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# The effect of vitamin K1 on VEGF levels in chick embryos with type 1 diabetes and Diabetic Retinopathy induced by streptozotocin

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## ABSTRACT

**Objective:** Hyperglycemia caused by Diabetes Mellitus (DM) is associated with long-term dysfunction such as diabetic retinopathy (DRP). The most effective growth factor in the development of DRP is the vascular endothelial growth factor (VEGF). Vitamin K1 reduces hyperglycemia and prevents the development of DM. In this study, we aimed to create streptozotocin (STZ) induced DM and DRP in chick embryos and to show whether vitamin K1 can prevent early-stage DRP by measuring VEGF levels.

**Material and Methods:** The 140 specific pathogen-free (SPF) fertilized chicken eggs were used in this study. Three different STZ doses were administered to 120 SPF eggs for an induced DM model. Three different vitamin K1 doses were administered in each STZ dose group. On the 12th day and 18th day the remaining 20 SPF eggs were separated as control groups. On the 18th-day, blood glucose, blood insulin and VEGF levels were measured.

**Results:** 0.45 mg/egg STZ dose (STZ3) was determined as the optimal/ideal dose for the DM model. When the group-administered STZ3 and vitamin K1 were evaluated among themselves; it was determined that there were significant changes in blood glucose, blood insulin, VEGF levels of the STZ3+K1-3 group compared to the STZ3+K1-1 and STZ3+K1-2 groups ($p<0.05$).

**Conclusion:** Vitamin K1 increased blood insulin levels and decreased blood glucose levels. When hyperglycemia reduced, the VEGF levels reduced. Vitamin K1 may protect from DRP by reducing VEGF levels.

**Keywords:** Chick Embryo, Type 1 Diabetes Mellitus, Vascular Endothelial Growth Factor, Vitamin K1

## INTRODUCTION

Diabetic retinopathy (DRP) is the most common complication of diabetes mellitus (DM) (1). The two main changes seen in DRP due to glucose metabolism disorder are increased vascular permeability and micro vascular occlusion. Blocking blood flow due to capillary occlusion causes retinal hypoxia and ischemia. Ischemia in the retina causes abnormal new vessel formation (neovascularization). The new vessel formation seen in DRP is driven by growth factors released from hypoxic retinal tissue (2–5). The primary growth factor responsible for these vascular changes seen in DRP is the vascular endothelial growth factor (VEGF) (6). Hypoxia is the major stimulus for VEGF expression (7). Because of retinal hypoxia and ischemia, VEGF levels increase in retinal and ocular fluids in patients with DRP (8). VEGF increases vascular permeability against macromolecules, increases monocyte chemotaxis and tissue factor production, causing diabetic micro vascular complications and DRP (9). Vitamin K is a vitamin that has two biologically active forms, phylloquinone (vitamin K1) and menaquinone (vitamin K2) (10). In previous studies, it has been reported that vitamin K1 reduces hyperglycemia and insulin resistance, has a hypoglycemic effect and prevents the development of type 2 DM (11–14).

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The aim of this study was to create streptozotocin (STZ)-induced early onset DM and DRP in chick embryos and to show whether vitamin K1 can prevent early-stage DRP by measuring VEGF levels.

## MATERIALS and METHODS

Ethics committee approval was obtained from Afyon Kocatepe University Animal Experiments Local Ethics Committee with the decision dated 07.10.2019 and numbered 49533702/122.. The experimental phase and morphological analysis of the study were carried out in Afyonkarahisar Health Sciences University, Faculty of Medicine, Department of Anatomy.

A total of 140 specific pathogen free (SPF) fertilized eggs were used in this study. After all the eggs were provided by the research coordinator, they were placed in the incubator in Anatomy laboratory. SPF eggs were kept in the incubators at 70% humidity and 37.5 $^{0}$C. STZ (N-(Methyl Nitroso Carbamoil)-a-D-glucosamine, CAS Number: 18883-66-4, Sigma-Aldrich Chemie GmbH, Germany) was dissolved in saline and a stock STZ solution was prepared. STZ doses given to chick embryos were determined according to the literature (**15–18**). There were 14 groups in total, with 10 SPF fertilized eggs in each group. The first group was the control group, whose blood glucose and blood insulin levels were measured on the 12th day. There were three main groups in which only STZ was applied on the 12th day (STZ1, STZ2, STZ3). It was desired to determine the most appropriate dose of STZ by applying three different doses of STZ. STZ1 dose was 0.15 mg/egg, STZ2 dose was 0.30 mg/egg, STZ3 dose was 0.45 mg/egg.

Nine more groups were formed to administer three different doses of vitamin K1(Konakion 10 mg/ml, Roche) to each group, to which STZ1, STZ2, and STZ3 doses were to be administered. Vitamin K1-1 dose was 0.005 mg/egg (0.1 mg/kg), Vitamin K1-2 dose was 0.025 mg/egg (0.5 mg/kg), Vitamin K1-3 dose was 0.050 mg/egg (1 mg/kg). One more control group was formed to measure blood glucose and blood insulin levels on the 18th day.

On the 12th day, the control group SPF eggs were sterilized by rubbing 70% ethyl alcohol on the egg shell and opened from the upper part of the air sac. The inner shell membrane was carefully removed and at least 200 µl of chick embryo blood was taken from the thickest vessel under it with 30GX13 mm diameter mesotherapy needle attached to the tip of the insulin injector. Insulin levels were measured by means of the chick embryo insulin kit (INS ELISA KIT, BT-LAB/EA0012Ch) from these blood samples. Then, another thick vessel was found under the inner shell membrane and a blood glucose strip used for glucose measurement was placed under the vessel in a way that would not damage the structures. Afterwards, the vessel was gently lifted with a strip to allow blood circulation in the vessel. The amniotic fluid around the vessel was cleaned with the cotton part of the ear cleaner so as not to damage the vessel. The vessel on the strip was fragmented through the mesotherapy needle tip. From the blood, dispersed on the strip, glucose level from was measured as mg/dl with the Accu-check blood glucose meter.

On the 12th day, the egg shells of the groups to be made Type1 DM with three different STZ doses were sterilized by applying 70% ethyl alcohol. Then, 0.5 mm hole was drilled through the egg shell on the air sac, through which the Hamilton needle could pass, and STZ at a dose of 0.15 mg/egg (STZ1, 40 eggs) was administered to the first group, and 0.30mg/egg STZ (STZ2, 40 eggs) to the second group. STZ was administered to the third group at a dose of 0.45 mg/egg (STZ3, 40 eggs). After STZ injections, the hole opened in the egg shell was closed with tape so that the egg would not get air. Groups (10+10+10=30 eggs) that were administered only STZ1, STZ2, STZ3 doses were put back into the incubator to be opened on the 18th day.

The remaining 30 eggs in each STZ group (STZ1, STZ2, STZ3) were divided into 3 subgroups. In the STZ1, STZ2, STZ3 groups, vitamin K1-1 at a dose of 0.005 mg/egg for the first 10 eggs, vitamin K1-2 at a dose of 0.025 mg/egg for the second 10 eggs, and vitamin K1-3 at a dose of 0.050 mg/egg for the third 10 eggs, administered via insulin injector and it was closed with a tape so that no air could enter. The eggs, which were closed with tape, were placed back in the incubator. On the 18th day, control group, STZ and vitamin K1 applied groups were sterilized by rubbing 70% ethyl alcohol on the egg shell and opened from the upper part of the air sac. With the same blood sampling techniques applied to the control group on the 12th day; blood glucose levels, blood insulin levels and VEGF levels were measured of STZ and vitamin K1 applied groups.

On the 18th day, the eyes of the chick embryos were centrifuged at 1500 rpm for 10 minutes in the Biochemistry department. Chicken vascular endothelial cell Growth Factor, VEGF ELISA Kit (BT-LAB/E0223Ch) was used to measure VEGF levels and studied with ELISA method.

Statistical analysis

Statistical analysis was performed with the IBM Statistical Package for the Social Sciences program. Kolmogorov Smirnov test was used to determine the normal distribution of data. Kruskal-Wallis test was used to compare the groups since the data were not normally distributed and $n \leqslant 30$. Dunn test were employed as post-hoc tests and $p<0.05$ were considered significant. Pearson's correlation analysis was used to determine whether there is a linear relationship between blood glucose and blood insulin measurements, and if so, the direction and severity of this relationship. Mean statistical values were expressed as Mean±Standard Deviation (Mean±SD).

## RESULTS

In this study, the effect of different doses of STZ on the development of DM were examined in the firstly. The most appropriate dose of STZ for the DM model in chick embryos was tried to be determined. Then, three different doses of vitamin K1 were given to chick embryos and their effects on VEGF levels were examined.

Mean blood glucose levels of chick embryos opened on the 12th day were 124.70±12.3 mg/dL and blood insulin levels were 2.05±0.11 mIU/L. Mean blood glucose levels of the control group chick embryos opened on the 18th day were 133.90±9.12 mg/dL, and blood insulin levels were

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5.88±2.01mIU/l. The mean blood glucose and insulin levels of the groups that were administered only STZ and tried to be DM on the 18th day are given in Table 1. According to these results, 0.45 mg/egg STZ dose (STZ3) was determined as the optimal/ideal dose for the DM model in our study.

The mean blood glucose levels, blood insulin levels and VEGF levels of the groups given vitamin K1 to prevent DRP after STZ application are given in Table 2.

When the group administered STZ3 and vitamin K1 were evaluated among themselves; it was determined that there were significant changes in blood glucose, blood insulin, VEGF levels of the STZ3+K1-3 group compared to the STZ3+K1-1 ($p<0.001$, $p<0.001$, $p<0.001$, respectively) and STZ3+K1-2 ($p<0.05$, $p<0.05$, $p<0.05$, respectively) groups. There was no significant difference in blood glucose, blood insulin and VEGF levels between STZ3+K1-1 and STZ3+K1-2 groups ($p=0.061$, $p>0.05$, $p=0.089$, respectively).

## DISCUSSION

In this study, we aimed to determine whether vitamin K1 has a DRP-reducing effect in chick embryos induced with STZ-induced Type 1DM by measuring VEGF levels. In literature, there are few chick embryo DM models induced by STZ in the literature and they used different STZ doses and different incubation days (15–18). In literature, STZ application day changed by 12th day to 14th day. We determined to inject STZ on the 12th day. The purpose of injecting STZ on the 12th day of incubation is completing the development of the pancreas, which started on the 5th day, by the 12th day (16).

When we searched the literature, Yoshiyama et al. stated in their study that 0.3 mg/egg STZ dose is the ideal dose to increase blood glucose levels and decrease serum insulin levels (17). Sivajothi et al., like the study of Yoshiyama et al., created DM model with dose of 0.3 mg/egg STZ (16). In the study of Shi et al., three different doses of STZ ranging from 250 to 300 mg/kg/egg were used (15).

Therefore, we used different doses of STZ on the 12th day of incubation to create a DM model. As in our previous DM model study (18), STZ3 dose statistically significantly increased both blood glucose levels and decreased blood insulin levels compared to the other STZ1 and STZ2 doses. We determined that the STZ3 dose was the most applicable dose to create a DM model in chick embryos.

In addition, serum insulin levels could not be measured until the 12th day of chick embryo development in the literature (16). We think that this is due to the small, fragile chick embryo vessels and insulin kit. However, we easily measured blood insulin levels. We determined that by using Atay et al.'s blood sampling technique (19) and chick embryo insulin kit (INS ELISA KIT, BT-LAB/EA0012Ch) blood insulin levels can be measured.

Vitamin K1 is the primary circulating form of Vitamin K and has been successfully measured worldwide in various population-based and clinical-based studies to assess circulating Vitamin K status. In previous studies, it has been shown that dietary vitamin K1 reduces hyperglycemia and reduces the risk of type 2 DM (20). In the study of Zwakenberg et al., higher circulating vitamin K1 levels were found to be causally associated with a lower risk of type 2 DM, emphasizing the importance of adequate vitamin K1 in the human diet (21).

In the study of Dihinga et al. in type 2 DM mice; they divided the mice with type 2 DM into two groups and gave only olive oil to the first group and olive oil and Vitamin K1 to the other group. They found that vitamin K1 decreased body weight, basal glucose and insulin levels, glycated hemoglobin A1c (HbA1C) and homeostasis model assessment-estimated insulin resistance (HOMA-IR) levels dose-dependently in the group receiving vitamin K1 compared to the control group (20). In the study of Ibarrola-Jurado et al., it was shown that higher dose vitamin K1 intake was associated with a decrease in the risk of new onset type 2 DM.

**Table 1.** Control and STZ groups' mean blood glucose and insulin levels

| Groups | Mean blood glucose levels (mg/dL) | Mean blood insulin levels (mIU/L) |
|---|---|---|
| **18th day control** | 133.90±9.12 | 5.88±2.01 |
| **18th day STZ1** | 151.30±9.15 | 5.34±2.11 |
| **18th day STZ2** | 178.80±9.89[a,b] | 4.83±1.62 |
| **18th day STZ3** | 191.56±17.74[a,b] | 3.21±1.18[a] |

aThere was a statistically significant difference with the control group. $p < 0.05$, Kruskal-Wallis test, Dunn test as post-hoc test.
bThere was a statistically significant difference with ASM 1 group. $p < 0.05$, Kruskal-Wallis test, Dunn test as post-hoc test.

**Table 2.** After vitamin K1, mean blood glucose, insulin and VEGF levels of STZ groups

| Groups | Mean blood glucose levels (mg/dL) | Mean blood insulin levels (mIU/L) | VEGF levels |
|---|---|---|---|
| **18th day control** | 133.90±9.12 | 5.88±2.01 | 41.3 |
| **STZ1+Vitamin K1-1** | 150.90±8.65 | 5.36±1.95 | 48.37 |
| **STZ1+Vitamin K1-2** | 136.76±9.08 | 5.81±1.76 | 40.33 |
| **STZ1+Vitamin K1-3** | 130.89±7.56* | 5.91±1.54* | 38.27* |
| **STZ2+Vitamin K1-1** | 179.90±13.15 | 4.81±1.1 | 54.27 |
| **STZ2+Vitamin K1-2** | 165.12±8.92 | 5.21±0.9 | 48.12 |
| **STZ2+Vitamin K1-3** | 134.90±7.15* | 5.75±0.8* | 39.59* |
| **STZ3+Vitamin K1-1** | 189.21±12.36 | 3.69±2.32 | 65.76 |
| **STZ3+Vitamin K1-2** | 165.00±12.57 | 4.86±3.35 | 58.28 |
| **STZ3+Vitamin K1-3** | 141.01±5.23* | 5.59±1.31* | 41.1* |

*In each STZ group, according to the dose of vitamin K1; there is a significant difference in blood glucose, insulin and VEGF levels. $p < 0.05$

Moreover, in this study, increased intake of vitamin K1 during follow-up was associated with a 51% lower risk of diabetes in elderly patients at high cardiovascular risk, after a median follow-up of 5.5 years (13). Varsha et al administered STZ to male Wistar rats for three days. Then, they administered vitamin K1 (5 mg/kg, twice a week) to DM treated rats for 2.5 months. At the end of their experiment, the pancreas of the rats was dissected and HbA1C, plasma insulin and islet areas were determined. Varsha et al. found that the treatment of vitamin K1 saved the endocrine pancreas cell from death caused by STZ, and vitamin K1 stimulated islet cell proliferation/regeneration. In addition, they determined that Vitamin K1 caused increased insulin secretion and normal blood glucose and HbA1c levels in diabetic rats. The main findings of this study demonstrated the anti-diabetic mechanism of vitamin K1 (22).

In this study, we applied vitamin K1 at doses of 0.005 mg/egg (0.1 mg/kg), 0.025 mg/egg (0.5 mg/kg), 0.050 mg/egg (1 mg/kg) to chick embryos treated with STZ. Like the literature, we found that vitamin K1 increased blood insulin levels and decreased blood glucose levels. However, in our study, we found that vitamin K1-3 (0.050 mg/egg (1 mg/kg)) was the most effective dose in reducing VEGF, blood glucose levels and increasing blood insulin levels.

We think that vitamin K1 does this by repairing or regenerating pancreatic islet cells. However, we think that larger experimental studies are needed to prove this. In addition, we think that experimental animals larger than chick embryos will need to be used to easily examine pancreatic histology. When hyperglycemia reduces with the effect of vitamin K1, vascular occlusion and hypoxia in the retina reduces. VEGF levels and neovascularization are reduced. So, vitamin K1 protects from new vessel formation and DRP by reducing VEGF levels.

## CONCLUSION

These results show that Vitamin K1 increased blood insulin levels and accordingly caused a decrease in blood glucose levels. It also shows that VEGF levels, one of the first indicators of DRP due to hyperglycemia, decreased with Vitamin K1 treatment. In this context, a sufficient dose of Vitamin K1 may prevent DRP by providing glucose homeostasis..

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