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ORIGINAL ARTICLE

Investigation of the protective effect of vitamin K1 on the heart in streptozotocin induced type 1 diabetes model

Abdülkadir Bilir[1] **Esra Aslan**[2]

1 Department of Anatomy, Faculty of Medicine, Afyonkarahisar Health Sciences University. Afyonkarahisar / Türkiye
2 Department of Histology and Embryology, Faculty of Medicine, Afyonkarahisar Health Sciences University. Afyonkarahisar / Türkiye

Abstract

Maternal and fetal diabetes are directly associated with increased morbidity and mortality risk. Along with this increased risk, the incidence of congenital malformations in newborns also increases depending on the mother's diabetes. Vitamin K1 is used as a therapeutic and protective agent in diabetes and various clinical conditions. For this reason, it was aimed to investigate the effect of vitamin K1 on chick embryo hearts immunohistochemically and morphologically by creating type 1 diabetes mellitus with streptozotocin in a chick embryo model. In our study, 5 different experimental groups will be created and a total of 50 SPF fertilized eggs, 10 in each group, will be used. The first group will be the control group, the second group will be the diabetes group, and the other three groups will be the treatment groups given different doses of vitamin K1. All solutions will be given on the 12th day of incubation, and the hearts of all embryos will be analyzed immunohistochemically and morphologically on the 18th day of incubation. It was determined that the weight, length and ventricular thickness of the chick embryo hearts were statistically significantly decreased in the streptozotocin group compared to the control group embryo hearts. It was determined that the heart weights, lengths, and ventricular thicknesses increased depending on the dose of vitamin K1 compared to the streptozotocin group in the groups therapeutically administered vitamin K1 (p<0.05). In addition, caspase-3 expression was also evaluated in our study, and a statistically significant increase was found in the streptozotocin group compared to the control group. Again, as a result of vitamin K1 administration, caspase-3 expressions decreased depending on the applied dose (p<0.05). In conclusion, it was concluded that the therapeutically applied vitamin K1 to diabetes mellitus reduces the degenerative and hyperplastic effects of diabetes mellitus.

Keywords: Chick embryo, diabetes mellitus, heart, streptozotocin, vitamin K1.

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Corresponding Author:
Abdülkadir Bilir
Email: fztabdulkadirbilir@gmail.com

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disease characterized by abnormalities in lipid, protein and carbohydrate metabolism causing from complete or relative insufficiency of insulin action and/or insulin secretion. The riskiest population for this disease is pregnant women. Because maternal and fetal diabetes are directly associated with increased morbidity and mortality risk [1]. These risks increase in the early and late stages of pregnancy and even may cause congenital malformations in newborns due to maternal diabetes [2–4]. Although central nervous system anomalies and cardiovascular defects are common among these malformations, they are associated with complications that occur especially in the early stages of organogenesis (the first 8 weeks of pregnancy) [5,6].

Cardiovascular defects due to DM include patent ventricular septal defects, ductus arteriosus, and endocardial cushion defects [7]. Despite all clinical interventions to ensure optimal glycemic control during and before pregnancy, the rate of cardiovascular defects in children of diabetic mothers is still higher than in non-diabetic mothers. The mechanisms underlying these defects are not also fully discovered [8]. However, in disorders caused by DM, factors such as changes in metabolic and signaling pathways, beta cell tropism, immunosuppressors, viral infectious agents or hypercaloric diets, and chemical, toxic agents or drugs come to the fore. Alloxan and streptozotocin (STZ), which are toxic glucose analogues specific to beta cells, are toxins that are frequently used to induce experimental diabetes by chemical method and have been used for a long time to create experimental DM in animals [9,10].

STZ is a potent alkylating agent that causes glucokinase function, multiple DNA strand breaks, and glucose transport. It is one of the most commonly used agents in non-surgical DM models. Even a single high dose can cause DM as a result of its toxic [8,11,12]. Vitamin K1 is a form of vitamin K that has proven its therapeutic and protective efficacy in different populations and various clinical conditions worldwide. In the literature, its effectiveness on DM and hyperglycemia has been emphasized [13,14]. For this reason, it was aimed to examine the therapeutic effect of vitamin K1 in chick embryo hearts by immunohistochemical and morphological methods by creating type 1 DM with STZ in the chick embryo model.

Materials and Methods

Ethical Consent

This study was approved by Afyon Kocatepe University Animal Experiments Ethics Committee (49533702/267).

Experimental Animals and Laboratory Conditions

In this study, 50 fertilized 0-day-old specific pathogen-free (SPF) eggs weighing 65±5 g obtained from the Veterinary Control and Research Institute (Bornova, İzmir, Türkiye) were used. The SPF eggs used in the study were incubated in the anatomy laboratory at 70% humidity and 37.5°C. During the incubation period, all eggs were automatically rotated at an angle of 45° to the vertical axis every 2 hours.

Chemicals and Doses

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. The dose of 0.30 mg/egg STZ was determined according to the literature to induce diabetes in chick embryos [10,15,16]. Three different doses vitamin K1 (Konakion 10 mg/ml, Roche) were administered for treatment [13].

Experimental Groups and Experimental Protocol

50 SPF eggs were placed in the incubator with their acute ends down. On the 12th day of incubation, all eggs were randomly distributed into 5 groups, with 10 eggs in each group. The first group was designated as the positive control group and was administered only saline. Other eggs were applied STZ. The first of the groups to which STZ was applied was determined as the negative control group (n=10). Three different doses of vitamin K1 (0.005 mg/egg for low dose, 0.025 mg/egg for medium dose, 0.050 mg/egg for high dose) were administered to the other groups in which STZ was aplied, 3 hours after

the injection. All solutions were administered to the air sack in 0.1 ml volumes with an insulin injector, and the window opened after each application was closed with sterile drape and put back into the incubator. On the 18th day of incubation, all of chick embryos were evacuated from the egg and quickly decapitated. After decapitation, the hearts of all embryos were dissected and routine protocols were performed for morphological and immunohistochemical examination.

Morphological Measurements

Firstly, the dissected hearts were weighed with precision balance. Photographs were then taken with a stereomicroscope (Carl Zeiss Stemi 2000-C). For better histopathological and immunohistochemical examination, hearts were rinsed in cold saline, and fixed in 10% formaldehyde solution and transversely cut. Cuts were made from the ventricular apex to approximately 60% of the length of the heart. Ventricular tissues were routinely processed and embedded in paraffin and 5 micromillimeter sections were taken. The septomarginal trabecula was used to maintain a relatively stable position to allow measurements of the ventricular wall at the same location in each heart. Hearts were stained with Hematoxylin and Eosin (H&E) solution for routine histology [17,18]. Image Analysis Software program (NIS Elements Nikon, Japan) used to measure the thickness of the ventricular wall. The right ventricular wall was chosen as the histological target and the thickness of the ventricular walls was measured from 5 different locations (Figure 1).

Immunohistochemical staining

Tissue samples were deparaffinized and dehydrated by taking 5 μm sections on polylyzed slides. It was stained immunohistochemically with caspase-3 primary antibody. Antigen retrieval was performed with heat for 20 min in a microwave oven in citrate buffer (pH 6.0). Endogenous peroxidase activity was inhibited by the application of 3% hydrogen peroxide (H_2O_2). The primary antibody caspase-3 (Anti Caspase-3, NB100-56708, Novus Biologicals, 1/200) was then dropped and incubated overnight. After incubation, horseradish peroxidase (HRP) secondary antibody kit was used as secondary antibody. Sections were incubated with biotinylated secondary antibody for 30 minutes and then incubated with HRP-conjugated streptavidin for 30 minutes. Aminoethyl carbazole (AEC) kit was used as chromogen. Finally, all sections were counterstained with Mayers hematoxylin and sealed with water-based sealing solution.

The histological scoring method used by Wang et al was used to evaluate caspase-3 expression. Scoring of expression was based on the intensity and extent of staining. The mean rate of stained cells was determined semi quantitatively (Figure 2) and scored as follows [19]:

0 for staining <1%, 1 for 1 to 25%, 2 for 26 to 50%, 3 for 51 to 75%, and 4 for >75% of the examined cells. Staining intensity was graded as follows: 0, negative staining; 1, weak staining; 2, moderate staining; 3, strong staining. The histological score (H score) for each specimen was computed by the formula: H score = intensity score × proportion score. All the sections were evaluated under light microscope (Eclipse E-600 Nikon, Japan) and Image analysis was made with Image Analysis Software for assessing the samples.

Statistical analysis

Statistical analysis of the study data was performed with the IBM Statistical Package for Social Sciences (SPSS) Statistics 25.0 program. The normal distribution of data was analyzed with the Shapiro Wilk test. After it was determined that the data were not normally distributed, the Kruskal-Wallis *H* test was used to determine the differences between multiple independent continuous variables, and the Mann-Whitney U test was used to compare the paired groups with Bonferroni correction. P <0.05 were considered statistically significant. Data were presented as median (minimum-maximum)

Results

In our study, 10 SPF eggs were randomly determined for each experimental group. However, due to developmental defects (developmental retardation, lack of vascularization, unfertilization) determined in the embryos during the incubation period or

their death during the incubation period, an average of two embryos from each group were lost. Depending on this loss, the analyzes were evaluated on 8 embryo hearts.

As a result of the study, it was determined that the weight, length and ventricular thickness of the chick embryo hearts were statistically significantly decreased in the STZ group compared to the control group embryo hearts. It was determined that the heart weights, lengths, and ventricular thicknesses increased depending on the dose of vitamin K1 compared to the STZ group in the groups therapeutically administered vitamin K1 (p<0.05). In addition, caspase-3 expression was also evaluated in our study, and a statistically significant increase was found in the STZ group compared to the control group. Again, as a result of vitamin K1 administration, caspase-3 expressions decreased depending on the applied dose (p<0.05). Statistical differences among study result data and groups are shown in Table 1.

Discussion

Diabetes mellitus is one of the common metabolic pregnancy complications related with an increased risk of neonatal and maternal morbidity. Undoubtedly, the cardiovascular system, which is one of the first systems to develop in the fetus, is vulnerable to exposure to hyperglycemia caused by gestational diabetes. Dysplasia of the cardiovascular system, which occurs during the cardiogenesis and organogenesis period as a result of uncontrolled diabetes, often results in neonatal and maternal morbidity. But the biggest concern with diabetes is cardiovascular morbidity rather than mortality [20,21]. Because diabetes-induced morbidity leads to cardiovascular defects and thus disrupts quality of life to a great extent [7,22]. In order to prevent that, the prevention of diabetes during pregnancy has become a basic principle by clinicians. For this reason, antioxidants are used as potential protectors during pregnancy [20,21].

Chick and human pancreatic islets are flimsy against streptozotocin. Therefore, chick embryos are used in experimental diabetic methods instead of mammals. It is also preferred due to its ease of manipulation and sensitivity in antioxidant mechanisms [23].

The primary circulating form of vitamin K is vitamin K1. The efficacy of circulating vitamin K has been successfully demonstrated worldwide in various clinical-based and population-based studies. In previous studies, it has been shown that dietary vitamin K1 reduces risk of type 2 DM and hyperglycemia [13,14]. Therefore, in this study, we aimed to immunohistochemically and morphologically examine vitamin K1 in STZ-induced chick embryo type 1 DM model.

In the literature, Memon and Pratten showed the effect of hyperglycemia on the heart in the chick embryo experimental model created with 20 mM glucose. As a result of the study, abnormal heart development (especially enlargement of ventricular chambers) was detected in embryos treated with glucose [20]. Likewise, Datar et al. examined the exposure of chick embryos to 30, 50 and 100 mM glucose for 24 hours and found that mortality increased with increasing glucose concentrations, especially in the early embryonic stages. However, at stage 21 of Hamburger-Hamilton 100 mM glucose caused 1-2% abnormal heart development [24]. Mohammed et al. investigated the potential cardiotoxic effect of hyperglycemia with hyperketonemia using two in vitro models. As a result of the study,

Table 1. Study result data and statistical differences.

Groups	Weight (g)	Lenght (mm)	Thickness (µm)	Caspase 3
STZ	0.26 (0.2-0.29) [a]	1.02 (0.92-1.3) [a]	647.92 (488.31-805.55) [a]	2 (1-3) [a]
Low Dose	0.13 (0.12-0.13) [a,b]	0.91 (0.84-0.97) [a]	485.01 (364.77-647.776)	5.67 (4-8) [a]
Medium Dose	0.16 (0.12-0.25) [a,b]	0.93 (0.74-0.99) [b]	578.55 (522.37-681.428) [b]	3 (3-6) [b]
High Dose	0.17 (0.15-0.2) [a,b]	1 (0.89-1.13) [b]	624.11 (499.01-724.806) [b]	2 (2-6) [b]
Control	0.19 (0.15-0.22) [b]	1.03 (0.89-1.24) [b]	615.65 (529.98-733.21) [b]	2 (1-4) [b]
***p* value**	<0.001	0.035	0.004	<0.001

a : There is a statistically significant difference with the control group.

b : There is a statistically significant difference with STZ Group.

Data shown as **median (minumum-maximum)**, **g:** gram, **mm:** millimeter, **µm:** mikrometer.

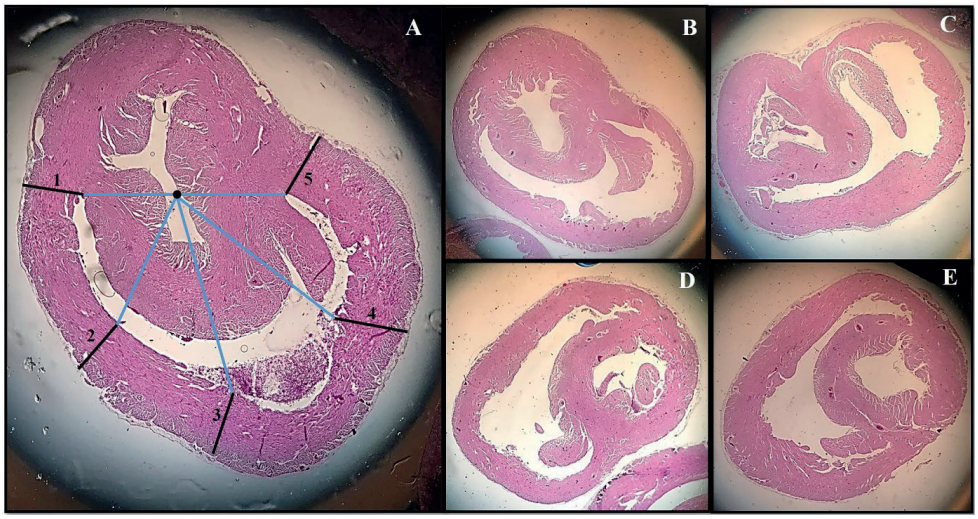

Figure 1. H&E histological sections showing the ventricular wall thicknesses of the experimental groups and morphometric measurements (indicated by radiating lines).
A: Control group, **B:** STZ, **C:** Low dose, **D:** Medium dose, **E:** High dose.

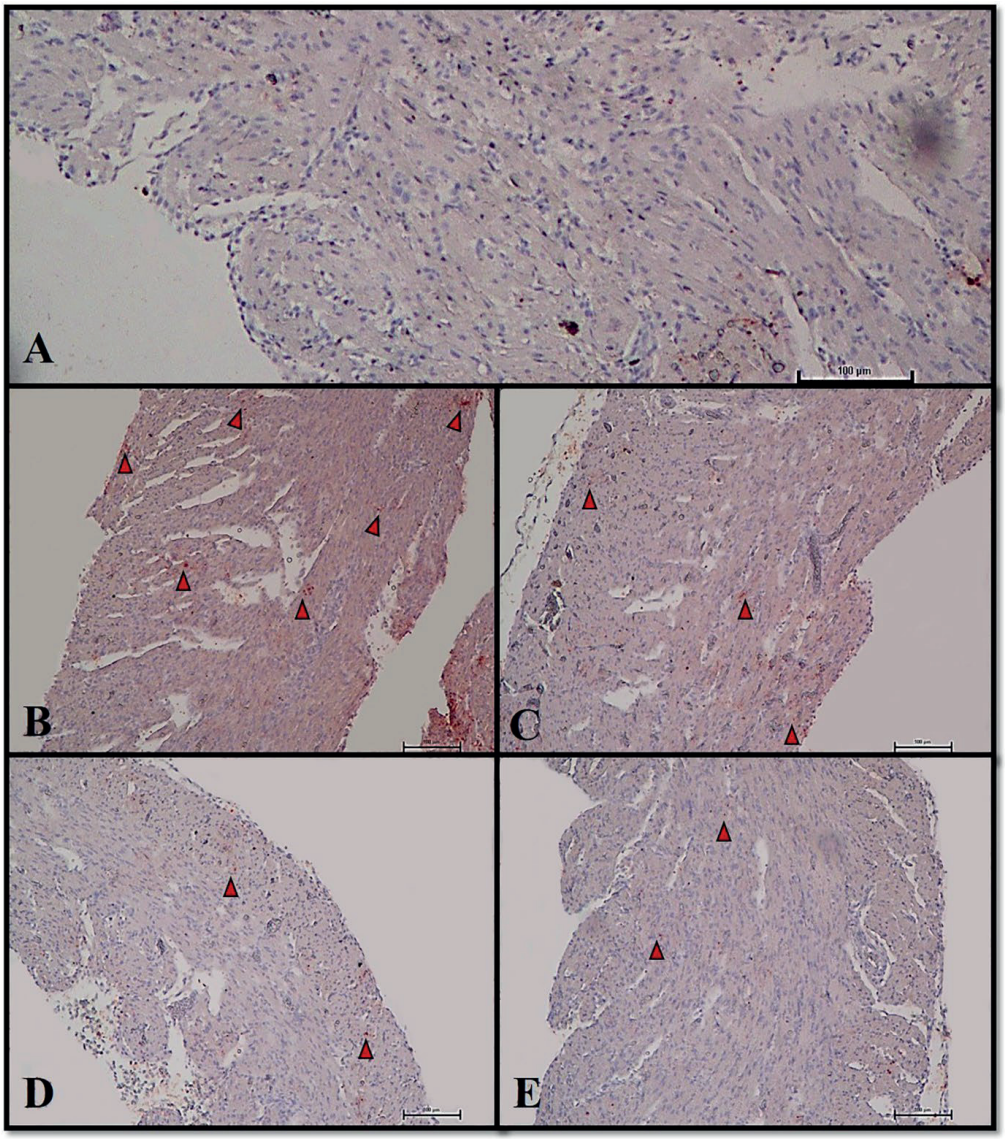

Figure 2. Caspase-3 stained immunohistochemical sections of the experimental groups.
A: Control group, **B:** STZ, **C:** Low dose, **D:** Medium dose, **E:** High dose.

they determined that there was an increase in DNA damage and a decrease in cell migration in mouse embryonic stem cells, which had a negative effect on the formation and development of the heart [22]. Based on this information, it was determined that muscle cells were hypertrophied in direct proportion to glucose consumption and abnormal heart formations were formed. As a matter of fact, in our study, the insulin mechanism was disrupted with STZ administration and sufficient glucose uptake of the heart cells was prevented. Accordingly, the morphometric values of the STZ group embryos were found to be lower than the control group In addition, vitamin K1 was administered as a potential therapeutic in STZ application in our study. An increase in heart morphometry was observed as a result of the administration of vitamin K1. This increase can be explained by the effects of vitamin K1 on pancreatic B cells on insulin secretion (increasing glucose tolerance) and its antioxidant properties that support the regeneration of pancreatic islets and increase insulin secretion [25]. In addition, active caspase-3 expression in diabetic apoptotic cells was also evaluated by immunohistochemical staining method in our study. As a result of the evaluation, it was determined that STZ application increased apoptosis, and vitamin K1 decreased it depending on the rate of vitamin K1 dose.

Similarly, in the literature, Dihinga et al applied olive oil and olive oil + vitamin K1 to type 2 DM mice. It was determined that vitamin K1 decreased basal glucose, insulin levels, body weight in a dose dependent manner compared to the control group [14]. Varsha et al. gave STZ to male Wistar rats for 3 days and attempted to treat them with vitamin K1 (5 mg/kg, twice a week) for 2.5 months. At the end of the study, they determined that vitamin K1 treatment reduced the deaths caused by STZ in endocrine pancreatic cells and vitamin K1 facilitated islet cell regeneration [26]. In other studies in the literature, it has been found that high-dose vitamin K1 intake reduces the risk of new onset type 2 DM [27], and vitamin K1 plays an important role in diabetes induced embryopathy due to its possible roles in various cellular activities such as apoptosis, proliferation and differentiation [22]. In addition, DM triggers oxidative stress that causes tissue pathogenesis, and oxidative stress situmulates the release of reactive oxygen species (ROS). It contributes to apoptosis in ROS under both physiological and pathological conditions. Thus, it is thought that unplanned apoptosis causes cardiomyocyte damage and indirectly causes compensatory hyperplasia of the heart walls through vascular dysplasia. We support the idea that vitamin K1 has an inhibitory effect on ROS due to its antioxidant effect [28], and accordingly, it has a protective/therapeutic effect on the heart wall.

Conclusion

It was concluded in our study that DM is an important factor in cardiovascular diseases and has serious irreversible destructive effects on heart morphometry and histopathology. We have also determined that the therapeutically applied vitamin K1 to DM also reduces the degenerative and hyperplastic effects of DM. However, although we rely on the outcome data of our study, we think that our study should be supported by studies in which the scope is expanded and different doses of vitamin K1 are applied.

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Conflict of interest

Authors declared no conflict of interest.

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