Osteopontin Expression in a Rat Model of Cardiovascular Calcification and the Possible Protective Role of Atorvastatin Versus Vitamin K\(_1\), Histological and Immunohistochemical Study

Ghada Galal Hamam and Mohamed Ahmed Abdou Hegazy

Histology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

ghada.hamam@yahoo.com

Abstract: Introduction: Pathological calcification in soft tissues (i.e. ectopic calcification) has severe consequences especially when it occurs in tissues as cardiovascular system. Aim of the work: to study the role osteopontin in cardiovascular calcification and to compare between the possible protective role of atorvastatin and vitamin K\(_1\) in male albino rats. Materials and methods: thirty four young male albino rats were divided into four groups. Group I (control group), group II (calcification group): they concomitantly received subcutaneous injection of vitamin D\(_3\) and oral warfarin daily for three days. Group III received oral atorvastatin daily for ten days beginning four days before induction of calcification as in group II, during induction (three days) and continued for three days after induction. Group IV received vitamin K\(_1\) orally for ten days as in group III. At the end of the experiment, abdominal aorta and heart were collected and processed for histological and immunohistochemical techniques and the results were statistically analyzed. Results: combined administration of vitamin D\(_3\) and warfarin induced histopathological changes in cardiac muscle fibers and aorta. Areas of hemorrhage and mononuclear cellular infiltration were noticed in-between cardiac muscle fibers. Significant increase in mean area percentage of calcium deposition and osteopontin expression were noticed in cardiac muscle of calcification group. An apparent increase in collagen fiber deposition was also seen in-between cardiac muscle fibers. Sections of the abdominal aorta showed areas of disorganized widely separated elastic lamellae. Irregularity of endothelial lining of the aorta, vacuolation and apoptosis of medial smooth muscle fibers were also recorded. A significant increase in mean area percentage of calcium deposition and osteopontin expression were noticed in the aorta. Atorvastatin and vitamin K\(_1\) significantly attenuated calcium deposition and osteopontin expression, but in vitamin K\(_1\) treated group, few smooth muscle cells in the aortic media were still seen vacuolated. Conclusions: either atorvastatin or vitamin K\(_1\) can be used to suppress calcifications in cardiac muscle, while atorvastatin is superior to vitamin K\(_1\) in suppressing calcifications in the aorta.

1. Introduction:

In the past few years, increased incidence of vascular calcification was noticed as a complication of many diseases as chronic kidney diseases [1,2], hyperparathyroidism [3] and diabetes mellitus [2]. Calcification is also a common finding with aging in human arteries. It is associated with several cardiovascular disease states [2,4,5]. Vascular calcification usually leads to gradual stiffening of arteries [4,6], thrombosis, arterial rupture and myocardial infarction [7]. It is also considered an important risk factor for atherosclerosis, stroke, renal disease [6] and all causal mortality [2].

Vascular calcification means deposition of calcium phosphate salts in cardiovascular tissues [8]. There are two types of soft tissue calcifications. Dystrophic calcification occurs after tissue injury in the presence of normal plasma calcium and phosphate concentrations. Metastatic calcification means deposition of calcium within soft tissues due to abnormal calcium and phosphate metabolism. This occurs in chronic renal failure, osteolytic bone tumors, hyperparathyroidism and hypervitaminosis D [3,9].

Vascular calcification, specifically arterial, has been recognized for many years as a common complication of end-stage renal disease. Patients on hemodialysis have an increased incidence of cardiovascular events 10 to 30-times greater than those of the general population [5,10]. These patients complain of calcium overload arising from hemodialysis, drug therapy [11] or high calcium diet [5]. They also usually present with secondary hyperparathyroidism, due to decrease calcitriol synthesis by the kidney [12]. So, they receive vitamin D in their treatment regimen. They also receive warfarin to prevent thrombotic events. It was demonstrated that each of these treatments is associated with an increased risk of arterial calcification [5].

High doses of vitamin D\(_3\) are accompanied by increase plasma calcium and phosphorus concentration to the level that is suitable for formation.
& precipitation of calcium phosphates [13,14]. This causes mineralization of tissues or organs like kidneys, GIT, cardiac muscle and blood vessels. It was reported that the cause of death reported in vitamin D3 toxicity includes cardiac and renal failure [13]. Warfarin is used clinically as an oral anticoagulant. It is implicated in vascular and valvular calcification in experimental animal models, as well as in patients. Rats treated with warfarin developed focal calcification of the elastic lamellae of the aorta and aortic valve. This calcification could be evident on radiographs after five weeks of treatment [15]. Several studies demonstrated that vascular calcification is an active phenomenon [4,6] controlled by serum and matrix proteins, as matrix G1a protein (MGP) [4]. Warfarin inhibits γ-carboxylation of MGP [5,15,16] and so causes extensive calcification of the elastic lamella in the media of the artery [16].

Osteopontin is a calcium-binding glycoprotein that regulates calcification [3]. It accumulates in the extracellular matrix of bone tissue where it binds to calcium and hydroxyapatite. Osteopontin is also found in atherosclerotic arteries and is abundant at sites of ectopic calcification [17-19]. Osteopontin is produced by a variety of cell types, as endothelial and vascular smooth muscle cells. Numerous clinical studies observed an association between osteopontin and extent of vascular calcification and stiffness [19]. It was also reported that osteopontin is important for the development of cardiac fibrosis and remodeling [3].

Statins, 3-hydroxy-3-methyl-glutarate coenzyme A (HMG-CoA) reductase inhibitors are widely prescribed for the treatment of hypercholesterolaemia. Statins also transform atherosclerotic plaque architecture making them less likely to rupture [20].

Vitamin K is an essential cofactor in the γ-carboxylation of glutamate residues in MGP [5,6] which is a potent inhibitor of arterial calcification [4-6,15]. Vitamin K consists of two forms, phylloquinone (vitamin K1) and the menaquinones (vitamin K2) [6]. Humans may obtain vitamin K2 from fermented dietary sources, as curd cheese and natto, or convert high doses of vitamin K1 into K2 in their bodies [6,14].

The animal model used in this experiment mimics arterial medial calcification, which is common in diabetes mellitus, end-stage renal disease, and aging [5]. This work was designed to study the role osteopontin in cardiovascular calcification and also to compare between the effect of atorvastatin and vitamin K1 on experimentally induced calcification in the cardiac muscle and aorta of male albino rats.

2. Materials and methods:

1-Animals and diet

Thirty four young male albino rats of six -seven weeks old [5] were purchased from the Medical Research Center in Ain Shams University. The rats were housed in plastic cages with wire mesh cover under standard experimental conditions. They had free access to water and standard diet. All animal procedures were carried out according to the guidelines of animal care and the ethical committee of faculty of Medicine, Ain Shams University.

2-Drugs and doses

Warfarin was supplied as warfarin sodium tablets (Marevan) from Glaxosmithkline Egypt, El salam City, A.R.E. One mg warfarin was suspended in one ml normal saline and was given orally to rats. The dose was modified from [4] to be 20 mg/kg/day for three days. Modification was done because of the high mortality rate.

Vitamin D3 was given to rats by subcutaneous injection in a dose of 300000 IU/kg/day for three days [5]. It was obtained in the form of Devarol-S ampoules (Memphis pharmaceutical and chemical industries El-Amirya, Cairo, Egypt) containing 200000 IU cholecalciferol.

Atorvastatin was obtained in the form of Ator tablets (Egyptian Int. Pharmaceutical industries. 10th of Ramadan city). Five mg of atorvastatin was suspended in five ml normal saline and was administered orally to rats in a dose of 10mg/kg/day [21].

Vitamin K1 was obtained in the form of Konakion MM ampoules containing 10mg phytonemadione per one ml. Konakion MM was made for F. Hoffmann-La Roche Ltd, Basel, Switzerland by CENEXI SAS, Fontenay-sous-Bois, France. High dose vitamin K1 was given orally to rats by gastric tube in a dose equivalent to 0.2mg/gm/day [22].

3-Induction of calcification

Induction of calcification was done by concomitant administration of warfarin and vitamin D3 for three days. Rats received subcutaneous injection of vitamin D3 300000 IU/kg/day for three days (at 0, 24 and 48 hours). Each animal also received oral warfarin daily for three days. Seventy two hours after the third injection rats from all groups were killed [5].

4-Animal grouping

After one week acclimatization period, animals were randomly divided into four groups:

Group 1 (Control group): consisted of ten rats that were further subdivided into two subgroups five animals each.

Subgroup 1a: concomitantly received subcutaneous injection of 0.2ml normal saline daily and one ml normal saline orally for three days, then they were sacrificed after further three days.

Subgroup 1b: received one ml oral saline daily by gastric tube for ten days. On day five they received subcutaneous injection of 0.2 ml saline daily for three days. Then they were sacrificed after further three days.
**Group II: (calcification group):** consisted of eight rats that received oral warfarin and subcutaneous injection of vitamin D$_3$ for three days. Then they were left without treatment for further three days then they were sacrificed.

**Group III: (Atorvastatin group):** consisted of eight rats that received intragastric atorvasatin daily for ten days, beginning four days before induction of calcification as in group II, continued during induction (three days) and for three days after induction of calcification.

**Group IV: (Vitamin K$_1$ group):** consisted of eight rats that received vitamin K$_1$ daily by gastric tube for ten days, beginning four days before induction of calcification as in group II, continued during induction (three days) and for three days after induction of calcification.

5-Histological study

At the end of the experiment, all rats were sacrificed under ether inhalation anesthesia. Laparotomy was done. The entire abdominal aorta, from above the renal branch to just above the femoral bifurcation was taken. Heart was also taken to examine the left ventricle. Specimens were fixed immediately in 10% formal saline solution and were processed to obtain paraffin sections of five µm thickness. Sections were then subjected to H&E stain [23] and Mallory’s triple stain [24]. Von Kossa stain was also done for detection of calcifications [25]; Areas with calcification appeared brown-black in color.

**Immunohistochemical stain:** sections of cardiac muscle and aorta were stained with anti-osteopontin antibodies (Lab vision/Neomarkers. Westinghouse Dr. Fremont, California). Osteopontin is also called secreted phosphoprotein 1 (SPP1). Positive reaction appeared as brown cytoplasmic reaction. Negative control was done by omitting the step of primary antibody. Positive control was done by staining a section of the cancellous bone.

6-Morphometric and statistical study

An image analyzer Leica Q win V.3 program installed on a computer in the Histology Department, Faculty of Medicine, Ain Shams University, was used. The computer was connected to a Leica DM2500 microscope (Wetzlar, Germany). Five different non overlapping fields from five different sections of different rats were examined in each group at objective lens X 20 for measuring each of the following:

- The mean area percentage of black-stained calcified areas in the aorta and cardiac muscle [20].
- The mean area percentage of positive immunoreactivity to osteopontin in the aorta and cardiac muscle.

All values were presented as mean ± standard deviation (SD). All data were collected, revised, and subjected to statistical analysis using one-way analysis of variance performed using SPSS.21 program (IBM Inc., Chicago, Illinois, USA). The calculations were considered significant if $P<0.05$.

3.Results:

**Histological results:**

**Cardiac muscle:**

Examination of H&E stained sections of the control group showed cardiac muscle fibers running in different directions. These fibers appeared as branching and anatomizing cylinders of uniform diameters. They were separated by scanty connective tissue. Cardiac muscle cells contained acidophilic sarcoplasm and central, oval, vesicular nuclei (Fig.1). In calcification group, areas of hemorrhage and mononuclear cellular infiltration were seen in-between cardiac muscle fibers (Figure:2). Cardiac muscle fibers in atorvastatin (Figure: 3) and vitamin K$_1$ (Figure: 4) treated groups was almost as that seen in the group I.

Examination of Von Kossa’s stained sections of cardiac muscle of the control group showed no brown calcium deposits (Figure: 5a). Brown calcium deposits were seen in the cardiac muscle fibers in calcification group (figure: 5b). No calcium deposits were seen in cardiac muscle fibers in atorvastatin (Figure: 5c) and vitamin K$_1$ (Figure: 5d) treated groups.

**Immunohistochemical stain for osteopontin** revealed negative reaction in cardiac muscle fibers of the control group. Expression of osteopontin was noticed in the extracellular matrix in-between cardiac myocytes (Figure: 6a). Positive reaction was seen in cardiac muscle fibers and the endothelial cells of blood vessels in calcification group (Figure: 6b). Negative reaction was noticed in cardiac myocytes in both atorvastatin (Figure: 6c) and vitamin K$_1$ (Figure: 6d) treated groups, while expression of osteopontin was noticed in the endomesium between cardiac myocytes.

Examination of Mallory’s triple stain sections of the control group showed minimal amounts of collagen fibers in-between cardiac muscle fibers and around blood vessels (Figure: 7a). In calcification group, increased deposition of collagen fibers was seen in-between cardiac muscle fibers (Figure: 7b). Minimal amounts of collagen fibers were seen in atorvastatin (Figure: 7c) and vitamin K$_1$ (Figure: 7d) treated groups between cardiac myocytes and around blood vessels.

**Aorta:**

Examination of H&E stained sections of the abdominal aorta of the control group showed the wall formed of three layers, tunica intima, media and adventitia. Tunica intima was formed of lining...
endothelial cells resting on a thin layer of connective tissue. The tunica media appeared to contain smooth muscle cells with rod shaped nuclei and numerous elastic membranes which appeared as acidophilic, wavy refractile lamellae. The tunica adventitia, the outermost part of the aorta, was formed of loose connective tissue (Figure: 8) and vasavasorum. In calcification group, irregularity in endothelial lining of tunica intima was noticed. The elastic lamellae of tunica media were seen disorganized and widely separated. Disorganized proliferation of vascular smooth muscle cells was also noticed between the elastic lamellae. Vacuolated smooth muscle cells with deeply stained pyknotic nuclei were frequently detected (Figure: 9). In atorvastatin treated group, the structure of abdominal aorta was seen nearly as those in rats of the control group (Figure:10) while, in vitamin K\textsubscript{1} treated rats, few vacuolated smooth muscle cells in the tunica media was seen (Figure: 11).

Examination of Von Kossa’s stained sections of the abdominal aorta in the control group showed no brown areas of calcification (Figure: 12a). Brown calcified materials were seen deposited between the elastic lamellae in calcification group (figure: 12b). No brown areas of calcification were detected in atorvastatin (Figure: 12c) and vitamin K\textsubscript{1} (Figure: 12d) treated groups.

**Immunohistochemical stain for osteopontin** in sections of the abdominal aorta showed negative reaction in the control group (Figure: 13a). In calcification group, positive reaction was seen in the smooth muscle cells of the media and in the endothelium of vasa-vasorum (Figure: 13b). Negative reaction was seen in the aorta of both atorvastatin (Figure: 13c) and vitamin K\textsubscript{1} treated group (Figure: 13d).

**Histomorphometric results:**

In the current study, a significant ($P<0.05$) increase in the mean area percentage of calcium deposition in aorta and cardiac muscle was noticed in calcification group (Group II) compared to control group. Atorvastatin (Group III) and vitamin K\textsubscript{1} (Group IV) treated groups showed a significant decrease compared to calcification group (table 1, histogram 1).

The mean area percentage of osteopontin expression showed a significant increase in calcification group (Group II) compared to control group, while atorvastatin (Group III) and vitamin K\textsubscript{1} (Group IV) treated groups showed a significant decrease compared to calcification group (table 1, histogram 2).

Table 1: showing the mean ± SD of the mean area percentage of calcium deposition and osteopontin expression in the aorta and cardiac muscle in different groups

<table>
<thead>
<tr>
<th></th>
<th>Area % of calcium deposition in aorta</th>
<th>Area % of calcium deposition in cardiac muscle</th>
<th>Area % of osteopontin expression in aorta</th>
<th>Area % of osteopontin expression in cardiac muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (Group I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calciification group (Group II)</td>
<td>12.3±1.5*</td>
<td>15.9±1.9*</td>
<td>13.1±2.1*</td>
<td>17.7±1.5*</td>
</tr>
<tr>
<td>Atorvastatin treated group (Group III)</td>
<td>0.8±0.3●</td>
<td>0.7±0.3●</td>
<td>0.7±0.4●</td>
<td>1.2±0.3●</td>
</tr>
<tr>
<td>Vitamin K\textsubscript{1} treated group (Group IV)</td>
<td>0.9±0.5●</td>
<td>0.6±0.2●</td>
<td>1.2±0.6●</td>
<td>2.0±0.4●</td>
</tr>
</tbody>
</table>

SD= Standard deviation; *Significant increase compared to control group; ●Significant decrease compared to group II

**Figure 1:** showing acidophilic cardiac myocytes running in different direction and containing central oval vesicular nuclei (↑). Group I (H&EX640)

**Figure 2:** showing area of hemorrhage (*) and mononuclear cellular infiltration in-between cardiac muscle fibers. Group II (H&EX640)

**Figure 3:** showing cardiac muscle fibers with acidophilic sarcoplasm and central oval vesicular nuclei (↑). Group III (H&EX640)

**Figure 4:** showing branching and anastomosing cardiac muscle fibers with vesicular nuclei (↑). Notice dilated blood vessel. Group IV (H&EX640)
Figure 5: showing no brown deposits of calcium in the cardiac muscle fibers of the control group (Figure: 5a). Brown calcified tissues are seen in the cardiac muscle of calcification group (figure: 5b). No calcified deposits are seen in cardiac muscle fibers in atorvastatin (Figure: 5c) and vitamin K\textsubscript{1} (Figure: 5d) treated groups. Von Kossa's stain X 640

Figure 6: showing negative reaction to osteopontin in cardiac myocytes of the control group. Expression of osteopontin (↑) is seen in the extracellular matrix and in the endomesium between the cardiac myocytes (Figure: 6a). Positive reaction is seen in the cytoplasm of some cardiac myocytes and the endothelial cells (↑) of blood vessels of group II (Figure: 6b). Osteopontin expression is seen in the endomesium while negative reaction is seen in cardiac muscle fibers of atorvastatin treated group (Figure: 6c) and in vitamin K\textsubscript{1} treated rats (Figure: 6d). Anti Osteopontin antibody X 640
Figure 7: showing minimal amounts of collagen fibers in-between cardiac muscle fibers and around the blood vessels (↑) in the control group (figure: 7a). In calcification group, increase collagen fiber deposition (↑↑) is seen in between cardiac myocytes (Figure: 7b). Minimal amounts of collagen fibers are seen between cardiac myocytes and around blood vessels in atorvastatin (Figure: 7c) and vitamin K<sub>1</sub> (Figure: 7d) treated groups.

**Mallory's trichrome stain X 640**

Figure 8: showing the wall of the abdominal aorta formed of tunica intima (I), tunica media (M) and tunica adventitia (A). Tunica intima appears with smooth surface and is lined with flattened endothelial cells (↑). Tunica media contains smooth muscle cells with rod shaped nuclei (▲) and numerous acidophilic, wavy elastic lamellae (↑↑). The tunica adventitia consists mainly of loose connective tissue. **Group I (H&EX640)**

Figure 9: showing irregularity of endothelial lining of tunica intima (↑). Elastic lamellae appear separated by disorganized proliferation of smooth muscle fibers (↑↑). Vacuolated smooth muscle cells are seen with pyknotic nuclei (▲). **Group II (H&EX640)**

Figure 10: showing the abdominal aorta formed of tunica intima (I), media (M) and adventitia (A). **Group III (H&EX640)**

Figure 11: showing vacuolated smooth muscles (↑↑) in the tunica media. **Group IV (H&E X640)**
Figure 12: showing no brown areas of calcification in the aorta of control group (Figure: 12a). Brown calcified deposits (↑) are seen between the elastic lamellae in calcification group (figure 12b). No brown areas of calcification are seen in atorvastatin (Figure: 12c) and vitamin K$_1$ (Figure: 12d) treated groups.  

\textbf{Von Kossa's stain X 640}

Figure 13: Showing negative reaction for osteopontin in the aorta of control group (Figure: 13a). In calcification group, positive reaction to osteopontin is seen in the smooth muscle of the media between the elastic lamellae (↑). In the adventitia, osteopontin is expressed in the endothelium (▲) of vasavasorum (Figure: 13b). Negative reaction is seen in atorvastatin (Figure: 13c) and vitamin K$_1$ (Figure: 13d) treated groups.  

\textbf{Anti Osteopontin antibody X 640}

4. Discussion:

Vascular calcification is a common complication in patients with chronic kidney diseases [12,14]. Treatment of these patients was associated with an increased incidence of vascular calcification as a side effect [12]. It was reported that calcification in soft tissues can cause structural damage of these tissues and decrease their functional capacity [13].

The aim of the present study was to study the role osteopontin in cardiovascular calcification and to compare between the effect of atorvastatin and vitamin K$_1$ on experimentally induced calcification in the cardiac muscle and aorta of young male albino rats.

Induction of calcification in the current study was done by combined administration of vitamin D$_3$ and warfarin in young rats. It was reported that
vascular calcification induced by warfarin is accelerated by growth and by vitamin D [5]. High doses of warfarin were administrated to prevent the carboxylation of MGP [4] and high doses of vitamin D accentuated arterial calcification [16] as it increases serum calcium level [5]. It was also reported that young rapidly growing rats -20 days or 42 days- are more sensitive to the effect of warfarin on arterial calcification and ten month-old rats are completely resistant [5,16]. This was the cause of choosing young rats in this experiment.

In the current study, cardiac muscle fibers of group II (calcification group) revealed areas of hemorrhage and inflammatory cells in between cardiac myocytes. Significant calcium deposition and significant increase in the area percentage of osteopontin expression were also noticed in calcification group compared to control group. It was reported that osteopontin is a matricellular protein. It is an inflammatory mediator secreted by inflammatory cells especially macrophages in necrotic and ischemic areas [17]. Moreover, Matsui, et al., 2004 recorded increased myocardial expression of osteopontin, in patients with heart failure [26]. In the current study, increased deposition of collagen fibers was also noticed between cardiac muscle fibers of calcification group. It was reported that osteopontin prompted cardiac fibrosis through over expression of galectin-3 gene. Galectin-3 was secreted by activated macrophage. It exerted paracrine effect on resident cardiac fibroblasts leading to their proliferation and differentiation. Moreover, it was reported that osteopontin was important for the development of cardiac fibrosis and remodeling in angiotensin II induced cardiac hypertrophy [26] and heart failure [17]. It was stated that osteopontin is produced by rat cardiac fibroblasts and can interact with extracellular matrix protein and integrin receptors on cell membrane [26], but there is no evidence of significant osteopontin expression in normal cardiomyocytes [18]. This could explain the presence of positive osteopontin reaction in the endomesium and on the cell membrane of cardiac muscle fibers of the control group.

In the current study, examination of sections of the abdominal aorta in calcification group showed several areas of intimal irregularities. The media showed widely separated elastic lamellae. Vascular smooth muscle fibers of the media showed disorganized proliferation, vacoulation and apoptosis. This was accompanied by calcium deposition and osteopontin expression in the smooth muscles of the media. Rocha et al., 2002 declared that osteopontin stimulated proliferation and migration of smooth muscle fibers from media to intima [18]. Other researchers reported that the process of vascular calcification is mediated by differentiation of resident vascular smooth muscle cells into osteocyte-like cellular elements [14], when they lose the expression of smooth-muscle lineage markers and begin to express osteogenic markers as osteopontin and deposit a mineralized bone-like matrix [27,28,29].

Researches have focused on the prevention or retardation of arterial calcification using lipid-lowering drugs as statins or bisphosphonates [6]. In the current study, the histopathological changes induced by warfarin and vitamin D3 administration, were ameliorated by pretreatment with atorvastatin. These findings coincide with other investigator who reported that simvastatin could inhibit inorganic phosphate induced vascular calcification or calcification induced by inflammatory mediators. Statins could also induce apoptosis of macrophages that are capable of differentiating into osteoclasts [20]. Statins have antioxidant effects [30] and suppress the production of inflammatory molecules, which are involved in the acceleration of atherosclerosis [21,30]. It was reported that atorvastatin reduces the gene expression of osteoblast markers [28] and patients on statin therapy had reduced serum osteopontin levels [28,30]. Statins are the most common lipid lowering drugs worldwide and are recommended for the prevention of cardiovascular diseases [30] and many physicians reported that high-dose statin pretreatment should be used for patients before major cardiac procedures to reduce the risk of myocardial infarction [31].

It was reported that at very high intakes of K1, (200-fold the daily requirement of the liver) both vitamin K1 and K2 had similar effects in preventing calcification during warfarin treatment probably through the conversion of K1 into K2 in the human body [6]. It was reported that vitamin K regulate gene transcription of proteins involved in calcification. It was found to reduce the levels of osteopontin mRNA, while increasing the expression of MGP which is a powerful inhibitor of vascular and other tissue calcification. It was also reported that vitamin K1, K2 decrease arterial calcification on cultured bovine aortic smooth muscle cells treated with inorganic phosphate [14].

In the current study, pretreatment with vitamin K1 attenuated the histopathological changes induced by combined administration of vitamin D3 and warfarin. Few smooth muscle cells in tunica media of the aorta still showed vacoulation. This was explained by investigators who postulated that inhibition of calcification provided by high vitamin K diet, is carried out by vascular smooth muscle cells which phagocytose the calcium deposits. This is consistent with investigators who reported that phagocytosis is a normal property of vascular smooth muscle cells [6].
Experiments showed that vitamin K helps guide calcium towards the areas of the body where it is needed, such as the skeleton, and away from areas where it could have a negative effect, as the cardiovascular system repairing what is called calcium paradox [32].

**Conclusion:**
In the current model of warfarin and Vitamin D$_3$ induced calcification, atorvastatin and vitamin K$_1$ pretreatment ameliorated cardiac muscle calcifications. On the other hand, rats received vitamin K$_1$ still showed few vacuolated smooth muscle cells in tunica media of the abdominal aorta. So, atorvastatin is superior to vitamin K$_1$ in suppressing calcifications in the aorta.

**Recommendations:**
Patients at high risk of developing metastatic calcification should be regularly monitored. These patients could receive prophylactic statins to avoid cardiovascular complications.

**Acknowledgments**
Special thanks are due to Prof. Dr. Amany Elshwarby and Prof. Dr. Sohier Kamal Ahmed for reviewing this paper.

**Conflict of Interest**
The Authors declare that they have no conflict of interests

**References:**


