



Vitamin D and L-Isoleucine Promote Antimicrobial Peptide hBD-2 Production in Peripheral Blood Mononuclear Cells from Elderly Individuals

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Abstract: Elderly individuals are susceptible to develop infectious diseases; promoting innate immunity to prevent infections is a key issue. Human β -defensin-2 (hBD-2) is an antimicrobial peptide with antimicrobial and immunomodulatory properties. L-isoleucine and vitamin D are important molecules that induce hBD-2. The Aim of this study was to determine the use L-isoleucine and Vitamin D to induce hBD-2 in cells from healthy elderly individuals and elderly individuals with recurrent infections. We explored three groups: young adults ($n = 20$) used as control group, elderly adults ($n = 18$) and elderly with recurrent infections ($n = 11$). PBMCs (peripheral blood mononuclear cells) were isolated from the different groups and then were treated with L-isoleucine or vitamin D3. hBD-2 concentration was assessed with a sandwich enzyme Immunosorbent assay by triplicate. Using the vehicle as a mock control. Our results showed that a percentage of the individuals responded to the treatments producing hBD-2 ($p < 0.05$). These results showed that both molecules induced hBD-2 in elderly individuals and can be potentially used as prophylactic therapy to decrease infection diseases rates in this vulnerable group.

Keywords: Defensins, elderly, aging, vitamin D, L-isoleucine, innate immunity

Introduction

Antimicrobial peptides (AMPs) are cationic endogenous antibiotic peptides expressed mainly by epithelium and cells of the innate immune system. AMPs exert antimicrobial activity in a concentration-dependent manner, making their expression a critical factor in host defense. In humans it has been reported that the main AMPs are cathelicidins, α -defensins and β -defensins [1].

β -defensins are at the interface between the adaptive and innate immune systems; besides its direct antimicrobial activity, β -defensins exhibit chemotactic function towards cells expressing the chemokine receptor CCR6 such

as immature dendritic cells, memory T cells, neutrophils and mast cells [2, 3]. Defensin are expressed in unstimulated cells in basal amounts, however, the expression of human beta-defensin-2 (hBD-2) and human beta-defensin-3 (hBD-3) is conditioned by pro-inflammatory cytokines stimuli such as TNF- α , interleukin (IL)-1 β , IL-17, and IL-22. hBD-2 is mainly concentrated in the epithelia of the lung, tonsils, trachea and blood innate immune cells, therefore plays a critical role in the prevention of infection. The inducible properties of hBD-2 suggest that it plays a significant role in innate immune defense [4], indeed it has been

reported that individuals with deficiency in the production of this AMP are prone to develop infectious diseases [5].

Thus AMPs have been suggested for therapeutic agents administered as recombinant or synthetic protein, as well as using inducers, which stimulate specific cells for high AMPs production and secretion. A good example of an inducer is L-isoleucine, which is an essential amino acid that it has been reported as a strong inducer of β -defensins in bovine kidney epithelial cells and lung epithelia [6]. On the other hand, it has been described that 1,25-dihydroxyvitamin D3 (1,25D) is an important hBD-2 inducer which promotes bacteria elimination [7], indeed deficiency

in the vitamin D-receptor leads to a reduced hBD-2 production and in consequence an impaired capacity to eliminate bacteria [8]. After sunlight exposure of the skin, 7-dehydrocholesterol converts to pre-vitamin D3, which is hydroxylated in the liver into 25-hydroxyvitamin D3 (25-(OH)D3) and in the kidney into 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) [9], however other cells of the monocyte/macrophage lineage, hydroxylate 25(OH)D3 into 1,25(OH)2D3. In particular, PBMCs (peripheral blood mononuclear cells) -derived macrophage also respond to vitamin D through vitamin D receptor with increased NFATc1 expression [10, 11].

Table I. Clinical characteristics

	Young Adults (n = 20)	Elderly Adults (n = 18)	Recurrent infection Elderly Adults (n = 11)	P value ^A
Age (Years)	28.29±3.43	70.30±6.22	77.21±9.93	<0.001 ^{B,D}
Gender (M/F)	10/10	9/9	6/5	N/D
Erythrocytes (million/ul)	5.14±0.52	4.93±0.47	4.57±0.65	0.051
Leukocytes (1x10 ³ cell/ μ l)	6.47±1.39	6.74±1.61	5.42±1.23	0.052
Lymphocytes (%)	35.84±6.46	32.93±6.57	28.88±10.27	0.054
Monocytes (%)	7.56±1.49	8.15±2.31	8.37±2.82	0.253
Eosinophils (%)	2.11±1.16	2.60±1.47	2.59±1.84	0.758
Basophils (%)	0.63±0.35	0.55±0.38	0.57±0.38	0.544
Neutrophils (%)	53.78±6.95	55.77±8.65	59.59±9.47	0.090
Hemoglobin (g/dl)	15.14±1.46	14.69±1.59	13.79±2.00	0.090
Ht, EVF (%)	45.36±3.91	45.20±4.48	41.28±5.71	0.055
Glucose (mg/dl)	76.66±8.62	90.25±12.59	78.27±24.04	<0.001 ^{B,E}
BUN (mg/dl)	13.48±2.94	16.10±5.22	20.11±10.23	0.051
Creatinine (mg/dl)	0.81±0.16	0.85±0.15	0.95±0.32	0.272
Uric Acid (mg/dl)	5.99±1.64	5.70±1.19	4.90±0.93	0.190
Cholesterol (mg/dl)	201.06±38.72	210.50±40.75	188.39±46.38	0.374
Triglycerides(mg/dl)	158.80±94.93	168.14±79.99	132.71±58.85	0.491
Urea (mg/dl)	28.99±6.17	34.41±11.15	43.10±21.88	0.051
Alanine Transaminase (IU/L) ^C	29.20±17.67	20.83±6.55	25.35±14.88	0.660
Aspartate Transaminase (IU/L) ^C	24.21±8.61	25.39±6.64	25.36±10.37	0.654

^A Group comparisons were calculated with a Kruskal-Wallis rank sum test for non-parametric data

^B P values of less than 0.05 were considered statistically significant

^C Data was available for these variables for at least 18 and 18 and 11 subjects of each group respectively.

^D Elderly groups showed no statistical differences between them, only when compared any of the elder groups with young adults.

^E Elderly groups showed no statistical differences between them, only when compared any of the elder groups with young adults.

BUN. Blood urea nitrogen

Ht, EVF. Hematocrit, erythrocyte volume fraction

Descriptive statistics used were mean \pm SD

Elderly population has been growing in the last decades, recent research suggests that aging comes with a decreased immune function [12] and therefore an increased incidence of several infectious diseases. The relationship between morbidity and decreased immune function clearly acknowledge the fact that with decreased or impaired immunity comes an increased susceptibility to infectious diseases, cancer and autoimmune disease [13]. In this pilot study we evaluated L-isoleucine and 1,25 D as hBD-2 inducers in PBMC from elderly individuals, thus promoting enhancement of innate immunity in this vulnerable group.

Material and methods

Study subjects

The present study was approved by the National Committee of Ethics and the National Commission of Scientific Research (R-2011-785-030) of the Mexican Institute of Social Security (IMSS). The study was also performed according to international treaty guidelines such as the declaration of Helsinki. We recruited 20 individuals from 25–34 years old (young healthy adults), 18 individuals from 65–84 years old (healthy elderly adults) and 11 individuals 65–84 years old with recurrent infections in a period of three to six months according to medical history (see Table I). The selection criteria used were those of the SENIEUR protocol [14] and the absence of infection and diabetes mellitus. Measurements of laboratory parameters were made by standard clinical laboratory procedures at the “Emilio Varela Luján” general hospital of IMSS, Zacatecas.

Peripheral blood mononuclear cells isolation and stimulation

Heparinized blood was obtained from each donor; samples were processed within 3 h after drawing. PBMCs were isolated using Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway). Cells were washed twice with phosphate-buffered saline (PBS, pH 7.3), and their viability was assessed by trypan blue dye exclusion. 5×10^5 PBMCs were cultured per well in 24 well-plates (Costar, Ontario, Canada) with RPMI-1640 containing L-glutamine and penicillin in 5% CO₂ at 37°C. For stimulation, cells were treated with 50 µg/ml of L-isoleucine (Sigma-Aldrich, St. Louis, USA) for 24 hours as reported previously [15]. Similarly,

cells were stimulated with 10^{-7} M of active form vitamin D₃ (1,25(OH)₂D₃) (Sigma-Aldrich, St. Louis, USA) or an equal amount of DMSO (Sigma-Aldrich, St. Louis, USA) (0.5% v/v, such as vehicle control) for 24 hours similar to other models reported elsewhere [16]. After incubation, the obtained supernatants were supplemented with protease inhibitor cocktails, divided into aliquots and stored at -70 °C until use. As control it was used cells stimulated only with the vehicle (Mock).

Human β-defensin-2 quantification from cell-derived supernatants

The supernatants obtained from stimulated cells were collected as PBMCs-conditioned medium (PBMCCM) and filtrated using Amicon Ultra-4 centrifugal filter devices with a cut-off 10 kDa (Millipore, Billerica, MA, USA) according to the manufacturer's instructions and the flow-through was used for further analyses. Subsequently, we performed Bradford protein assay to measure total proteins and performed ELISA assay using equal amounts of total proteins for each assay. It was used a commercial ELISA kit from Peptotech (Rocky Hill, CT, USA) following manufacturer's recommendations.

Statistical analysis

Normality of all data was analyzed using a Kolmogorov-Smirnov normality test for each data set. Followed by non-parametric multiple comparison test Kruskal-Wallis to identify differences between groups. In the case of finding statistical significance ($p < 0.05$) a Dunn's post hoc test was performed. Two-sided p values of < 0.05 were considered statistically significant. Statistical analysis was performed using the GraphPad Prism Software (Graph Prism Software version 5.02, San Diego, CA).

Results

Clinical characteristics of participants

The clinical data analysis for each group showed that there were only differences regarding age and glucose between groups, worthwhile to notice that the mean values found in this group are not higher than those values reported as pathological. We analyzed several other variables associated

with study subjects that have been reported to be associated with the healthy state but found no differences. All this data is summarized in Table I.

Proportion of responders to stimulation

The results from ELISA assay showed that not all individuals produced hBD-2 after stimulation either with L-isoleucine or 1,25D. The proportion of individuals varies among groups. In young adults, 12 responded to L-isoleucine and 9 to 1,25 D, in elderly adults 9 responded to L-isoleucine and 9 to 1,25D whereas in the group of elderly with recurrent infection, 2 responded to L-isoleucine and 1 to 1,25 D (Table II). The hBD-2 concentration obtained for each group of responders was similar for both treatments. Young adults-responder group showed statistical differences for both L-isoleucine and 1,25D (Figure 1A), similar results were observed for elderly adults (Figure 1B). For the case of the group of elderly with recurrent infections

Table II. Proportion of responders to in vitro stimulation with L-isoleucine and Vitamin D

	Young Adults	Elderly Adults	Elderly Recurrent** Infection
Total subjects stimulated	20	18	11
Number of responders* to L-isoleucine/percentage	12/60	9/50	2/18 ^A
Number of responders* to vitamin D/percentage	9/45	9/50	1/9 ^A

* Defined as an increase of 2 pg/ml with respect to control or mock stimulated sample

** Defined as subjects that in a period of 4 months presented with at least two recurrent clinical manifestations of urinary, gastrointestinal or upper tract infection. Samples were obtained from these individuals when no clinical manifestations of disease were present.

^A The percentage of responders to L-isoleucine or vitamin D were statistically different only in the group of elderly with recurrent infections $p < 0.01$.

there were no statistical differences (Figure 1C).

Discussion

Several reports have suggested the utility of Vitamin D and L-isoleucine to improve innate immunity through the induction of antimicrobial peptides such as cathelicidin and

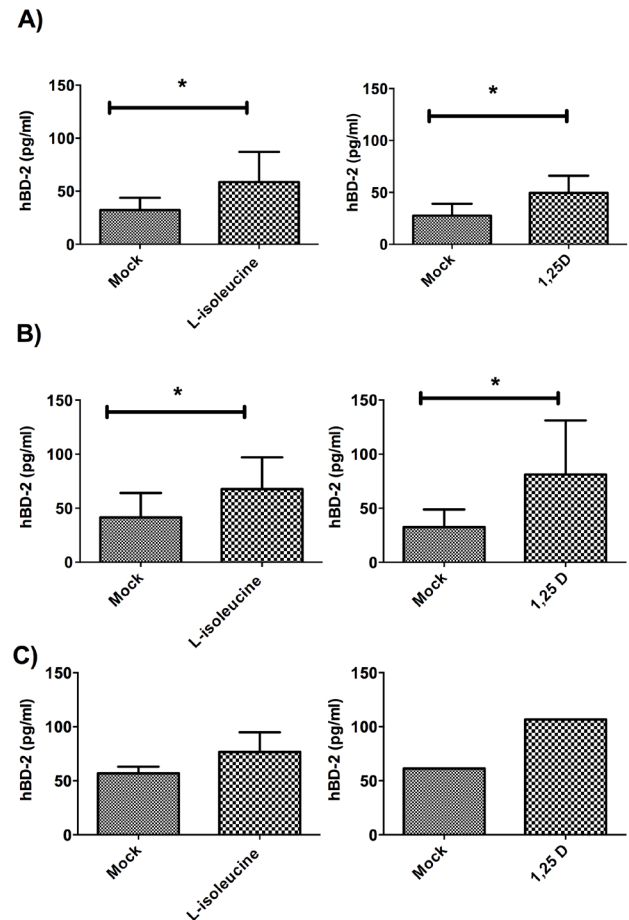


Figure 1. human β -Defensin-2 (hBD-2) production in individuals that responded to stimuli. PBMCs from 3 groups were harvested and treated for 24 h with 50 μ g/mL of L-isoleucine or with 10⁻⁷ M of 1,25(OH)2D3. hBD-2 production of individuals who responded to the treatment (defined as an increase of 2 pg/ml with respect to control) was graphed. As control we used cells stimulated only with the vehicle (Mock). Young healthy adults (Panel A), healthy elderly individuals (Panel B) and elderly individuals with recurrent infection (Panel C) were evaluated for production of hBD-2 after treatment. Data is expressed as mean \pm SD. * $p < 0.05$. $n = YA/20, EA/18, ERI/11$

hBD-2 in cell line cultures [8, 15–20]. It has been widely described the direct antimicrobial activity of these two peptides, as well as their immunomodulatory properties which provide a link between innate and adaptive immunity promoting an efficient response against invading pathogens; the deficiency in the production of these peptides is associated with the susceptibility to infectious disease [5]. Thus, in the last few years a big effort has been made to induce these peptides in vulnerable groups such as in diabetic mellitus patients, tuberculosis patients and neonates [15, 16, 18]. Indeed, L-isoleucine has been used as experimental treatment in children suffering acute diarrhea [21]. In the present study, we focused to the elderly individuals, due to the fact that it is known that aging comes with a decreased immune function [12] and therefore an increased

incidence of several infectious diseases. The decreased immune function in aged patients is associated with increased susceptibility to infectious diseases [13]. Thus we decided to explore the induction of hBD-2 in this group with L-isoleucine and vitamin D.

First, we analyzed the clinical aspects of the different groups, results showed only statistical significance in age and glucose levels, however these glucose levels are considered as normal. Since gender is homogeneous among groups, it can be discarded hormonal influence in our results. Thereafter, we measured the concentration of hBD-2 in the supernatants from PBMCs, no-stimulated and stimulated with L-isoleucine or 1,25D, our results showed that there were no differences in the baseline production of hBD-2 between groups, which correlates with previous studies, where it was reported that hBD-2 production is preserved during aging [22]. For the case of stimulated PBMCs, not all individuals responded to the treatment; the percentage of responders were variable according to the group and with the treatment (L-isoleucine or 1,25D). Altogether these results showed that only a percentage of the individuals treated with L-isoleucine or 1,25D responded to the treatment with the production of hBD-2, worthwhile to mention that individuals who responded to one treatment had a trend to respond to the other as well. Although it was most desirable to find a major responsiveness in the group of recurrent infections, it cannot be discarded the potential use of these two molecules as prophylactic in both groups of elderly, because inducing hBD-2 will provide beneficial effects to prevent other pathologies and improve nutritional fitness. Whether this treatment is functional to improve elderly's health needs to be further studied in a preclinical and multi-centric study using a much bigger number of volunteers for each group.

Previous studies by our group have shown similar results in diabetic patients, where only a percentage of the individuals responded to the treatment with 1,25D, the non-responder-patients showed an evident Vitamin D Receptor (VDR) downregulation [8]. We hypothesize that similar phenomena could be seen in the present study but need to be elucidated; though this is a possible explanation, other molecules should be also explored such as CYP27B1, which is involved in vitamin D hydroxylation. Besides, it is needed to identify the L-isoleucine receptor, which is involved in the antimicrobial-mediated immune response. Although only less than 60% of the individuals responded to the treatment, it is worthy to use both molecules alone or in combination in elderly individuals mainly for prophylaxis, since both molecules are cheap and have no toxic or side effects, however it needs to be previously tested. Since this is a descriptive pilot study, it is worthy for future studies, to increase the number of subjects for each group and correlate the hBD-2 production with the combination

of a low CD4:CD8 ratio (<1), an expansion of CD8+CD28-T-cells of 50% of the peripheral blood lymphocytes and the presence of CMV specific IgG antibodies which altogether lead to an increased risk of mortality among octogenarians (Immune Risk Profile) [23].

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Conflict of Interest

Authors declare no conflict of interest

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