

## Protective effect of vitamin D&K against arterial calcification in ovariectomized rats

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**Abstract:** Background: Objective: The objective of this study was to assess associations between the daily oral dose of vitamin D, vitamin K, both vitamins D & K supplementation and serum concentration of Matrix Gla Protein (MGP), interleukin 6 (IL6) and interleukin 10 (IL 10). Aim of the work: Inflammation is regarded as a risk predictor for metabolic syndrome and atherogenesis. The objective of this study was to conduct a systematic review and a meta-analysis to confirm the effect of vitamin supplementation on cytokine levels. **Methods:** forty ovariectomized rats with an average weight of 150 g were used in this study. They were equally divided into four groups. Group I: control ovariectomized group. Group II: Ovariectomized & vitamin D supplementation group, Group III: ovariectomized & vitamin K supplementation group, Group IV; ovariectomized & both vitamins D&K supplementation group, the animals were given daily dose of vitamin D and K or both vitamins daily by gavage for nine weeks. Blood samples were taken from all groups. We investigated the circulating concentrations of MGP, IL6 and, IL 10, and their relationships to vitamins D, K and both vitamins supplementation. **Results:** Vitamin D supplementation alone, Vitamin K Supplementation alone or combined with vitamin D and K Supplementation to ovariectomized rats produce significant increase in MGP in versus to control ovariectomized rats. While Ovariectomized rats with vitamin K supplementation and ovariectomized rats with vitamin D+K supplementation induced significant decrease of IL6 and significant increase of IL10, ovariectomized rats with vitamin D supplementation induced insignificant decrease of IL6 and insignificant increase of IL10 compared to control ovariectomized rats. **Conclusions:** vitamins D & K stimulate MGP, a strong protein inhibitor of vascular calcification. Vitamin K supplementation was associated with lower concentrations of inflammatory markers suggests that a possible protective role of vitamin K in inflammation.

[Aziza Khalil and Gehan A. Youssef. **Protective effect of vitamin D&K against arterial calcification in ovariectomized rats.** *Nat Sci* 2013;11(12):239-246]. (ISSN: 1545-0740). <http://www.sciencepub.net/nature>. 34

**Key words:** Vitamin D, Vitamin K, MGP, IL6 and IL10.

### 1. Introduction:

Vascular calcification is largely divided into two types. One is atherosclerotic intimal layer calcification and other is medial layer calcification. Evidence is growing that vascular calcification is an active process as well as passive process resulting from elevated serum phosphate and an increase in calcium phosphate product leading to oversaturated plasma. Proving the active process, *in vitro* studies have demonstrated that the transformation of vascular smooth muscle cells (VSMCs) into osteoblast like cells is a crucial mechanism in the progression of vascular calcification.

#### Vitamin D and arterial calcification:

The active form of vitamin D, 1, 25 dihydroxyvitamin D or calcitriol, is the end product of two hydroxylation steps of vitamin D: a hepatic 25-hydroxylation and a subsequent renal 1 $\alpha$ -hydroxylation. Calcitriol exerts genomic and nongenomic effects through a cytosolic vitamin D receptor (VDR) and a membrane bound receptor. (Somjen *et al.*, 2005).

There is accumulating evidence that the vitamin D hormone calcitriol exerts important physiological

effects in cardiomyocytes, vascular smooth muscle cells, and the vascular endothelium.

The link between vitamin D and vascular calcification is complex. Experimental & clinical researches have revealed that both vitamin D excess and vitamin D deficiency have been shown to be associated with vascular calcification (Watson *et al.*, 1997). Recent experimental data demonstrate that physiologic vitamin D action include the inhibition of the process that are important for intimal & medial layer calcification such as pro-inflammatory cytokine release, adhesion molecule release, proliferation and migration of vascular smooth muscle cells. (Konradsen *et al.*, 2008)

Deficient calcitriol concentrations probably contribute to the massive vascular calcification such as pro-inflammatory cytokine release, adhesion molecule release, proliferation and migration of vascular smooth muscle cells. Low cellular availability of calcitriol enhanced synthesis and/or enhanced deleterious effect of matrix metalloproteinase (MMPs), tumor necrosis factor TNF, IL-1, IL-6, contracting factors, and advanced glycation end products (AGEs). Low calcitriol reduced synthesis of

matrix Gla protein (MGP), osteopontin, IL-10. (Holick, 2007)

In postmenopausal women, calcitriol concentrations were reduced by approximately one-third in patients with deficient 25(OH)D levels (<25 nmol/l) compared with adequate levels (>80 nmol/l) [Rejnmark *et al.*, 2008]. Data are in line with the fact that some postmenopausal women patients, has an enhanced risk for vascular calcification. (Rahman *et al.*, 2007)

#### **Vitamin K and arterial calcification:**

Vitamin K is essential for blood coagulation, vascular calcification and bone metabolism in mammals. Vitamin K and vitamin K-dependent proteins may be involved in regulation of calcification, energy metabolism, and inflammation.

Matrix Gla Protein (MGP) and Growth Arrest Specific Gene 6 (Gas-6) are two particularly important of Vitamin K-dependent proteins (VKDPs). These proteins have many diverse biologic functions, yet with the recognition that they are produced by vascular smooth muscle cells, their roles in vascular biology are being increasingly explored. MGP functions primarily as a vascular calcification inhibitor. Gas-6 affects vascular smooth muscle cell movement and apoptosis. Together, these proteins constitute a new mechanism of local vascular regulation, where the blood vessel defends itself against injury and participates in self-repair. A failure of these local mechanisms might be an important first step in a cascade of events culminating in vascular calcification, and supports the notion that vascular calcification is an active, regulated process. (John, 2008)

The process of converting VKDPs to their biologically active forms requires the carboxylation of glutamic acid residues (Furie *et al.*, 1999). As a final step,  $\gamma$ -glutamyl carboxylase (GGC) facilitates the addition of a CO<sub>2</sub> molecule to the  $\gamma$ -carbon of glutamic acid, forming  $\gamma$ -carboxyglutamic acid. This requires the presence of the reduced form of vitamin K. Because vitamin K naturally occurs in the oxidized form, it must be converted into a reduced form, a reaction catalyzed by vitamin K epoxide reductase (VKOR). In this process of carboxylation, vitamin K is returned to its oxidized state, and a cycle of vitamin K reuse ensues (Yabe *et al.*, 1997).

Recently, it was observed that the dietary vitamin K requirement for the synthesis of the coagulation factors is much lower than for that of the extra-hepatic Gla-proteins. This forms the basis of the triage theory since bleeding to deaths the major and acute threat of vitamin K deficiency, vitamin K entering our blood stream is preferentially Transport to the liver, the place where the coagulation factors are synthesized. Only after hepatic vitamin K

requirement has been met, the excess vitamin K is transported to extra hepatic tissues. This explains why in the healthy population all clotting factors are synthesized in their active form, whereas the synthesis of other Gla-proteins is sub-optimal in non-supplemented subjects. Prolonged sub-clinical vitamin K deficiency is a risk factor for osteoporosis, atherosclerosis, and cancer. Present recommendations for dietary intake are based on the daily dose required to prevent bleeding. Accumulating scientific data suggests that new, higher recommendations for vitamin K intake should be formulated (McCann *et al.*, 2009) MGP also has important functions in vascular biology. It composes of 84-amino acid protein, it is produced in bone and vascular smooth muscle cells and prevents vascular calcification (Murshed *et al.*, 2004).

The clinical importance of MGP in preventing vascular calcification, its exact mechanism of action remains unknown. MGP complexes with ambient calcium, preventing calcium supersaturation and crystallization within vessel walls (Roy and Nishimoto 2002), and by binding to hydroxyapatite crystals, inhibits their crystalline growth. MGP also inhibits vascular smooth muscle cells from dedifferentiating into osteoblast-like cells, a well-established mechanism for vascular calcification (Zebboudj *et al.*, 2003). Recent studies suggest that the activity of MGP is affected by bone morphogenic protein-2 (BMP-2) (Zebboudj *et al.*, 2003).

Although both normal and calcified vessels express MGP, normal vessels have a predominance of carboxylated MGP, whereas calcified vessels have a predominance of the uncarboxylated version (Schurgers *et al.*, 2005). It seems that the ratio of carboxylated to uncarboxylated protein ultimately determines biologic activity of both MGP and Gas-6 (Hasanbasic *et al.*, 2005). These findings suggest a potential mechanism. To help protect blood vessels from injury, vascular smooth muscle cells continuously produce MGP and Gas-6, which then undergo carboxylation. The carboxylated proteins form a defense against injury: MGP preventing crystalline supersaturation and Gas-6 regulating both apoptosis and migration of smooth muscle cells into areas of injury. (Pearson, 2007)

However, other functions of vitamin K have been reported recently. We previously found that vitamin K suppresses the inflammatory reaction induced by lipopolysaccharide (LPS) in rats and human macrophage. (Kyla *et al.*, 2011)

#### **2. Methods:**

Forty ovariectomized rats with an average weight of 150 g were used in this study. They were equally divided into four groups.

**Group I:** control ovariectomized group, Ovariectomy was done for all rats under complete sanitary conditions using single dorsal midline incision (*Olson et al., 1986*).

**Group II:** Ovariectomized & vit. D supplementation They received 0.1ug/kg body weight of vitamin D daily by gavage (*Yuji Kasukawa et al., 2010*) for nine weeks.

**Group III:** ovariectomized & vitamin K supplementation group, the animals were given 0.0009 mg/g body weight of vitamin K daily by gavage (*El-Morsy et al., 2011*) for nine weeks.

**Group IV:** ovariectomized with both vitamin D&K supplementation group, the animals were given daily dose about 0.1ug/kg body weight of vitamin D and given 0.0009 mg/g body weight of vitamin K daily or both vitamins daily by gavage for nine weeks

**Blood samples** were taken from all groups. We investigated the circulating concentrations of matrix Gla protein (MGP), IL-6 and IL-10 and their relationships to vitamin D, vitamin K and compination between vitamin D and K supplementation of overectomized rats

1- Enzyme-linked Immunosorbent Assay Kit For Matrix Gla Protein (MGP) (*O'Donnell et al., 2006*)

2-Serum IL 6 according to (*Heinrich et al., 2003*)

3--Serum IL10 according to (*Peyron et al., 1994*)

**Statistical Analysis:**

All statistical analysis was computed by SPSS version 14.

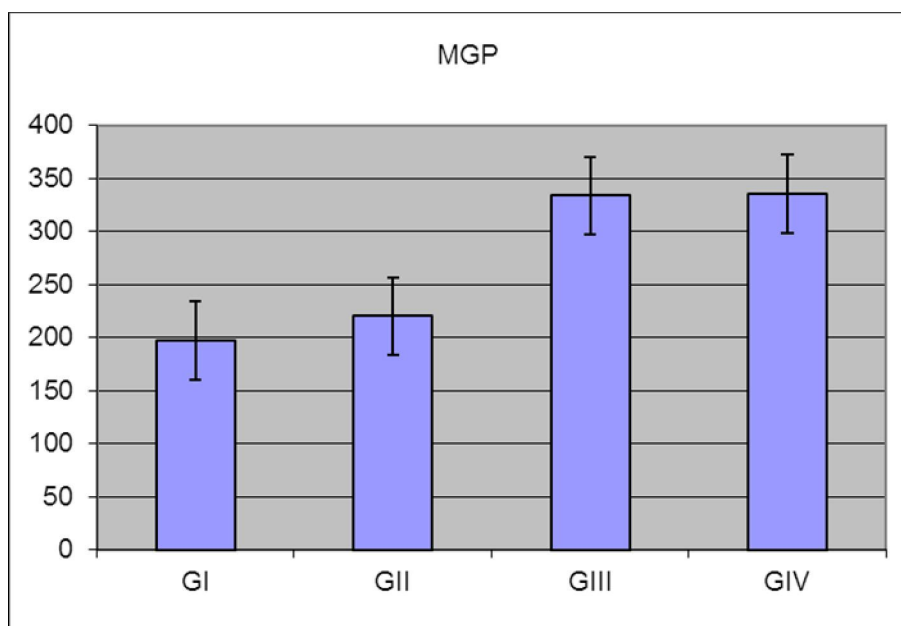
The values obtained were revealed as mean  $\pm$  S.D. Data were analyzed using student's 't'-test and results were considered significant at  $P < 0.05$ . and ANOVA test.

### 3. Results

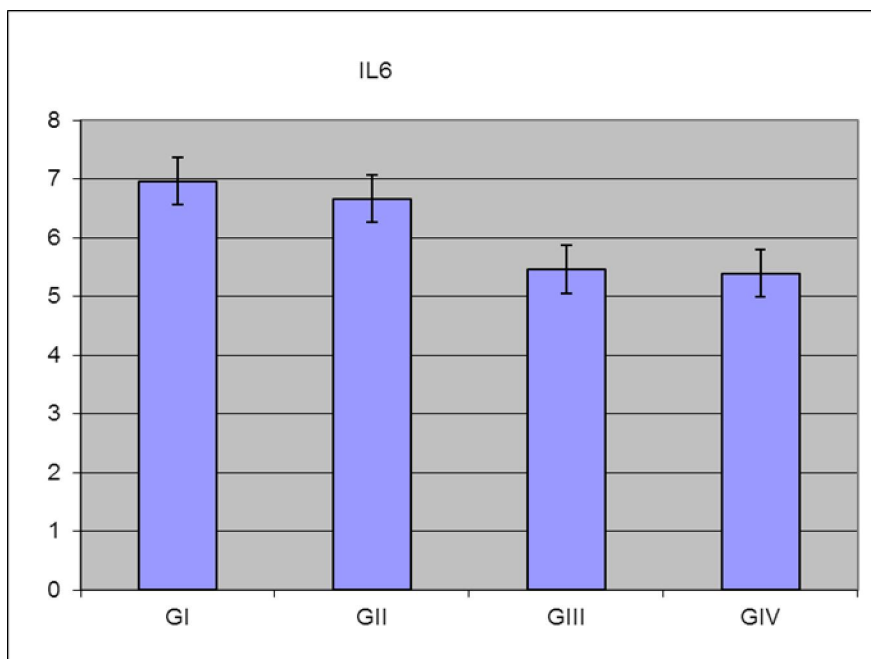
Regarding the vascular calcification marker such as matrix Gla protein (MGP) wa significant increased in Vitamin D supplementation alone, Vitamin K Supplementation alone or combined between vitamin D and K Supplementation to overictomized rats in versus to control overictomized rats. Table (1), Fig (1)

The proinflammatory cytokines such as interleukin IL-6 was significant decreased in Ovariectomized rats with vitamin K supplementation and ovariectomized rats with vitamin D+K supplementation, insignificant decreased in overictomized rats with vitamin D supplementation compared to control overictomized rats. Table (1), Fig (2).

While the anti-inflammatory cytokine IL-10 was significant increased in Ovariectomized rats with vitamin K supplementation and ovariectomized rats with vitamins D+K supplementation, insignificant increased in overictomized rats with vitamin D supplementation in comparison to control overictomized rats. Table (1),Fig (3).



**Fig(1): Serum MGP (ng/ml) in all studied groups**



Fig(2): Serum IL6(ng/ml) in all studied groups

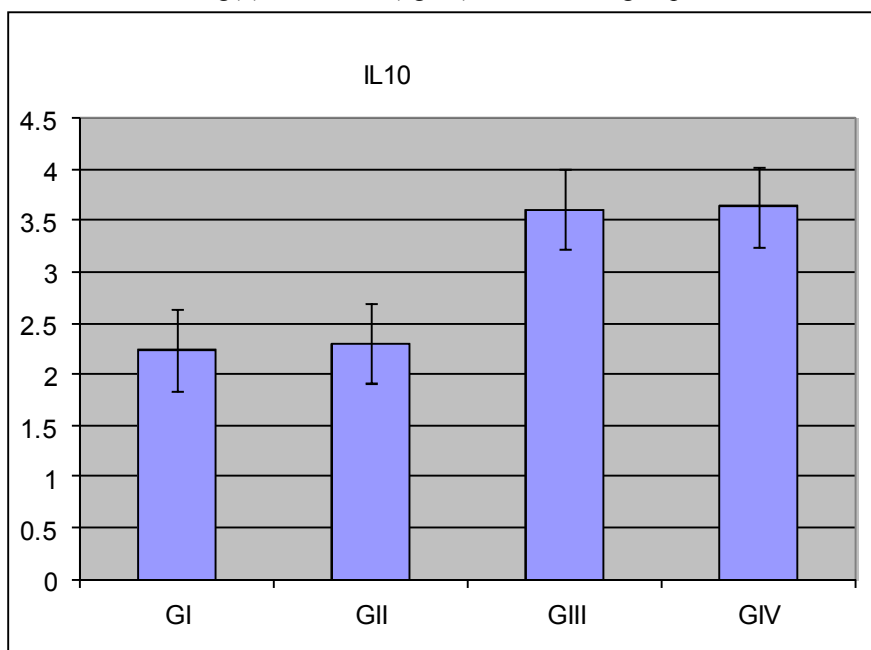


Fig (3): Serum IL10(ng/ml) in all studied groups.

Table (1): Mean ±SD of measured parameters in studied groups compared to control.

Groups parameters	Group (GI)	vit D(GII)		vit K(GIII)		vits D+K(GIV)		ANOVA F=
	Mean ±S.D	Mean ± S.D	P value	Mean ±S.D	P value	Mean± S.D	P value	
<b>MGP ng/ml</b>	197.00 ±14.41	220.00 ±19.66	<i>P</i> <0.05*	333.71 ±23.59	<i>P</i> <0.05*	335.57 ± 31.53	<i>P</i> <0.05*	70.41
<b>IL6 ng/ml</b>	6.96 ±0.59	6.66 ±0.40	<i>P</i> >0.05†	5.46 ±0.46	<i>P</i> <0.05*	5.39 ±0.27	<i>P</i> <0.05*	22.96
<b>IL 10 ng/ml</b>	2.24 ±0.50	2.30 ±0.37	<i>P</i> >0.05†	3.61 ±0.43	<i>P</i> <0.05*	3.64 ±0.15	<i>P</i> <0.05*	28.98

#### 4. Discussion:

The present study demonstrated the protective effects of vitamin D, vitamin K and both against the risks of arterial calcification in adult female rats of estrogen-deprivation condition.

As women enter menopause, they simultaneously lose calcium from bone and increase its deposition in arteries a negative double whammy called " calcification paradox" which greatly increases the risk of both osteoporosis and cardiovascular disease (Adams and Pepping,2005).The dropping estrogen causes both problem,but both vitamins D and K can help rectify them.

The results of the present study showed that ( MGP ) was significantly increased in ovariectomized and vitamin D supplementation group, which is in agreement with Konradsen *et al.* ( 2008 ) they found that vitamin D increases the production of Matrix Gla Protein (MGP), vitamin D inhibits the process that are important for intimal and medial artery calcification such as proinflammatory cytokine release, adhesion molecule release,and proliferation and migration of vascular smooth muscle cells.

Zittermann *et al.*, 2007 also demonstrated that physiologic vitamin D actions include the inhibition of processes that are important for intimal and medial artery calcification such as pro- igration of vascular smooth muscle cells.

Zittermann and Koerfer ( 2008) found that low level of calcitriol precursor 25-hydroxy vitamin D are associated with myocardial infraction, congestive heart failure and calcific aortic stenosis. Deficient calcitriol concentrations probably contribute to the massive vascular calcification seen in chronic kidney disease.In patients with end stage renal disease and end stage heart failure.Very low circulating calcitriol levels of nonuse of active vitamin D or both indently associated with high mortality rats.

In contrast to our result,Price *et al.*,(2000) found that vitamin D treatment enhance the extent of artery calcification in rats given sufficient doses of walfarin to inhibit gamma-carboxylation of matrix Gla Protein, a calcification inhibitor known to be expressed by smooth muscle cells and macrophages in the artery wall. The synergy between walfarin and vitamin D is probably best explained by the hypothesis that warfarin inhibits the activity of matrix Gla protein as a calcification inhibitor and vitamin D may induce artery calcification through its effect on serum calcium.

In this study, serum matrix Gla protein (MGP) level was significantly increased in overiectomized and vitamin K supplementation group.

In agreement with our results Beulens *et al.* (2009), found that high dietary vitamin K intake is

associated with significant increased in matrix Gla Protein( MGP ) which reduced coronary calcification.

Also Seyama and Wachi, 2004 detected that matrix Gla protein (MGP) is produced by small muscle cells in our blood vessels where once activated by vitamin K it prevents calcium deposits.

Vitamin K activated matrix Gla Protein(MGP) which also needed to prevent the excessive proliferation and mineralization of muscle cells in the walls of the veins that causes varicose veins (Cario *et al.*, 2007).

Demer *et al.* (2002) who found that one of the vitamin K dependant proteins, matrix Gla protein (MGP) is the strongest inhibition of tissue calcification presently known. matrix Gla protein (MGP) is importance for blood vessel health was first demonstrated in animals bred to be matrix Gla protein (MGP)- deficient, all of which died of massive arterial calcification within 6-8 after birth.

Fodor *et al.* ( 2010)who discussed that Vitamin K-dependent proteins in the body act as potent inhibitors of vascular calcification. So adequate vitamin K will help to ensure calcium ends up in bone where it belongs, instead of calcifying various soft tissues. Vitamin K – dependent proteins (VKDPs) require carboxylation to become biologically active matrix Gla Protein ( MGP ) and growth Arrest specific gene 6 ( Gas-6) are two particularly important VKDPs,and their roles in vascular biology are just beginning to be under stood.Both function to protect the vasculature; matrix Gla Protein ( MGP ) prevents vascular calcification and Gas-6 affects vascular smooth muscle cell apoptosis and movement.

Roy and Nishimoto( 2002) showed that MGP complex with ambient calcium, preventing calcium supersaturation and crystallization within vessel wall by binding to hydroxyapatite crystals,inhibits their crystalline growth.

MGP also inhibits vascular smooth muscle cells from differentiating into osteoblast –like cell a well established mechanism for vascular calcification ( Zebboudj *et al.*, 2003).

Yao *et al.* (2006) suggest that the activity of matrix Gla Protein (MGP) is affected by bone morphogenic protein -2 (BMP-2) is a known osteoinductive factor increased calcification and osteogenic differentiation in calcifying vascular cells.

Matrix Gla Protein ( MGP ) inhibits calcification by binding to and inhibiting the activity of BMP-2,and potent bone morphogen whose expression triggers the induction of an osteogenic gene expression profile in vascular smooth muscle cells (VSMC), which causes them to transform into osteoblast-like cells, a transformation known to precede arterial calcification

Also, **Cramenburg et al. (2008)** reported that serum matrix Gla Protein (MGP) concentration were higher in this study among the vitamin K treatment group had less progression of coronary artery calcification (CAC).

One of vitamin K's primary actions in the body is the carboxylation (activation) of Matrix Gla Protein (MGP). Critical to maintaining the health of the entire cardiovascular system. Matrix Gla Protein (MGP) is known to prevent calcification in the arteries. Vitamin K promotes blood vessel elasticity by safeguarding elastin primarily responsible for the elasticity of the arterial wall. Existing elastin is damaged and new production is inhibited by calcium deposits (**Seyama and Wachi, 2004**)

Recent studies suggested that MGP suppresses the inflammatory reaction induced by lipopolysaccharide (LPS) in rats and human macrophages (**Shea et al., 2011**)

In contrast to our results **Reily et al., (2004)** found that increased concentration of circulating MGP have been associated with arterial calcification in the rats and in patient with severe atherosclerosis. MGP is found at levels in the vicinity of calcium deposits in mice and humans (**Spronk et al., 2001**). The protective effect of vitamin K on progression of arterial calcification would be associated with a concomitant decrease in serum MGP concentration. Elevated serum concentration of MGP have been reported in patients with severe atherosclerosis (**Schurgers et al., 2005**).

The results of the present study showed that MGP was significantly increased in ovariectomized and vitamin D and K supplementation group, which is in agreement with (**Iwamoto et al., 2003**) who found that the combination of vitamin K and D is more effective in increasing MGP and preventing blood vessel calcification than either nutrient alone. Vitamin D increases both the demand of vitamin K and potential for benefit from K – dependent protein, such as MGP in blood vessels and osteocalcin in bone.

Only a few studies have addressed the effect of vitamin D&K supplementation on MGP, IL6 and IL10

The results of the present study showed that insignificant decrease in IL6 and insignificant increase in IL 10 in ovariectomized and vitamin D supplementation group. Which is in agreement with (**Zittermann and Koerfer 2008**) who found that vitamin D decrease proinflammatory cytokines such as interleukin 1 ( IL- 1 ), interleukin 6 (IL-6) and Tumor necrosis factor  $\alpha$  ( T NF-  $\alpha$  ), vitamin D increase the anti-inflammatory cytokine interleukin 10 ( IL-10 ) and the inhibitor of vascular calcification such as ( MGP)

**Zhu et al., ( 2005)** who found that daily supplement of vitamin D for 9 month able to

increase serum concentrations of anti-inflammatory cytokine IL-10 and to prevent an increase in serum concentrations of proinflammatory cytokine TNF- $\alpha$  in chronic heart failure patient.

Also, **Zittermann et al. ( 2007 )** found that vitamin D is able to suppress the release of TNF - $\alpha$  and IL-6 while it enhance IL-10 synthesis.

In line with our hypothesis, significant negative correlations between serum concentration of 25 (OH) D and IL -6 (**Shea et al., 2008**).

Moreover, earlier epidemiologic data indicate that high blood concentration of 25(OH) D were associated with high IL -10 concentration (**Zittermann et al., 2004**). Because IL-10 is able to suppress the production of proinflammatory cytokine, this anti-inflammatory cytokine seems to have important cardioprotective action (**Ohtsaka et al., 2001**). In addition, experimental studies have shown that IL-10 deficiency leads to severe atherosclerosis and arterial calcification (**Mallat et al., 1999**).

The results of the present study showed that IL-6 was significantly decreased while IL- 10 was significantly increased in ovariectomized rats with vitamin K supplementation group which is in agreement with **Ohsaki et al. ( 2006)** and **Katsuyama et al. (2005)** who found that low plasma phylloquinone was associated with high circulating concentration of pro-inflammatory markers C-reactive protein (CRP) and interleukin – 6 (IL-6) and low concentration of osteoprotegerin in older men and women. **Shea et al. (2008)** showed that plasma phylloquinone was inversely associated with overall inflammation as well as with individual proinflammatory biomarkers, including IL-6, intercellular adhesion molecule -1, and tumor necrosis factor receptor 2.

Also **Shea et al. (2008)** found that the circulating concentration of cytokines (IL-6, osteoprotegerin and CRP) in old men and women are inversely associated with vitamin K supplementation.

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12/12/2013