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Research Article

Role of the Vitamin D in Maintaining Integrity of the Intestinal Mucosal Barrier and its Potential Therapeutic Effect in Ulcerative Colitis Model: Experimental Study

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Abstract

Background: Ulcerative colitis (UC) is a chronic inflammatory bowel autoimmune disease characterized by T-cell infiltration of the colon. The conventional therapy for UC (5-aminosalicylates, corticosteroids, immune-suppressive drugs, monoclonal antibodies and antitumor necrosis factors) sometimes fails to produce satisfactory results. Moreover, it involves many side effects, which turn into more complications at later stages of the disease. So, new therapeutic strategies are needed for patients who do not respond to currently available treatments and to reduce side effects associated with conventional combined therapy**.** Vitamin D has been shown to regulate adaptive immune responses and enhance innate immunity through Vitamin D Receptors (VDR). **Aim of the work:** to detect usefulness of Vit D supplementation in active ulcerative colitis for reduction of disease severity. **Methods:** Experimental induction of ulcerative colitis in virgin female rats and comparing the effect of vitamin D alone, the effect of conventional therapy 5ASA alone and that of combined therapy. Histological evaluation will be done on the distal colon for assessing the activity. Toll-like receptors (TLRs) will be measured in tissue lysate using RT PCR, ELISA and Western blot. Epidermal growth factor (EGF) and vitamin D receptor will be assayed in fixed sections from cecum, proximal and distal halves of the colon by immunohistochemistry. ELISA will determine tissue Interleukin-18 (IL-18) and 25-OH Vitamin D. Tumor necrosis factor-alpha (TNF-α) and its receptors will be determined in tissue lysate by Western Blot. **Results:** Disease activity index was higher in the group of vit D free diet than that of rats with normal diet. There was no observed statistically significant difference concerning the histopathological activity between vit D free and normal diet group. TNF is the least in rats receiving ASA and VitD combined therapy in the group of normal diet. VDR is higher in normal diet group than Vit D free diet especially in the subgroup of Vit D enema and ASA. VDR expression was statistically significant higher in vitamin D normal diet group than that of vitamin D free diet especially in the subgroup receiving both vit D enama and ASA. TL4 was found to be higher in vitamin D free than in normal diet group. On the other hand, IL10 showed statistically higher level in the normal diet group especially after taking both ASA and vit D enema as treatment than in vitamin D free group. **Conclusion:** Inspite there is no statistical difference concerning activity between groups, VDR, TL4 suggest that vitamin D may represent a potential therapeutic agent for the treatment of active UC.

Introduction

Both forms of IBD can increase the incidence of gastrointestinal and colon cancers, and both are associated with significant morbidity and mortality worldwide [1]. The conventional therapies for IBD include 5-aminosalicylates, sulfasalazine, antimicrobial therapy, corticosteroids, immunosuppressive agents (azathioprine, cyclosporine, etc), and monoclonal antibodies (mAbs) as natalizumab, which is a mAb directed against alpha4-integrin, and the three antitumor necrosis factor (TNF) antibodies, namely, infliximab, adalimumab, and certolizumab pegol [2,3].

This conventional therapy commonly fails to produce satisfactory results. Moreover, conventional therapy involves many side effects, which turn into more complications at later stages of the disease [1]. So, new therapeutic strategies are needed for patients who do not respond to currently available treatments and to reduce side effects associated with conventional combined therapy [4].

Over the past years, there has been a rapid resurgence of interest in vitamin D outside of its traditional role in metabolic bone disease. Some non-traditional roles ascribed to vitamin D include anti-inflammatory and immune-modulating effects. These effects have led to possible implications in the pathophysiology of immune-mediated diseases including IBD. Recent evidence suggests that the vitamin D receptor (VDR) is present in more than 30 different types of tissue, including peripheral blood monocytes and leukocytes, antigen presenting cells, and activated CD4+ and CD8+T cells [5]. In addition, vitamin D insufficiency has been linked to higher rates of cancers including cancer colon [6].

Objectives

We aim to study the effect of Vit D diffeciency on Toll-like receptors, TNF-α, Interleukin-18 (IL-18), and to detect usefulness of Vit D supplementation to reduce the need for expensive biological therapy or decrease the total dose needed.

Methods

In this experimental intervention study, total number of 64 rats was included: Virgin female SD rats (160~180 g), maintained in a restricted access room with controlled temperature (23°C) and light/dark (12 h/12 h) cycle. The animals housed in rack-mounted, wire cages with a maximum of 10 animals per cage.

They were divided into 2 main groups : Vitamin D free diet and Vitamin D normal diet. Vitamin D free diet consists of 20% casein, 50% sucrose, 15 % corn starch, 5% fat, 5 % fiber, and a standard salt and vitamin-D-free vitamin mixture. The rats were kept away from the sunlight until full induction of UC.

Pilot study on animal model

5 Virgin female SD rats were exposed to induction of ulcerative colitis by aceto acetic acid 4% to check activity before starting experiment.

Each group was divided into 4 subgroups (each subgroup n=8 rats)

- Sub group 1 no treatment
- Subgroup 2 will be treated by vit D
- Subgroup 3 will be treated by 5 ASA
- Subgroup 4 will be treated by vit D and 5 ASA

1. Subgroup 1 considered control group (no treatment only induction of UC)
2. Subgroup 2 treated by vitamin D 50 ng dissolved in 20 ul of corn oil given as enema 1 day before induction of ulcerative colitis.
3. Subgroup 3 treated by conventional treatment 5-aminosalsylic acid (5-ASA) 100 mg/kg).
4. Subgroup 4 treated by both(vitamin D and conventional)

Each group was exposed to the following after treatment

a- Clinical evaluation by Disease Activity Index was done before and after treatment (DAI); Scoring of colitis activity index as described by Cooper et al. [7].

b- Laboratory study was done in Specialized Medical Hospital, Mansoura university including:

1) C- reactive protein
2) Serum albumin
3) Serum Na, serum k
4) Vitamin D: by vitamin D 96 test Elisa kit in rat (Bioassay).
5) Toll like receptor-4, IL 10, Vit D receptor and TNF : by Real time PCR of amplification CDNAs: total RNA was isolated from whole blood using (Invitrogen kit). Expression of mRNAs for mouse GAPDH, IL 10, VDR, INF, TLr4 was assessed using Taqman mouse gene expression assay : VDR (MM0043797), TNF (Mm 00443258), IL10 (Mm 01288386), TLr4 (Mm 00445273) and mouse GAPD (4362339E). All reaction were performed using the 2-ΔΔCT method (Relative quantification=Rq) which was used to quantify the relative amount of target gene expression in each sample.

c- Histopathological evaluation (scoring for the activity according to Iba et al. [8]. Histological evaluation was done on the distal colon for activity. Biopsy specimens were fixed in 10% buffered formalin and subsequently the tissues were processed and embedded in paraffin by using routine methods. Tissue blocks were serially sectioned to obtain consecutive levels. Sections were stained with hematoxylin and eosin for diagnosis, and assessment the degree of activity of inflammation by pathologist without knowing clinical characteristics.

Statistical analysis

After exclusion of the dead rats (18), data were fed to the computer and analyzed using SPSS software package version 19. Qualitative data were described using number and percent. Quantitative data were described using median (minimum and maximum) for non-parametric variables and mean. The student t-test was used to compare continuous data, and the chi-square test was used for categorical data. P-Value <0.05 was considered statistically significant. Some analyses required the Fisher exact test due to small sample size limitations.

Results

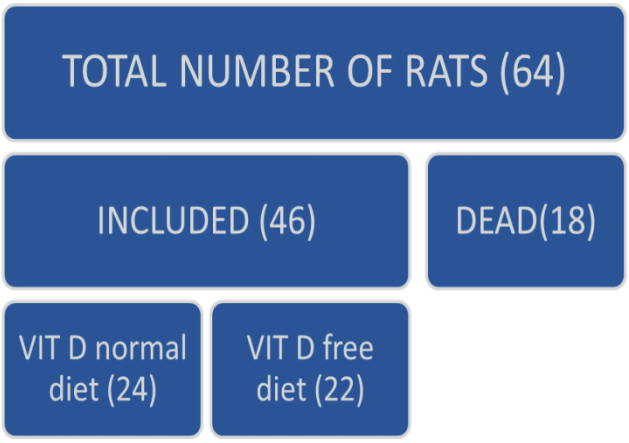


Figure 1: Scheme of all rats included in the study.

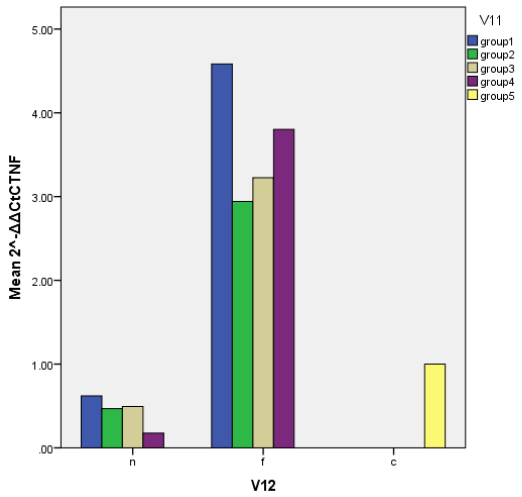


Figure 2: TNF is the least in rats receiving ASA+VitD combined therapy in the group of normal diet.

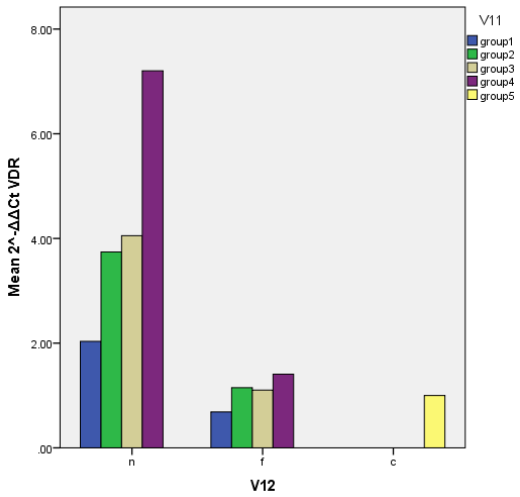
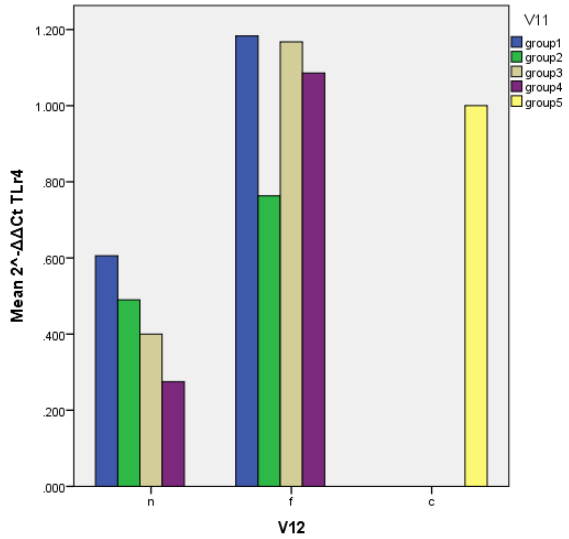


Figure 3: VDR is higher in normal diet group than Vit D free diet especially in the subgroup of Vit D enema and ASA.



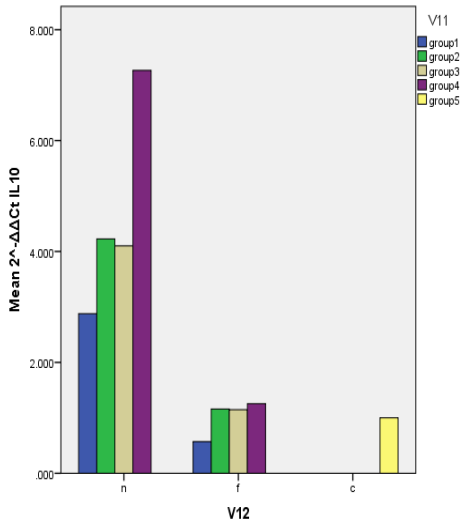


Figure 4: Differences in TL4 and IL10 among different groups

Discussion

Normal levels of vitamin D are approximately 30 ng/mL. Levels between 20 and 30 ng/mL are considered insufficient, and level below 20 ng/mL is considered deficient [9].

The prevalence of vitamin D deficiency in inflammatory bowel disease (IBD) varies in different studies, it may reach up to 60% to 70% of people with IBD, especially those with active disease and requiring corticosteroids [10].

Maruotti et al. in 2010 have shown that 1.25 $(OH)_2D_3$ inhibits Th1 and Th17 cell proliferation and stimulates T regulatory cell activity [11]. Also, it inhibits the differentiation of monocytes to dendritic cells, thereby reducing the number of antigen presenting cells available to stimulate T cells [12]. This declares the possible links between vitamin D and IBD i.e.; vitamin D level is related inversely to UC disease activity concerning symptom scores and quality-of-life as shown by many studies [10,13,14]. So, it may be helpful to supply vitamin D in patients with IBD, although not yet widely proven [10].

In our study, disease activity index [7] was higher in the group of vit D free diet than that of rats with normal diet; indicating the presence of more inflammation in that group. There was no statistical significant relationship between vitamin D status and disease activity when rats were stratified into four subtype groups (likely related to relatively small sample size after death of some rats), but still it is notable that there is increase in the disease activity as vitamin D levels decrease (Table 1). Surprisingly there was no observed statistical significant difference concerning the histopathological activity between vit D free and normal diet

group. This might be attributed to the short duration between induction of inflammation and rat scarification.

	VIT_D	
	Vitamin D normal diet (n=24)	**Vitamin D free diet (n=22)**
	Mean	Mean
VIT D	25.78	11.91
2^-ΔΔCt VDR	3.87	1.05
2^-ΔΔCt IL10	4.21	1.01
2^-ΔΔCtCTNF	0.49	3.61
2^-ΔΔCt TLr4	0.48	1.04
Disease Activity Index	4.06	6.19
Macroscopic score	1.66	2.34
Microscopic score	4.91	5.97
Na	146.68	132.52
K	4.63	3.16
ALBUMIN	3.71	2.46
CRP	9.27	91.59

Table 1: Effect of vitamin D level and disease progression in all groups.

In our study it was found that mean of TNF expression was statistically significant higher in group of vitamin D free diet than that in normal diet (Table 1), also it was very low in subgroup of rats receiving ASA and vitamin D combined therapy (figure1). This raises the suspicious of whether vitamin D deficiency is a marker of severity influencing response to therapy or this is a direct effect of vitamin D augmenting response to therapy.

VDRs have been found to affect transcription of at least 913 genes [15,16]. VDR is necessary for proper control of bone formation, renal excretion of calcium and it is implicated in IBD as mucosal VDR proteins are lower in ulcerative colitis (UC) patients compared to healthy controls according to Wada et al. [4]. Thus it has role in controlling intestinal homeostasis and regulating the adaptive immune responses and enhance innate immunity [17,18].

In our study we found that VDR expression was statistically significant higher in vitamin D normal diet group than that of vitamin D free diet especially in the subgroup receiving both vitamin D enema and ASA as combined therapy rather than other subgroups receiving vitamin D enema alone or ASA treatment alone or neither (Figure 3). This goes with the study of Li et al. in 2015; that showed VDR levels were reduced by more than 50%, and the pro-inflammatory cytokines, TNFα and IL-1β were elevated, as proved by colonic biopsies taken from patients with CD and UC, indicating that VDR can be repressed by inflammatory mediators [19]. Another study was conducted on 112 UC patients and it concluded that VDR is an important receptor in the pathogenesis of UC, so optimizing vitamin D levels could have a therapeutic role in UC [20].

In our study, TL4 was found to be higher in vitamin D free than in normal diet group. On the other hand, IL10 showed statistically higher level in the normal diet group especially after taking both ASA and vitamin D enema as treatment than in vitamin D free group (Figure 4). Studies on wild-type, specific pathogen-free IL-10 KO mice, and VDR KO mice showed impaired T cell homing to the gut in the absence of vitamin D signaling, with low levels of IL-10 and consequent increase of inflammation [21].

Systemic inflammatory markers in IBD as CRP, was found higher in vit D free group than in normal diet group and rats with higher levels of CRP were more susceptible to activity and worse in both macroscopic and microscopic score. Also they showed lower level of Na, K and albumin (Table 2).

		DAI	**Macro**	**Micro**
Vitamin D normal diet	Without Vitamin D enema	6.0000	2.5000	8.5000
	With Vitamin D enema	3.2500	1.5000	4.6250
	St. Sig.	**0.001**	**0.100**	**0.016**
Vitamin D free diet	Without Vitmin D enema	10.5000	3.7500	10.2500
	With Vitamin D enema	6.3750	1.8750	5.2500
	St. Sig.	**0.001**	**0.001**	**0.005**

Table 2: Effect of Vitamin D enema as monotherapy in both Vitamin D normal and vitamin D free diet groups.

In our study rats were subjected to treatment of the induced colitis either by vitamin D enema alone, ASA alone or received both treatments. The group of rats that have received both lines of therapy showed better DAI, macroscopic and microscopic score than other groups, and it was statistically better than the control group which has received no treatment (Table 3). This is almost the same as a clinical study conducted in UC patients, which concluded that low vitamin D levels (35 ng/mL) correlate with endoscopic and histologic inflammation and are associated with an increased risk of subsequent clinical relapse during periods of clinical remission [22].

		Vitamin D normal diet	**Vitamin D free diet**	**St. sig.**
Diseased group without therapy (n=10)	DAI	6	10.5	**0.001**
	Macroscopic score	2.5	3.75	**0.02**
	Microscopic score	8.5	10.25	0.22
Diseased group received Vitamin D enema(n=14)	DAI	3.25	6.38	**0.001**
	Macroscopic score	1.5	1.88	0.4

	Microscopic score	4.63	5.25	0.69
Diseased group received 5.ASA(n=11)	DAI	3.63	4.25	0.5
	Macroscopic score	1.88	2	0.8
	Microscopic score	4.38	5.25	0.7
Diseased group received both Vitamin D enema and 5.ASA(n=11)	DAI	3.38	3.63	0.7
	Macroscopic score	0.75	1.75	0.07
	Microscopic score	2.13	3.13	0.4

Table 3: Evaluation (DAI, macroscopically and histopathologically) in all treatment groups.

Conclusion

We conclude that, inspite that there was no observed statistically significant difference concerning the histopathological activity between vitamin D free and normal diet group, the group of rats that have received both lines of therapy showed better DAI, macroscopic and microscopic score than other subgroups, also it showed less expression of TNF and more expression of VDR and IL10.

So, combining vitamin D with the traditional therapy of UC (ASA) can decrease the severity of inflammation and will augment patient response to therapy.

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