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Experimentally Induced Hyperlipidemia and Associated Atherosclerosis in the Aorta and Vascular Expression of CD 31, CD44, Beta-catenin and E Cadherin in Wistar Male Rats

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ABSTRACT

Background: Hyperlipidemia is the disorder of lipid metabolism, characterized by elevated serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and very low-density lipoproteins cholesterol (VLDL-C) and decreased high-density lipoprotein cholesterol (HDL-C). Atherosclerosis is a cardiovascular and fibroproliferative inflammatory disease commonly associated with dyslipidemia.

Methods: The study was conducted using 24 Wistar male rats, divided into two groups of 12 rats each. Hyperlipidemia and atherosclerosis were induced by the addition of 1% cholesterol and 15% saturated oil to the 1000 g of standard rat diet and given to group II rats. Group I kept as control and maintained for 90 days.

Result: The present experiment established hyperlipidemia with a significant increase of TC, TG, LDL-C and decreased HDL-C cholesterol with initiated atherosclerotic lesions in the aorta and showed varying degrees of positivity with CD31, CD44, Cadherin and Beta-catenin proteins.

Key words: Atherosclerosis, Beta-catenin, Cadherin, CD31, CD44, Hyperlipidemia.

INTRODUCTION

Hyperlipidemia is the elevation of serum TC, TG, LDL-C and VLDL-C and decreased HDL-C levels (Rahaman *et al.,* 2013). It is associated with cardiovascular diseases (CVD), including coronary heart disease and stroke. Hyperlipidemia is considered as the primary mediator cascade of atherosclerosis (Balakumar *et al.*, 2007), pancreatitis, renal injury (Attia *et al.,* 2002) and hereditary familial hypercholesterolemia (Frederick 2009). Understanding of lipid profile, alteration of aortic changes in hyperlipidemia helps in the formulation of various therapeutic agents to counter act hyperlipidemia and its associated conditions.

Vascular adhesion molecules, including selectins, vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1), PECAM-1, JAMs and connexins play a role in atherosclerosis (Elena and Klaus, 2007).

Platelet-endothelial cell adhesion molecule (PECAM)-1 is a type-1 transmembrane glycoprotein of the immuno-globulin super family that is expressed on platelets, most leukocyte subsets and at endothelial cells junctions. PECAM-1 has both pro and anti-inflammatory roles in atherosclerosis (Harry *et al.,* 2008 and Goel *et al.,* 2008). PECAM-1 is expressed at high density at the lateral borders of EC and lower density on the surface of hematopoietic and immune cells, including macrophages, neutrophils, monocytes, mast cells, natural killer cells, lymphocytes and platelets.

CD44 molecule mediates through endothelial adhesion of lymphocyte and monocyte and stimulates the release of various cytokines. CD44 also acts in atherosclerotic plaque destabilization and neointimal proliferation (Protasiewicz and Adamiec 2005). Altered CD44 expression might influence the extrinsic coagulation cascade and therefore, may affect thrombus formation (Sojberg, 2007).

Cadherins are a family of cell adhesion molecules that mediate Ca2+dependent hemophilic cell-cell interactions (Angst *et al.*, 2001). Endothelial cells express E-cadherin (Lampugnani *et al.,* 1992), N cadherin (Alexendar *et al.*, 1993) and T cadherin (Wyder *et al.,* 2000 and Ivanov *et al.,* 2001), The pattern of E Cadherin expression is different in different types of atherosclerotic lesions suggest that E-cadherin is

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involved in the formation and progression of lesions. No E-cadherin expression was found in normal non-atherosclerotic intima (Yuri *et al.,* 1998).

Catenins belong to the Armadillo family proteins. They are members of cell-adherent junctions and bind the cytosolic tail of cadherin. The cadherin-catenin complex is a target for many cell signaling pathways involved in adhesion, proliferation and cell motility (Hoschuetzky *et al.,*1994).

Role of the β-catenin pathway in SMC proliferation induced by oxLDL and the immunohistochemical staining of the human atherosclerotic aorta with anti-active β-catenin antibody showed an increased active β-catenin level in disrupted plaque associated with more intense positive staining in SMC layers located under the core when compared to bare expression in fibrous plaque (Aurelie *et al.* 2008).

MATERIALS AND METHODS

Procurement of experimental animals

Male wistar rats (n=24) weighing around 200 g were procured from Sri Venkateswara Agencies, Bangalore and kept for acclimatization for one week. The rats were grouped into two (n=12 each) and housed in standard polypropylene rat cages and maintained at 25±10°C and a 12:12 h interval light/dark cycle throughout the experimental period of 90 days. Experiment was carried out in the Department of Veterinary Pathology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati in the year 2017-18. The approval of the institutional animal ethical committee was obtained before the commencement of the experiment.

Source of chemicals and IHC kits

Cholesterol extra pure, AR grade with product code No: 77 97900 was procured from the SRL Fine Chemicals, Indian Scientific, Tirupati, Andhra Pradesh. CD31, CD44, Cadherin and Beta-catenin kits purchased from Biogenic ltd, Bangalore.

Experimental design

Hyperlipidemia was experimentally induced by feeding 1% cholesterol and 15% hydrogenated oil in 1000 g standard rat chow diet (High cholesterol diet).

Clinical observations

Health condition, behavior, feed and water intake of all the rats were monitored every day and recorded clinical signs and body weight.

Hematology

Blood samples were collected in 10% EDTA at each sacrifice from all the sacrificed rats and used for the estimation of TEC, TLC, PCV by microhematocrit method Jain (1986) and Hb by Sahli's method (Coles, 1986).

Biochemical parameters

At each sacrifice, blood samples from all the groups were collected into the sterile test tubes. After the blood clots formation, clear serum samples were separated without RBC and stored at 4°C. Estimation of TC, LDL-C, VLDL-C, HDL-C and TG was carried out by using commercially available biochemical kits (Auto Span Diagnostics, Bangalore).

Histopathology

Small tissue pieces of the aorta were collected in neutral buffered formalin for routine histoprocessing by paraffin embedding technique and section were stained with Haemotoxylin and Eosin [H&E] (Culling,1974).

Immunohistochemistry

Paraffin sections were cut at the 3-4 µ thickness and mounted on APES coated slides and incubated overnight at 37°C. Deparaffinized through xylene 15 min for two changes and two alcohol dips to remove xylene. Washed under running tap water for 10 min and distilled water rinsing for 5 min. Kept in the citrate buffer for 20 min (10 min at medium power and 10 min at high powering the micro oven). Cooled at room temperature and then kept in the distilled water for 5 min and in PBS for 5 min. The slides were held in the humid chamber and in the peroxidase block solution for 30 min to block the endogenous peroxidase and washed in PBS for 5 min for three changes. The power block solution was put on the tissue section for 15 min. Primary antibodies of CD31, CD44, Cadherin and β-catenin were added on the tissue sections of the aorta; then, slides were kept in room temperature for two hr, washed in PBS for 5 min for three changes. Added super-enhancer solution: The slides were kept in PBS for 5 min for three changes and added secondary antibody with HRP for 30 min. Washed in PBS for 5 min for three changes. DAB coloring reagent was prepared by adding one drop of DAB in 1 ml of a substrate. The sections were kept in the coloring reagent for 5-8 min and washed in PBS for 2 min and in tap water for 2 min. Stained with Harris haematoxylin for 1 min and washed in tap water for 5 min, dried and mounted in DPX.

Statistical analysis

The results were analyzed statistically by performing one-way ANOVA (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

Clinically obesity, sluggishness, poor hair coat and a non-significant increase in body weight were observed in group II rats in comparison with the control group I rats (Faheemuddin *et al.,* 2013). Results of TEC, PCV and Hb% of group I rats was normal and non-significant throughout the experimental period. Total leukocyte count in the hyperlipidemic diet-fed group II was non-significantly higher when compared to the control group. Mohamed Anwar *et al.* (2008), who observed increased WBC and lymphocyte levels in rabbits that were fed with high cholesterol diet (Table 1) (Huang *et al.,* 2001).

Rats on hyperlipidemic diet showed a significant ($P<0.05$) increase in serum TC, TG, LDL-C, VLDL-C and a significant decrease in HDL-C compared with control rats received

standard basal diet throughout the experimental period (Naiel Abbass *et al.*, 2012; Rahaman *et al.,* 2013). Increased serum lipid parameters in the present study might be due to hyperlipidemic diet and it indicated that the diet under trial had established hyperlipidemia in this group of rats.

Hyperlipidemic diet significantly (P<0.05) increased the atherogenic index by the end of experiment in the hyperlipidemic diet-fed group II rats compared to control rats fed on the standard diet (Rahman *et al.,* 2013; Harini *et al.,* 2016). It indicates that HDL-C (Good cholesterol) is decreased in proportion to increased TC, LDL-C and it is significantly (P<0.05) evidenced by all serum biochemical parameters (Table 1).

Hyperlipidemic diet-fed group II rats revealed grossly pale and enlarged liver in most of the rats (Fig 1). Microscopically, microvesicular hepatic steatosis was observed predominantly and macrovesicular fatty change in a few rats (Fig 2) (Olubukula *et al.,* 2012). Liver steatosis was absent in the control group.

Aorta from hyperlipidemic diet-fed group II rats revealed moderate initiation of atherosclerotic lesions with degeneration of endothelial cells, subintimal lipid-laden macrophages (foam cells), slight thickening of the tunica intima with the proliferation of few SMCs. Disruption of the elastic lamina of tunica media with structural and directional changes in the myocytes was also seen in a few cases. Atheromatous plaque consisted of foam cells and SMCs and in some thrombus formation in the aorta was also evident. Similar changes were also observed in the aorta of rabbits fed with 1% cholesterol for seven weeks (Olubukula *et al.,* 2012). Disruption of the elastic lamina of tunica media with structural and directional changes in the myocytes was also seen in a few cases. Coleman *et al.* (2006) Stary *et al.* (1995) also described thrombus formation in the aorta of humans as a type VI atherosclerotic lesion. No atherosclerotic changes were observed in the control group I rats.

CD 31 expression was noticed in ECs, platelets and most of the leukocyte subsets and at the endothelial cellular junctions (Fig 3) It might be due to the high level of platelets and other leukocytes in the atherosclerotic lesions and at

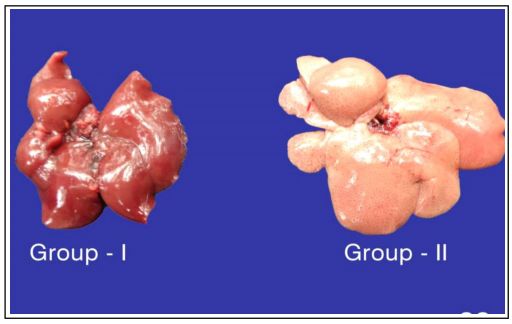

Fig 1: Liver: group II: Enlarged and pale liver compared to normal liver of group I.

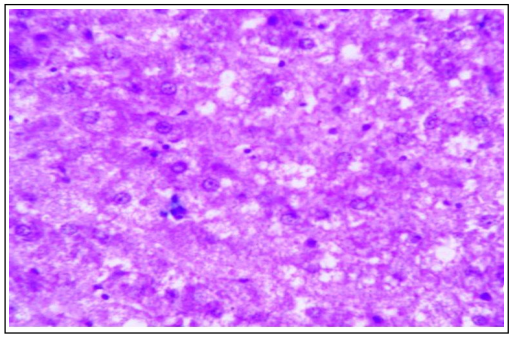

Fig 2: Liver: group II: Section of liver showing mild to moderate micro and macro vascular fat vacuoles in the hepatocytes. H&E×400.

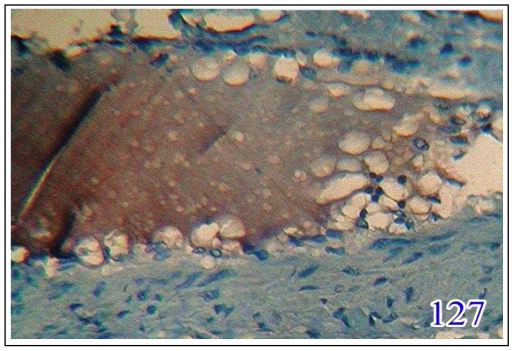

Fig 3: Aorta: group II: Note small blood vessel showing endothelial degeneration, sub intimal fat cells with attached thrombus stained positively with CD31. CD31×400.

Table 1: Mean values of clinical, serum biochemical and hematological parameters of rats by 90th day of experiment.

Parameters	Group I	Group II
Body weight (grams)	307.16±10.1	392.33^{a}±21.5
Total erythrocyte count (Million/mm^3)	6.31±0.2	6.81±1.1
Total leukocyte count (x^{mm3} µl)	10.20±0.56	16.2±0.71
Packed cell volume (%)	37.84±1.4	32.4±2.0
Haemoglobin (Hb) (g%)	12.62±0.5	10.9±0.7
Total cholesterol (mg/dl)	51.66±7.03	154.3^{a}±10.57
Triglycerides (mg/dl)	67.16±11.06	177.2^{a}±39.83
Low density lipoprotein cholelsterol (mg/dl)	20.15±2.7	95.9^{a}±9.1
Mean value of VLDL cholesterol (mg/dl)	13.42±2.21	31.0^{a}±8.79
High density lipoprotein cholesterol (mg/dl)	27.12±5.16	18.4^{a}±3.95
Atherogenic index (TC/HDL-C)	3.12±0.64	5.55^{a}±0.88

Mean values with different superscripts differ significantly (P<0.05).

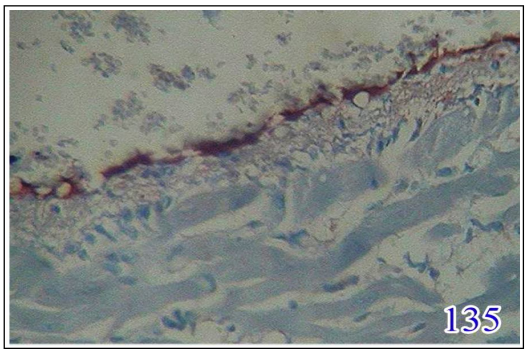

Fig 4: Aorta: ×roup II. Section of aorta showing endothelial cell denudation, sub endothelial foam cell accumulation under the endothelium stained positively with CD44 antibody. CD44×400.

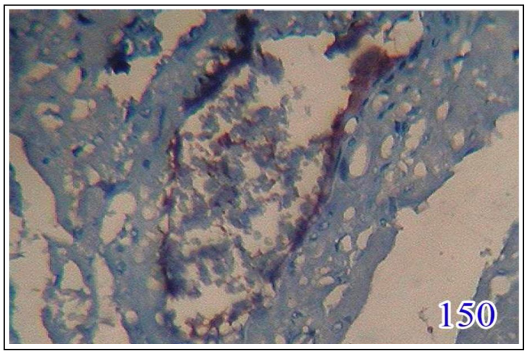

Fig 5: Aorta: group II: Note small blood vessel in between cardiac muscle fibers showing E cadherin positive cells with mild endothelial degeneration. E cadherin×400.

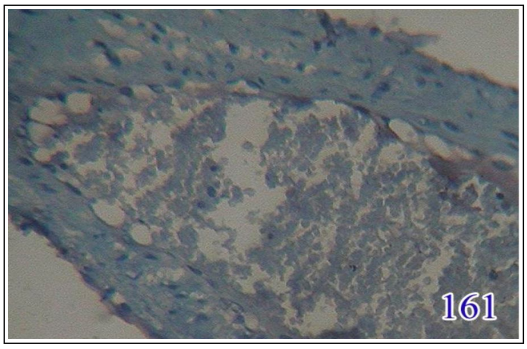

Fig 6: Aorta: group II: Section of the aorta showing endothelial degeneration, sub endothelial fat cells stained positively with β-catenin. Beta catenin×400.

the junction of thrombus in the hyperlipidemic diet-fed group II. CD31 is proatherogenic and expressed throughout the atheroma of the aorta (Newman 2003; Woodfin *et al.,* 2007). PECAM-1 has both pro and anti-inflammatory roles in atherosclerosis (Harry *et al.,* 2008; Goel *et al.,* 2008).

CD 44 IHC antibody positivity was observed in the endothelial cell degeneration, subendothelial foam cell accumulation and in the thrombus also in the small blood vessels of cardiac muscle fibers with a few fat cells with attached microthrombus (Fig 4). The complete absence of CD 44 reaction was observed in the control group I. CD 44 mediates endothelial adhesion of lymphocyte and monocyte and stimulates cytokine release from macrophages and participates in the dedifferentiation of smooth muscle cells. CD44 also acts in atherosclerotic plaque destabilization and neointimal proliferation (Protasiewicz and Adamiec 2005; Sojberg 2007).

E cadherin positivity was observed in the endothelial damaged regions, foam cell accumulated areas, thrombus attached parts and the initiated atherosclerotic plaques of the large aorta and in the small cardiac vessels of the group II rats (Fig 5). Other than lesion parts of the vessel showed no reaction with E cadherin IHC antibody indicating its selective staining of lesions. Control group I revealed typical histological structures of the aorta without E cadherin positivity. Selective expression of E cadherin was observed in the atherosclerotic lesions of the group II rats indicates that it is playing a role in the initiation and progression of the atherosclerotic lesions. Yuri *et al.* (1998) reported that the pattern of E cadherin expression is different in different types of atherosclerotic lesions.

Subendothelial degeneration, subintimal fat vacuolation, small atheromatous plaque consisting of various necrotic cells and thrombus are majorly stained with Beta-catenin immunohistochemical antibody (Fig 6). No positive staining of β-catenin in the control group I aorta. Aurelie *et al.* (2008) reported modest Beta-catenin positive staining in the disrupted human atherosclerotic plaques. The cadherin-catenin complex is a target for many cell signaling pathways involved in adhesion, proliferation and cell motility (Hoschuetzky *et al.,* 1994).

CONCLUSION

The hyperlipidemic diet of the present study has established the hyperlipidemia evidenced by increased TC, TG, LDL-C, VLDL-C and low HDL-C and elevated the total leukocyte count. Hepatic steatosis and degenerated endothelium with initiated atherosclerotic lesions were evidenced microscopically. CD 31, CD44, E cadherin and Beta-catenin positivity was observed in the lesions of endothelial degeneration, subendothelial lipid-laden macrophages and in the thrombus. Detection of various inflammatory and immune cell markers of atherosclerosis gives an insight into the tissue expression of these proteins, thereby the role of involved cells. It needs further quantification both in serum and at tissue levels for further understanding of these markers.

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