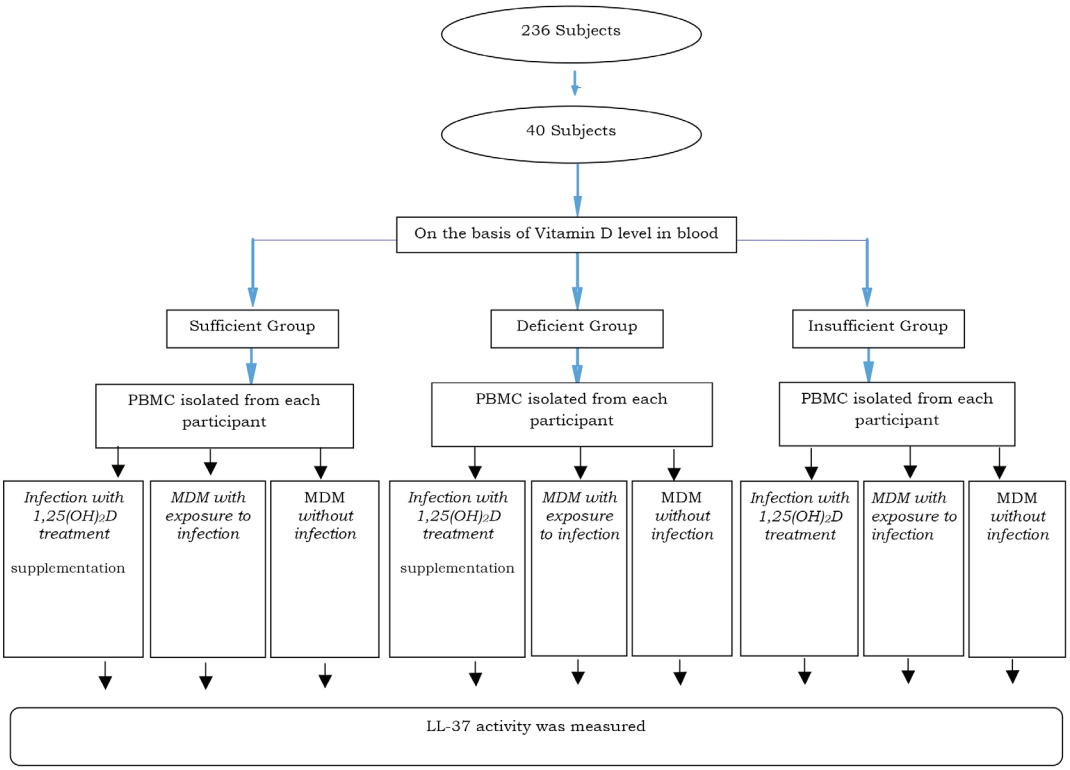

Figure 1. Details of work flow

$1,25(OH)_2D$ supplementation was discovered more than a decade ago (Liu *et al.*, 2007, Martineau *et al.*, 2007). It was also demonstrated that *in vitro* TLR 2/1 (Toll Like Receptor) signalling by a synthetic 19-kD *M. tuberculosis*–derived lipopeptide enhanced the antimicrobial capacity of monocytes via a vitamin D and VDR-dependent pathway (Liu *et al.*, 2007). Several reports have shown that such incidence was involved in the induction of CAMP gene (LL-37 gene) and its protein expression (Liu *et al.*, 2007, Martineau *et al.*, 2007, Nursyam, Amin & Rumende, 2006).

While these reports prove that vitamin D boosts anti-mycobacterial immunity *in vitro,* there are also many *in vivo* reports where vitamin D supplementation trials were conducted to find out its immunomodulatory role in CAMP induction (Gombart, Borregaard & Koeffler, 2005; Nallelyl *et al.*, 2014; Adams *et al.*, 2009). Most immune cells in the human body have in-built VDR expressions that initiate production of antimicrobial peptides after receiving stimulation from 25(OH)D (Schwalfenberg *et al.,* 2011). Epidemiological studies in the United States revealed a positive relationship between serum 25(OH)D and cathelicidin LL-37 levels among acute septicaemia patients (Jeng *et al.*, 2009; Routsias *et al.*, 2010). One interesting study by Adams *et al.* (2009) showed that during vitamin D insufficient conditions, cultured monocytes showed increased expression of the vitamin D-activating enzyme CYP27b1, but decreased expression of cathelicidin LL-37 antimicrobial peptide (hCAP) mRNA. Again, vitamin D

supplementation increased hCAP mRNA expression significantly (Adams *et al.,* 2009).

Our study revealed significant increase of cathelicidin LL-37 levels in cultured macrophages isolated from elderly women having sufficient and insufficient serum 25(OH)D levels when infected with *V. cholerae*. This was not observed in the serum 25(OH)D deficient group, which is in accordance with earlier reports (Jeng *et al.*, 2009; Liu *et al.*, 2006; Adams *et al.,* 2009). This indicates that cathelicidin LL-37 expression can be down-regulated owing to very low levels of serum 25(OH)D (Jeng *et al.*, 2009, Adams *et al.,* 2009), that is associated with higher susceptibility to nosocomial infections like pneumonia, sepsis, and central line infections (Bikle, 2008).

Though the cathelicidin LL-37 levels in macrophages isolated from serum 25(OH)D deficient group did not significantly increase after exposure to *V. cholerae* infection, *in vitro* supplementation of $1,25(OH)_2D$ increased the levels significantly ($p>0.05$). Similar observations were made in earlier report as well (Adams *et al.*, 2009). Thus, vitamin D plays a key role in innate immunity by maintaining localised production of anti-microbial LL-37 following TLR activation of monocytes/macrophages (Adams *et al.*, 2009).

Similar work was published demonstrating that $1,25(OH)_2D$ treatment of macrophages infected with *M. tuberculosis in vitro* enhanced the production of an endogenous anti-microbial peptide, cathelicidin LL-37 and ameliorate the killing of the microorganisms (Liu *et al.*, 2007). In another study, it was observed that most of the diabetes mellitus 2 (DM2) patients with low VDR had low antimicrobial peptides (AMPs) expression, but when monocyte derived macrophages (MDMs) from patients having DM2 and having low VDR expression were supplemented with $1,25(OH)_2D$, MDMs eliminated more *M. tuberculosis* (Nallelyl *et al.*, 2014). The authors suggested the use of vitamin D as a prophylaxis for tuberculosis in high DM2 endemic countries (Nallelyl *et al.*, 2014). According to Hacıhamdioğlu *et al.* (2016), children with vitamin D insufficiency may not be able to increase their urine cathelicidin LL-37 levels during urinary tract infection caused by *Escherichia coli* (Hacıhamdioğlu *et al.,* 2016). Again, according to Adams *et al.* (2009), the ability of human macrophages to induce cathelicidin LL-37 level in response to TLR-activation is directly proportional to serum 25(OH) D status; thus it can be enhanced in vitamin D insufficient patients with supplementary vitamin D.

CONCLUSION

The present study revealed that vitamin D status has a strong influence on cathelicidin LL-37 level of macrophages and subsequent protection against infection in elderly women, but up to the stage of insufficiency. Further study may demonstrate the efficacy of higher $1,25(OH)_2D$ supplementation doses in elevating the cathelicidin LL-37 level in macrophages among elderly with vitamin D deficient status.

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Authors' contributions

JG, conducted the study, prepared the draft of the manuscript and reviewed the manuscript; also led the data collection and did all the biochemical, microbiological experiments; ANC, conceptualised, designed and conducted the study, reviewed

the manuscript, reviewed the data analysis and interpretation, and assisted in drafting of the manuscript; IS, reviewed the manuscript, and reviewed the data analysis and interpretation; DC, conceptualised and designed the study, reviewed the manuscript, reviewed the data analysis and interpretation, and assisted in drafting of the manuscript.

Conflict of interest

There are no conflicts of interest.

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