ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com
Journal DOI: 10.21474/IJAR01

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

INVITRO STUDY OF ROLE OF VITAMIN D ON MACROPHAGES DYSFUNCTION IN PATIENTS WITH DIABETIC FOOT INFECTION.

Balram Gupta, *S K Singh.
Department of Endocrinology & Metabolism, Institute of Medical Sciences, Banaras Hindu University, Varanasi - 221005

Manuscript Info

Manuscript History:

Received: 12 April 2016
Final Accepted: 22 May 2016
Published Online: June 2016

Key words:
Type 2 Diabetes, Foot infection, Vitamin D, Phagocytosis, In vitro, Cell culture, Monocytes derived macrophage.

Corresponding Author
S K Singh.

Abstract

In addition to macro and micro vascular complication, hyperglycemia also causes dysfunction of immune system that protects the individuals against infection. Vitamin D deficiency, which has very high prevalence among the diabetic population, might contribute to impairment of macrophage dysfunction. The present study aimed to accesses the effect of vitamin D on phagocytic activity of macrophages in patients with diabetic foot infection in an in-vitro experimental model. The subjects included in the study were divided in to three groups. Serum vitamin D level of all groups was measured by Radioimmunoassay methods. Human PBMC were isolated by density-gradient centrifugation and cultured for 5 days in presence and absence of vitamin D. Phagocytosis was assessed by Confocal and fluorescent microscopy. Comparisons were performed using ANOVA and data were expressed as mean ± SD. The increased percentage of phagocytosis was observed in macrophages treated with $1X10^{-7}$M concentration of vitamin D3 in comparison to untreated sample among the diabetic foot group. In conclusion this study demonstrated that vitamin D deficiency lead to impaired phagocytosis of macrophages from diabetic subjects. Addition of vitamin D in culture medium of the macrophages improves phagocytosis significantly. This remarkable observation strengthens the concept of vitamin D supplementation to the diabetic foot for improving innate immunity.

Introduction:-

In addition to macro- and micro vascular complications, hyperglycemia affects immune system rendering diabetic patients susceptible to infection. 1, 25-dihydroxyvitamin D_3 (1, $25(OH)_2$ D_3), active form of vitamin D, has important role in calcium homeostasis and displays immunomodulatory effects [1]. It regulates the differentiation, growth and function of a broad range of cells including cells of the immune system [2]. Modulation of macrophages and dendritic cells, by inhibition of their differentiation, maturation and T-cell-stimulatory activity in vitro as well as in vivo has been well recognized [3-4].

Vitamin D deficiency has very high prevalence among the diabetic population [5]. Assumption can be made regarding its contribution to impairment of macrophage function in diabetes mellitus. Improvement of phagocytosis with Vitamin D supplementation in such situation would be an interesting area of research. The present study aimed to assess the effect of 1, 25-dihydroxyvitamin D_3 on phagocytic activity of Monocytes derived macrophages (MDM) in patients with diabetic foot infection in an in-vitro experimental model.

Materials & Methods:-

Patients with type 2 diabetes with and without foot infection were selected from Department of Endocrinology & Metabolism & wound clinic of General Surgery unit of the university Hospital. The subjects enrolled in the study

were divided in to three groups. Group I included 50 patients with Diabetic foot Infection. Group II and Group III included 50 patients with Diabetes without foot infection and 25 Healthy volunteers respectively. A detailed clinical history including age, sex, height, weight, body mass index (BMI), duration of diabetes and anti diabetic medications was recorded. Written and informed consent was obtained from every patient participated in the study prior to collection of blood samples and the study was approved by the institutional ethics committee.

Biochemical analysis:-

Biochemical parameters like Fasting plasma glucose, triglycerides, total cholesterol, high density lipoproteins (HDL) and low density lipoproteins (LDL) were measured by spectrometry (ERBA TRANSASIA BIO-MEDICALS LTD) from the blood plasma obtained after 8-12 hours of fasting of subject in the study groups. Post prandial plasma glucose was measured after 2hours of 75g glucose challenge given per oral to the subjects. HbA1c was measured using HbA1c Now+ kit, Bayer Health care, Sunnyvale, CA. and Serum 25-hydroxyvitamin D (25-OH-D) of all the groups were measured by Radioimmunoassay (RIA) kit Diasorin, USA.

Culture and Labeling of the E. coli with fluorescein isothiocyanate (FITC):-

Labeling of Escherichia coli with fluorescein isothiocyanate (FITC) was done by previous described methods [6]. Briefly, E. coli was grown in a shaker (Orbitek, Scigenics Biotech) overnight at 37^0C in 250 ml of LB broth. After 16 hours, bacteria were heat-killed at 65^0C for 45 min. and the cultures were centrifuged at 10,000 g for 10 min. and the pellets were resuspended in phosphate buffered saline (PBS). This process was repeated for 3 times and finally the pellets were resuspended in 1 ml PBS. The bacterial cultures were incubated with FITC (1 mg) in a 1ml of carbonate-bicarbonate buffer, pH 9.5 (9.5 ml 0.2 M Na_2CO_3 mixed with 41.5 ml 0.2 M $NaHCO_3$, solution made up to 200 ml) on a shaker for 30 min at room temperature in the dark. The cells were rinsed 4 times as above. To cheque proper heat killing of organisms, one loop of suspension was cultured on nutrient agar and growth was not detected at 37°C after 48 hours of incubation. Fluorescence was analyzed by flow cytometry (BD Biosciences, Erembodegem, Belgium), and 5000 events were collected that contained $2.5X10^7$ FITC-labeled E. coli per ml of PBS and stored at -20^0C in 0.5 ml aliquots to be used for all the experiments.

Isolation of Peripheral blood mononuclear cells:-

Peripheral blood mononuclear cells (PBMC) were isolated from all the groups by Ficoll-Paque™ Plus [density = 1.077] (GE Healthcare Bio-Sciences AB, Uppsala) density- gradient centrifugation as per the manufacturer's protocol. Purification of monocytes from freshly isolated PBMCs was done by positive selection using a magnetic cell separator and LS column (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated monocytes were washed 3 times with pre warmed RPMI 1640 (HiMedia Laboratories Pvt. Ltd) medium. With the help of Hemocytometer, viability of isolated cells was ascertained by treating cells with trypan blue stain which was always greater than 95%. Dead cells stain blue, while live cells exclude trypan blue stain.

Monocytes culture and infection with FITC-labeled E. coli:-

Cells were plated ($1.0X10^5$/cover slip) on sterile glass cover slips (10-mm diameter round) placed on to 12 well culture plates (HiMedia Laboratories Pvt. Ltd) supplemented with RPMI-1640 media containing 2mM L-Glutamine (HiMedia Laboratories Pvt. Ltd) and 10% Fetal bovine serum (FBS) (HyClone, Logan, UT). Triplicate cell culture plates were processed for each experiment. The Plates were incubated in a humidified CO2 incubator (Thermo Scientific) in presence of 5% CO_2 at 37^0C for 2hours. The non adherent cells were washed two times with pre warmed RPMI 1640 medium at 37^0C and the remaining adherent monocytes were cultured in final volume of 2ml with 10% FBS and L-glutamine (2mM) both in the absence and presence of gradient concentration ($1X10^{-7}$M and $1X10^{-9}$M) of 1, 25-dihydroxy vitamin D_3 (Sigma Chemical Co, St. Louis, MO). Vitamin D_3 was dissolved in 95% ethanol and the concentration of ethanol did not exceed 0.5% of total cell culture medium volume. After 5 days of incubation monocytes convert in to macrophages (monocytes derived macrophages), were infected with FITC-labeled E. coli in the proportion of 10 bacteria per monocytes.

Assessment of Phagocytosis by Fluorescent and Confocal microscopy:-

Phagocytosis was initiated by adding 1.5 µl containing $1.0X10^5$ FITC-labeled E. coli to each well. Culture plates were incubated for 2hrs at 37^0C in an incubator with 5% CO_2. Following this, cells were washed with PBS for 3times to remove any non adherent bacteria. After incubation, plates were treated with 0.4% trypan blue stain to quench external bacteria present in the medium or attached on the surface of phagocytic cells. The percentage of phagocytizing MDM was done by counting cells under fluorescence microscope (Nikon) and defined as the number of MDM engulfing at least one bacterium per 200 phagocytic cells in the culture.

The phagocytic index (PI) was calculated as the mean number of bacteria per macrophages according to the formula: phagocytic index = (total number of engulfed bacteria/total number of counted macrophages) × (number of macrophages containing engulfed bacteria/total number of counted macrophages) [7]. Phagocytic index was assessed after 2 hours of infection with E. coli by confocal microscopy (Zeiss) and fluorescent phase-contrast microscopy in several independent experiments. Fresh media was supplemented 4 hrs prior to infection with E. coli.

Statistical Analysis:-

Data were expressed as mean ± SD. Comparisons were performed using One-way analysis of variance (SPSS, Chicago, IL, USA) followed by the post-hoc test to demonstrate statistically significant differences. A p value of <0.05 were considered statistically significant.

Results:-

Baseline clinical and biochemical parameters of all the groups were shown in Table 1. There was significant difference in age, duration of diabetes, body mass index, fasting and post prandial blood sugar, HbA1c and serum vitamin D3 Level among all groups. Flow cytometry analysis showed $2.5X10^7$ FITC labeled E. coli per ml of PBS (Not shown). Monocytes count after isolation was $>2.4X10^5$ cells/ml and viability >95% in all experiments (not shown). Confocal microscopy at baseline showed significantly lower phagocytic activity ($p < 0.05$) and phagocytic index ($p < 0.05$) in Diabetic foot group and Diabetic control group in comparison with Healthy group (Figure 1). Increased phagocytosis percentage was observed in MDM treated with $1X10^{-7}$M concentration of 1, 25 dihydroxy vitamin D_3 in comparison to untreated sample among the diabetic groups only. When compared between the two groups, the phagocytosis was increased more in diabetic foot group than in diabetic control with vitamin D addition to culture medium (Figure 2). Based on 200 MDM examined, the percentage of phagocytically active MDM was similar for Diabetic foot, Diabetic control and Healthy group (Table 2; 72.13%, 67.59% versus 69.69 %, respectively).

Table 1:- Baseline clinical and biochemical parameters in Diabetic control, Diabetic foot and Healthy group.

Characteristics	Diabetic foot	Diabetic control	Healthy volunteer	P value
N (Male/Female)	50 (37/13)	50 (26/24)	25 (11/14)	
Age (years)	54.56 ± 9.62	49.62 ± 9.88	47.84 ± 9.02	0.006
Duration of Diabetes (years)	6.71±4.49	2.36± 2.79	NA	0.000
FPG (mg/dl)	207.48 ± 65.56	193.59±75.41	96.68 ± 10.37	0.000
PPPG (mg/dl)	291.76 ± 83.05	282.78±122.19	120.28 ± 17.64	0.000
HbA1C (%)	11.04 ± 2.55	8.80± 1.88	5.03 ± 0.44	0.000
BMI (kg/m^2)	22.57± 3.84	24.90±4.90	26.34 ± 4.88	0.002
Vitamin D (ng/ml)	14.25± 8.46	21.28 ± 10.98	42.03± 8.97	0.000
Phagocytosis (%)	22.08±4.56	36.00±11.03	56.19±11.34	0.00

BMI = body mass index, FPG=Fasting plasma glucose, PPPG= Post prandial plasma glucose

Figure 1. Confocal microscopy comparison of monocytes-macrophages phagocytosis among Healthy, Diabetic control and Diabetic foot subjects. Blood monocytes were isolated from whole blood and cultured for 5days in the absence of Vitamin D. Scale bar represents 10 µm.

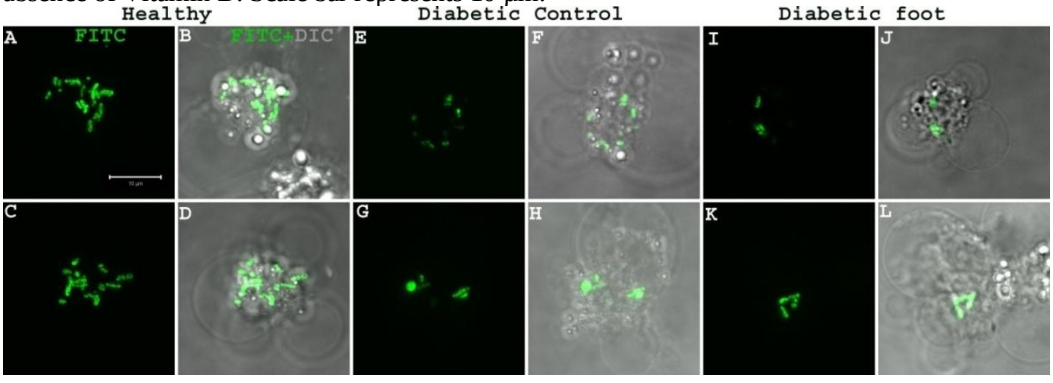

Figure 2:- Confocal microscopy study of 5 days cultured macrophages isolated from Diabetic foot patients, diabetic control and healthy volunteer grown in presence of 10^{-7}M 1, 25 dihydroxy vitamin D_3, ingested FITC tagged E coli after 2 hours of infection.

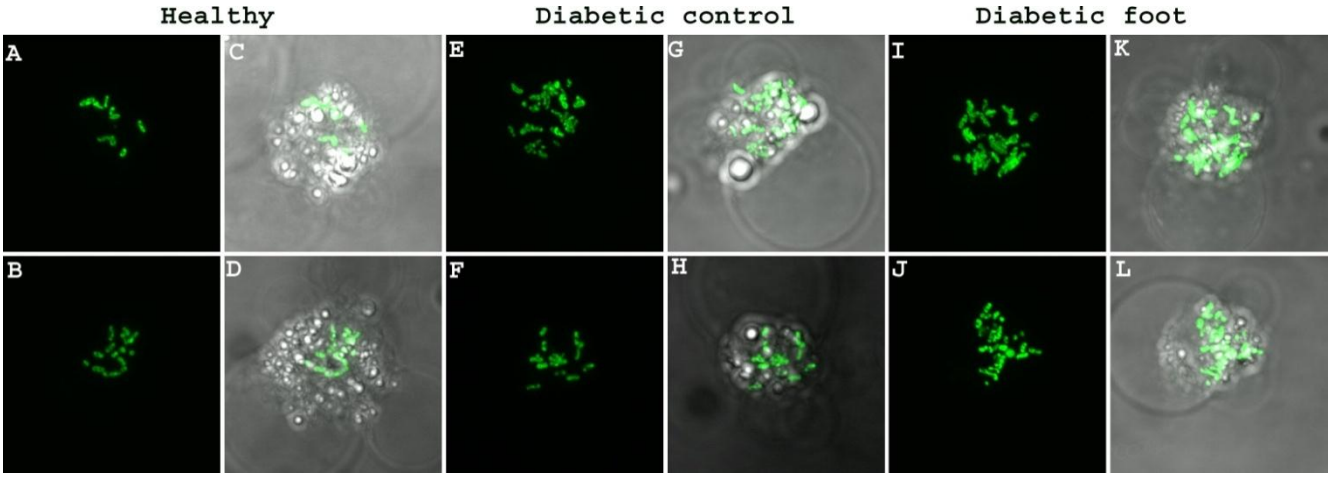

Table 2:- Comparison of phagocytosis of monocytes derived macrophages among all the groups treated with 10^{-7} M of 1, 25 dihydroxy vitamin D_3 in culture medium. (Based on 200 cells).

	Diabetic foot	**Diabetic control**	**Healthy group**	**P value**
Phagocytosis (%)	72.13 ± 8.28	67.59 ± 13.60	69.69 ± 11.56	.138
Phagocytic index	11.52 ± 1.93	8.53 ± 2.31	7.68 ± 1.53	.000

Discussion:-

Phagocytosis is a primary function of macrophage cells which requires receptor mediated recognition and endocytosis of pathogen, fusion of phagosomes with lysosomes for enzymatic digestion of pathogen, as well as presentation of antigens through MHC (8-10). Hyperglycemia in diabetes has been shown to affect phagocytosis (11). This defect is partially corrected by control of hyperglycemia [12].

Vitamin D influences innate immunity and its deficiency is very common in diabetic and non diabetic population [5]. Its relationship with diabetic foot ulcer and diabetic foot infection was reported earlier and the link between the vitamin D deficiency and innate immunity was cytokine and chemokines (13-14).

However the basic question was not answered if vitamin D receptor on macrophage could presumably be responsible for the phagocytosis [15-17]. The present study was designed in vitro to demonstrate that the MDM from the diabetic patient having decreased phagocytic activity, improved their function on addition of vitamin D. The improvement was more marked with monocytes from diabetic foot group than from diabetic control. Such improvement was not observed in monocytes with normal phagocytic activity from healthy volunteers. The outcome of the study indicated association between severity of vitamin D deficiency and impairment of phagocytosis in diabetics.

The strength of our experiment was ex vivo in vitro design as it was not the monocytic culture cell line and the hyperglycemic milieu in the culture medium. The weakness of the study was that we did not demonstrate vitamin D receptors on the monocytes cell surface in the experiment.

Conclusion:-

This study demonstrated that vitamin D deficiency had negative correlation with decreased phagocytosis activity of the macrophages from diabetic subjects. Addition of vitamin D in the culture medium of the macrophages of the diabetic patients significantly improved phagocytosis. This remarkable observation strengthened the concept of vitamin D supplementation to the diabetic foot for improving innate immunity. Intervention study may be designed to translate the laboratory observation to bed site clinical outcome.

Author's Contribution:-

SKS was responsible for the conception, design of the study, interpretation of the results, editing and reviewing of the manuscript. BG contributed in manuscript preparation, patient's enrollment, sampling, experimentation and data analysis. None of the authors had any personal or financial conflicts of interest.

Acknowledgements:-

The authors are thankful to the University Grant Commission, New Delhi for providing grant to perform this study. University Grant Commission letter no. F. No. 37-59/2009 (SR). SKS and BG are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

References:-

1. Lemire JM. Immunomodulatory actions of 1, 25-dihydroxyvitamin D3. J Steroid Biochem Mol Biol 1995;53:599–602.
2. Piemonti L, Monti P, Sironi M et al. Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. J Immunol 2000;164:4443–4451.
3. Holick MF. Vitamin D: a millenium perspective. J Cell Biochem 2003;88:296–307.
4. Baeke F, Takiishi T, Korf H et al. Vitamin D: modulator of the immune system. Curr Opin Pharmacol 2010;10:482–496.
5. Tiwari S, Pratyush DD, Gupta B et al. Prevalence and severity of vitamin D deficiency in patients with diabetic foot infection. Br J Nutr 2012;109: 99 – 102.
6. Rohloff L-H, Weisner A, Gotz P. A fluorescence assay demonstrating stimulation of phagocytosis by haemolymph molecules of Galleria mellonella. J Insect Physiol 1994;40:1045–1049.
7. Sano H, Hsu DK, Apgar JR et al. Critical role of galectin-3 in phagocytosis by macrophages. J Clin Invest 2003;112:389–397.
8. Doyle SE, O'Connell RM, Miranda GA et al. Toll-like receptors Induce a Phagocytic Gene Program through p38. J. Exp. Med 2004;199: 81–90.
9. Beal RK, Stuart LM, Ezekowitz RAB. Phagocytosis: Elegant Complexity. Immunity 2005;22: 539–550
10. Randolph GJ, Jakubzick C, Qu C. Antigen Presentation by Monocytes and Monocyte-derived Cells. Curr Opin Immunol 2008;20: 52–60.
11. Bing-Fen Liu, Satoshi Miyata, Hiroshi Kojima et al. Low Phagocytic Activity of Resident Peritoneal Macrophages in Diabetic Mice: Relevance to the Formation of Advanced Glycation End Product. Diabetes 1999;48:2074–2082.
12. Lecube A, Pacho´n G, Petriz J et al. Phagocytic Activity Is Impaired in Type 2 Diabetes Mellitus and Increases after Metabolic Improvement. PLoS ONE 2011;6: e23366.
13. Mosser DM. The many faces of macrophage activation. J Leukoc Biol 2003;73: 209-212.
14. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 2009;27: 451-483.
15. Takahashi K, Nakayama Y, Horiuchi H et al. Human neutrophils express messenger RNA of vitamin D receptor and respond to 1alpha,25- dihydroxyvitamin D3. Immunopharmacol Immunotoxicol 2002;24:335-347
16. Baeke F, Korf H, Overbergh L et al. Human T lymphocytes are direct targets of 1,25-dihydroxyvitamin D3 in the immune system. J Steroid Biochem Mol Biol 2010;121:221-227.
17. White JH. Vitamin D signaling, infectious diseases and regulation of innate immunity. Infect Immun 2008;76:3837–3843.