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Original Article

Effects of Moringa Oliefera on Thickness of Tunica Intima and Tunica Media In Aorta of Diabetic Rat

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ABSTRACT

Objective: To study the effects of Moringa Oliefera on thickness of Tunica intima and media in aorta of Streptozotocin induced diabetic rat.

Study Design: Laboratory-Based Experimental Study.

Place and Duration of Study: Army Medical College Rawalpindi, Pakistan and National Institute of Health Islamabad, Pakistan from Jun to Dec 2020.

Methodology: Ninety Sprague Dawley rats were randomly divided into a control group A, and two experimental groups B and C, each consisting of thirty rats. In both experimental groups High fat diet was given, diabetes mellitus was induced by injecting Streptozotocin at a dose of 35mg/kg body weight. Aqueous extract of Moringa Oliefera leaves at a dose of 200 mg/kg body weight was administered orally for 8 weeks to experimental Group-C. The animals were sacrificed at the end of experiment, aorta dissected out, fixed and stained with Elastin Von Geison (EVG) for histological analysis. Thickness of Tunica Intima and Tunica Media were measured on histological slides using image J software.

Results: Thickness of Intima of rat aorta in experimental Group-C was significantly less than Group-B but more than control Group A. Similarly, in intergroup comparison difference in mean thickness of tunica media of rat aorta in experimental Group-C was much less than Group-B but more than control Group A.

Conclusion: Moringa Oliefera has ameliorative effects against diabetes induced increase in tunica intima and media thickness.

Keywords: Diabetes Mellitus, Moringa Oliefera, Oxidative stress, Streptozotocin.

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INTRODUCTION

Diabetes Mellitus (DM) poses a substantial global health challenge, with projections indicating a rise to 300 million affected individuals by 2025[1]. This complex metabolic disorder involves chronic hyperglycemia resulting from reduced insulin secretion, insulin resistance, or both, impacting carbohydrate, protein, and lipid metabolism[2]. This sustained elevation in blood sugar levels triggers an overproduction of reactive oxygen species, leading to oxidative stress—a fundamental mechanism behind DM's detrimental effects[3].

Alarmingly, many individuals remain unaware of their diabetic status until complications arise, categorized as microvascular (retinopathy, nephropathy, neuropathy) and macrovascular (myocardial infarction, stroke, peripheral vascular disease)[4]. DM-induced atherosclerosis emerges as a primary factor contributing to these vascular complications. Atherosclerosis, a multifaceted disease, involves thickening of intima, smooth muscle cell proliferation, cholesterol deposition, primarily occurring post-endothelial injury in specific arteries[5].It stands as a leading cause of cardiovascular disorders, attributing to elevated mortality and morbidity rates[6].

The incidence of atherosclerosis escalates in DM due to impaired lipid metabolism, hypercholesterolemia, and exacerbated by chronic hyperglycemia. This sustained hyperglycemic state fosters an excessive production of reactive oxygen species, triggering cellular and molecular alterations in vascular cells, exacerbating structural and functional damage[7]. Unchecked, atheroma expansion can impede blood flow, causing vessel occlusion or ruptures leading to vascular thrombosis, compromising tissue perfusion. Therefore, prevention and proactive measures are pivotal in mitigating atherosclerosis progression induced by DM[7,8].

Moringa Oleifera, known as the "miracle tree," boasts abundant nutritive and medicinal properties. Its leaves harbor various natural antioxidants like

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ascorbic acid, flavonoids, carotenoids, saponin, tannic, phenolic, and alkaloid phytoconstituents[9,10]. While prior studies have highlighted Moringa's positive impact on DM by reducing blood sugar levels and preventing diabetic nephropathy, its potential anti-atherosclerotic effects in diabetes require further exploration[11,12]. If this study reveals Moringa's preventive effects on diabetes-induced atherosclerosis, it could become a valuable recommendation for diabetic patients.

METHODOLOGY

The lab based experimental research took place at the Department of Anatomy, Army Medical College in Rawalpindi, in collaboration with the National Institute of Health (NIH) in Islamabad and the Armed Forces Institute of Pathology (AFIP) in Rawalpindi. All animal procedures adhered to the ethics committee's approval from Army Medical College in Rawalpindi.

Inclusion Criteria: Sprague Dawley rats 70-80 days old of either gender with an average weight of 250±50 grams were included in the study.

Exclusion Criteria: Rats with any gross abnormalities or with blood glucose in the pre-diabetes range were excluded from the study

Ninety healthy Sprague Dawley rats were sourced from NIH, Islamabad, and housed in the NIH animal facility under standardized conditions. Non-probability convenience sampling was performed, and the rats were randomly divided into three groups as shown in Figure-I: a control group (Group-A) and two experimental groups (Groups B and C), each comprising thirty rats. Control Group-A received normal pellet diet whereas both experimental groups were subjected to a high-fat diet, 2 weeks before and 8weeks after inducing diabetes mellitus through the administration of Streptozotocin (STZ) obtained from Sigma, St. Louis, Mo, USA. A single intra-peritoneal injection of STZ 35 mg/kg body weight dissolved in 0.1M Sodium Citrate buffer; pH 4.5 was administered. Following the STZ injection, the rats were provided with a 5% glucose solution for overnight consumption. Diabetes development was confirmed 72 hours later by measuring random blood glucose levels above 250 mg/dl using a glucometer (On-Call® Plus, Model G113-111, USA) through tail vein puncture.[13]

Fresh leaves of MO plant were obtained from Department of Botany, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi. Leaves were washed under running water and air dried. The aqueous extract from these dried and powdered leaves was prepared in laboratory by mixing 1.5g of air dried, powdered leaves with 15ml pre heated water for 15min at 60^{o}C. Cooled mixture was filtered twice through a 2µm pore sterile filter paper into a sterilized tube. This extract was freshly prepared and administered once daily by oral gavage, to Diabetic rats in Group-C, for 8 weeks.[14]

The animals were sacrificed after three months of the experimental period. The blood samples for blood glucose level were collected at the start (72hrs after administration of Inj STZ) and end of experiment. The samples were labeled according to the groups. 24hrs after administration of last dose of MO extract to Group-C, all rats were euthanized by over dose of diethyl ether anesthesia. The animals were fixed on a dissection board with pins. A longitudinal midline incision was given, extending from upper end of sternum to pubic symphysis. The ribcage was opened, retracted and fixed with pins to the dissection board. The lungs were dissected and aorta was dissected out. The tissues after excision were put in 10% Formaline for fixation. The sample tissue obtained was enclosed in labelled cassette and passed through series of increasing concentrations of Alcohol from 70% to 100%, cleared in Xylene. The samples were then infiltrated and embedded with in Paraffin wax at a temperature of 58C. The Paraffin tissue blocks thus prepared were labelled similar to the cassettes. 5µm thick sections were cut using rotary microtome and mounted on a glass slide. The sections were stained with Elastin Von Geison stain (EVG). For histological parameters, slides were observed using Olympus CX21FSI light microscope. Images were taken using Olympus DP27 Camera and Cell Sense Entry software was used. For analysis, the image was opened in morphometric computer software Image J Version 1.49g. Micrometry was performed to calculate distance of one division of the ocular 40x magnification. This distance was then fed into the Image J program, and the scale was set for readings.

Three locations exhibiting the highest intimal thickness were chosen to capture images along the circumference of the aortic cross-section, positioned perpendicular to the internal elastic lamina. Using a linear tool, the measurement was taken from the endothelial margin to the internal elastic lamina. Three separate measurements of Tunica Intima were noted, and their mean value was computed as the final reading for each animal.[15]

For measuring Tunica Media, similar method was used. Three points were photographed in continuation with those captured for the intima, measuring from the internal elastic lamina to the external elastic lamina using a linear tool. Three readings were taken, and their mean value was calculated as the final reading for each animal.[15]

Data was analysed using computer software IBM SPSS (Statistical package for social sciences) version 21. Quantitative variables were expressed as Mean±Sd deviation. Analysis of variance (ANOVA) test was used to determine difference among various groups for quantitative and biochemical variables followed by Tukey's Post Hoc test. A *p*-value of 0.05 or less was considered statistically significant.

RESULTS

A total of 90 Sprague Dawley rats, without any gross abnormality were included in the study. The ameliorative effects of Moringa Oliefera were observed on blood glucose levels and thickness of TI and TM in aorta of diabetes affected rats. The BGL at the start of experiment (72hrs after administration of Inj STZ), showed statistically significant results (< 0.001) with profound hyperglycemia in Group-B (274.80 ± 14.03 mg/dl) and Group-C (277.07 ± 15.76 mg /dl) that confirmed the development of DM (blood glucose level >250mg/dl) in both the groups as compared to the control Group-A (75.80 ± 10.39 mg /dl). At the end of experiment, average final blood glucose levels of all the animals were assessed again. The mean final blood glucose level of Group-B (477.70± 59.24 mg /dl) was significantly high (p< 0.001) as compared to Group-A (109.10 ± 6.27mg /dl) and C(182.43 ± 27.48 mg /dl). However, mean final blood glucose level of Group-C was much less than Group-B but slightly raised than Group-A (p< 0.001) as shown in Table-I

One Way ANOVA was conducted among all groups to analyze the effects of DM and ameliorative effects of MO on Mean±SD thickness of Intima and Media of rat aorta. Control Group-A exhibited smooth endothelium; experimental Group-B showed disorganized, interrupted endothelium with vacuolated cells, thickened intima, increased sub-endothelial tissue, and mononuclear cell infiltration. Group-C displayed minimal histological changes. The intergroup Comparison, as shown in Table-II and Figure-I, it was observed that thickness of Intima of rat aorta in experimental Group-B (17.73±3.02 µm) with DM, was much increased and statistically significant (p< 0.001) than control Group-A (4.31±0.76 µm) and experimental diabetic-Group-C (7.11±1.36 µm) treated with MO. However, thickness of Intima of rat aorta in experimental Group-C was much less than Group-B (p< 0.001) but more than control Group-A (p< 0.001)

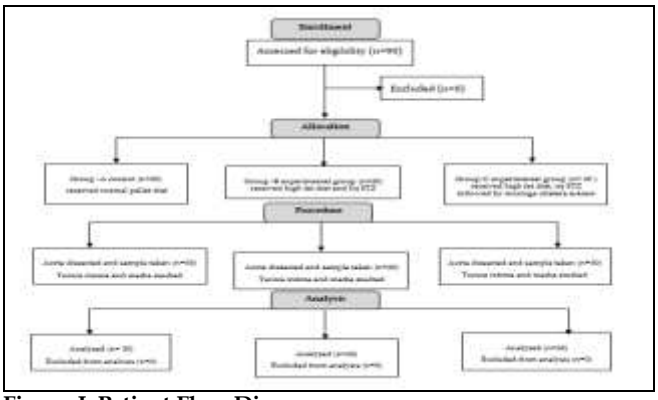

Figure-I: Patient Flow Diagram

Tunica Media of control Group-A showed well defined closely packed, concentric elastic lamella. However, TM of experimental Group-B was markedly increased in thickness with disrupted, distant elastic lamella, increased ECM proliferation with lipid deposition. The animals treated with MO extract in experimental Group-C showed normal to minimal change in histological architecture. In intergroup comparison, as shown in Table III and Figure-II,

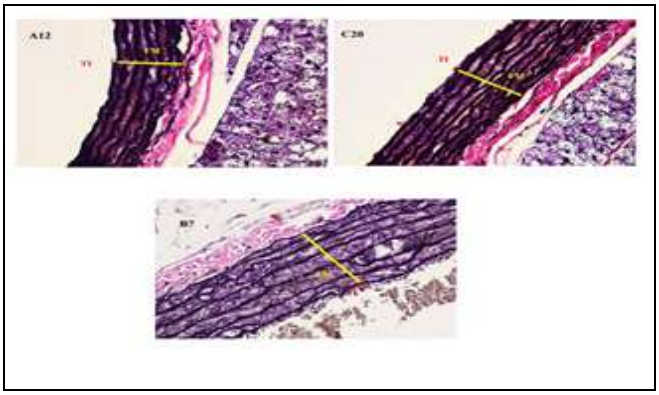

Figure-II: Photomicrograph showing comparison of thickness of Tunica Intima(TI) and Tunica Media(TM) of aorta of animals A12, B7, C20 from control Group-A, experimental groups B and C respectively 40X EVG stain

it was observed that mean thickness of Tunica Media of rat aorta in experimental Group-B (199.59±15.05 µm) with DM, was much increased and statistically significant (p< 0.001) than control Group-A (88.56±8.34 µm) and experimental diabetic Group-C (126.54±12.73 µm) treated with MO. However,

Table-1 Comparison of Blood Glucose Level (BGL) between control Group-A, experimental Group-B and Group-C (n=90)

Parameters	Groups(n=90)	Mean ±SD	Statistical Significance (*p*-value)		
			Group A/B	Group A/C	Group-B /C
Blood Glucose level (mg/dl) at the start (72hrs after Injection Streptozotocin)	A(n=30)	75.80±10.39	< 0.001	< 0.001	>0.05
	B(n=30)	274.80±14.03			
	C(n=30)	277.07±15.76			
Blood Glucose Level (mg/dl) at the end	A(n=30)	109.10±6.27	< 0.001	< 0.001	< 0.001
	B(n=30)	477.70±59.24			
	C(n=30)	182.43±27.48			

Table-II Comparison of Thickness of Tunica intima of rat aorta in control Group-A, experimental Group-B and Group-C (n=90)

Parameter	Groups	Mean ± SD	Statistical Significance (*p*-value)		
			Group A/B	Group A/C	Group-B /C
Thickness of Tunica intima (µm)	A (n=30)	4.31±0.76	< 0.001	< 0.001	< 0.001
	B (n=30)	17.73±3.02			
	C (n=30)	7.11±1.36			

Table-III Comparison of Thickness of Tunica Media of rat Aorta in control Group-A, Experimental Group-B and Group-C (n=90)

Parameter	Groups	Mean ± SD	Statistical Significance (p value)		
			Group A/B	Group A/C	Group-B /C
Thickness of Tunica Media (µm)	A (n=30)	88.56±8.34	< 0.001	< 0.001	< 0.001
	B (n=30)	199.59±15.05			
	C (n=30)	126.54±12.73			

difference in mean thickness of Tunica Media of rat aorta in experimental Group-C was much less than Group-B (p< 0.001) but more than control Group-A (p< 0.001)

DISCUSSION

In our study blood glucose levels of animals were taken before and after administration of STZ. A cut off value of 250mg/dl for blood sugar was taken as DM. Final blood glucose level was taken at the end of study. The mean final blood glucose level of Group-B was significantly high as compared to Group-A and C. However, mean final blood glucose level of Group-C was much less than Group-B but slightly raised than Group A. The results of our study demonstrated that low dose STZ administration developed sustained hyperglycemia in HFD-fed animals. However, administration of MO extract for 8 weeks significantly reduced and maintained the glycemic levels of diabetic rats, which clearly indicates its anti-hyperglycemic effect. The results were consistent in relation to previous studies published, documenting that MO leaf extract has significantly reduced glucose levels in type 2 DM.[16] The proposed mechanism being radical scavenging potential of MO which is able to reduce the chronic hyperglycemia induced oxidative stress and IR produced by HFD.[17]

The present study showed MO has protective effect against histo-morphological changes observed in diabetes induced atherosclerosis. The slides were observed for histological analysis of TI and TM. Normal histology was observed for both TI and TM. Smooth continuous endothelium with squamous cells was observed in aorta of normal control group. There was negligible sub endothelial connective tissue. TM showed well defined closely packed, concentric elastic lamella. In STZ induced diabetic HFD fed rats, the endothelium was disorganized and interrupted. The endothelial cells showed vacuolated appearance. TI was significantly thick with increased sub endothelial connective tissue with mononuclear cells infiltration. TM was also markedly increased in thickness with disrupted, distant elastic lamella, increased ECM proliferation with lipid deposition. The animals treated with MO extract showed normal to minimal change in histological architecture. The endothelium was continuous and organized with less sub endothelial connective tissue than diabetic non treated animals. Thickness of both TI and TM in aorta of MO treated animals was significantly less than STZ induced diabetic HFD fed rats, but more than control group rats. The results and findings of this study are consistent with previous researches on diabetic

animals which were treated with Vit E18 and Centella Asiatica extract[19] respectively, exhibiting similar antioxidant mechanism as MO extract. Hyperglycemia induced oxidative stress results in non-enzymatic glycation of proteins forming AGE and peroxidation of lipids such as polyunsaturated fatty acids from cellular membranes resulting in advanced lipoxidation end products, contributing to the vascular damage in DM. Free ROS and dyslipidemia caused by poor glycemic control is responsible for histopathological changes in blood vessels[20]. Hence, concluding that Intimal thickening is the earliest sign for micro and macro vascular damage.

CONCLUSION

Chronic hyperglycemia generates oxidative stress that causes vascular walls to thicken. Moringa Oliefera having rich antioxidant properties not only maintains glycemic levels but also has ameliorative effects against diabetes induced increase in tunica intima and media thickness.

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Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

MR & SH: Data acquisition, data analysis, drafting the manuscript, critical review, approval of the final version to be published.

SR & MOA: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

FS & MA: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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