Experimental Model of Subclinical Vitamin K Deficiency

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Abstract

Introduction: Vitamin K (VK) is a co-factor in the post-translational gamma glutamic carboxylation of Gla-proteins. VK-dependent coagulation factors are carboxylated in the liver by VK1. Osteocalcin and Matrix-Gla protein (MGP) are carboxylated in extrahepatic tissues by VK2. A model of VK deficiency would be suitable for studying extrahepatic Gla-proteins provided that severe bleeding is prevented.

Aim: The aim of this work was to adapt an established protocol of vascular calcification by warfarin-induced inactivation of MGP as a calcification inhibitor, in an attempt to create a broader state of subclinical VK deficiency and to verify its safety.

Materials and methods: Two consecutive experiments, each lasting 4 weeks, were required to modify the dosing schedule of warfarin and VK1 and to adapt it to the Wistar rats used. The original high doses of warfarin used initially had to be halved and the protective dose of VK1 to be doubled, in order to avoid treatment-induced hemorrhagic deaths. The second experiment aimed to confirm the efficacy and safety of the modified doses.

To verify the VK deficiency, blood vessels were examined histologically for calcium deposits and serum osteocalcin levels were measured.

Results: The original dosing schedule induced VK deficiency, manifested by arterial calcifications and dramatic changes in carboxylated and uncarboxylated osteocalcin. The modified dosing regimen caused similar vascular calcification and no bleeding.

Conclusion: The modified protocol of carefully balanced warfarin and VK1 doses is an effective and safe way to induce subclinical VK deficiency that can be implemented to investigate VK-dependent proteins like osteocalcin.

Keywords

osteocalcin, rats, vascular calcification, warfarin

INTRODUCTION

Vitamin K is the family name of fat-soluble vitamins – the naturally occurring vitamin K1 (phylloquinone) and vitamin K2 (menaquinones, Mqs). Vitamin K is a cofactor of the gamma-glutamyl carboxylase, an enzyme required for the carboxylation of glutamic acid (Gla) residues of target proteins, known as vitamin K dependent or Gla-proteins.¹ There are 17 currently known Gla-proteins.² Seven of them are involved in coagulation – the four well known vitamin K-dependent clotting factors and three endogenous anticoagulants (antithrombin III, protein C and protein S). They undergo a reaction of post-translational carboxylation in the liver. The reaction is essential to their activity and they
are indispensable for life. The overt deficiency of vitamin K impairs seriously blood coagulation and causes bleeding. Vitamin K, responsible for the coagulation, is phyloquinone, stored mainly in the liver. It is widely found in plant-based foods (leafy green vegetables); therefore, the diet is the major supply of it.2

Other Gla-proteins, known as extrahepatic vitamin K-dependent factors, are involved in a variety of physiological roles. Among them, osteocalcin and MGP are relatively well studied. Osteocalcin is a bone-derived Gla-protein, but its skeletal function remains controversial.4,6 Matrix Gla-protein (MGP) is a potent inhibitor of vascular calcification.7 Both osteocalcin and MGP undergo post-translational modification by carboxylation of their gamma glutamic moieties – osteocalcin in the bones, and MGP in the vascular smooth muscle cells, with the participation of vitamin K2, which is the major vitamer outside the liver.2,5

Unlike phyloquinone, menaquinones are relatively low in the common Western diet, mostly found in some fermented foods like cheese. The popular belief that colonial bacteria provide the necessary amount of vitamin K2 for the body is currently debatable.2 Nevertheless, it is now known that phyloquinone can be converted endogenously into some menaquinones (particularly MQ-4).5 The extrahepatic functions of vitamin K appear to require higher body stores of vitamin K compared to those needed for activation of coagulation factors due to different sensitivity to the vitamin available. In a condition of a low dietary intake, the vital effects of the vitamin in the liver are preserved, but the carboxylation of extrahepatic Gla-proteins will be impaired.9 As a result, the concentrations of the uncarboxylated forms of the extrahepatic Gla-proteins in plasma will rise.10,11

The active form of MGP capable of preventing vascular calcification is the carboxylated one.5,7,10 In 1998, Price et al. designed an experimental model of vascular calcification, based on drug-induced vitamin K deficiency. In this model, the authors used high doses of warfarin, a vitamin K antagonist, to inhibit the carboxylation of MGP, with supplementation with phyloquinone to prevent bleeding.

Recent experimental studies on mice have implicated osteocalcin in physiological effects outside the bones, namely regulation of glucose homeostasis,5,13,14 behaviour and other functions. Surprisingly, these functions of osteocalcin require the undercarboxylated form of the protein.12

The newly identified physiological role of osteocalcin attracted our attention. In order to study its metabolic and CNS effects in rats we needed a method allowing changes in the physiological levels of the undercarboxylated osteocalcin. To achieve an increase in the undercarboxylated osteocalcin level, we relied on the established above mentioned model of Price et al. (1998) of subclinical vitamin K deficiency.12

**AIM**

Thus the aim of the current study was: 1) to adapt the model of Price et al. in young healthy rats in terms of safety (lack of bleeding) and to verify it by demonstrating vascular calcification in the warfarin treated animals; 2) to prove that the created subclinical vitamin K deficiency will result in the expected changes in the serum levels of both forms of osteocalcin – carboxylated and undercarboxylated.

**MATERIALS AND METHODS**

**Experimental animals**

We performed two consecutive experiments. In every one, we used 20 young healthy male Wistar rats (a total number of 40 rats), allocated into two groups of 10 animals each – a control group (C) and an experimental group (W/K1). In the first experiment, the initial mean body weight of the rats was 231±6 g in group C and 232±9 g in group W/K1. In the second experiment, the initial body weights were 233±7 g in group C and 234±5 g in group W/K1. Both experiments lasted 4 weeks. Animals were kept under standard laboratory conditions (temperature of 20°-25°C, 12-hour light-dark cycle) and had free access to food and drinking water.

**Treatment**

The rats from W/K1 group received the vitamin K antagonist warfarin (Sigma-Aldrich, Germany) dissolved in saline, aiming to induce a state of vitamin K deficiency, and vitamin K1 (Alfa Aesar, Thermo Fisher Scientific, UK) dissolved in sunflower oil, aiming to prevent bleeding. Warfarin was administered by subcutaneous injections, and vitamin K1 was given by orogastral probe. Rats from C group received the solvents – saline and sunflower oil by the corresponding routes of administration. The treatment with vitamin K1 was started 48 hours before the first warfarin injection to ensure the stores necessary for coagulation.

Initially, in the first experiment, we used the drug doses administered by Price et al.12 in their original model, namely 15 mg/kg body weight of vitamin K1, administered in 24 hours, and 300 mg/kg body weight of warfarin daily divided in two doses, administered every 12 hours. We alternated the sites of injection to reduce the trauma. At the end of the first week, however, we had several cases of deadly hemorrhage, which made us increase the dose of vitamin K1 by 50% to 22.5 mg/kg body weight, and reduce the dose of warfarin by half to 150 mg/kg body weight, administered as a single daily dose. We continued the experiment with the described dosing regimen.

To confirm the safety of the doses used, we performed a second experiment with the same treatment groups and duration, but with the modified dosing schedule: 22.5 mg/
kg vitamin K1 orally, and 150 mg/kg warfarin subcutaneously, administered once daily.

After 4 weeks of treatment, the rats from both experiments were sacrificed under light ether anesthesia. Blood samples were collected from sublingual veins of the rats. The blood was centrifuged and serum was stored at -20°C for biochemical measurement of osteocalcin levels.

Portions of aorta, pulmonary veins, renal arteries, and iliac arteries of each animal were dissected out and preserved in 10% neutral formalin for histological evaluation.

**Vascular calcification**

After 24 hour fixation, samples from the arterial blood vessels were cut into appropriate portions and placed in embedding cassettes. The process continued with dehydration in progressively concentrated ethanol baths, followed by a clearing agent (xylene) and finally molten paraffin wax infiltrated the samples. The next steps were to embed tissues into paraffin blocks, cut 5 µm slices and mount them on a glass microscope slide. Finally the sections were stained with hematoxylin and eosin.

**Biochemical measurements**

The serum levels of carboxylated and uncarboxylated osteocalcin were determined after the first experiment. High sensitive sandwich-type enzyme immunoassay (EIA) kits were used (Takara Bio, Inc., Japan), following the producer’s instructions. The capture antibodies recognize the C terminal region of the osteocalcin polypeptide chain and are the same in both EIA kits. The detection antibodies, recognizing the uncarboxylated osteocalcin fraction, bind specifically to glutamic acid residues at positions 21 and 24 in the osteocalcin polypeptide chain.

**Statistical analysis**

The results are presented as a mean ± standard error of the mean (SEM). The groups were compared by Student's two-tailed unpaired t-test. Differences were considered significant at \( p < 0.05 \). The statistical software GraphPad Prism 5 was used (GraphPad Software, Inc., CA, USA).

**Ethical approval**

The experiments were approved by the Commission of Foods Safety of the Bulgarian Ministry of Agriculture and Foods and were conducted in agreement with the National Policies and the Council Directive (2010/63/EU).

**RESULTS**

**Safety**

The modified doses of warfarin and vitamin K1 resulted in no cases of hemorrhagic deaths during the second experiment, neither in nonfatal or minor bleeding, as revealed by the macroscopic postmortem inspection of the organs and tissues.

**Vascular calcification**

Calcium deposits were found in the arteries of rats from the W/K1 groups, but not of the control groups. The vascular calcification of the different arterial blood vessels of each rat was evaluated histologically as present or absent. The results are presented as percentage of rats manifesting signs of vascular calcification (Table 1).

![Figure 1. Vascular calcification.](image)

Calcium deposits in medial layer of aorta (A first experiment, B second experiment), pulmonary veins (C), and renal arteries (D) in rats subjected to a model of subclinical vitamin K deficiency.
Table 1. Presence of vascular calcification in rats subjected to a model of subclinical vitamin K deficiency

|          | Aorta | Pulmonary veins | Renal arteries | Iliac arteries |
|----------|-------|----------------|---------------|---------------|
| Experiment 1 | 66.7% | 50%            | 83.3%         | 16.7%         |
| Experiment 2 | 60%   | 60%            | 60%           | 20%           |

The morphological changes of arterial vessel walls are presented in Fig. 1. Calcium deposits were found in the medial (muscular) layer of the aorta (Fig. 1A, B), pulmonary veins (Fig. 1C), renal arteries (Fig. 1D), and iliac arteries (not shown). The hematoxylin and eosin staining demonstrated calcium deposits as amorphous basophilic structures with extracellular localization. The deposits were situated focally and did not affect the whole circumference of the vessel wall. There were no morphologically detectable differences in the amount of calcium deposits between the first (Fig. 1A) and second (Fig. 1B) experiment.

**DISCUSSION**

Uncarboxylated osteocalcin attracts significant research interest in the last decade. Our previous study showed that its level is lower in rats subjected to diet-induced metabolic syndrome than in intact animals. We found negative correlation between uncarboxylated osteocalcin and fasting blood glucose level, as well as correlations between uncarboxylated osteocalcin and behavioral indices of anxiety and depression, thus supporting the hypothesis of osteocalcin hormonal role.13

In our further effort to evaluate the hormonal function of the uncarboxylated osteocalcin in rats we needed a way to increase its endogenous level. In this respect, the experimental model of subclinical vitamin K deficiency, designed by Price et al.12 to induce vascular calcification, appeared to be a logical and suitable tool for elevating the concentration of the uncarboxylated form of the protein. The model was based on the assumption that it is possible to selectively in-

Osteocalcin levels

The changes in serum osteocalcin levels are presented in Fig. 2. Warfarin treatment increased dramatically the level of uncarboxylated osteocalcin (Fig. 2A). Its serum concentration was 570.3±47.93 ng/ml in W/K1 group, compared to 75.60±4.997 ng/ml in C group (p<0.0001). The level of carboxylated osteocalcin (Fig. 2B) was significantly reduced: 56.28±1.473 ng/ml in the W/K1 group versus 317.4±31 ng/ml in the C group (p<0.0001). The ratio between uncarboxylated and carboxylated osteocalcin (Fig. 2C) was increased in the W/K1 group: 10.10±0.703 versus 0.2438±0.0094 (p<0.0001).

Figure 2. Osteocalcin levels.

Changes in the serum level of uncarboxylated osteocalcin (ucOC) (A), carboxylated osteocalcin (cOC) (B), and their ratio (ucOC/cOC) (C) in control rats (C, n=10) and rats subjected to a model of subclinical vitamin K deficiency (W/K1, n=6); ***p<0.001 vs. C.
scribed arterial calcification that started after two weeks of warfarin treatment and progressively increased with time. This model has been subsequently used in different experimental studies to induce vascular calcification by either the same scientific group\textsuperscript{15,16}, or by others, and deaths have not been reported. Some authors have modified the model – Schurgers et al.\textsuperscript{17} have administered the experimental substances mixed with food, and Bouvet et al.\textsuperscript{18} have dissolved warfarin in the drinking water. Vascular calcification has been successfully induced in these studies, showing that the oral route of administration is as effective for this purpose as the injectable one.

We attempted initially to use the original doses described by Price et al.\textsuperscript{12}, using the oral route of administration for vitamin K\textsubscript{1} instead of the subcutaneous injection, to limit the traumatic injury to the animals and the risk of bleeding. Nevertheless, until the end of the first week of treatment, we recorded four deaths of animals from the W/K\textsubscript{1} group: one on the 4\textsuperscript{th} day and 5\textsuperscript{th} day and two on the 7\textsuperscript{th} day. All the cases were associated with massive hemorrhages in the subcutaneous tissue. In order to prevent further deaths, from day 8 on we increased the dose of vitamin K\textsubscript{1} by 50%, and reduced the dose of warfarin by 50%. The modified dosing regimen was kept till the end of the experiment, and no more lethal or bleeding cases were observed.

Among the papers on vascular calcification, where this or similar models were used, no evidence of injection, to limit the traumatic injury to the animals and the risk of bleeding. Nevertheless, until the end of the first week of treatment, we recorded four deaths of animals from the W/K\textsubscript{1} group: one on the 4\textsuperscript{th} day and 5\textsuperscript{th} day and two on the 7\textsuperscript{th} day. All the cases were associated with massive hemorrhages in the subcutaneous tissue. In order to prevent further deaths, from day 8 on we increased the dose of vitamin K\textsubscript{1} by 50%, and reduced the dose of warfarin by 50%. The modified dosing regimen was kept till the end of the experiment, and no more lethal or bleeding cases were observed.

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The vascular calcification in arterial vessel walls results, at least partly, from impaired carboxylation of MGP.\textsuperscript{7,10,12} The congenital deficiency of MGP results in death in early life associated with massive vascular calcification, followed by aortic rupture.\textsuperscript{19} The carboxylated form of the protein is the active one. Because of the high affinity of Gla-residues to calcium, the carboxylated MGP, containing 5 such residues, is able to bind tightly and selectively to hydroxyapatite crystal nuclei of calcium depositions in the vessel wall, and to prevent their growth and ability to seed daughter crystals.\textsuperscript{7,12} At present, the elevated level of uncarboxylated MGP is used clinically as a biomarker detecting vascular calcification.\textsuperscript{10,20}

In all the studies, utilizing the original model of Price et al. or its modifications, the authors have aimed at induction of vascular calcification.\textsuperscript{12,14,18} In our study, we used the presence of calcium deposits in arterial vessel wall as a means to verify the state of vitamin K deficiency. In the first experiment we found signs of vascular calcification in the experimental group, proving that warfarin treatment was responsible for the observed changes (Fig. 1).

As for the two forms of osteocalcin in the serum, warfarin treatment caused more than 7-fold increase of uncarboxylated osteocalcin level, and more than 5-fold decrease of carboxylated osteocalcin level. These significant changes in both forms of osteocalcin (Fig. 2) demonstrated that the model of subclinical vitamin K deficiency could be successfully used to study the functions of osteocalcin and possibly other extrahepatic Gla-proteins.

The results from the first experiment showed that warfarin treatment induced a state of subclinical vitamin K deficiency. However, we were not convinced in the safety of the model. Therefore, we conducted a second experiment, where we used the dosing regimen that had already demonstrated lack of bleeding. Indeed, the modified doses of warfarin and vitamin K\textsubscript{1} proved to be well tolerated and no hemorrhages were observed in the experimental rats any more.

In order to confirm the ability of the reduced dose of warfarin to induce a state of vitamin K deficiency, we similarly examined the arterial blood vessels of the rats from the second experiment for calcification. Calcium deposits were demonstrated again in the blood vessels and the arteries were similarly affected in both experiments. Thus the modification of the dosing regimen was as effective as the original one in terms of generating a state of subclinical vitamin K deficiency, being safer at the same time. Of note, similar dosing schedules have been used by other authors, e.g. Howe and Webster\textsuperscript{21} have successfully induced vascular calcification with a dose of warfarin of 100 mg/kg body weight daily, while preventing bleeding with 10 mg/kg body weight vitamin K\textsubscript{1}.

CONCLUSION

A safe model of vitamin K deficiency could be successfully induced in young healthy Wistar rats with daily subcutaneous injections of warfarin at a dose of 150 mg/kg body weight. Oral supplementation with vitamin K\textsubscript{1} at a dose of 22.5 mg/kg body weight per day was sufficient to prevent bleeding. The state was verified by demonstrating the presence of vascular calcification associated with reciprocal changes in the serum level of carboxylated and uncarboxylated osteocalcin. The model can be used to study the newly identified physiological roles of osteocalcin.

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Экспериментальная модель субклинической недостаточности витамина К

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Резюме

Введение: Витамин К (ВК) является ко-фактором в посттрансляционном гамма-глутамилкарбоксилировании Gla-белков. BK-зависимые факторы свёртывания карбоксилируются в печени BK1. Остеокальцин и матричный белок Gla (MGP) карбоксилируются во внепечёночных тканях из BK2. Модель недостаточности BK будет подходящей для изучения в непечёночных Gla-белков, если серьёзное кровотечение предотвращено.

Цель: Цель настоящего исследования состояла в том, чтобы адаптировать установленный протокол для кальцификации сосудов от индуцированной варфарином инактивации MGP в качестве ингибитора кальцификации в попытке достичь более общего субклинического дефицита BK и подтвердить его безопасность.

Материалы и методы: Два последовательных эксперимента, каждый продолжительностью 4 недели, были необходимы для изменения режима дозирования варфарина и BK1 и для адаптации к используемым крысам линии Вистар. Первоначально использованные дозы варфарина должны были быть уменьшены вдвое, а защитная доза BK1 должна была быть удвоена, чтобы избежать вызванной лечением геморрагической смерти. Целью второго эксперимента было подтвердить эффективность и безопасность модифицированных доз.

Чтобы проверить дефицит ВК, кровеносные сосуды были исследованы гистологически на наличие отложений кальция и измерены уровни остеокальцина.

Результаты: Начальный режим дозирования вызвал дефицит ВК, что проявлялось в кальцификации артерий и резком изменении уровней карбоксилированного и некарбоксилированного остеокальцина. Модифицированный режим дозирования вызвал аналогичную кальцификацию сосудов и отсутствие кровотечений.

Вывод: Модифицированный протокол тщательно сбалансированных доз варфарина и BK1 является эффективным и безопасным способом вызвать дефицит ВК в субклинических условиях, который может быть реализован для изучения BK-зависимых белков, таких как остеокальцин.

Ключевые слова
остеокальцин, крысы, кальцификация сосудов, варфарин