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**RESEARCH ARTICLE**

# Effect of vitamin D in a rat model of pregnancy induced hypertension

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## *Manuscript Info* *Abstract*

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Preeclampsia is an important cause of maternal mortality, particularly in the developing world. There is a long-standing belief that maternal nutrition and preeclampsia are related. Recently, vitamin D deficiency was claimed to be a risk factor for developing preeclampsia.Preeclampsia is an important cause of maternal mortality, particularly in the developing world. There is a long-standing belief that maternal nutrition and preeclampsia are related. Recently, vitamin D deficiency was claimed to be a risk factor for developing preeclampsia.

**Aim**: This study was designed to examine the effect of vitamin D supplementation during pregnancy using a rat model of pregnancy induced hypertension, with a trial to explore possible involved mechanism (s).

**Design**: L-Nitroargininemethylester (L-NAME; 50 mg/kg body weight/day) orally from day 14-19, was used to induce hypertension during pregnancy.24 pregnant Wistar rats were assigned to control , pregnancy induced hypertension (PIH) and PIH treated with vitamin D3(50 IU/Kg i.p) daily from day 1 to day 19 of pregnancy. Protein in urine was estimated, blood pressure was recorded and pregnant rats were dissected at day 20 of gestation. Plasma was collected for estimation of malondialdehyde (MDA), tumor necrosis factor–alpha (TNF-alpha) and angiotensin II (Ang II). Finally, histopathological studies of the placenta and kidney were done.

**Results**: Animals from the PIH group demonstrated higher MAP which was associated with proteinuria; higher plasma MDA, higher plasma TNF–alpha and higher Ang II levels as compared to control ($p<0.001$). In contrast, vitamin D3 supplementation significantly ($p<0.001$) lowered MAP, proteinuria, plasma MDA , TNF-alpha and Ang II levels in dams as compared to PIH group. This was associated with marked improvement in results of histopathological studies of the placenta and kidney in the same group.

**Conclusion**: In the current study, vitamin D supplementation to PIH showed beneficial effects in terms of reducing: blood pressure, proteinuria, inflammation and oxidative stress, along with the negative effect on angiotensin II levels.

Effective preventive or therapeutic strategies for preeclampsia do not exist to date. Therefore, Vitamin D supplementation could benefit these women greatly by interacting with multiple pathways. Whatever, more studies are required to explore the molecular mechanisms underlying this multifaceted effectsof vitamin D.

## INTRODUCTION

Preeclampsia (PE) is a serious complication of pregnancy defined by new-onset hypertension and proteinuria that occurs in 3–8% of all pregnant women **(Roberts et al., 2003**). Preeclampsia is an important cause of maternal mortality, particularly in the developing world, and a leading cause of infant mortality, preterm delivery, and fetal growth restriction **(Ilekis et al., 2007&Chaturvedi et al., 2013).** The cause of preeclampsia is not well understood. The cause is believed to be multifactorial, and many pathophysiologic factors like, inflammation, endothelial dysfunction , and oxidative stress have been implicated **(Oken et al., 2007&Kalinderis et al., 2011&Firoz et al., 2011)** along with the uteroplacental ischemia **(Roberts and Escudero, 2012&Henao and Saleem, 2013)**.

There is a long-standing belief that maternal nutrition and preeclampsia are related. Some researchers, reported that ,vitamin D deficiency may be a risk factor for developing preeclampsia **(Bodnar et al., 2007&Haugen et al., 2009& Liu et al., 2013&Tabesh et al., 2013&Mohaghegh et al., 2015),** while others denied this **(Oken et al., 2007&Powe et al., 2010&Shand et al., 2010)**. Whatever the condition is, the underlying mechanisms are unclear **(Brodowski et al., 2014)**.

Classical actions of Vitamin D have been described in bone and kidney; however, Vitamin D also has important effects on the immune system andcardiovascular system **(Bikle, 2009).** Importantly, Vitamin D blunts adaptive immune responses while increasing innate immunity. Therefore, Vitamin D analogs may be useful in the prevention and treatment of human autoimmune or hypertensive disorders (**Adorini, 2005).**

The vitamin D receptor (VDR) is expressed in human placenta, endothelial cells and in cord endothelial colony forming cells (ECFCs) **(Pospechova et al., 2009&Grundmann et al., 2012)**. Potentially relevant to preeclampsia, vitamin D regulates key target genes associated with implantation, trophoblast invasion and anti-inflammatory responses in maternal decidua and fetal trophoblast **(Evans et al., 2004, 2006& Barrera et al., 2011).** Furthermore, vitamin D regulates angiogenesis through direct effects on vascular endothelial growth factor (VEGF)gene transcription**(Cardus et al., 2006, 2009&Grundmann et al.,2012).**

Vitamin D effects on the renin angiotensin aldosterone system (RAAS) have been investigated by many experimental studies (**Li , 2011a)**. Vitamin D receptor (VDR) knockout mice exhibit an increased renin expression, arterial hypertension and myocardial hypertrophy **(Bouillon et al., 2008 & Li, 2011a).** Subsequent studies confirmed the molecular mechanisms by which VDR activation down-regulates renin expression, but it is not clear whether these significant effects observed in vitro are also of relevance in vivo, i.e. in a clinical setting **(Kienreich et al., 2013**).

In light of the above mentioned interrelationship between many pathophysiologic factors of PE and non-classical actions of vitamin D, the objective of this study was to examine the effect of vitamin D supplementation during pregnancy using a rat model of pregnancy induced hypertension, with a trial to explore possible involved mechanism (s).

## ANIMALS AND METHODS

All the experimental procedures were conducted in accordance with the guiding principles for the care and use of research animals and were approved by the Institutional Review Board of Faculty of Medicine -Zagazig University.

**Animals**

Female Sprague–Dawley rats (12 weeks old, 180-220 g) were purchased from the animal house of Faculty of Veterinary Medicine-Zagazig University. Animals were housed in plastic cages with wood chips as bedding in a controlled environment at 20–25°C and 12 hour light/dark cycles. Rats were fed a standard laboratory diet and water ad libitum. After 3 days of adaptation, the rats were mated overnight with adult male rats. The next day was taken as day 1 of pregnancy if spermatozoa were found in vaginal smears. Weights of pregnant rats were recorded at day 1 and day 20 of gestation to calculate the weight gain.

**Methods**

**Experimental protocol**

24 pregnant female Wistar albino rats were used for the present study and were randomly divided into 3 groups (n=8 per group) as follows: Control group (I);Pregnancy induced hypertension (PIH) group (II) and PIH + Vitamin $D_3$ (excess vitamin $D_3$) supplemented group (III).

L-NAME was used to induce hypertension in the pregnant rat. The dose of L-NAME (Sigma-Aldrich , (St. Louis, Missouri, USA) used was 50 mg/kg body weight/day and was administered by gavage dissolved in distilled water from day $14^{th}$ to $19^{th}$ of gestation **(Kemse et al., 2014)**.

Vitamin $D_3$ (Puritan Co., Cairo, Egypt) in a dose of 50 IU/kg, was given intraperitoneally form day 1 until day 19 of gestation **(Mehta et al., 2014)** .

**Determination of urinary albumin excretion**

On day 19 of pregnancy, the rats were placed in metabolic cages for 24-hour urine collection. Urine protein concentrations were determined by the principle of turbidimetry by adding 5% trichloroacetic acid and using Microlab300 according to **Abuel , (1983).**

**Blood pressure measurement**

The blood pressure of the overnight fasting pregnant rats was measured using the power lab device (AD Instruments Pty Ltd, Australia) according to**Parasuraman and Raveendran, (2012)**. The systolic and diastolic BP was recorded on day 19 of gestation for all dams. Three measurements with 30 s intervals were recorded and the average of these readings was calculated followed by calculation of the mean arterial blood pressure (MAP).

**Specimen collection**

On day 20 of pregnancy, rats were anaesthetized between 9:00 a.m. and 10:00 a.m. Maternal blood was collected by cardiac puncture and put in polyethylene tubes pre-rinsed with EDTA. Plasma was prepared by centrifugation for 10 min at 3000 r.p.m and was stored at −80°C until analysis.

**Biochemical assay**

-Plasma Angiotensin II were measured using enzyme linked immunosorbent assay kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA ) according to **Nussberger et al. (2001).**

-Plasma oxidative stress marker (MDA) levels were estimated using Oxis kits (MDA586, Oxis International, Foster City, CA, USA) according to **Niehaus and Samuelsson, (1968)**.

-Plasma TNF-α were measured using enzyme linked immunosorbent assay kit (Abcam, Catalog No. ab100785, USA) according to **He and Ting, (2002).**

**Histopathological studies**

After the rats were killed, pups were delivered by caesarean section and parts of the kidneys and placentas were harvested and fixed horizontally in 10% neutral-buffered formaldehyde solution.

After dehydration, the samples were embedded in paraffin, and 4-μm sections were cut by a microtome and collected for routine H&E .

**Statistical analysis:**

Data were presented as mean ± S.D Statistical significance was determined by one way analysis of variance (ANOVA) followed by LSD test, P values less than 0.05 were considered to be significant. In statistical analysis, SPSS version 18 program for Windows (SPSS Inc. Chicago, IL, USA) was used.

## RESULTS

Body weight (BW) gain, the biochemical and haemodynamic characteristics of the studied groups are summarized in table 1.

**As regards group II**, it showed a non significant ($p>0.05$) change in BW gain along with a significant ($p<0.001$) increase in MAP which was accompanied with a significant ($p<0.001$) increase in plasmaAng II levels and proteinuria in comparison with that of control group ( group I) .

As regards the oxidative stress there was a significant ($p<0.001$) increase in plasma MDA levels, also there was a significant ($p<0.001$) increase in plasma TNF-alpha levels in comparison with that of control group (group I).

In addition, In PIH group the histopathological studies of the placenta showed degenerative changes with vacuolization and swelling of the decidua (Fig.1-B). Moreover, Kidney of the preeclamptic group showed glomerular endotheliosis and thickening of the media of renal vessel walls, in addition to vacuolated cytoplasm and dark nuclei of the cells lining tubules(Fig 1-F).

**As regards the effect of chronic Vitamin D supplementation in the PIH rats (Group III)**, there was a non-significant ($p>0.05$) change in BW gain, along with a significant ($p<0.001$) decrease in MAP which was accompanied with a significant ($p<0.001$) decrease in plasma Ang II and proteinuria in comparison with that of PIH group ( group II).

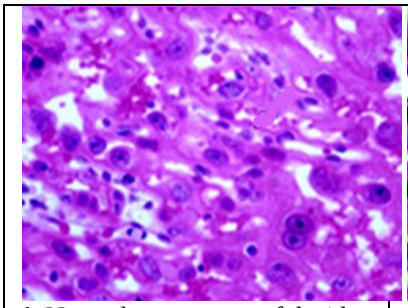
As regards the oxidative stress there was a significant ($p<0.001$) decrease in plasma MDA levels, also there was a significant ($p<0.001$) decrease in plasma TNF-alpha levels in comparison with that of PIH group (group II).

In addition, vitamin D supplementation in group III produced a marked improvement in results of the histopathological studies as regards both the placenta and the kidney (Fig.1- C, G).

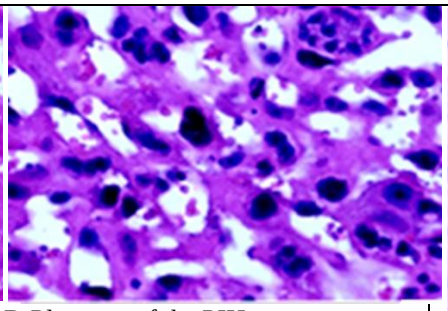
**Table (1):** BW gain , biochemical and hemodynamic parameters of the studied groups.

| Groups / Parameters | Group I (Control) | Group II (PIH) | Group III (PIH +vitamin D) |
|---|---|---|---|
| **Weight gain(gm)** | 205±15.9 | 212±13.5 | 217±10.7 |
| **MAP (mmHg)** | 101±6 | 143±8***a | 122±5***a,b |
| **Protein in urine (gm/dl)** | 0.41±0.05 | 1.38±0.19***a | 0.8±0.07***a,b |
| **Ang II (ng/ml)** | 0.79±0.03 | 1.5±0.21***a | 0.92±0.06 ***a,b |
| **MDA (nmol/mg protein)** | 3.99±0.5 | 8.78±1***a | 5.5±0.4***a,b |
| **TNF-α (ng/ml)** | 40.99±1.99 | 61.64±2.71***a | 49.53±1.86***b |

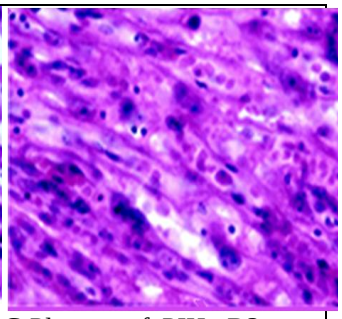
***=significant (P<0.001) ,a (versus group I), b (versus group II).



A-Normal appearance of decidua of the control group ( H&E, ×200) .



B-Placenta of the PIH group showing degenerative changes with vacuolization and swelling of the decidua ( H&E, ×200).



C-Placenta of PIH +D3 group showing marked improvement in comparison to the PIH group : Marked improvement in vascularization, nucleus decreased size & multinucleated cyto&syncytiotrophoblast ( H&E, ×200).

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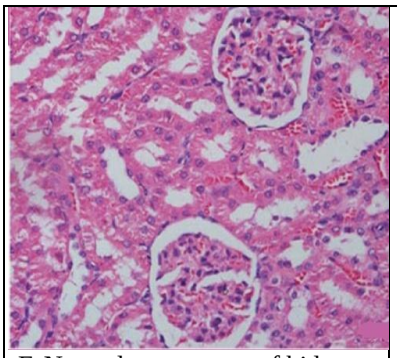
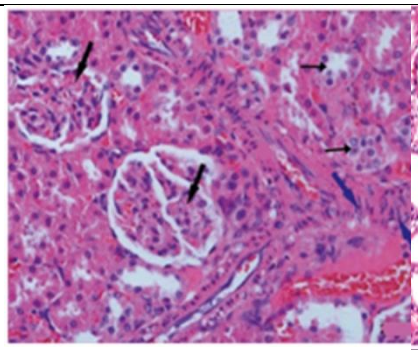
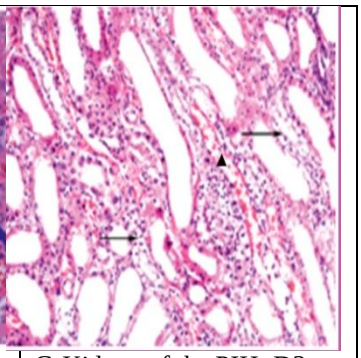
|  |  |  |
| E-Normal appearance of kidney of the control group ( H&E, ×400). | F-Kidney of the PIH group showing glomerular endotheliosis (black arrows) and thickening of the media of renal vessel walls (blue arrows), in addition to vacuolated cytoplasm and dark nuclei (thin arrows) of the cells lining tubules ( H&E, ×400). | G-Kidney of the PIH+D3 group showing Vacuolated cytoplasm and dark nuclei (thin arrows) of the cells lining fewer tubules compared to Fig.F and congestion of some peritubular capillaries (pyramid) (H&E, ×200). |

**Figure(1):**Results of the histopathological examination

## DISCUSSION

Preeclampsia remains one of the most common causes of maternal and fetal morbidity and mortality in the developed world **(Wang et al., 2004&Ilekis et al., 2007)**.

Although the pathogenesis of preeclampsia is still not fully understood, a multi-stage model is generally accepted **(Brodowski et al., 2014)**. The utero-placental syndrome with impaired placental development in the first stage of the disease causes generalized maternal endothelial dysfunction as a main clinical feature of preeclampsia in the second stage **(Redman and Sargent, 2005)** . An array of placenta-derived factors are candidate contributors to endothelial dysfunction in preeclampsia **(Maynard et al., 2003)**.

The term 'animal model of preeclampsia' is commonly and consistently used when nitric oxide synthase inhibitor NG-nitro-L-arginine methylester (L-NAME) was administered from day14 of gestation to induce preeclampsia-like syndrome in rats (**Ma et al., 2010,2011&Neerhof et al., 2011&Nassar et al., 2012& Brown et al., 2013)** . It has been reported that although chronic treatment with L-NAME may not reproduce the entire disease entity, it produces virtually all the pathophysiologies of preeclampsia in the animal model **(Takie et al., 2007)**. In view of this the L-NAME induced rat model of pregnancy induced hypertension (PIH) was used.

In the present study PIH induction did not affect the total weight gain of the rats, furthermore, vitamin D supplementation in group III did not produce any significant change in final body weight gain. This is similar to earlier reported studies **(Gairard et al., 2004 & de Moura et al., 2007)**. In contrast others report lower body weights of PIH rats as compared to control **(Fernandez Celadilla et al., 2005)**.

PIH induction using L-NAME administration in this work increased blood pressure and is consistent with other recent reports **(Adamcova et al., 2013&Gad, 2013& Sung et al., 2013&Zhou et al., 2013).**However, vitamin D supplementation was able to reduce significantly MAP.

In humans it has been reported that supplementation of multivitamins containing vitamin D is associated with reduced risk of preeclampsia **(Wen et al., 2008&Walker et al., 2011)**.Furthermore, Vitamin D supplementation decreased hypertension in response to placental ischemia during pregnancy in PE rat model **(Darby et al., 2013&Kemse et al., 2014)**. A recent meta-analysis found that decreased circulating Vitamin D is associated with an increased risk of hypertension in healthy populations **(Zhang et al.,2013)**.

Mechanisms linking vitamin D and blood pressure may be related to direct vitamin D effects on the vasculature **(Brewer et al., 2011)**. In experimental studies, VDR activation has been shown to exert a variety of anti-atherosclerotic effects **(Tare et al., 2011)**. These involve, amongst others, vitamin D induced decrease of endothelial adhesion molecules, increase of nitric oxide (NO) production and inhibition of macrophage to foam cell formation **(Oh et al., 2009&Brewer et al., 2011& Tare et al., 2011&Molinari et al., 2011)**. Data from clinical studies addressing these issues are, however, less clear, but there are at least some studies suggesting a relationship of vitamin D deficiency and endothelial dysfunction (**Harris et al., 2011& Witham et al., 2012)**. Taken together, several plausible pathophysiological mechanisms exist that may account for the link between vitamin D and regulation of blood pressure.

In addition, vitamin D supplementation produced a significant decrease in proteinuria in PIH group (group III).Vitamin D is also responsible for kidney protection and the regulation of several renal physiological activities **(Li, 2011b)**. Thus, vitamin D deficiency (VDD) (<10 ng/mL) or insufficiency (10–30 ng/mL) can accelerate the progression of kidney disease **(Gonzalez et al., 2004&Ulerich , 2010&Cuppari et al., 2011)**. Furthermore, vitamin D deficiency itself has been associated with increased prevalence of proteinuria in adults **(Li, 2011b)**.

Our findings are supported by experimental data which suggest that VDR activation may exert anti-proteinuric effects *e.g*. by protection against podocyte damage and induction of megalin expression, which is required for tubular reabsoprtion of albumin **(Dusso andTokumoto, 2011&Mirković et al., 2011)**. Various other nephroprotective actions of vitamin D have also been described by other researchers, these partially involve anti-inflammatory actions of vitamin D by suppression of nuclear factor-κB (NF- κB) **(Bouillon et al., 2008).**

Results of this study also revealed a significant increase in plasma angiotensin II levels in PIH rats, however, vitamin D supplementation in group III produced a significant decrease in the above mentioned parameter. Vitamin D is a negative endocrine regulator of the renin-angiotensin-aldosterone-system (RAAS), which plays a key role in the development of kidney disease **(Petchey et al., 2011)**. ***Li et al. (2002)*** and **Kong et al. (2008)** demonstrated that VDR- knockout mice presented higher renin and angiotensin II levels leading to hypertension. Moreover, low levels of vitamin D also induce podocyte loss and the development of glomerulosclerosis through direct cellular effects, compromising the integrity of the glomerular filtration membrane **(Kuhlmann et al., 2004 &Li, 2011b)**.

Previous studies conducted in humans and animals have shown strong evidences that vitamin D deficiency led to an up regulation of the renin-angiotensin system **(Li, 2003&Tamez et al., 2013 &Schroten., et al., 2013)**. In addition, **Canale et al. (2014)** findings also revealed increased activation of RAAS in VDD rats which could be explained by the increased mRNA expression of renin, angiotensinogen (AGT), angiotensin converting enzyme (ACE), and angiotensin receptor (AT1a) associated with augmented levels of aldosterone. In addition, they showed higher expression of angiotensin II and its receptor in the kidney of VDD animals, reinforcing the role of vitamin D on blood pressure control.

It is well known that oxidative stress is also implicated in the development of hypertension. Angiotensin II activates NADPH oxidase leading to the generation of superoxides**(Patzak andPersson ,2007& Finch et al., 2012)**. In this context, **Zhang et al. (2015)** concluded that, 1,25(OH)2D3 normalizes the altered central RAS in 1α(OH)ase(-/-)mice, at least partially, through a central antioxidative mechanism.

In this work elevated MDA levels were observed in the PIH group. Oxidative stress may play a central role in the pathophysiology of preeclampsia and higher levels have been reported by many investigators in animal **(Tanir et al., 2005& Yang et al., 2011)** and human **(Menon and Rozman, 2007&Patil et al., 2007 &Mehendale et al., 2008&Gohil et al., 2011 &Kulkarni et al., 2011 &Pimentel et al., 2013)** studies. It has been reported that increased free radicals lead to cellular dysfunction, oxidative damage of biomolecules and endothelial dysfunction **(Valko et al., 2006)**. A recent review highlights the need to supplement preeclamptic women with antioxidants during pregnancy to counteract oxidative stress to prevent or delay the onset of preeclampsia and improve the health of mother and baby **(Jones et al., 2014)**.

Our findings also revealed that, vitamin D supplementation in PIH rats (group III) was able to reduce the oxidative stress by lowering plasma MDA levels. It has been suggested that an vitamin D- micronutrient cocktail and even alone can modulate biomarkers of oxidative stress and inflammation in humans under different pathological conditions **(Hopkins et al., 2010&Shab-Bidar et al., 2014)**. In addition, ***Tarcin et al*. (2009)** demonstrated that vitamin D deficient individuals presented higher plasma levels of Thiobarbituric Acid Reactive Substances (TBARS) as a result of increased lipid perioxidation .

Preeclampsia is considered to have a multifactorial etiology associated with inflammatory dysfunction **(Bayram et al., 2012)**. In the present study, higher levels of plasma TNF-alpha was observed in PIH induced dams and is similar to earlier reported human studies **(Ellis et al., 2001&Bernardi et al., 2008&Guven et al., 2009&Catarino et al., 2012&Lau et al., 2013)** as well as in animals **(Murphy et al., 2013)**. In contrast there are some studies which show no significant differences in the serum levels of TNF-alpha between control and preeclamptic patients **(Freeman et al., 2004&Afshari et al ., 2005&Ozler et al., 2012)**.

In this context, vitamin D supplementation in PIH rats (group III) produced a significant decrease in plasma TNF-alpha in our study. This finding is supported by those of other investigators who reported increased inflammation in association with hypovitaminosis D in many settings, such as diabetic, after hip fracture, and hemodialysis patients **(Miller et al., 2007&Giulietti et al., 2007&Bednarek-Skublewska et al., 2010)**. In vitro studies support these observations. Vitamin D inhibits TNF-alpha induced inflammatory cytokines in human coronary endothelial cells **(Suzuki et al., 2009)**.Vitamin D deficiency was also connected with increased interleukin-6 (IL-6) concentrations through a stress –related kinase, p38 inactivation, in human prostatic epithelial cells **(Casart et al., 2007)**. These studies are all consistent with an anti-inflammatory role of vitamin D.

Finally, those potentially beneficial effects of vitamin D supplementation in PIH rats, in this work is supported by the marked improvement in results of the histopathological studies as regards both the placenta and the kidney (Fig.1- C, G).

**Conclusion**: In the current study, Vitamin D supplementation to PIH rats showed beneficial effects in terms of reducing: blood pressure, proteinuria, inflammation and oxidative stress, along with the negative effect on angiotensin II levels.

**Recommendations**: Effective preventive or therapeutic strategies for preeclampsia do not exist to date. Therefore, Vitamin D supplementation could benefit these women greatly by interacting with multiple pathways. In addition, more studies are required to explore the molecular mechanisms underlying this multifaceted effects of vitamin D.

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