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Original Research Article

Effect of Type 2 Diabetes Mellitus on Expression of Vascular Endothelial Growth Factor-A (VEGF-A) in various grades of Stenosis of Coronary Artery Segments: An Autopsy-based Study

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ABSTRACT

Background: VEGF has been reported as an important factor promoting coronary collateral formation in Ischemic Cardiac diseases. However, the complete mechanism and factors affecting VEGF expression during Acute myocardial infarction are not fully understood. The present study aims to correlate the effect of Diabetes Mellitus on VEGF expression in endothelial cells in various grades of Coronary artery stenosis.

Materials and Methods: Selective coronary angiography of both right and left coronary arteries were done to know the grade of occlusion in autopsy samples with a history of sudden cardiac death.5cm slice, of coronary artery segments were taken at the level of the highest grade of stenosis. Decedents hearts were selected as the control and cases based on patients history and 3D image build after selective coronary Angiography. In control group (without any coronary occlusion) segments were taken from the proximal part of both the Right and left coronary arteries for Analysis. A routine histo-technique was done to grade the stenosis in case study group on sections taken at the level of maximum occlusion. Immunohistochemical staining was done in all sections to know the VEGF expression of endothelial cells and infiltrated Monocyte. VEGF expression was then correlated based on the grades of stenosis, presence of cardiovascular risk factors like diabetes mellites and hypertension.

Results: The Coronary artery with less than 90% stenosis showed no substantial VEGF expression. A statistical significance was found when the VEGF expression was correlated with the various grade of stenosis and the presence of cardiovascular risk factors like diabetes Mellitus.

Conclusion: Considerable variation was shown in the Monocyte migration and expression of the VEGF in the endothelial cells of the coronary artery in Diabetic patients with higher grade of stenosis. Impaired monocyte migration might explain the reduced arteriogenic potential in diabetic patients when corelated with the control group.

KEYWORDS: Coronary artery disease, Selective Coronary Angiography, VEGF (Vascular endothelial growth factor), Arteriogenesis, Angiogenesis, Diabetes mellitus.

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INTRODUCTION

Coronary artery disease is a common heart condition that involves atherosclerotic plaque formation in the lumen of the coronary artery, it leads to impairment in blood flow and oxygen delivery in the myocardium [1].

It is a major cause of mortality and morbidity worldwide [2]. Within India, the rates of coronary artery disease vary markedly with higher rates in the states of Kerala, Tamil Nadu and Punjab [3]. The number of CAD deaths in South Asia is predicted to increase by another 50% in 2030 unless aggressive preventive efforts are taken [4].

Type II diabetes Mellitus is a prime risk factor for cardiovascular disease [5]. Compared with non-diabetic, Diabetic patients have diffused coronary atherosclerotic lesions [6]. Percutaneous coronary intervention and surgical revascularization in Diabetic patients experience worst outcomes than non-diabetic patients [7].

Coronary collateral circulation acts as an alternative source of blood supply to the myocardium in the presence of Advanced Coronary Artery Disease [8]. Well Developed coronary collaterals has a potential to alleviate Myocardial Ischemia and preserve residual contractility and reduce cardiovascular diseases [9]. Collateral scores were found less in diabetic patients when compared with non-diabetic patients [10].

Vascular Endothelial growth factor is potent mitogen for Endothelial cells. VEGF-A has been reported to promote collateral formation [11]. In several in vivo studies of regional myocardial Ischemia, VEGF-A is an inducer of collateral blood flow [12]. Previous studies done on collateral score correlating the serum level VEGF in Diabetic and Non diabetic patients showed no statistical significance [13]. The study of the effect of VEGF as a mitogen in the endothelial cells at the level of occlusion is an extremely difficult task in living subject and an autopsy study is the best way to work on it.

The present study was conducted to know the effect of Demographic and Cardiovascular risk factors on expression of VEGF in endothelial cells of Coronary artery segments with Various grades of stenosis in both Diabetic and Non diabetic Patients.

MATERIALS AND METHODS

Analytical cross-sectional study was conducted in 35 heart specimens of decedents recommended for autopsy in the Department of Forensic Medicine, District Hospital Palakkad. The study was done in the department of Anatomy, Government Medical college Palakkad and Department of Forensic medicine and Radiodiagnosis, District Hospital Palakkad. Ethical clearance for the study was obtained from the Institute of Integrated Medical Sciences (Government Medical College, Palakkad) Reference Number-**IEC/GMCPKD17/19**. The study period was from August 2020 to September 2023.

Subjects for the study were selected through consecutive sampling from the Department of Forensic Medicine, (District Hospital Palakkad) recommended for autopsy. 50 consecutive samples with mean age of 59 ±4.05 with complete clinical history taken before one month of death were included in the study. Only the patients, who died within 48hrs were recommended for the Autopsy were included for the study. Decedents with a history of malignancy, pulmonary disease, previous history of PCI and renal disease were excluded from the study.

Among the 50 autopsy samples 40 patients were with history of sudden cardiac deaths were taken as the cases and 10 with the history of death other than CAD taken as the control group. Post-mortem selective coronary CT angiography of the Isolated heart was done in 50 subjects parallel with the autopsy. Written informed consent was taken according to the rates of the Institutional Ethics committee which approved the study.

Selective coronary angiography:

The heart was removed and selective coronary angiography was performed. The heart was isolated with aorta 2-3cm above the coronary ostium. The tissue surrounding the coronary artery was removed and 6 Fr coronary Angio catheters which are shortened to 5cm were inserted into the right and left coronary ostium respectively and secured with the ligature at the side

of the aorta. After ligature, the catheter was pulled inside to a specific point. High viscosity contrast material composed of 15 ml of opaque 350 in 500 ml of Polyethyline glycol 250 were prepared. The solution was infused into an empty drip infusion set and put in a pressurised bag and the bag was pressurised to 120 -150 mm of Hg. CT was performed by continuous injection for 3 min (enhanced CT was performed without stopping perfusion) [14].

Left and right coronary angiography was performed separately. Pre image review and 3-D image were built up. The level of the maximum coronary lesion was noted from the 3D built-up image. It was then compared to dissection findings made during the autopsy. A 5 mm slice of the coronary artery was removed at the site of the maximum lesion (comparing both dissection findings of autopsy and angiogram). The heart was then handed over to the Forensic department after the evaluation within the time frame stipulated for completion of the autopsy.

After performing selective coronary CT angiography of 10 controls only 5 were found with normal coronary arteries (without coronary occlusion) and of 40 cases 30 were observed with occlusion in any one of the major coronary arteries.

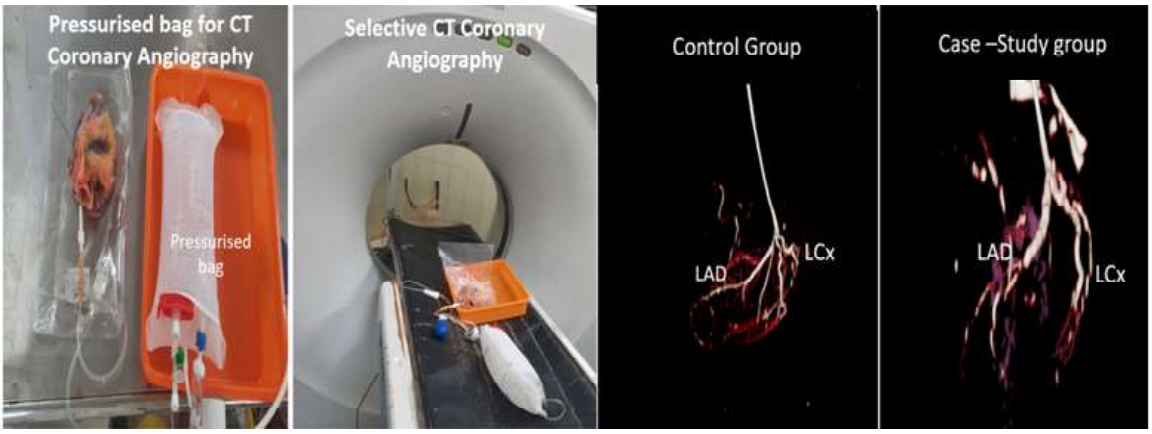
Documentation of the demographic factors and clinical history: Baseline data regarding clinical history, age, weight, and sex were obtained from the medical history after getting consent from the relatives of the patients. Diabetes Mellitus (DM) was defined as a history of DM, which is the use of antidiabetic drugs or fasting plasma glucose level of $\geq$ 7 mol/L. Hypertension (HTN)was defined as a history of Hypertension (HTN) or use of antihypertensive drugs, or blood pressure $\geq$140/90mm of Hg [15].

Histopathological Examination and Grading of A Stenosis:

Specimen size and fixation: The section of 5mm size was taken at the level of maximum occlusion of the coronary artery correlating the angiographic and forensic findings. Sections are then transferred to a tissue processing cassette. Each cassette was separately labelled with a diamond pencil. Fixation is done using 10% buffered formaldehyde solution. And routine biotechniques were done.

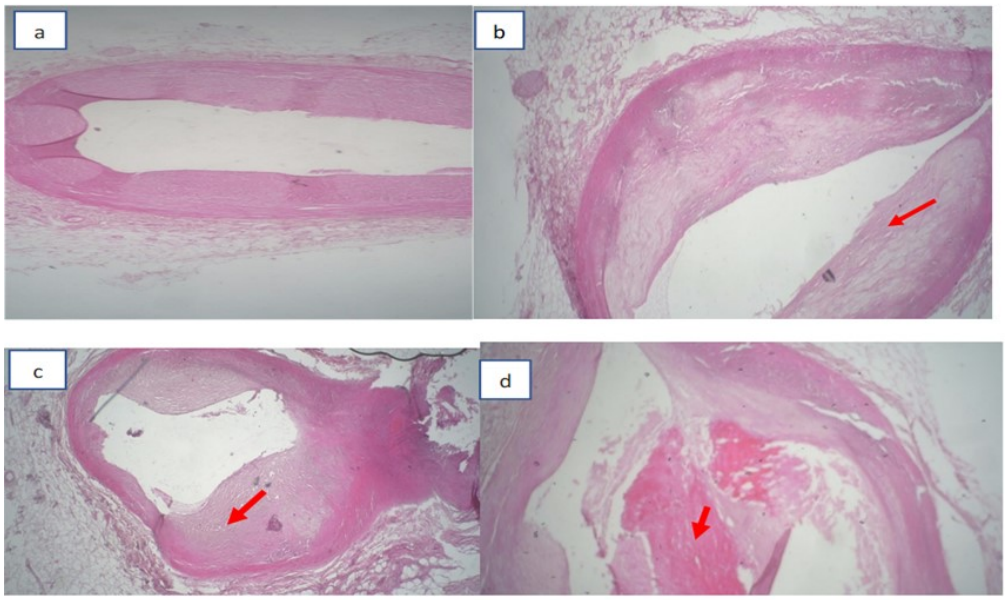
Grading of stenosis in coronary arteries: The grading of coronary artery lesions was done by the two experienced pathologists who were blind to the study protocol, clinical history and medical report of the patients. Microscopic assessment of the stenosis was done after taking digital photographs of coronary stenosis.

Grading of coronary stenosis was done under a four-point scale. Grade 0-normal coronary artery, Grade I-Hypercellular atherosclerotic plaque, Grade II-advanced atherosclerotic plaque and Grade III -Total occlusion in the lumen of the coronary artery [16].



The solution was infused in a drip Infusion set which was put in a Pressurised bag and the Bag was pressurized to 120-150 mm of Hg. CT was performed without stopping perfusion for 3 min. The 3D image was built up after Selective Coronary Angiography.

Fig. 1: Coronary CT angiography in Autopsy Samples.



a) *Grade 0-normal coronary arteries)*

b) *Grade I-hypercellular atherosclerotic plaque- well-defined lipid core with the luminal surface covered by intima (arrow show atherosclerotic plaque in the tunica intima* ***c)***

c) *Grade II- advanced atherosclerotic plaque-lipid core with fibrous cape (shows red arrow)*

d) *Grade III-total occlusion – Fibroatheroma with haemorrhage or thrombosis occluding the arterial lumen (Red arrow thrombus in the Lumen of the Artery)*

Fig. 2: Grading of coronary stenosis in coronary artery segments.

Immunohistochemistry Manual Staining:

For immunohistochemistry, the slides were deparaffinised before staining, with xylene I and xylenes II. The slides were then placed in 90% and 70% ethanol for 5 minutes. After deparaffinisation, the slides were washed with distilled water for 5 minutes. The washed slides were kept in Tris -EDTA buffer and were allowed to cool. After cooling, the slides were kept in Tri-EDTA buffer (pH 8.5-9.5) autoclaved (15 minutes) and were allowed to cool. After cooling, the slides were incubated in 3% hydrogen peroxide (H_2O_2) for ten minutes. The tissue on the slide was marked after washing in Tris Buffered Saline (pH 7.4-7.6) for 3-5 minutes, followed by incubation with primary antibody for 1 hour at room temperature VEGF-A antibody (ImmunoTag -Cat Ltt05513) 1:200 was used as the primary antibody. The slides were washed twice in Iris buffered saline (3 minutes each). Tissues in the slides were flooded with secondary antibodies (HRP conjugate -polymer-horseradish peroxidase anti-rabbit) and were incubated for 30 minutes at room temperature. After incubation, the slides were washed with TrisBuffered saline four times followed by the addition of development chromogen (DAB-3,3'-diaminobenzidine) to visualize the protein. The slides were further washed in distilled water four times. The slides were counterstained with Haematoxylin for one minute and were washed with distilled water, hydrated, cleaned and mounted using D.P.X. The slides were finally observed under a phase contrast microscope (Olympus CKX 41 with Optika Pro5 CCD) camera and microscopic observations were captured.

Quantification of VEGF expression on Endothelial cells:

The surface area containing VEGF-positive endothelial cells was quantified by the use of computer-aided planimetry and expressed as a percentage of the total surface area of Endothelial cells. The area is termed as VEGF -positive endothelial area [17].

$$\text{VEGF positive endothelial cell area} = \frac{\text{Area occupied by the VEGF + endothelial cells X 100}}{\text{Area occupied by the Endothelial cell}}$$

$$\text{VEGF-positive cell area} = \frac{\text{Area occupied by the VEGF - cells in tunica intima X 100}}{\text{Area occupied by the Endothelial cells}}$$

The statistical package for social science (SPSS) 21 for windows was used for statistical

analysis. All the p-values are considered with a significant level <0.05.

Of the total number of 35 subjects, 30 were with occlusion in any one of the major coronary arteries and in the remaining 5 subjects (control) were with no significant occlusion was found in angiogram (with normal coronary artery).

Continuous variables are presented as mean ± standard deviation and categorical data are summarized as frequencies or percentages. The differences between groups were analysed by Chi-square test for categorical clinical variables and independent sample students **T** - test for continuous variables. Correlation between VEGF expression and morphological and cardiovascular risk factors on patients with various grades of stenosis was done by ANOVA (Analysis of Variance). All p -values are considered with a significant level of < 0.05.

RESULTS

Grading of coronary stenosis in coronary artery segments: Sections of the coronary arteries were taken from both case and control groups. In the case study group sections were taken from the proximal part of coronary occlusion. Of the 35 sections taken 5 had grade 0 stenosis,7 sections had grade 1 stenosis and 15 had grade 2 stenosis and 8 had grade 3 stenosis

Quantification of VEGF-positive endothelial cell area: Area of Quantification of VEGF-positive endothelial cells was quantified using computer-aided planimetry and expressed as a percentage of the total surface area occupied by VEGF-positive Endothelial cells. *And the VEGF positive cell area expressed as a percentage of total surface area occupied by VEGF positive cells.*

VEGF positivity in endothelial cells of normal coronary artery sections:

Normal Coronary arteries -Grade -0 Control group n=5: The VEGF staining was found virtually negative in almost all 5 sections of the coronary artery .VEGF $^{+}$ endothelial cellsarea of 5 samples were taken and the mean was taken as the true value.Endothelial cells and smooth muscle cells were virtually negative for VEGF staining

The VEGF $^{+}$endothelial cell area was **0.350±0.196 %.** And VEGF $^{+}$ cell area was **0.551±0.06 %**

Hypercellular Atherosclerotic Lesion - Grade 1 lesion: n=7

Macrophages were observed scattered throughout the intima showed no VEGF positivity. VEGF-positive staining was seen in some of the endothelial cells (occasionaly)and in some macrophages and smooth muscle of Tunica media .

The VEGF $^{+}$endothelial cell area was =**23.08±1.720 %** And VEGF $^{+}$ cell area was **25.532±0.26 %**

Advanced Atherosclerotic Lesion:

Grade 2 lesion: n=15

In advanced atherosclerotic plaque a distinct lipid core with abundant macrophages and smooth muscles were observed. Endothelial cells show distinct VEGF positivity Macrophages in the tunica intima showed VEGF positivity. The VEGF $^{+}$endothelial cell area was **27.48±3.382%.** The VEGF $^{+}$cell area was **30.132 ± 0.216%.**

Total occlusion >90% of occlusion n=8

Grade -3 n=3

Advanced atheromatous plaque with a distinct core of lipid and fibrous cap were observed. A distinct VEGF-positive endothelial cells with VEGF-positive macrophages in the tunica intima were noted. The VEGF^{+}endothelial cell area was=**33.217±1.2746%.** Infiltrated macrophage cells of tunica intima and tunica media show VEGF positivity. The VEGF $^{+}$cell area was=**34.22 ±0.56%.**

Effect of diabetes mellitus on VEGF expression in different grades of Stenosis: Among the 35 patients,5 patients had normal coronary arteries without any occlusion. In these 5 patients, 4 are without diabetes mellitus and 1 with diabetes mellitus. In the grade one lesion, 4 are without DM and 3 with DM. In grade 2 stenosis group, 2 are without diabetes mellitus and 13 with DM. In 8 patients with grade 3 stenosis, 3 are without diabetes mellitus and 5 were with DM.

In 35 patients 5 were the control group with normal coronary arteries of which no patients had hypertension. In patients with grade 1 stenosis 6 had normal blood pressure and 1 had high

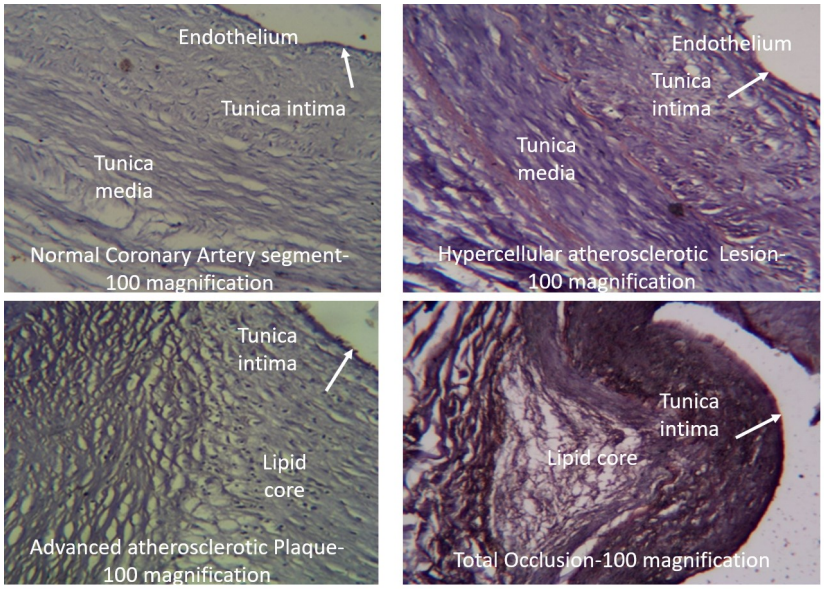


Fig. 3: VEGF positivity in endothelial cells after Immunohistochemical staining in coronary artery Segment.

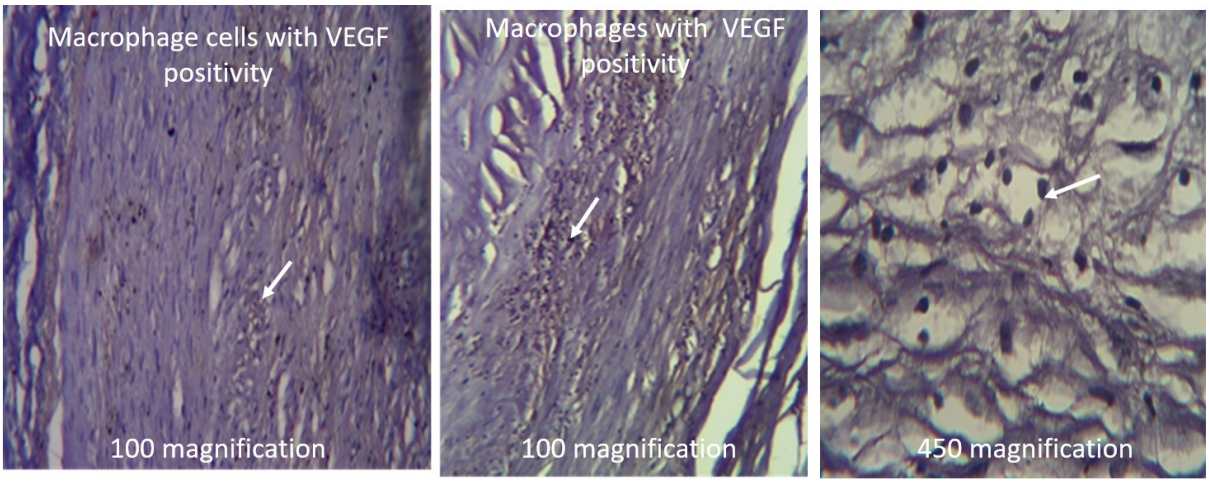


Fig. 4: Infiltrated macrophage cells of tunica intima and tunica media showing VEGF positivity (reddish Brown Stain).

blood pressure. Of patients with grade 2 stenosis five had normal blood pressure and nine with high blood pressure. In patients with grade 3 stenosis,5 patients had normal blood pressure and 3 had high blood pressure.

Correlation of VEGF expression with cardiovascular risk factors in various grades of stenosis: Effect of Demographic factors on VEGF expression on various grade of stenosis:

Table 1: Effect of age on VEGF expression in various grades of coronary stenosis.

Grade of stenosis	Age	N	Mean	Std. deviation	p-value
0	<60	3	0.387	0.197	0.564
	≥60	2	0.2945	0.258	
1	<60	5	23.28	1.765	0.699
	≥60	2	22.6	2.156	
2	<60	7	27.105	3.2413	0.291
	≥60	8	27.81	3.685	
3	<60	6	32.86	1.272	0.131
	≥60	2	34.27	0.565	

When the VEGF expression is correlated with grades of stenosis in old age and middle age group no statistical p-value was obtained, which shows age has no correlation with VEGF expression in various grades of stenosis. ($p<0.05$ was considered statistically significant)

Effect of sex on VEGF expression in various grades of coronary stenosis:

Table 2: Effect of sex on VEGF expression in various grades of coronary stenosis.

Grade of stenosis	Sex	N	Mean	Std. Deviation	p-value
0	Male	6	0.35	0.1966	-------
	Female	0	------	-------	
1	Male	5	22.59	1.397	0.245
	Female	2	24.315	2.397	
2	Male	10	27.69	3.804	0.853
	Female	5	24.315	2.669	
3	Male	6	33.148	1.277	0.502
	Female	2	33.425	1.76	

When the grade of stenosis is correlated with the VEGF expression with sex no statistically significant correlation was found($p>0.05$ was non-significant, and a value of $p<0.05$ was considered statistically significant).

Table 3: Effect of diabetes mellitus on VEGF expression in different grades of stenosis.

Grade of stenosis		Mean ±SD	Median (mini-Max)	p-value
0	NDM (4)	0.393±0.1974	0.447 (0.112-0.567)	0.48
	DM (1)	……	……	
1	NDM (4)	23.23±0.989	22.31 (22.16-24.130)	0.48
	DM (3)	22.90±2.706	21.61 (21.08-26.01)	
2	NDM (2)	28.15±0.663	28.15 (24.15-32.16)	0.864
	DM (13)	27.37±3.25	26.13 (23.14-32.16)	
3	NDM (3)	32.73±0.981	32.18 (32.16-33.87)	0.022
	DM (5)	26.738±2.62	26.13 (24.13-31.17)	

A statistical significance in p-value is obtained when the expression of VEGF in the endothelial cells is correlated with Diabetes mellitus in grade 3 stenosis. ($p<0.05$ was considered statistically significant) (NDN-Non diabetes mellites -Diabetes mellitus)

Correlation Diabetes with VEGF expression of infiltrated monocyte in the Tunica intima in different grades of stenosis:

Table 4: Effect of Hypertension on VEGF expression in different grades of stenosis.

Grade of stenosis		Mean ±SD	Median (mini-Max)	p-value
0	NBP (5)	0.350±0.196	0.418 (0.112-0.567)	
	HTN (0)	……	……	
1	NBP (6)	22.60±1.250	23.91 (21.08-24.130)	0.134
	HTN (1)	……	……	
2	NBP (5)	28.08±3.79	26.13 (24.15-32.16)	0.33737
	HTN (9)	27.62±3.19	26.13 (24.13-32.16)	
3	NBP (5)	28.34±3.05	26.13 (26.13-32.160)	0.445
	HTN (3)	30.06±5.20	32.18 (24.13-33.87)	

Table 5: Effect of diabetes mellitus on VEGF expression in macrophages in tunica intima in different grades of stenosis.

Grade of stenosis		Mean ±SD	Median (mini-Max)	p-value
0	NDM (4)	0.551±0.06	0.467 (0.212-0.801)	0.46
	DM (1)	……	……	
1	NDM (4)	25.69±0.929	24.32 (24.16-27.27)	0.95
	DM (3)	24.78±0.030	23.26 (22.28-28.21)	
0	NDM (2)	31.32±0.68	31.32 (27.38-35.26)	0.028
	DM (13)	28.90±3.25	27.74 (27.13-34.24)	
1	NDM (3)	35.18±0.621	35.18 (34.26-36.87)	0.018
	DM (5)	33.64±0.62	33.64 (32.18-35.29)	

A significant p-value was found in VEGF expression and VEGF-positive cells infiltrated in the tunica intima of blood vessels when correlated in Diabetic and Non-diabetic subjects in Grade 2 and 3 stenoses.

The above study on live patients shows that morphological factors like proximal location of the lesion in RCA, higher grade of lesion and severity of the lesion affect collateral formation. Diabetes mellitus causes low collateralization. Studies in **S**erum level VEGF shows high collateral formation is directly related to high serum VEGF level. Autopsy samples show low collateral scores compared to live patients. VEGF expression in endothelial cells was found to correlate with the grade of stenosis and severity of disease and Artery affected. Impaired VEGF expression was found in the Diabetic patient with higher grade of stenosis.

Univariate analysis of Variance -independent effect of factors on VEGF expression in the Endothelial cells of the occluded artery:

Table 6: Univariate analysis of variance -The independent effect of factors on VEGF expression in the endothelium. (VEGF endothelial area).

Source	Type III sum of squares	df	Mean square	F	sig
Intercept	9449.90	1	9449.90	1686.30	0.001
Grades of stenosis	740.79	3	246.93	44.06	0.001
DM	29.64	1	29.64	5.29	0.039
HTN	2.22	1	2.22	0.40	0.540

A higher grade of stenosis showed a significant p-value 0.001 (p-value <0.05) when correlated with the expression of VEGF on endothelial cells. Cardiovascular risk factor-Diabetes mellitus also showed a significant p-value 0.039 when correlated with the VEGF expression of endothelial cells

From the above it was understood that the VEGF expression of the endothelial cells are mainly depended on factors like grade of stenosis, Diabetes mellitus. Thus, diabetes mellitus can be considered as an important variable which effect the collateral formation in coronary artery disease.

DISCUSSION

The autopsy study provides a means of better understanding the basic process which sets the stage for clinically significant atherosclerotic cardiovascular disease [18]. There is no valid method of sampling the living population. The study of the effect of VEGF as a mitogen in the endothelial cells is an extremely difficult task in living subjects and an autopsy study is the best possible way to work on it.

The study conducted on post-mortem specimens by Vandana et al., (2016) concluded that males are more prone to cardiovascular disease than females and the common type of atherosclerosis was type III [19].

It was also concluded that the left anterior descending artery was the most frequently involved vessel. In the present study, the number of male patients was more when compared to female subjects. In comparing the artery most affected by CAD it was LAD which was similar to the previous studies. Inukochi et al., (2013) studied the utility of post-mortem computerized angiography as supportive evidence for finding ischemic heart disease during autopsy [14].

In the present study, the same method was found to be an effective technique to detect the level of coronary occlusion.

Studies done on 38 coronary artery segments by Inoue et al., (1998) concluded that a distinct expression of VEGF and its receptors was found in various grades of atherosclerotic lesions in coronary arteries [17]. In the present study also an increase in the expression of VEGF in the endothelial cells was found in various grades of stenosis. Endothelial cells are the prime targets of VEGF.

Herttuala et al., (2007) in their study concluded that VEGF -A receptors are not found in normal coronary artery segments, but their expression increases in Endothelial cells of micro capillaries in atherosclerotic lesions [20]. Chronic stress induces atherosclerosis in tunica intima and plaque instability which promote angiogenesis this in turn is related to an increase in serum VEGF -A level. In the present study also a potent increase in the expression of VEGF -A in Endothelial cells were

observed when the grade of stenosis increased [21].

Macrophages seem to play a central role in inducing the proliferation of vascular wall cells as in vascular wall remodelling. Furthermore, it has been shown that the release of growth factors such as fibroblast growth factor -2 (FGF-2) by macrophages directly enhances the collateral walls [22].

Briefly, growth factors released from macrophages induce the proliferation of Endothelial and smooth muscle. VEGF-A has shown to be an inducer of enhanced collateral blood flow as it is considered to be an inducer of monocyte migration. In the present study on autopsy samples, great variation in the expression of VEGF-A in the endothelial cells was found between the subjects with and without diabetes mellitus, especially in the cases of high-grade stenosis and severity of the disease. These data support the hypothesis that impaired monocyte migration might explain a reduced arteriogenic potential in the diabetic heart when compared to the non-diabetic heart which causes reduced expression of VEGF-A in the endothelial cells. As diabetes mellitus is associated with impairment of collateral vessel formation as well as an impairment of VEGF-A-induced monocyte function, any treatment strategy using VEGF -would likely be less effective in diabetic individuals compared to non-diabetic individuals.

CONCLUSION

In autopsy samples a distinct expression of VEGF -A was found in samples with a higher grade of stenosis, the severity of disease and in the presence of cardiovascular risk factors like diabetes mellitus. Reduced expression of VEGF-A in the endothelial cells of Diabetic patients may be due to endothelial dysfunction in diabetes mellitus caused by high glucose levels in the endothelial cell. Impaired monocyte migration might explain the reduced arteriogenic potential in diabetes hearts when compared to non-diabetic patients.

As diabetes mellitus is associated with impairment of VEGF-A-induced monocyte function any treatment strategy using VEGF –A would likely be less effective in diabetic individuals compared to non-diabetic individuals. In sections of the coronary artery, VEGF-A expressions and monocyte recruitment were found impaired in diabetic patients when compared with non-diabetic. This may be the reason for the low collateral score in diabetic patients and thus treatment strategies with VEGF -A are found less effective in diabetic patients.

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Author Contributions

Sheeja Balakrishnan: Contributed to the concept, Design, analysis and interpretation of the Data.
Senthil Kumar B: substantial role for revising the manuscript.
P B Gujral and Jerry Joseph K contributed valuable guidance to the Autopsy part of the study.

Conflicts of Interests: None

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