

## Potential protective effect of vitamin D on the aortic tissue of streptozotocin-induced diabetic vascular impairment in adult male rats.

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### Abstract

**Background:** Diabetes mellitus is a major risk factor of cardiovascular disease. There is evidence that vitamin D decreases type 1 diabetes mellitus risk during early adulthood and improves insulin secretion and resistance in diabetic patients. Low vitamin D level was observed to increase the cardiovascular disease.

**Objectives:** This study aimed to assess the protective effect of vitamin D on diabetic vascular damages in aorta.

**Materials and Methods:** 40 adult male rats were randomly divided into: group I (control), group II (vitamin D), group III (diabetic) and group VI (diabetic plus Vitamin D) (n = 10 rats /each group). Injection of streptozotocin (60 mg/kg) as a single dose intraperitoneal to induce diabetes. Vitamin D was administered orally every other day in a dose of 12.5 mg/kg. After 12 weeks of treatment period, aortic samples were collected for histological examination.

**Results:** Morphological changes of aortic tissue in diabetic untreated group in the form of reduction of tunica media thickness and areas of tunica intima detachment. The elastic lamellae became irregular, fragmented or branched. Shrunken dark or lysed nuclei of smooth muscle fibers were seen in tunica media of diabetic group. The diabetic treated group with vitamin D showed more or less normal structure of the layers of aortic tissue with comparable thickness to the control group.

**Conclusion:** Vitamin D may reduce the vascular complications and tissue injuries induced by diabetes in aorta. This effect has a positive influence on the function of the cardiovascular system.

**Keywords:** Diabetes, Vitamin D, Aorta.

### Introduction

The diabetes mellitus prevalence is 8.5 % among adult people (WHO, 2013) and it is a major risk factor for the development of atherosclerotic cardiovascular damages and coronary diseases (Beckman et al., 2013). The atherosclerosis in diabetes are heavily influenced by oxidative stress (Li et al., 2014). Type 1 diabetes mellitus was found to be caused by a complex autoimmune process,

related to altered humoral and cellular immunity. The autoimmune response includes both cell-specific autoantibodies and autoreactive pathogenic CD4 and CD8T cells infiltrating the pancreatic islets (insulinitis). (Bizzarri et al., 2010).

Streptozotocin (STZ) is an antibiotic and an analogue of N-acetylglucosamine (GlcNAc) which is transported rapidly into pancreatic  $\beta$ -cells by GLUT-2 (glucose transporter 2) and become the

main cause of  $\beta$ -cell toxicity, resulting in insulin deficiency (Lee et al., 2006).

Vitamin D is considered as one of fat-soluble vitamins group that causes intestinal absorption of calcium, magnesium and phosphate, and multiple other biological effects (Holick et al., 2004). The most important compounds in this group in humans are vitamin D<sub>2</sub> (ergocalciferol) and vitamin D<sub>3</sub> (also called cholecalciferol) which can be ingested from the diet and supplements (Holick et al., 2004).

A low vitamin D status is related to increase the risk of type 1 diabetes mellitus (Mathieu et al., 2005), type 2 diabetes mellitus (Forouhi et al., 2008) and cardiovascular disease (Wang et al., 2008).

Supplementation of vitamin D to subjects with diabetes improves insulin secretion and insulin resistance (Borissova et al., 2003) and its administration during infancy decreases the risk of type 1 diabetes mellitus during early adulthood (Hypponen et al., 2001).

The previous studies suggested that the potential effect of vitamin D can modulate the immune response and help in preservation of B cell function after type 1 diabetes onset (Bizzarri et al., 2010).

## Materials and Methods

### Animals

This prospective study was conducted on 40 adult male albino rats weighed (180-350 gm) and were obtained from Laboratory Animal Resource Unit, south valley University, Egypt. Ethical approval from the Institutional Animal Ethics Committee was obtained. The rats were kept in isolated cages under air-conditioned room with adequate ventilation, a 12-hrs light/dark cycle and had free access to rat chow and water and along the study period. All rats housed in an acclimatization period for 1 week before the commencement of the study. The animals were equally divided into four groups (ten rats for each):

Group I (control group): control healthy group.

Group II (Vitamin D group): the rats were given cholecalciferol only in a dose of (12.5 mg/kg), every other day for twelve weeks.

Group III (diabetic group): rats were given streptozotocin (supplied by Pharmacia Company) in a single intraperitoneal dose (60mg/kg). The dose was freshly dissolved in 0.9 % NaCl solution and blood samples were taken after 48 hours from the tail vein and measured by Accu-check glucometer. Rats with blood glucose level more than 15mmol/L were considered diabetic.

Group IV (STZ+vitamin D) group: rats were given 60mg/kg of streptozotocin intraperitoneal as a single dose. Diabetes was confirmed because mean of blood glucose level was  $263.33 \pm 64.39$  mg/dl, then rats were given cholecalciferol [500 IU] dissolved in 0.3ml olive oil manufactured by Pharma Company in India orally in a dose (12.5  $\mu$ /kg) every other day for a period of twelve weeks. The rats were sacrificed after 12 weeks with diethyl ether and the thoracic cavity was opened and the thoracic aorta (approximately 2 cm long) was dissected and sectioned transversely for the histological examination.

### Tissue preparation

a- The specimens were taken from aorta of the four groups and were fixed in 10% formalin for at least 24h and then proceeded for histological examination by passing in ascending grades of alcohol. The specimens were cleared in Cedar wood oil and impregnated in Paraffin wax, sectioned (5 microns in thickness) and stained with Hematoxylin and Eosin for general histological structure study and with Orcein stain for study of the elastic fibers also with Masson Trichrome for collagen fibers of the aorta in different groups.

b- Morphometric study and Statistical analysis:

The thickness of tunica intima (TI) and tunica media (TM) and the ratio of intima to media (TI/TM) were

measured in different groups, using magnification x1000 by Image J. Data presented as mean±S.D.

Differences between groups were statistically analyzed using one-way analysis of variance (ANOVA). All analyses were performed using the Statistical Package for Social Sciences (SPSS) software, version 18.0. P -value < 0.05 is considered significant.

## Results

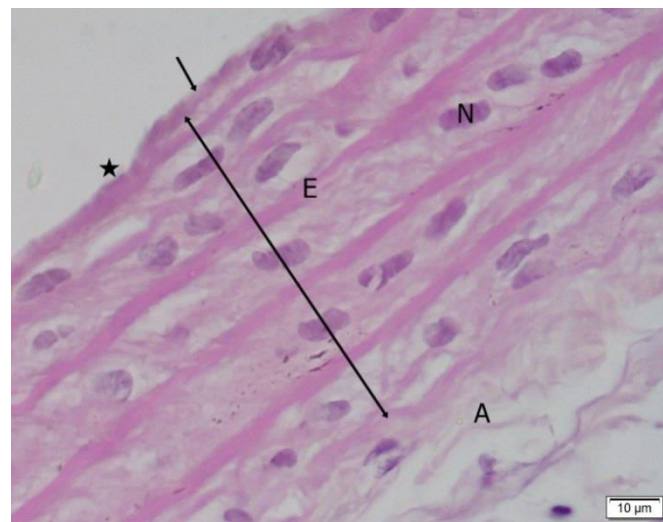
Histological examination of the aorta stained by H&E in the control and vitamin D groups revealed no structural difference in the layers of aorta of the control (Figure1) and vitamin D (Figure2) groups. The tunica intima appeared thin with intact endothelial cells of elongated flattened nuclei. The tunica media appeared the thickest layer, composed of elastic fibers and smooth muscle cells with oval vesicular nuclei in between. The Orcein stained sections revealed normal regular parallel elastic fibers in tunica media (Figures 5&6). The tunica adventitia showed its fibrous content of loose connective tissue (Figures1&2). Using Masson Trichrome both groups have normal distribution of collagen fibers (Figure 10,11).

In the aorta of the diabetic group by H&E stain showed detached areas in the intima. The tunica media in diabetic group showed irregular, disrupted and sometimes thinning in the elastic lamina. Degenerative changes in the smooth muscle fibers with vacuolations and shrunken dark nuclei were also observed in tunica media of this group (Figs.7&8). Using Masson Trichrome there was an increase in the distribution of collagen fibers were also seen in tunica media (Figure 12,13)

No obvious damages of the aorta were observed in diabetic treated (STZ+vitamin D) group and the layers preserved its normal structure. The tunica media of this group appeared with normal thickness compared to control (Figure 4) and the elastic lamina showed regular and parallel arrangement with normal smooth muscle cells and vesicular

nuclei in between (Figure 9) and more or less normal distribution of collagen fibers were seen in this group (Figure 14,15).

## Sections stained by H&E:



**Figure 1:** a photomicrograph of a cross-section of aorta of rat of group I (control group) showing the three layers of the aorta: intact intima (arrow) with normal small flattened nuclei(\*) and the tunica media (long arrow with double heads) appeared with regular parallel elastic fibers E and oval vesicular nuclei N of smooth muscle fibers in between. Note the fibers in the adventitia layer A, H&E X1000.

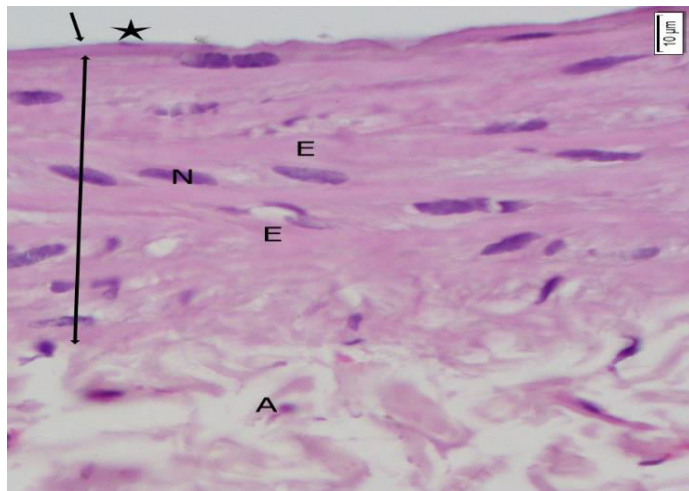


**Figure2:** a photomicrograph of a cross-section of aorta of rat of groupII (vitamin D group) showing

three layers of aorta, normal intact intima (**arrow**) with normal small flattened nuclei (\*) and the tunica media (**long arrow with double heads**) appeared with regular parallel elastic fibers **E** and oval vesicular nuclei **N** of smooth muscle fibers in between. Note the fibers in the adventitia layer **A**, H&E X1000.

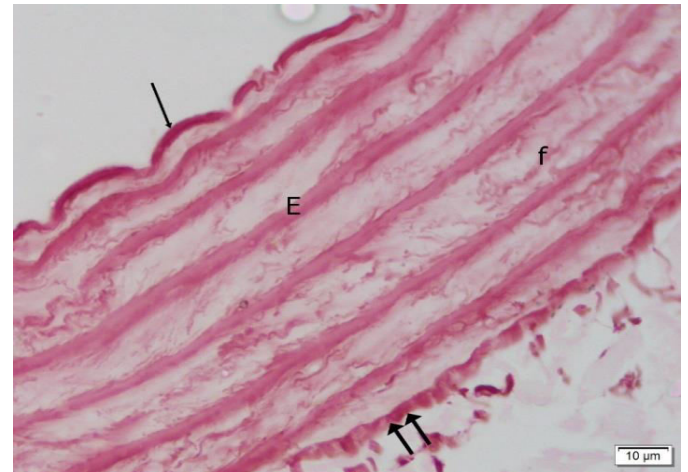


**Figure 3:** a photomicrograph of a cross-section of aorta of rat of group III (diabetic group) showing the three layers of aorta, the tunica intima I showed detached endothelium (**broken arrow**). The tunica media **M** appeared with vacuolations **V**, irregular and disarranged elastic fibers **E** and dark shrunken nuclei **N1** or lysed nuclei **N2**. Note dark nucleus in adventitia layer **A**. There is decrease in thickness (**long arrow with double heads**) of media compared by control group, H&Ex1000.

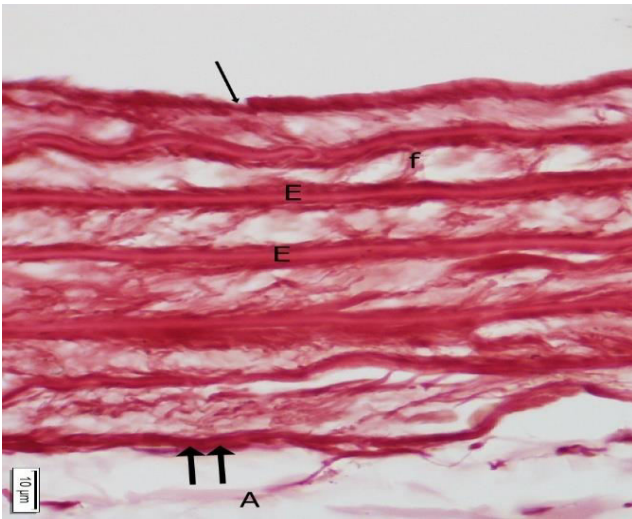


**Figure4:** a photomicrograph of across-section of aorta of rat of group VI (STZ+ vitamin D) group showing normal intact intima (**arrow**) with normal small flattened (\*) and the tunica media (**long arrow with double heads**) appeared with regular parallel elastic fibers **E** and oval vesicular nuclei **N** of smooth muscle fibers in between and thickness of media more or less normal compared to control group (figure1). Note the fibers in the adventitia layer **A**,H&E X1000.

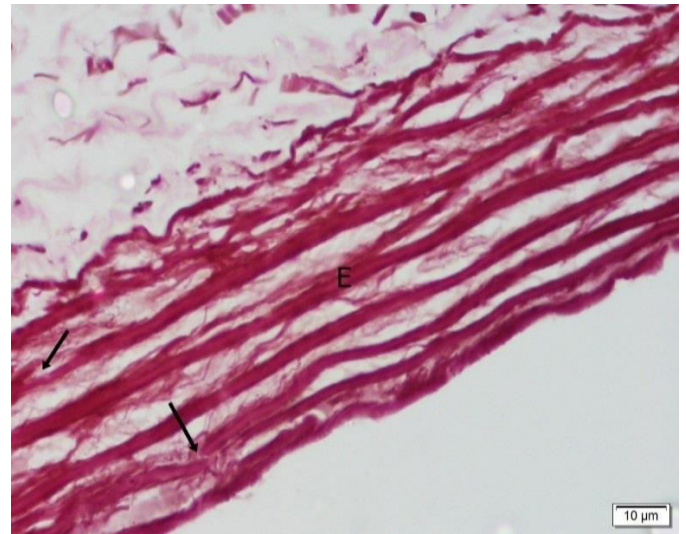
**Sections stained by Orcein stain:**



**Figure5:** a photomicrograph of a cross-section of aorta of rat of group I showing regular parallel arranged red stained elastic lamina **E** with fine elastic fibers **f** distributed in tunica media. Note the internal elastic lamina (**arrow**) in tunica intima and external elastic lamina (**double arrow**) in outer part of media, Orceinx1000.



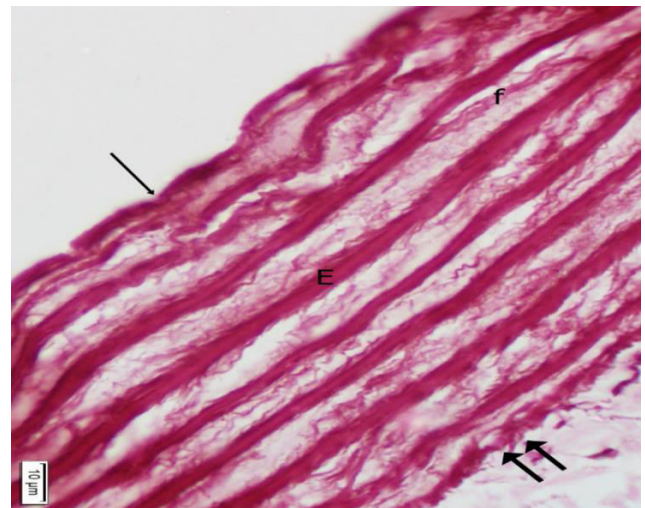
**Figure 6:** a photomicrograph of a cross-section of aorta of rat of group II showing regular parallel arranged red stained elastic lamina **E** with fine elastic fibers **f** distributed in tunica media. Note the internal elastic lamina (**arrow**) in tunica intima and external elastic lamina (**double arrow**) in outer part of media, Orcein x1000.



**Figure 8:** a photomicrograph of a cross-section of aorta of rat of group III showing degenerated and distorted elastic fibers (**arrow**), Orcein x1000.



**Figure 7:** a photomicrograph of a cross-section of aorta of rat of group III showing irregular **E1** or branching **E2** elastic fibers. Note interrupted internal elastic lamina in tunica intima (**arrow**) and thinning of external elastic lamina (**double arrow**), Orcein x1000.

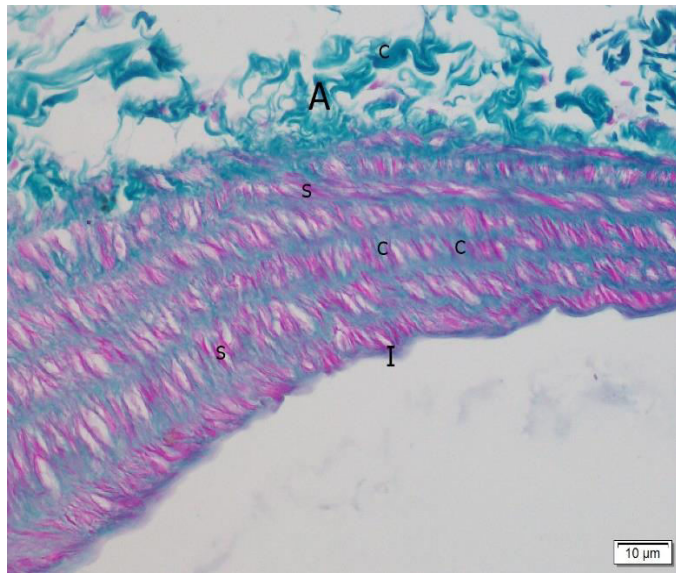


**Figure 9:** a photomicrograph of a cross-section of aorta of rat of group VI showing regular parallel arranged red stained elastic lamina **E** with fine elastic fibers **f** distributed in tunica media. Note the internal elastic lamina (**arrow**) in tunica intima and external elastic lamina (**double arrow**) in outer part of media, Orcein x1000.

Sections stained by Masson Trichrome:

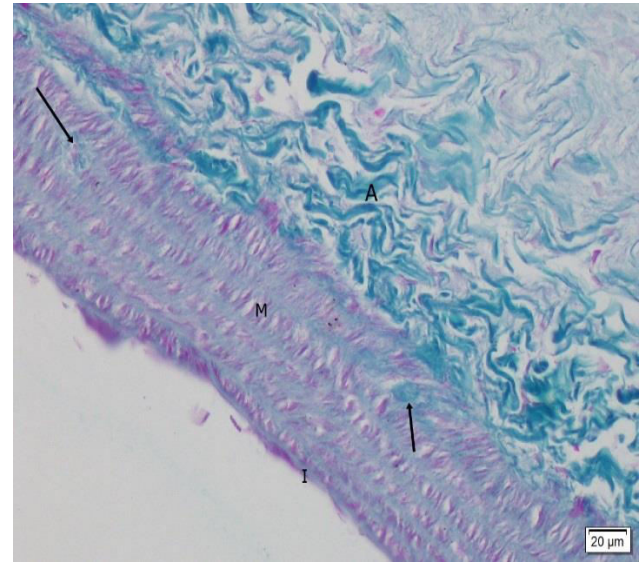


**Figure 10:** a photomicrograph of across-section of aorta of rat of group I showing the three layers of aorta with blue stained collagen C intermingled with red smooth muscle fibers S. Note the clear blue staining of collagen fibers in adventitia layers A, Masson Trichrome x1000.

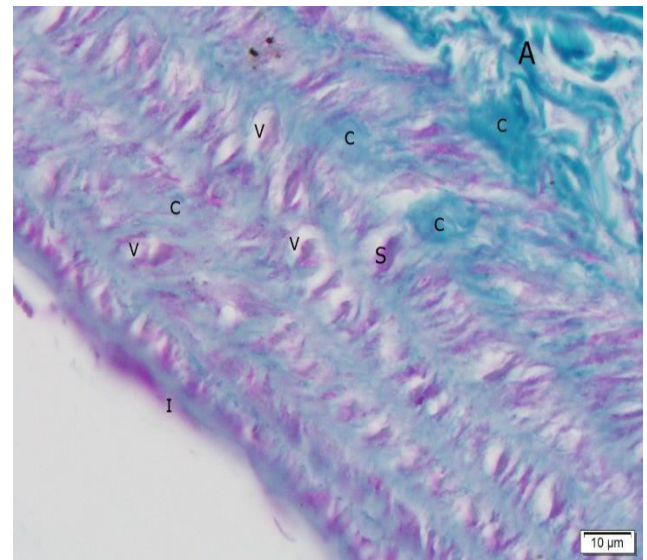


**Figure 11:** a photomicrograph of a cross-section of aorta of rat of group II showing the three layers of aorta with blue stained collagen C intermingled with red smooth muscle fibers S. Note the clear blue

staining of collagen fibers in adventitia layers A, MassonTrichromeX1000.

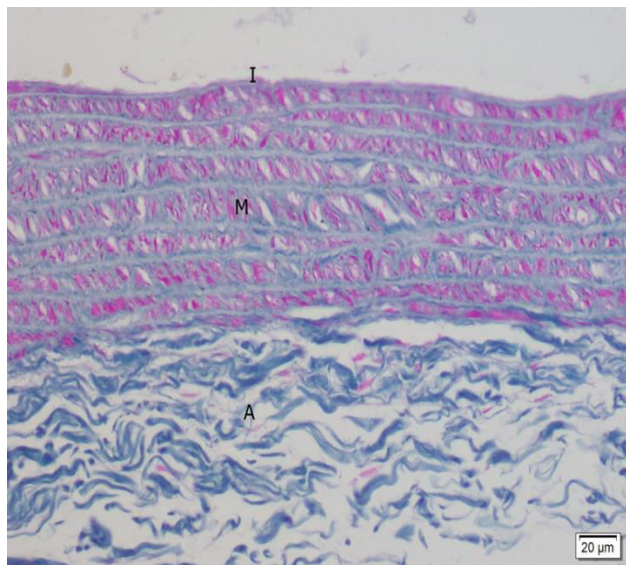


**Figure 12:** a photomicrograph of a cross-section of aorta of a rat of group III showing focal fibrosis (arrow) also there is increase in collagen distribution stained blue in media M and in adventitia layer A. Note the degenerated tunica intima I, Masson TrichromeX400.

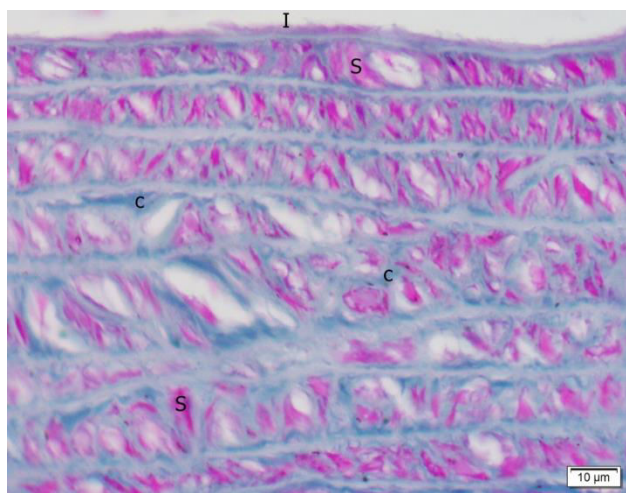


**Figure 13:** a photomicrograph of a cross-section of aorta of rat of group III showing degeneration and vacuolations V of smooth muscle fibers S, also there is increase in collagen distribution C in media

**M** and in adventitia layer **A**. Note the degenerated tunica intima **I**, Masson Trichrome X 1000.



**Figure 15:** a photomicrograph of a cross-section aorta of a rat of group VI showing the three layers of aorta with more or less normal blue stained collagen **C**. intermingled with red smooth muscle fibers **S**. Note the clear blue staining of collagen fibers in adventitia layers **A**, Masson Trichrome X400.



**Figure14:** a photomicrograph of a cross-section of aorta of a rat of group VI showing more or less normal blue stained collagen **C** intermingled with red smooth muscle fibers **S**, Masson Trichrome X1000.

### Morphometric results

The mean thickness of the intima layer of aorta in the adult control rat was ( $3.266 \pm 0.176 \mu\text{m}$ ), in vitamin D group was ( $3.273 \pm 0.314 \mu\text{m}$ ) in diabetic group was ( $3.274 \pm 0.146 \mu\text{m}$ ) diabetic treated (STZ +Vitamin D) group was ( $3.265 \pm 0.199 \mu\text{m}$ ) (Table 1 and Chart1) and p-value in all group is not significant compared to control groups (Table 2).

The mean thickness of the media layer of aorta in the adult control rat was ( $85.20 \pm 0.374 \mu\text{m}$ ), in vitamin D group was ( $85.43 \pm 0.64 \mu\text{m}$ ), in diabetic group was obviously decreased ( $47.55 \pm 0.518 \mu\text{m}$ ) and in diabetic treated (STZ +Vitamin D) group was returned to normal pattern ( $85.34 \pm 0.922 \mu\text{m}$ ) (Table 1 and Chart1).

**Table1. Measurements of thickness (Mean± SD) of the tunica intima and tunica media of the aortic wall (n = 10) and their ratio.**

SD= (standard deviation) n=number

TI=Tunica Intima TM=Tunica Media

There was significant difference in diabetic compared to control group (P-value < 0.001). Table 2 and chart1). Also (P-value < 0.000) is highly significant in diabetic treated group compared to diabetic untreated group. But (P-value < 0.3) is not significant different in diabetic treated group compared to control group (Table2) (chart1). There was no significant difference in the media thickness of the layers between the control and vitamin D groups (p-value < 0.463).

The ratio is obviously increased in diabetic group ( $0.0639 \pm 0.0109$ ) (Table1 and Chart2), and (P-value < 0.001) (Table 2) is highly significant different (increased) compared to control group ( $0.0398 \pm 0.0027$ ) (chart2) (Table1). Ratio in diabetic treated group ( $0.0402 \pm 0.0046$ ) is decreased return to normal and (p-value < 0.002) compared to diabetic (Table2) and it had no significant difference (p-value < 0.3) compared to control.

**Table1. Measurements of thickness (Mean± SD) of the tunica intima and tunica media of the aortic wall (n = 10) and their ratio.**

Group	Control	Vitamin D	STZ	STZ+vita min D
TI	3.266±0.176	3.273±0.314	3.27±0.147	3.265±0.1997
TM	85.20±0.37	85.43±0.64	47.55±0.518	85.34±0.922
Ratio TI/T M	0.0398±0.0027	0.0387±0.00362	0.0639±0.00109	0.04±0.0046

**Table2.P-value of Tunica Intima (TI) and Tunica Media (TM) and TI/TM ratio.**

P value	Control group	Vitamin D group	STZ group	Vitamin D +STZ
Intima p-value		0.412	0.531	0.482
Media p-value1		0.1	0.001	0.3
media P-value2	0.001	0.001		0.000
media P-value3	0.3	0.421	0.000	
media P-value4	0.463		0.000	0.511
Ratio p-value1		0.341	0.001	0.3
Ratio p-value 2	0.001	0.000		0.002

Ratio pvalue3	0.412		0.000	0.523
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By using one-way ANOVA test:

Intima p-value: compare all groups to control in intima thickness

Media p-value1: compare all groups to control in media thickness

Media p-value2: compare all groups to STZ (diabetic group) in media thickness

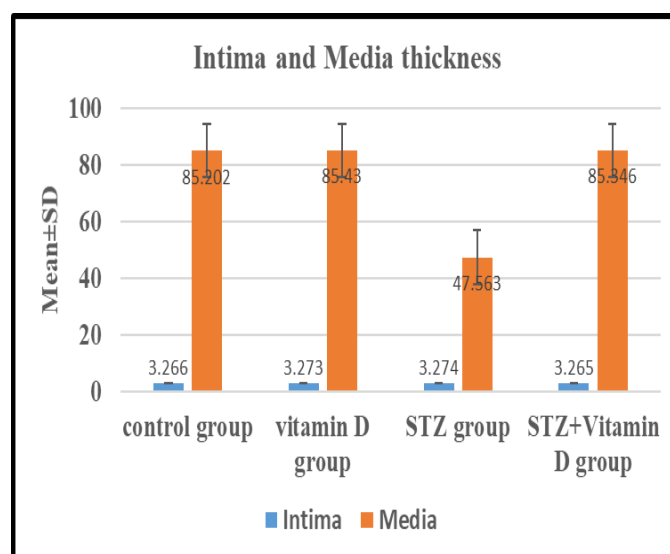
Media p-value3: compare all groups to (Vitamin D+STZ) diabetic treated groups in media thickness.

Media p-value4: compare all groups to (Vitamin D) group in media thickness.

Ratio p-value 1: compare all groups to control in TI/TM ratio.

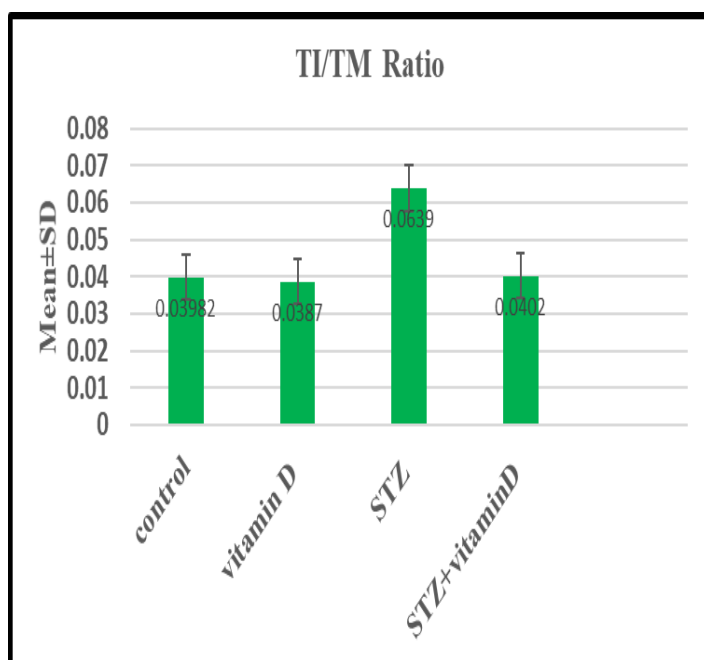
Ratio p-value2: compare all groups to STZ group in TI/TM ratio.

Ratio p-value3: compare all groups to vitamin D in TI/TM ratio.



**Chart 1: showing mean±SD of Tunica Intima and Tunica Media in all groups.**





**Chart 2: Showing mean±SD of TI/TM Ratio in all groups.**

## Discussion

The current study by H&E showed that in control and vitamin D groups the aortic tissue showed a normal morphology; the tunica intima was observed with intact endothelial cells and their spindle shaped nuclei. The tunica media appeared the thickest layer, with smooth muscle cells possessing normal vesicular oval nuclei in between the parallel arranged elastic fibers. The outer fibrous layer, tunica adventitia was also observed in these groups. Also, in the diabetic group showed detached areas in the intima. The tunica media showed many spaces between elastic fibers and smooth muscle fibers, with reduced thickness of the media, compared to control. Some of the smooth muscle nuclei were shrunken dark and lysed. Using Orcein stain in the diabetic group revealed irregularly arranged, distorted or branched elastic fibers but in group four appeared with regularly arranged elastic fibers. Using Masson Trichrome showing normal distribution of collagen fibers in control and vitamin D groups but increased collagen distribution in diabetic group while in (STZ+vitamin D) group nearly resembles the control group.

Previous studies were done by (Salum et al., 2012) reported that the aortic tissue of control group shows normal vascular morphology.

It was mentioned that the intima layer has been reported to form one-fourth of the entire aortic wall, with the media layer, (Aymen, 2000).

(Salum et al., 2012) reported that more changes were observed in diabetic group like elastic fibers showing focal irregular arrangement with decrease in intensity of staining of elastic fibers.

The changes observed along with time were in the extracellular matrix, particularly collagen content increased over time while the elastin steadily declined; an alteration known to increase vessel stiffness (Kolade et al., 2012)

The mechanism that possibly involved in the effect of diabetes on aortic stiffness may involve change in structural integrity and proportions of elastic fibers this explained by (Fiordaliso et al., 2006).

This study reported that significant decrease in thickness of TM was observed in the diabetic group compared to control group. In (STZ+ vitamin D) group, the thickness of TM was significantly increased (P-value < 0.002) compared to the diabetic group. However, TI thickness did not show significant differences among all the four groups. The ratio of TI: TM was found to be significantly increased in diabetic group compared to the control group and this disagreed with (Thent et al., 2012) there was marked increase in thickness of media in diabetic group compared to control group and (STZ+vitamin D) group so the TI:TM was found to be decreased in diabetic group compared to other groups.

However, there are studies with experimentally different durations of diabetes induced by STZ had shown that at an early stage, the thickness of the medial layer of aorta may be decreased mentioned by (Akgün-Dar et al., 2007) and, gradually increase with the developing of the disease due to vascular smooth muscle cell proliferation (Fukuda et

al.,2005)and this explain the results of the present study.

(Salum et al., 2014), reported that the width of the medial layer was decreased in the diabetic rats compared by control rats.

Astudy was done by(Thent et al.,2012) reported that under orcein stain, the elastic fibers were found to be distorted in the diabetic group than in the control and diabetic treated by vitamin D group and these results agreed with the present study.

There are clinical tools done by (Heilman et al.,2009) such as measurement of the intima-media thickness to detect structural changes in the wall of the vessel. The morphometric parameters showing changes early occur in the progression of type 1 diabetes and tend to become more dangerous and severe with the persistence of the disease, changes generally agreed with present study. Mildaortic wall changes, particularly medial elastic fibers did not have focal disarrangements in diabetic treated group. Untreated diabetes was also associated with decreased abundance of elastin that was prevented by vitamin D supplementation present this was reported by (Salum et al.,2012).

Vitamin D preserved structure of elastic fibers and ratio of elastic fibers to collagen fibers in the media of aorta, (Salum et al.,2012).

## Conclusion

According to the results of this study Vitamin D may play an important role to delay the degenerative changes of diabetes on cardiovascular system andand this improve cardiovascular function.

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