











Effect of Vitamin K2 on Blood Rheology and Vascular Responses in Diabetic Rats

Serra Öztürk Bostancı¹ , Nur Özen² , Ege Alkan¹ , Özge Güzelad¹ , Hande Salım³ , Ahmet Yıldırım⁴ , Pınar Ülker⁴ , Filiz Basralı⁴ , Deniz Erbaş⁵ , Muzaffer Sindel¹ 

¹ Department of Anatomy, Faculty of Medicine, Akdeniz University, Antalya, Türkiye

² Department of Basic Medical Sciences, Faculty of Dentistry, Antalya Bilim University, Antalya, Türkiye

³ Department of Anatomy, Faculty of Medicine, Hitit University, Çorum, Türkiye

⁴ Department of Physiology, Faculty of Medicine, Akdeniz University, Antalya, Türkiye

⁵ Department of Physiology, Faculty of Medicine, University of Kyrenia, Northern, Cyprus

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Corresponding Author

Muzaffer Sindel

Address: Department of Anatomy,
Faculty of Medicine, Akdeniz University,
Antalya, Türkiye

E-mail(s): sindelm@akdeniz.edu.tr

ABSTRACT

Objective: Diabetes is manifested by endothelial dysfunction and an imbalance between vasoconstriction and vasodilation. The aim of our study is to examine the effect of vitK2 application on vascular and rheological parameters in a rat diabetes model.

Materials and Methods: A total of 60 male Wistar Albino rats were used to examine vascular responses and hemorheological parameters. A total of 6 groups were: control (C), control+vehicle (Cv), control+vitK2 administered (C + K2), diabetes (D), diabetes+vehicle (Dv), and diabetes+vitK2 (D + K2) group. After the animals were sacrificed, blood and vascular samples were taken and the contraction and relaxation responses of the aorta and erythrocyte deformability and aggregation were examined.

Results: When KCl dose-response curves are evaluated; Increased vasoconstriction response was found in the Dv group compared to the Cv group. The increase in vasoconstriction observed in the Dv group decreased with the application of vit K2. D+vitK2 group thoracic aorta contraction responses returned to Cv group levels. In response to increasing cumulative doses of Phe, a significant increase in vasoconstriction response was observed in the Dv group compared to the Cv group. VitK2 application reduced the Phe-mediated contractile response, which was increased in the Dv group, and returned the contraction response to Cv conditions except for two intermediate Phe doses. In the Dv + K2 group, a significant decrease was observed in the aggregation index, which was tended to increase.

Conclusion: Considering the cardiovascular complications frequently observed in diabetes, it can be suggested that vitK2 therapy may yield positive outcomes in diabetes.

Keywords: Diabetes Mellitus, Vitamin K2, Menaquinone-7, Endothelial dysfunction



INTRODUCTION

Type 2 diabetes mellitus (T2DM) occurs with consequences such as microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (cardiovascular diseases) complications, hyperglycemia and insulin resistance [1]. Diabetes mellitus (DM) is characterized by endothelial dysfunction and decreased nitric oxide (NO) bioavailability [2,3]. Endothelial dysfunction is a critical and initiating factor in the formation of diabetic vascular complications.

Endothelium stimulates the relaxation of vascular smooth muscle by secreting vasodilator substances. The important relaxing factors are; NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF). The functional importance of the three substances varies depending on their location. While NO can be effective in large-diameter arteries, EDHF can be a vasodilator in smaller-diameter blood vessels or in some arteries such as coronary arteries when NO release is stopped [4].

The endothelium plays an important role in regulating vascular tone by synthesizing and releasing substances such as prostaglandin I₂ and EDHF [5]. Endothelial dysfunction is an expected finding associated with vascular damage in DM and depends in part on the balance between oxidative stress and NO. Endothelial dysfunction results in decreased NO bioavailability. Changes in NO release are important as they play a major role in the disruption of vascular homeostasis and the development of endothelial dysfunction associated various cardiovascular disorders [6,7].

Vitamin K (vitK) corresponds to 3 separate compounds: vitK1 (phylloquinone), vitK2 (menaquinones) and vitK3 (menadione),

which contain methylated naphthoquinone [8]. It functions in the carboxylation of glutamic acid (Gla) residues of proteins that require vitK for their activation. Known as a cofactor for clotting factors such as prothrombin. It also carboxylate some proteins such as osteocalcin [9]. It is stated that osteocalcin concentrations may affect insulin sensitivity and T2DM by regulating the expression of insulin genes and cell proliferation markers. Osteocalcin has been shown to increase insulin secretion and insulin sensitivity and reduce the severity of T2DM in mice [10]. Uncarboxylated osteocalcin increased cell proliferation, insulin secretion, and insulin sensitivity by stimulating adiponectin expression. However, it has been reported in the literature that vitK supplementation (vitK1 and vitK2), which causes a decrease in uncarboxylated osteocalcin levels and an increase in carboxylated osteocalcin, reduces insulin resistance in patients with high risk of T2DM [11].

VitK2 is generally of microbial origin. Important food sources are cheese, curd and natto (a traditional Japanese dish made from fermented soybeans) [12]. It has functions such as protecting the liver and nerves, preventing cardiovascular calcification, relieving menopausal symptoms, bone homeostasis, cognition and energy production. It is effective in reducing the risk of developing T2DM [13]. Many diseases accompanied by inflammation (inflammatory bowel disease, pancreatitis, chronic kidney disease and osteoporosis) are linked to vitK deficiency. It also contributes to endothelial health by reducing inflammation markers [13]. Low dose vitK2 positively affects endothelium-dependent vasodilation and NO release [14]. VitK2 is stored in most tissues; but relatively high in the brain, kidneys and pancreas [15]. Administration of vitK2 increases insulin sensitivity [16]. Its deficiency is responsible for the “calcium paradox”, characterized by low calcium deposition in bone and its deposition in the vascular wall [17]. Based on this information, we aimed to examine the effect of vitK2 application on vascular response and rheological parameters in our study.

MATERIALS AND METHODS

Total of 60 male Wistar Albino rats were used in the experiments examining vascular responses and hemorheological parameters carried out in the laboratories of Akdeniz University Faculty of Medicine, Department of Anatomy and Physiology. Akdeniz University Animal Experiments Local Ethics Committee approval was received (Application Form ID/Protocol no; 1036/2020.02012). The rats were kept in a room at 23 ± 2°C with a 12-hour daylight - 12-hour dark period and were fed with

Main Points:

- Endothelial dysfunction and decreased nitric oxide bioavailability have been observed in rats with type 2 diabetes.
- Vit K2 treatment has reduced increased vasoconstriction induced by phenylephrine and restored the contractile response to normal levels in diabetic rats.
- Vit K2 has reduced increased erythrocyte deformability and aggregation in diabetic rats.
- Vit K2 may be considered a potential therapeutic option in diabetes management.

rat chow without restriction. The animals were left for a one-week adaptation period before being taken to the experimental procedure, and their blood glucose levels were measured during this period to ensure that they had blood glucose values within normal limits. Weekly water and feed consumption and weight measurements of all rats were made until they were sacrificed (Table 1 and Table 2).

Control group, (C): Rats in this group were used to determine their normal morphological and physiological characteristics.

Control+Vehicle, (Cv): Rats in this group received an intraperitoneal injection of citrate buffer solution (0.1M, pH 4.5). Since vitK2 is soluble in corn-oil, citrate-buffered corn oil (0.2 ml per 100 g body) was applied via gavage.

Control+VitK2, (C+K2): VitK2 in corn-oil was given at a dose of 35 mg/kg via gavage, 5 days a week, once a day for 8 weeks.

Diabetes group, (D): A single dose of streptozotocin injection (50 mg/kg, dissolved in 10 mmol/L citrate buffer, pH 4.5) was administered intraperitoneally.

Diabetes+Vehicle, (Dv): A single dose of streptozotocin injection (50 mg / kg, dissolved in 10 mmol / L citrate buffer, pH 4.5) was administered intraperitoneally. Since vitK2 is soluble in corn-oil, citrate-buffered corn-oil (0.2 ml per 100 g body) was administered via gavage.

Diabetes+Vit K2, (D+K2): One week after a single dose of intraperitoneal streptozotocin injection, Vit K2 in corn oil was administered at a dose of 35 mg/kg via gavage, 5 days a week, once a day for 8 weeks.

Table 1. Average and standard deviation values of daily feed (g) and water (ml) consumed by the groups. C: control, Cv: control+vehicle, C+K2: control+vit K2, D: diabetes, Dv: diabetes+vehicle, D+K2: diabetes+vit K2.

		Daily feed (g)		Daily water (ml)	
		Mean	Std. deviation	Mean	Std. deviation
Control groups	C	65,59	13,88	58,59	7,74
	Cv	69,06	18,58	53,59	6,64
	C+K2	66,04	14,50	58,44	14,45
Diyabetes groups	D	77,14	22,37	182,26	79,19
	Dv	78,24	21,32	173,93	43,05
	D+K2	90,61	27,79	168,53	36,28

Table 2. Weekly average weight (g) values of the groups. C: control, Cv: control + vehicle, C + K2: control + vitK2, D: diabetes, Dv: diabetes + vehicle, D + K2: diabetes + vitK2.

		0. day	7. day	14. day	21. day	28. day	35. day	42. day	49. day
		Control groups	C	226,83	245,00	249,33	260,67	266,00	274,33
Cv	243,83		255,17	263,17	271,17	300,00	302,00	303,17	299,33
C+K2	232,50		246,83	256,33	263,80	266,00	275,80	282,60	288,80
Diyabetes groups	D	273,42	251,55	247,18	242,64	242,09	239,18	238,09	234,55
	Dv	251,89	248,14	252,86	257,00	259,71	256,00	257,86	259,86
	D+K2	279,88	248,75	243,88	231,50	229,75	225,63	224,75	224,13

Creating Diabetes

Streptozotocin (STZ, 50 mg/kg) dissolved in citrate buffer (0.1M, pH 4.5) (Sigma-Aldrich Cat#S0130) was administered to the animals in the experimental group by intraperitoneal injection under isoflurane anesthesia. Rats with blood glucose levels of 250 mg dl⁻¹ or higher were considered diabetic [18].

Application of Vitamin K2

VitK2 applications were administered via gavage at a dose of 35 mg/kg (by preparing a solution in 0.2 ml corn oil per 100 g body weight), once a day, 5 days a week for 8 weeks [19].

After measuring the blood glucose levels of the rats, their thoracic aorta tissues were placed in a cold Krebs solution. The tissue was divided into rings of appropriate length (2-5 mm) and placed in an organ bath containing 10 ml of Krebs solution, oxygenated with a gas mixture containing 95% O₂ - 5% CO₂, temperature 37°C and pH = 7.4, to be studied.

Endothelium-dependent and independent relaxation responses and contraction responses of the thoracic aorta suspended in the organ bath were examined.

Potassium Chloride (KCl) Mediated Contractile Response: Contraction responses of the vessels to 20, 40 and 80 mM KCl were obtained.

Phenylephrine (Phe) Mediated Contractile Response: Contractile responses were obtained by administering cumulatively increasing doses of Phe (10⁻⁹ – 3x10⁻⁵ M) to the vessels.

Following contraction with a submaximal dose of Phenylephrine (Phe), cumulative doses of ACh (10⁻⁹ – 3x10⁻⁵ M) were administered to the vessels and ACh-mediated relaxation responses were obtained.

Following contraction with a submaximal dose of Phenylephrine (Phe), cumulative doses of SNP were administered to the vessels (10⁻¹⁰ – 3x10⁻⁴ M), and SNP-mediated relaxation responses were obtained.

Erythrocyte deformability was assessed by laser diffraction analysis at various fluid shear forces using an ektacytometer (LORRCA, RR Mechatronics). Erythrocyte deformability was calculated as elongation index (EI) by the system. EI values were measured between nine shear forces (0.30 – 75.02 Pa), and using

these values, the shear force (SS1/2) that caused a deformation of half of the maximum EI was evaluated for each sample. Erythrocyte aggregation was measured in autologous plasma at 37°C using a photometric aggregometer.

Statistical Analysis

Statistical evaluations were made and the results are shown as ± mean standard deviation. The significance level was accepted as 0.05. Parametric two-way and one-way analyzes of variance (ANOVA) were used for data conforming to normality distribution, and Post-Hoc Bonferroni and Tukey tests were used for pairwise comparisons. Non-parametric Kruskal-Wallis Variance Analysis was used for data that did not comply with normality distribution and Dunn Test was used for pairwise comparisons.

RESULTS

In the vehicle study applied to control (C) and diabetes (D) groups; it was shown that a single injection of citrate buffer solution (0.1M, pH 4.5) into the groups followed by corn-oil (0.2 ml per 100 g body) application for 8 weeks affected conduction type vascular functions (thoracic aorta contraction and relaxation responses). Due to the effects of STZ and Vit K2 solvents, subsequent procedures were continued with Control-vehicle (Cv) and Diabetes-vehicle (Dv) groups instead of C and D groups.

When KCl dose-response curves at 20, 40 and 80 mM concentrations are compared; While vehicle application to group C caused a decrease in the contraction response to KCl (p<0.05 and p<0.01; Figure 1a), vehicle application to group D caused an increase in the contraction response to the highest dose of KCl (p<0.01; Figure 1b).

When comparing Phe dose-response curves with increasing doses; Vehicle application to group C caused a decrease in the contraction response to high doses of Phe (p<0.05; Figure 1c). Application of vehicle to group D caused an increase in the contractile response to all Phe doses except the lowest doses (p<0.001; Figure 1d).

When comparing ACh dose-response curves with increasing doses; No difference in relaxation response was observed after vehicle application in group C. (p>0,05; Figure 2a). Application of vehicle to group D resulted in decreased relaxation responses to all ACh doses except the highest doses. (p<0,05 and p<0,001; Figure 2b).

When the dose-response curves obtained in response to increasing cumulative doses of ACh are examined; A statistically significant decrease in the vasodilation response, thus an impairment in the endothelium-mediated relaxation response, was observed in the Dv group compared to the Cv group ($p < 0,001$). VitK2 application increased the diminished vasodilation response in Dv group vessels ($p < 0,01$ and $p < 0,001$) and returned to the responses observed in the Cv group ($p > 0,05$). Administration of vitK2 to the Cv group resulted in a decrease in relaxation responses compared to the Cv group ($p < 0,05$, $p < 0,01$ and $p < 0,001$) (Figure 4a).

When the dose-response curves in response to increasing cumulative doses of SNP are compared; it was observed that there was a decrease in the relaxation response of the Dv group compared to the Cv group ($p < 0,05$, $p < 0,01$ and $p < 0,001$).

Application of vitK2; it was found that it corrected the decrease in the vasodilation response observed in the Dv group and caused an increase in the relaxation response ($p < 0,001$). The relaxation response observed in the D + K2 group was greater than in the Cv group ($p < 0,01$ and $p < 0,001$). Administration of Vit K2 to the Cv group increased the relaxation response to SNP at intermediate doses ($p < 0,001$) (Figure 4b).

In the diabetes group compared to the control group; an increase in vascular contraction responses and a decrease in relaxation responses were observed. VitK2 partially or completely corrected these changes in contraction and relaxation responses. Considering the cardiovascular complications that frequently occur in diabetes, it can be said that vitK2 treatment will provide positive results.

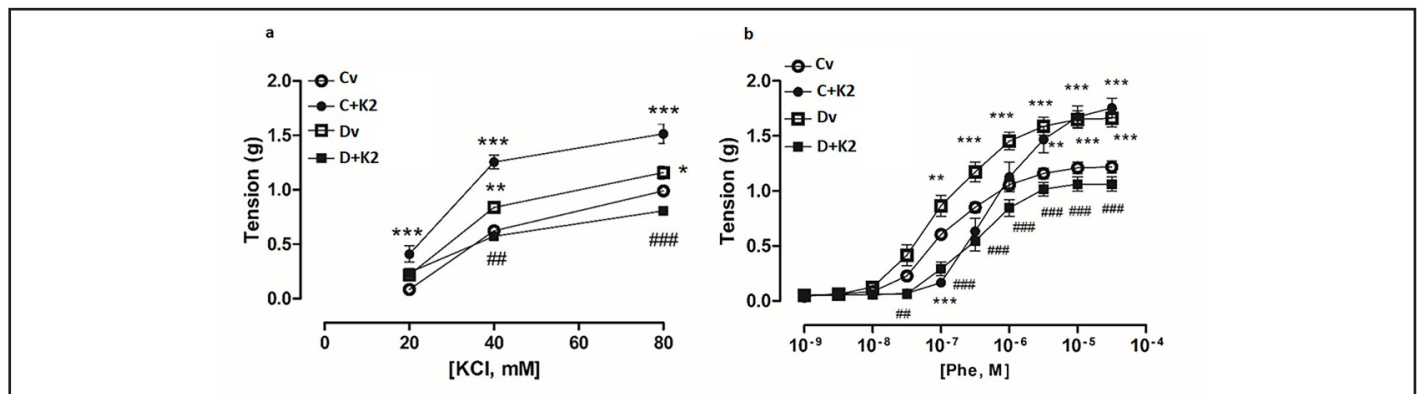


Figure 3. a) KCl-mediated contraction dose-response curve of thoracic aorta in Cv, C + K2, Dv and D + K2 groups. * $p < 0,05$, ** $p < 0,01$ and *** $p < 0,001$, different from Cv; ## $p < 0,01$ and ### $p < 0,001$, different from Dv, b) Phe-mediated contraction dose-response curve of thoracic aorta in Cv, C + K2, Dv and D + K2 groups. * $p < 0,01$ and *** $p < 0,001$, different from Cv; ## $p < 0,01$ and ### $p < 0,001$, different from Dv.

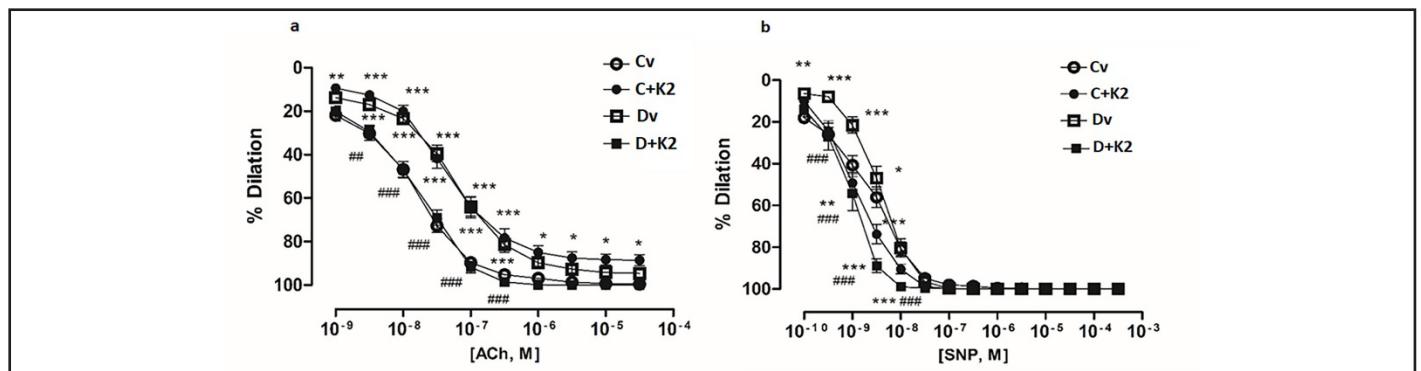


Figure 4. a) ACh-mediated relaxation dose-response curve of thoracic aorta Cv, C + K2, Dv and D + K2 groups. * $p < 0,05$, ** $p < 0,01$ and *** $p < 0,001$, different from Cv; ## $p < 0,01$ and ### $p < 0,001$, different from Dv, b) SNP-mediated relaxation dose-response curve of thoracic aorta in Cv, C+K2, Dv and D + K2 groups. * $p < 0,05$, ** $p < 0,01$ and *** $p < 0,001$, different from Cv; ### $p < 0,001$, different from Dv.

The results obtained by applying vitK2 to the control group suggest that vitK2 may have different effects on vascular responses under physiological conditions. In line with these results, more studies are needed on the effects of vitK2 under physiological conditions.

In the vehicle study applied to groups C and D; it was shown that the application of corn-oil (0.2 ml per 100 g body) for 8 weeks after a single injection of citrate buffer solution (0.1M, pH 4.5) in the groups did not affect the erythrocyte responses. (Figure 5, Figure 6 and Figure 7). However, since vehicle operation affects the thoracic aorta responses; procedures were continued with Cv and Dv groups instead of C and D groups.

Erythrocyte responses were obtained in Cv and Dv groups after gavage administration of Vit K2 in corn oil (0.2 ml per 100 g body) for 8 weeks. Statistical evaluation of the effects of Vit K2 on vascular responses was performed compared to vehicle groups. Evaluation took place between Cv, C + K2, Dv and D + K2 groups.

When erythrocyte deformabilities were compared, no difference was observed in both EImax and SS 1/2 values between the groups. ($p>0,05$; Figure 8).

When the erythrocyte aggregation indexes of the groups are compared; no significant difference was observed in erythrocyte aggregation in the Dv group compared to the Cv group ($p>0.05$), but the aggregation index of erythrocytes of diabetic rats tended to increase. Application of vitK2 to the Dv group caused a significant decrease in the aggregation index, which tended to increase ($p<0,01$; Figure 9).

The fact that no difference was observed in erythrocyte deformabilities between groups may be due to the fact that erythrocytes are in an intact state. Erythrocyte aggregation increased in the diabetes group; This suggests that blood viscosity increases, resulting in increased resistance to blood flow and deterioration in vascular perfusion in these groups. Application of Vit K2 to the diabetes group had a positive effect.

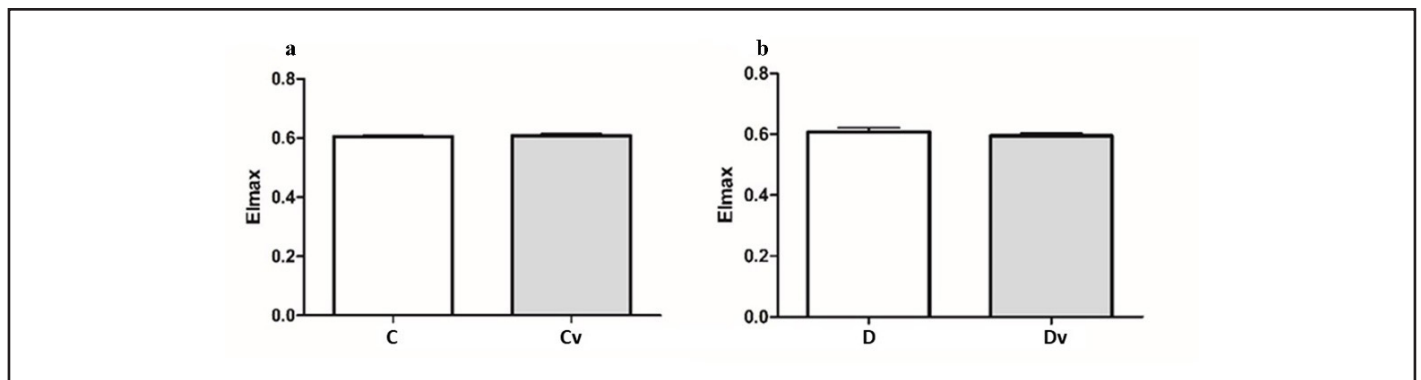


Figure 5. Erythrocyte deformability (EImax) changes a) C and Cv groups, b) D and Dv groups.

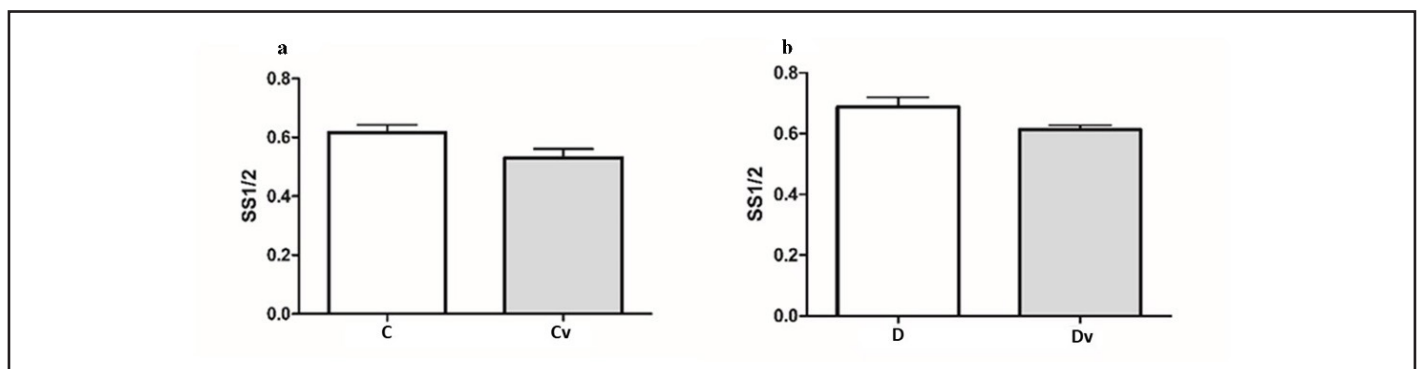


Figure 6. Erythrocyte deformability (SS1/2) changes a) C and Cv groups b) D and Dv groups.

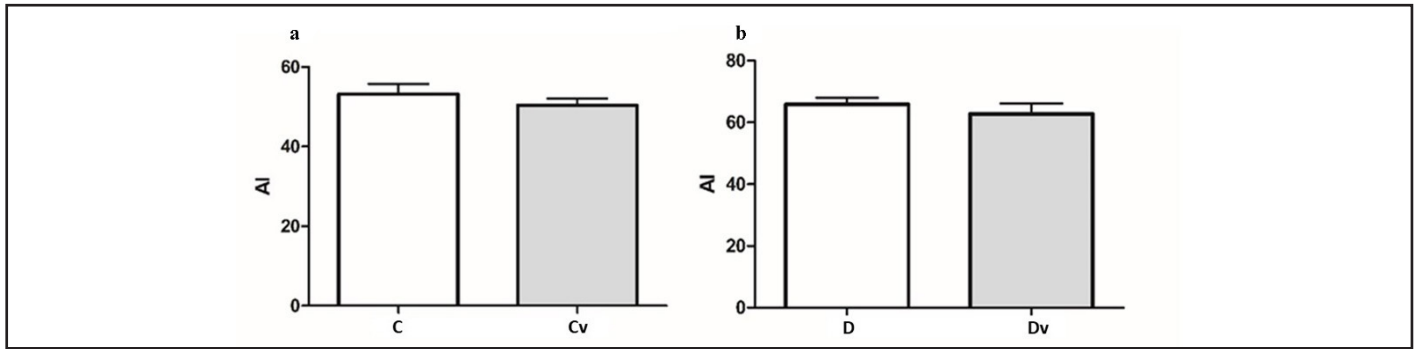


Figure 7. Erythrocyte aggregation changes a) C and Cv groups, b) D and Dv groups.

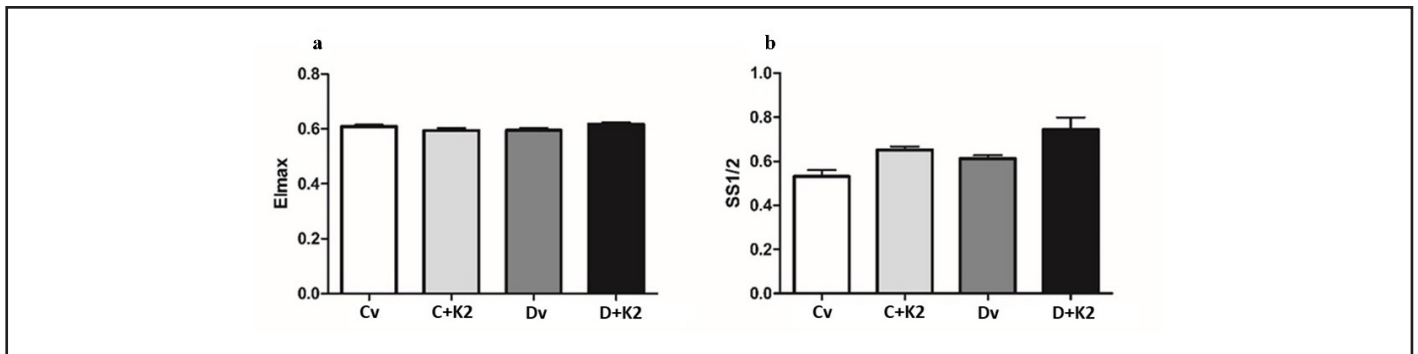


Figure 8. Erythrocyte deformability changes in Cv, C + K2, Dv and D + K2 groups a) EImax, b) SS1/2.

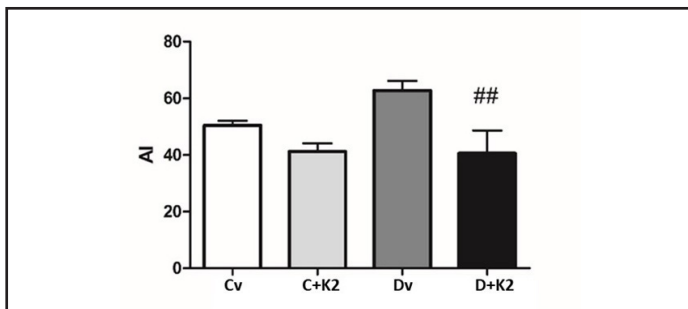


Figure 9. Changes in erythrocyte aggregation in groups Cv, C + K2, Dv and D + K2. ##p<;0,01, different from Dv.

DISCUSSION

The aim of the study was to examine the endothelium-dependent and independent relaxation responses of vitK2 on the aorta throacica, in T2DM rats. In addition, contraction responses were also examined in our study.

The literature, there are studies investigating the effects of vitK2 on intervertebral discs and fibrochondrocytes in diabetic mice, as well as its impact on glycemic status [20,21]. To the best of

our knowledge, our study is the first to examine the effect of vitK2 dissolved in corn oil on the vascular endothelium in a rat diabetes model.

Gaertner et al. investigated the effect of corn oil on vascular endothelial responses, they did not find any change in the relaxation response that develops due to low concentrations of ACh in the arteria femoralis. They saw a reduction in SNP-mediated relaxation response at similar doses [22]. In our study, consistent with this study, no change was observed in the vascular relaxation response to increasing doses of ACh in the Cv group given corn oil, while a decrease in vascular relaxation responses was observed in the Dv group, except for the highest doses. Similarly, in our study, the SNP-mediated relaxation response at low doses decreased in both the Cv and Dv groups. These results suggest that the decrease is associated with endothelial dysfunction.

In a placebo-controlled study in individuals with T2DM and cardiovascular disease, the amount of calcification was measured with 18F-NaF PET and CT scans and it was evaluated whether

Vit K2 reduced calcification. In the study, they reported that vascular calcification is an active process and these techniques do not measure it, that the ^{18}F -NaF PET method is a promising technique to detect early changes before calcifications become visible on CT, and that their study also supports that vitK2 supplementation inhibits vascular calcification [23]. Meer et al. in their study, investigated the effect of vitK2 on tendency time (T50) in T2DM individuals, based on the hypothesis that serum calcification T50 may be a new marker of arterial calcification tendency [24]. They reported that further research is needed on the effect of vitK2 supplementation on arterial calcification and the validity of T50 as a marker of arterial calcification [24]. In our study, as expected, an increase in KCL-mediated contractile response was observed in rats in the diabetes group compared to rats in the control group. We found that in vitK2 injected group to diabetic rats, the KCL-mediated endothelial contraction response decreased and returned to the control group level. This showed the positive effect of vitK2 application on vascular endothelial function. An increase in vascular endothelial response was observed in the control group administered vitK2 compared to the control group. It was observed that the vitK2 dose we applied to the diabetes group caused an increase in the vascular endothelial relaxation response. In the ACh-mediated relaxation response, a decrease in the vasodilatation response was observed in the diabetic group compared to the control group. This showed us that there was dysfunction in the endothelium-mediated relaxation response. The decreased vasodilatation response increased in the vitK2 administered group and returned to control levels.

In a study of 16,057 women, Gast et al. showed that individuals with high vitK2 intake had a lower risk of heart disease. They stated that every 10 micrograms of vitK2 consumed per day reduced the risk of heart disease by 9% [25]. A study was conducted to investigate the effects of vitK on calcification and TLR2 (Toll-like receptor 2) and TLR4 (Toll-like receptor 4) expression in aortic tissues and smooth muscle cells in ApoE^{-/-} mice.

They showed that vitK2 could reduce the expression of TLR2 and TLR4 in the aorta and smooth muscle cell, and inhibited high-fat diet-induced intimal calcification and sodium glycerophosphate-induced smooth muscle cell calcification in ApoE^{-/-} mice [26].

When the erythrocyte deformabilities of the groups were compared in our study, no difference was observed in both

EImax and SS1/2 values between the groups. When erythrocyte aggregation indexes were compared, although no significant difference was observed in erythrocyte aggregation in the Dv group compared to the Cv group, a tendency to increase in the aggregation index of erythrocytes of diabetic rats was observed. Administration of vitK2 to the Dv group caused a significant decrease in the aggregation index, which tended to increase.

CONCLUSION

As a result, in our study, in the diabetes group compared to the control group; An increase in conduction-type vascular contraction responses and a decrease in relaxation responses were observed. VitK2 application partially or completely corrected these changes in contraction and relaxation responses. Considering the cardiovascular complications that frequently occur in diabetes, it can be said that vitK2 treatment can produce positive results in diabetes. The fact that no difference was observed in erythrocyte deformabilities suggests that this may be due to the fact that erythrocytes are in an intact state. Erythrocyte aggregation increased in the diabetes group; This suggests that blood viscosity increases, increasing resistance to blood flow and causing deterioration in vascular perfusion in these groups. Application of vitK2 to the diabetes group had a positive effect. It was observed that vitK2 administered to the subjects in the control group had a negative effect on vascular responses. The negative effect of this dose, which is curative in diabetes, on the control group showed that dose studies are needed.

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Conflict of Interest Statement

Authors have no conflicts of interest to declare.

Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Declaration Statement

Akdeniz University Animal Experiments Local Ethics Committee approval was received (Application Form ID/Protocol no; 1036/2020.02012). All authors should also disclose any conflict of interest that may have influenced either the conduct or the presentation of the research.

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