**Evaluation of antiatherosclerotic and antihyperlipidemic effect of Cyclosporine and Ashwagandha in high fat diet induced atherosclerosis in rats**

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**ABSTRACT**

**Background:** Atherosclerosis is a chronic inflammation of the vascular wall typified by the accumulation of lipid and macrophage derived foam cells. As the endothelial cells become activated and adhesive, monocytes stick to them, pass between them, and enter the intimal layer of the vessel wall. With the use of Immunomodulators. Majority of immunomodulators like cyclosporine, Sirolimus and Mycophenolate mofetil are being used in renal transplant patients. Some of clinical evidences indicated that use of immunomodulators causes hyperlipidemia and increases the chances of atherogenesis. Whereas in preclinical studies various researchers reported that immunomodulators reduces the chances of atherogenesis. So because of non uniform results in clinical and preclinical studies, this study was designed with a purpose of evaluating role of cyclosporine and ashwagandha in atherosclerosis. **Method:** Cyclosporine and Ashwagandha at 3 different doses have been evaluated in High Fat diet (1% Cholesterol) induced atherosclerosis in rats. Atorvastatin 2 mg/kg was used as standard treatment. Up to day 28 High fat diet was administered and then from day 29 to day 56 treatment and High fat diet was administered. Biochemical estimations of Serum lipid profile, C Reactive Protein, Malondialdehyde and histopathology of aorta was performed. **Results:** There was significant reduction in Triglyceride, LDL cholesterol, Total Cholesterol, Atherogenic Index, HMG to mavelonate, C reactive protein and MDA level was observed with treatment of Cyclosporine and ashwagandha. There was significant elevation in HDL cholesterol level and was observed with treatment of Cyclosporine and ashwagandha. **Conclusion:** Cyclosporine and ashwagandha effectively reduce the risk of development of atherosclerosis.

**KEYWORDS:** Atherosclerosis, Immunomodulators, Ashwagandha, Cyclosporine, High Fat Diet, Rats

**INTRODUCTION**

Atherosclerosis is a chronic inflammation of the vascular wall typified by the accumulation of lipid and macrophage derived foam cells. As the endothelial cells become activated and adhesive, monocytes stick to them, pass between them, and enter the intimal layer of the vessel wall. If the monocytes become activated, they remain in the intimal layer, mature into macrophages, take up lipid to become foam cells, and release a variety of inflammatory mediators, as well as oxidants and proteases. At this point, the inflammation has become chronic and the fatty streak is now well on its way to becoming an atherosclerotic lesion. As lesions mature, they become necrotic and calcified. And at last this lesion may rupture and initiate thrombosis and block the artery and leading to wards other Cardio Vascular System (CVS) complications. Hence role of immune system, Innate and adaptive, is highly important in formation and progression of Atherosclerotic plaque.

Monocytes- macrophages and lymphocytes are major immune cells within atherosclerotic lesions. Lymphocytes are the key and versatile regulators of the immune system. So there is opportunity for novel and highly specific target for diagnostic, management, treatment and prevention for atherosclerosis. Soluble phospholipase A2 type IIA is an acute phase reactant that is markedly increased in inflammatory cardiovascular diseases. They stimulate the differentiation of monocytes in to dendriatic cells which are link between adaptive and innate immune system. Some studies have demonstrated that the each heterogenicity of immune associated cells contribute to atherogenic and atheroprotective axis. T cells are highly involved in atherogenesis and it is well known that different type of T cells can drive or dampen inflammatory process. T cells are responsible for
early progression and not for initiation of atherosclerosis. The fine tuning between proinflammatory and anti-inflammatory factors determine whether an atherosclerotic lesion will develop in to silent stable plaque or cascade of activating events will lead to immune activation. T cells plays important role in balancing both the activities. This study mainly is focused on immunomodulators and atherosclerosis. Majority of immunomodulators like cyclosporine, Sirolimus and Mycophenolate mofetil are being used in renal transplant patients. Some of clinical evidences indicated that use of immunomodulators causes hyperlipidemia and increases the chances of atherogenesis. Whereas in preclinical studies various researchers reported that immunomodulators reduces the chances of atherogenesis. So because of non uniform results in clinical and preclinical studies, this study was designed with a purpose of evaluating role of cyclosporine and ashwagandha in atherosclerosis.

MATERIALS AND METHODS:

Drugs and Plant material: Ashwagandha (Withania somnifera) was procured from Neemuch, M.P., India. It was authenticated by various morphological and chemical tests. Atorvastatin was procured from Intas pharmaceuticals as a gift sample. Cyclosporine was purchased as soft gel capsules of Ranbaxy laboratories ltd.

Animals: 54 Healthy adult Female Sprague Dawley rats weighing between 150-200 gm were selected for the study. Animals were kept in polypropylene cages (three in each cage) at an ambient temperature of (25±2 ºC) and 55%-65% relative humidity. A 12 h light and dark schedule was maintained. Animals had free access to water. They were fed with either commercially available rat chow (Pranav Agrochem, Gujarat, India) or High Fat Diet as per experimental protocols. High fat diet was prepared by adding 1% cholesterol, 0.5% cholic acid and 5% lard oil in normal rat chow diet.

54 Animals were divided in 9 groups of 6 animals each. These groups include Normal Control (Saline + Normal Pallet Diet), Diseased Control (High Fat Diet), Standard Treatment (HFD and Atorvastatin 2 mg/kg per oral), 3 of Cyclosporin Treated Groups (HFD + Cyclosporin at 1, 5 and 10 mg/kg per oral dose) and 3 Ashwagandha treatment groups (HFD + Ashwagandha at 50, 100 and 200 mg/kg per oral dose). Cyclosporine at 01 mg/kg, 05 mg/kg and 10 mg/kg are denoted as C1, C2 and C3 respectively for quick referencing. Ashwagandha ethanolic extract at 50 mg/kg, 100 mg/kg and 200 mg/kg are denoted as A1, A2 and A3 respectively for quick referencing. All the Animals except Normal control group were fed High fat diet from day 1 to up to day 56th. Treatment with test and standard drug were initiated from day 29th upto day 56th. On day 1st, day 28th and day 56th blood was collected from all the animals from retro orbital plexus under light ether anaesthesia for the estimation of various biochemical parameters i.e. Total Cholesterol, HDL-Cholesterol, LDL-Cholesterol, Triglycerides, C Reactive Protein, HMG to Mavelonate ratio, Malondialdehyde. Weekly body weight was measured. All the animals were sacrificed on day 57th and aorta were isolated for histopathology. All the experimental protocols were approved as per CPCSEA - IAEC of KBIPER under protocol no: KBIPER/2011/220.

Statistical Analysis

Results are expressed as Mean ± SEM. Statistical analysis was performed by applying One Way ANOVA test followed Post Hoc Tukey’s test and Student’s t test. The 0.05 level was selected as a point of minimal statistical significance in every comparison.

RESULTS AND DISCUSSION:

Physico-chemical analysis of plant material: Identification and authentication of ashwagandha plant material was done by Dr Y. T. Jasra (Professor and Head, Department of Botany, University School of Science, Gujarat University, Ahmedabad) Various physico-chemical test results were in accordance to standards mentioned by Indian Pharmacopoeia 2010. For Ethanol soluble extractive values 14%, Water soluble extractive values 20%, Ash value 3.5%, Acid insoluble ash 0.8%, Loss on drying 9% were permissible and hence plant material passed the Pharmacopoeia standards. Ashwagandha sample showed presence of alkaloids and sterols in chemical tests i.e. Libermann burchard test, Mayer’s Reagent, Dragendorff’s Reagent, Wagner’s Reagent. Thin layer chromatography was carried out to check presence of withaferin A in plant extract and rf value matched for standard withaferin A with our plant material extract i.e. 0.39. All these results directly indicated that plant material procured was authentic.

When % change in body weight of Day 56 Vs Day 28 among all the groups were calculated and compared there was no any significant change in body weight observed with treatment.

With High Fat Diet for 28 days there was no any significant change in body weight observed when control group was compared with all other groups. (Fig. 1) With the treatment with when diseased group was compared with all other treatment groups there was significant reduction in weight was observed with Ashwagandha at the dose of 200 mg/kg (Fig. 2). Saxena et al reported significant elevation of weight with treatment of withania coagulants at the dose of 1000 mg/kg. Treatment with Ashwagandha ethanolic extract for 28 days with High fat diet showed significant reduction in body weight when compared with Diseased control group.
Effect on Serum Biochemical Parameters

**Effect on Serum Triglyceride Level:**

By performing one way ANOVA and post Tukey’s test to compare all pairs of mean there was significant elevation of Triglyceride level at day 28 in Diseased control (152.4 ± 13.48) and A2 (153.6 ± 11.73) and A3 (162.1 ± 8.340) groups when compared with Control group (83.73 ± 8.19). By performing biochemical estimation of Triglyceride on Day 56 and applying student’s T- test between control group (80.37 ± 5.22) and Diseased control group (236.5 ± 14.54); significant elevation in Triglyceride was observed where \( P < 0.05 \), student’s t-test (Fig. 3). When performing One Way ANOVA and comparing all pair of means with Diseased control group (236.5 ± 14.54), there was significant reduction in Triglyceride level with C1 (191.7 ± 10.47), C2 (97.43 ± 11.32), C3 (103.8 ± 13.27), A1 (128.1 ± 13.96) and A3 (158.7 ± 6.31); where \( P < 0.05 \), by applying Post Hoc Tukey’s Test (Fig. 4). High fat diet elevates Triglyceride level which was observed by Braun et al. Administration of aqueous extract of fruits of withania coagulans at dose of 01 g/kg per oral reduced the elevated serum triglyceride level. Withania somnifera was also found to reduce the elevated Triglyceride level at the dose of 100 mg/kg and 200 mg/kg as it interferes with ability of hepatocytes to synthesize cholesterol in liver. Saxena B has also reported that Ashwagandha extract increases elimination and metabolism of cholesterol and triglyceride. Drew et al reported Cyclosporine treatment reduces early atherosclerosis in the cholesterol-fed rabbit. They reported inhibition of T cell activation by cyclosporine A prevents their localisation in plaques and reduces the extent of early lesions. He also confirms that it reduces triglyceride, Total cholesterol and LDL cholesterol levels.
Effects on Serum Cholesterol Levels

With administration of High fat Diet significant elevation of total cholesterol was observed in all other groups when compared with Control group on Day 28 by performing one way ANOVA (P<0.05, by applying Post Hoc Tukey’s Test) (Fig. 4). By performing biochemical estimation of Total Cholesterol on Day 56 and applying student’s T-test between control group (29.23 ± 3.53) and Diseased control group (368.9 ± 20.51); significant elevation in Total Cholesterol was observed where P<0.005, student’s t-test. When performing One Way ANOVA and comparing all pair of means with Diseased control group (368.9 ± 20.51), there was significant reduction in Total Cholesterol level with Standard (196.8 ± 23.76), C1 (134.2 ± 1.89), C2 (160.8 ± 4.2), C3 (115.5 ± 7.22), A1 (211.9 ± 14.79), A2 (224.9 ± 15.71) and A3 (246.4 ± 23.63); where P<0.05, by applying Post Hoc Tukey’s Test (Fig. 6). Hemlata et al also reported treatment with Withania for 7 weeks reduces total cholesterol, triglyceride and lipoprotein levels. Ferrero et al reported that cyclosporine increases lipid levels when administered at the dose of 15 mg/kg i.p. This could be related to drug induced damage to pancreas islets and by degeneration of fatty tissues.

Effects on LDL Cholesterol Levels:

With administration of High fat Diet significant elevation of LDL cholesterol was observed in all other groups when compared with Control group (32.62 ± 4.45) on Day 28 by performing one way ANOVA (P<0.05, by applying Post Hoc Tukey’s Test) (Fig. 7). By performing biochemical estimation of LDL Cholesterol on Day 56 and applying student’s T-test between control group (17.51 ± 4.39) and Diseased control group (313.5 ± 19.56); significant elevation in LDL Cholesterol was observed where P<0.005, student’s t-test (Fig. 8). When performing One Way ANOVA and comparing all pair of means with Diseased control group (313.5 ± 19.56), there was significant reduction in LDL Cholesterol level with Standard (139.8 ± 22.52), C1 (88.20 ± 7.79), C2 (128.3 ± 5.94), C3 (65.17 ± 7.94), A1 (167.2 ± 16.48), A2 (180.1 ± 15.20) and A3 (191.8 ± 24.10); where P<0.05, by applying Post Hoc Tukey’s Test. Houssen et al reported cyclosporine A significantly decreases serum level of lipids, C Reactive proteins and Interleukin 6 in cholesterol fed groups. He also concluded low doses of cyclosporine could not have generalized systemic anti-inflammatory or immunosuppressive effects.
Effects on HDL Cholesterol Levels

With administration of High fat Diet significant reduction of HDL cholesterol was observed in all other groups when compared with Control group (48.91 ± 1.91) on Day 28 by performing one way ANOVA (P<0.05, by applying Post Hoc Tukey’s Test) (Fig.9). By performing biochemical estimation of HDL Cholesterol on Day 56 and applying student’s T- test between control group (30.67 ± 3.83) and Diseased control group (8.14 ± 1.38); significant reduction in LDL Cholesterol was observed where P<0.005, student’s t-test (Fig.10). When performing One Way ANOVA and comparing all pair of means with Diseased control group (8.14 ± 1.38), there was significant elevation in HDL Cholesterol level with Standard (26.60 ± 2.91), C1 (14.26 ± 1.04), C2 (13.01 ± 2.31), C3 (29.61 ± 1.69), A1 (18.63 ± 1.22), A2 (19.23 ± 0.98) and A3 (22.90 ± 1.3); where P<0.05, by applying Post Hoc Tukey’s Test. HDL cholesterol causes reverse transport of cholesterol from systemic circulation to liver. It is causing elimination and metabolism of serum cholesterol and hence we can conclude that treatment with Cyclosporine at 10 mg/kg and Ashwagandha at all three representative doses causes significant elevation of HDL cholesterol when compared to Diseased group.

Effects on VLDL Cholesterol Levels

With administration of High fat Diet significant elevation of VLDL cholesterol was observed in Diseased control (30.47 ± 2.69), A2 (30.71 ± 2.35), A3 (32.43 ± 1.67) groups when compared with Control group (16.75 ± 1.64) on Day 28 by performing one way ANOVA (P<0.05, by applying Post Hoc Tukey’s Test) (Fig.11). By performing biochemical estimation of LDL Cholesterol on Day 56 and applying student’s T-test between control group (16.07 ± 1.04) and Diseased control group (47.31 ± 2.91); significant elevation in VLDL Cholesterol was observed.
where \( P < 0.005 \), student’s t-test (Fig. 12). When performing One Way ANOVA and comparing all pair of means with Diseased control group (47.31 ± 2.91), there was significant reduction in LDL Cholesterol level with Standard (30.39 ± 1.15), C1 (38.34 ± 2.09), C2 (19.49 ± 2.26), C3 (20.75 ± 2.65), A1 (26.04 ± 1.59), A2 (25.62 ± 2.79) and A3 (31.74 ± 1.263); where \( P < 0.05 \), by applying Post Hoc Tukey’s Test.

Atherogenic Index was observed where \( P < 0.005 \), student’s t-test. When performing One Way ANOVA and comparing all pair of means with Diseased control group (40.66 ± 5.3), there was significant reduction in Atherogenic Index with Standard (6.73 ± 1.08), C1 (8.65 ± 0.7), C2 (13.40 ± 2.47), C3 (2.93 ± 0.26), A1 (10.67 ± 1.28), A2 (10.81 ± 1.82) and A3 (10.01 ± 1.42); where \( P < 0.05 \), by applying Post Hoc Tukey’s Test (Fig. 13). Risk of Atherosclerosis was found to be significantly reduced with treatment of cyclosporine and ashwagandha.

**HMG to Mavelonate Ratio**

Hepatic cholesterol synthesis is direct indicator of hyperlipidemia and atherosclerosis. Increased level of this ratio indicates that HMG Co A is not getting converted into mavelonate and hence endogeneous hepatic cholesterol synthesis is inhibited. By calculating HMG to Mavelonate Ratio on Day 56 and performing One Way ANOVA and comparing all pair of means with Diseased control group (0.48 ± 0.12), there was significant elevation in HMG to Mavelonate Ratio with Standard (4.53 ± 0.37), C2 (13.37 ± 1.59), C3 (13.17 ± 1.59), A2 (3.44 ± 0.27) and A3 (7.90 ± 0.48); where \( P < 0.05 \), by applying Post Hoc Tukey’s Test (Fig. 14).

**Effect on C Reactive Protein**

By performing biochemical estimation of CRP on Day 56 and applying student’s T- test between control group (2.68 ± 0.88) and Diseased control group (11.02 ± 0.95); significant elevation in CRP was observed where \( P < 0.005 \), student’s t-test (Fig. 15). When performing One Way ANOVA and comparing all pair of means with Diseased control group
(11.02 ± 0.95), there was significant reduction in CRP level with Standard (2.056 ± 0.44), C1 (3.46 ± 0.97), C2 (1.42 ± 0.49), C3 (1.61 ± 0.30), A1 (1.9 ± 0.37), A2 (1.26 ± 0.29) and A3 (1.13 ± 0.15); where P<0.05, by applying Post Hoc Tukey’s Test.

**C Reactive Protein Vs Groups on Day 56.** (* indicates significantly elevated from control group (P<0.005, student’s t-test; # indicates significantly decreased from Diseased control group (P<0.05, by applying Post Hoc Tukey’s Test)

**Fig. 15. Effect on C Reactive Protein Vs Groups on Day 56**

**Effect on MDA level**

Free radicals cause lipid peroxidation and MDA is one of the final products of polyunsaturated fatty acids peroxidation. It is commonly known as a marker of oxidative stress and antioxidant status in animals. By performing biochemical estimation of MDA on Day 56 and applying student’s T-test between control group (0.65 ± 0.11) and Diseased control group (3.23 ± 0.08); significant elevation in MDA was observed where P<0.005, student’s t-test. When performing One Way ANOVA and comparing all pair of means with Diseased control group (3.23 ± 0.08), there was significant reduction in MDA level with Standard (1.06 ± 0.28), C1 (0.94 ± 0.14), C2 (0.64 ± 0.21), C3 (1.23 ± 0.38), A1 (0.21 ± 0.04), A2 (0.35 ± 0.15) and A3 (0.23 ± 0.1); where P<0.05, by applying Post Hoc Tukey’s Test (Fig. 16). *Withania somnifera* at the dose of 300 mg/kg reduced oxidative stress caused by doxorubicin induced cardiac toxicity in rats. Thus it is a useful adjuvant therapy when oxidation of LDL cholesterol acts as proatherogenic stimuli. Proatherogenic stimuli like injury, hyperglycemia will increase the expression of Thrombospondin 1 which will lead to endothelial dysfunction and apoptosis and increases the smooth muscle cell proliferation. Thus initiation of atherosclerotic lesion is there. It is also involved in progression and maturation of atherosclerotic plaque.

**HISTOPATHOLOGY:**

By use of Hematoxylin and eosin stain histopathology was performed. In control group (Fig. 17) there was no any accumulation of fatty streaks and proliferation of smooth muscle cell observed. Where as in diseased control group (Fig. 18) there was accumulation of fat deposits and disrupted intimal lining. With standard treatment Atorvastatin (Fig. 19) there is inhibition of adhesion and recruitment of monocytes and other immune cells was observed. Smooth muscle proliferation and recruitment of T cell and immune cell was inhibited in Cyclosporine (Fig. 20 and 21) and Ashwagandha (Fig. 22 and 23) treated groups. Henrik et al investigated effect of Cyclosporine on arterial balloon injury lesions in Cholesterol-Clamped Rabbits. They reported Cyclosporine did not affect aorta cholesterol accumulation or neointimal proliferation in balloon-injured aortas; however, it attenuated both in transplanted aortas. They also found out cyclosporine had no effect on endothelial cells at balloon-injured sites, but protected these cells in the transplanted aortas. Infiltration of smooth muscle cells, T lymphocytes, and macrophages were unaffected by cyclosporine in balloon-injured aortas; however, in transplanted aortas, cyclosporine reduced the relative number of T lymphocytes and macrophages but increased the relative number of smooth muscle cells. On final notes they concluded, cyclosporine does not attenuate neointimal proliferation after balloon dilatation, and that T lymphocyte-mediated immune responses are not involved in neointimal proliferation after balloon dilatation. Ferns et al reported that in vitro, cyclosporine A reduced the rate of proliferation of rabbit aortic smooth muscle and endothelial cells in a dose-dependent fashion, and induced smooth muscle cell vacuolation.
cyclosporine blocks T helper cell infiltration its role in reduction of initiation of atherosclerosis if well established by Drew et al. Rabbits treated with Cyclosporine developed significantly less extensive plaques after 4 weeks of treatment at the dose of 16 mg/kg body weight.\(^7\) \textit{W. somnifera} derived flavonoids and phenolic acid such as gallic acid, rutein, vanillic acid, quercetin and kaempferol block distinct signal transduction events necessary for NF-κB activation. The molecular activities of flavonoids and phenolic acids include inhibition of transcription factors such as NF-κB and activating protein-1 (AP-1), as well as activation of nuclear factor-erythroid 2-related factor 2 (Nrf2). Hence inhibition of TNF-α is responsible for anti-inflammatory activity and antiathrosclerotic activity.\(^{16}\) Sherry et al reported that cyclosporin A caused inhibition of monocyte recruitment and activation. They also concluded cyclosporin A caused inhibition of smooth muscle proliferation and migration with decreasing expression of Matrix metalloproteinase.\(^{17}\) While its immunosuppressive effects are closely linked to its effects on T cell activation via the inhibition of the nuclear factor of activated T cells (NFAT) pathway, the precise mechanisms underlying its cardiovascular effects appear to involve multiple pathways additional to those relevant for
immunosuppression. These include inhibition of calcineurin activity and intracellular cyclophilin peptidylprolyl isomerase and chaperone activities, inhibition of pro-inflammatory extracellular cyclophilin A, and NFAT-independent transcriptional effects. Cyclosporin A demonstrates complex effects on lipoprotein metabolism and bile acid production, and affects endothelial cells, smooth muscle cells and macrophages, all of which are critical to the atherosclerotic process. \textit{W. somnifera} derived flavonoids and phenolic acid such as gallic acid, rutein, vanillic acid, quercetin and kaempferol block distinct signal transduction events necessary for NF-\kappa B activation. The molecular activities of flavonoids and phenolic acids include inhibition of transcription factors such as NF-\kappa B and activating protein-1 (AP-1), as well as activation of nuclear factor-erythroid 2-related factor 2 (Nrf2). Hence inhibition ofTNF-\alpha is responsible for anti-inflammatory activity and antiatherosclerotic activity.\textsuperscript{16}

**CONCLUSION:**

There was significant reduction in Triglyceride, LDL cholesterol, Total Cholesterol, Atherogenic Index, HMG to mavelonate, C reactive protein and MDA level was observed with treatment of Cyclosporine and ashwagandha. There was significant elevation in HDL cholesterol level and was observed with treatment of Cyclosporine and ashwagandha. Cyclosporine and ashwagandha effectively reduce the risk of development of atherosclerosis.

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**REFERENCES:**


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Evaluation of antiatherosclerotic and antihyperlipidemic effect of Sirolimus and Mycophenolate mofetil in high fat diet induced atherosclerosis in rats

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ABSTRACT

Background: Atherosclerosis is a chronic inflammation of the vascular wall typified by the accumulation of lipid and macrophage derived foam cells. As the endothelial cells become activated and adhesive, monocytes stick to them, pass between them, and enter the intimal layer of the vessel wall. With the use of Immunomodulators. Majority of immunomodulators like Sirolimus and Mycophenolate mofetil are being used in renal transplant patients. Some of clinical evidences indicated that use of immunomodulators causes hyperlipidemia and increases the chances of atherogenesis. Whereas in preclinical studies various researchers reported that immunomodulators reduces the chances of atherogenesis. So because of non uniform results in clinical and preclinical studies, this study was designed with a purpose of evaluating role of Sirolimus and Mycophenolate mofetil in atherosclerosis. Methods: Sirolimus and Mycophenolate mofetil at 3 different doses have been evaluated in High Fat diet (1% Cholesterol) induced atherosclerosis in rats. Atorvastatin 2 mg/kg was used as standard treatment. Up to day 28 High fat diet was administered and then from day 29 to day 56 treatment and High fat diet was administered. Biochemical estimations of Serum lipid profile, C Reactive Protein, Malondialdehyde and histopathology of aorta was performed. Results and Discussion: There was significant reduction in Triglyceride, LDL cholesterol, Total Cholesterol, Atherogenic Index, HMG to mavelonate, C reactive protein and MDA level was observed with treatment of Cyclosporine and ashwagandha. There was significant elevation in HDL cholesterol level and was observed with treatment of Sirolimus and Mycophenolate mofetil. Conclusion: Sirolimus and Mycophenolate mofetil effectively reduce the risk of development of atherosclerosis.

KEYWORDS: Atherosclerosis, Immunomodulators, Sirolimus, Mycophenolate mofetil, High Fat Diet, Rats

INTRODUCTION

Atherosclerosis is a chronic inflammation of the vascular wall typified by the accumulation of lipid and macrophage derived foam cells. As the endothelial cells become activated and adhesive, monocytes stick to them, pass between them, and enter the intimal layer of the vessel wall. If the monocytes become activated, they remain in the intimal layer, mature into macrophages, take up lipid to become foam cells, and release a variety of inflammatory mediators, as well as oxidants and proteases. At this point, the inflammation has become chronic and the fatty streak is now well on its way to becoming an atherosclerotic lesion. As lesions mature, they become necrotic and calcified. And at this time this lesion may rupture and initiate thrombosis and block the artery and leading to wards other Cardio Vascular System (CVS) complications. Hence role of immune system, Innate and adaptive, is highly important in formation and progression of Atherosclerotic plaque.

Monocytes- macrophages and lymphocytes are major immune cells within atherosclerotic lesions. Lymphocytes are the key and versatile regulators of the immune system. So there is opportunity for novel and highly specific target for diagnostic, management, treatment and prevention for atherosclerosis. Soluble phospholipase M2 type II A is an acute phase reactant that is markedly increased in inflammatory cardiovascular diseases. They stimulate the differentiation of monocytes in to dendriatic cells which are link between adaptive and innate immune system. Some studies have demonstrated that the each heterogenicity of immune associated cells contribute to atherogenic and atheroprotective axis. T cells are highly involved in atherogenesis and it is well known that different type of T cells can drive or dampen inflammatory process. T cells are responsible for early progression and not for initiation of atherosclerosis. The fine tuning between proinflammatory and anti-inflammatory factors
determine whether an atherosclerotic lesion will develop in to silent stable plaque or cascade of activating events will lead to immune activation. T cells plays important role in balancing both the activities. This study mainly is mainly focused on immunomodulators and atherosclerosis. Majority of immunomodulators like Sirolimus and Mycophenolate mofetil are being used in renal transplant patients. Some of clinical evidences indicated that use of immunomodulators causes hyperlipidemia and increases the chances of atherogenesis. Whereas in preclinical studies various researchers reported that immunomodulators reduces the chances of atherogenesis. So because of non uniform results in clinical and preclinical studies, this study was designed with a purpose of evaluating role of Sirolimus and Mycophenolate moetil in atherosclerosis.

**MATERIALS AND METHODS:**
Sirolimus, Mycophenolate mofetil and Atorvastatin was obtained as gift sample form Intas Pharmaceuticals Ltd.

Animals: 54 Healthy adult Female Sprague Dawley rats weighing between 150-200 gm were selected for the study. Animals were kept in polypropylene cages (three in each cage) at an ambient temperature of (25±2 °C) and 55%-65% relative humidity. A 12 h light and dark schedule was maintained. Animals had free access to water. They were fed with either commercially available rat chow (Pranav Agrochem, Gujarat, India) or High Fat Diet as per experimental protocols. High fat diet was prepared by adding 1% cholesterol, 0.5% cholic acid and 5% lard oil in normal rat chow diet.

54 Animals were divided in 9 groups of 6 animals each. These groups include Normal Control (Saline + Normal Pallet Diet), Diseased Control (High Fat Diet), Standard Treatment (HFD and Atorvastatin 2 mg/kg per oral), 3 of Sirolimus Treated Groups (HFD + Sirolimus at 0.25, 0.5 and 1 mg/kg per oral dose) and 3 Mycophenolate mofetil treatment groups (HFD + Mycophenolate mofetil at 10, 20 and 40 mg/kg per oral dose). Sirolimus at 0.25 mg/kg, 0.5 mg/kg and 1 mg/kg are denoted as S1, S2 and S3 respectively for quick referencing. Mycophenolate at 10 mg/kg, 20 mg/kg and 40 mg/kg are denoted as M1, M2 and M3 respectively for quick referencing. All the Animals except Normal control group were fed High fat diet from day 1 to up to day 56. Treatment with test and standard drug were initiated from day 29th upto day 56th. On day 1, day 28th and day 56th blood was collected from all the animals from retro orbital plexus under light ether anaesthesia for the estimation of various biochemical parameters i.e. Total Cholesterol, HDL-Cholesterol, LDL-Cholesterol, Triglycerides, C Reactive Protein, HMG to Mavelonate ratio, Malondialdehyde. Weekly body weight was measured. All the animals were sacrificed on day 57th and aorta were isolated for histopathology. All the experimental protocols were approved as per CPCSEA - IAEC of KBIPER under protocol no: KBIPER/2012/342.

**Statistical Analysis**
Results are expressed as Mean ± SEM. Statistical analysis was performed by applying One Way ANOVA test followed Post Hoc Tukey’s test and Student’s t test. The 0.05 level was selected as a point of minimal statistical significance in every comparison.

**RESULTS AND DISCUSSION:**
When % change in body weight of Day 56 Vs Day 28 among all the groups were calculated and compared there was no any significant change in body weight observed with treatment.

**Effect on Serum Biochemical Parameters**

**Effect on Serum Triglyceride Level:**
By performing biochemical estimation of Triglyceride on Day 56 and applying student’s T- test between control group (99.12 ± 3.23) and Diseased control group (279.6 ± 5.63); significant elevation in Triglyceride was observed where P<0.005, student’s t-test. When performing One Way ANOVA and comparing all pair of means with Diseased control group (279.6 ± 5.63), there was significant dose dependent reduction in Triglyceride level with Standard (117.4 ± 6.79), S1 (170.8 ± 4.65), S2 (135.3 ± 3.70), S3 (102.9 ± 4.29), M1 (214.1 ± 6.79), M2 (174.3 ± 7.60) and M3 (133.3 ± 2.72); where P<0.05, by applying Post Hoc Tukey’s Test (Fig. 2). Sirolimus prevented the effect of high fat diet on the rate of accretion in body weight via reducing lipid...
accumulation despite of greater food intake. Reis et al evaluated Sirolimus activity in rats and observed elevation of LDL cholesterol and triglyceride level.

**Effects on Serum Cholesterol Levels:**

By performing biochemical estimation of Total Cholesterol on Day 56 and applying student’s T- test between control group (94.1 ± 1.76) and Diseased control group (405.4 ± 4.31); significant elevation in Total Cholesterol was observed where P<0.005, student’s t-test. When performing One Way ANOVA and comparing all pair of means with Diseased control group (405.4 ± 4.31), there was significant reduction in Total Cholesterol level with Standard (130.6 ± 3.18), S1 (356.0 ± 4.92), S2 (252.6 ± 2.36), S3 (154.0 ± 4.27), M1 (238.2 ± 10.42), M2 (177.0 ± 5.29) and M3 (110.3 ± 7.26); where P<0.05, by applying Post Hoc Tukey’s Test. Hemlata et al also reported treatment with Withania for 7 weeks reduces total cholesterol, triglyceride and lipoprotein levels. Ferrero et al reported that cyclosporine increases lipid levels when administered at the dose of 15 mg/kg i.p. This could be related to drug induced damage to pancreas islets and by degeneration of fatty tissues. Mycophenolate mofetil (MMF) has emerged as a non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) that exerts cytostatic effects, particularly on proliferating T-lymphocytes. In addition, MMF has other immune-modulating effects, such as down regulation of the expression of adhesion molecules and attenuation of monocyte and macrophage responses. Given the added benefit that MMF is well tolerated, this immunosuppressive agent constitutes an attractive candidate for the modulation of inflammatory activation in atherogenesis.
Effects on HDL Cholesterol Levels

By performing biochemical estimation of HDL Cholesterol on Day 56 and applying student’s T- test between control group (29.73 ± 2.02) and Diseased control group (8.68 ± 0.56); significant reduction in LDL Cholesterol was observed where P<0.005, student’s t-test. When performing One Way ANOVA and comparing all pair of means with Diseased control group (8.68 ± 0.56), there was significant elevation in HDL Cholesterol level with Standard (31.26 ± 1.84), S1 (15.08 ± 0.62), S2 (23.29 ± 2.25), S3 (33.51 ± 1.52), M1 (15.03 ± 0.81), M2 (21.14 ± 1.45) and M3 (24.79 ± 1.6); where P<0.05, by applying Post Hoc Tukey’s Test. HDL cholesterol causes reverse transport of cholesterol from systemic circulation to liver.

Atherogenic Index

AI is logarithmically transformed ratio of molar concentration of Total Cholesterol to HDL cholesterol. It is a useful predictor of cardiovascular risk. [10] By calculating Atherogenic Index on Day 56 and applying student’s T- test between control group (2.24 ± 0.22) and Diseased control group (46.93 ± 3.74); significant elevation in Atherogenic Index was observed where P<0.005, student’s t-test. When performing One Way ANOVA and comparing all pair of means with Diseased control group (46.93 ± 3.74), there was significant reduction in Atherogenic Index with Standard (4.96 ± 3.74), S1 (22.84 ± 1.17), S2 (10.37 ± 1.07), S3 (3.67 ± 0.33), M1 (14.98 ± 0.71), M2 (7.64 ± 0.82) and M3 (3.58 ± 0.52); where P<0.05, by applying Post Hoc Tukey’s Test. Risk of Atherosclerosis was found to be significantly reduced with treatment of cyclosporine and ashwagandha.
Hepatic cholesterol synthesis is direct indicator of hyperlipidemia and atherosclerosis. Increased level of this ratio indicates that HMG Co A is not getting converted into mavelonate and hence endogeneous hepatic cholesterol synthesis is inhibited. By calculating HMG to Mavelonate Ratio on Day 56 and performing One Way ANOVA and comparing all pair of means with Diseased control group (0.55 ± 0.04), there was significant elevation in HMG to Mavelonate Ratio with Standard (3.59 ± 0.15), S3 (2.97 ± 0.2), M2 (2.86 ± 0.32) and M3 (3.99 ± 0.39); where P<0.05, by applying Post Hoc Tukey’s Test.

Effect on C Reactive Protein

Vitiello et al evaluated effect of everolimus on the immunomodulation of the human neutrophil inflammatory response and activation. They reported that mammalian target of rapamycin (mTOR) inhibitors (sirolimus and everolimus) had the most potent anti-inflammatory effect.9 CRP elevation usually occurs at the time of endothelial injury induced inflammation. By performing biochemical estimation of CRP on Day 56 and applying student’s T- test between control group (2.07 ± 0.41) and Diseased control group (15.99 ± 0.80); significant elevation in CRP was observed where P<0.005, student’s t-test. When performing One Way ANOVA and comparing all pair of means with Diseased control group (3.37 ± 0.06); significant elevation in MDA was observed where P<0.005, student’s t-test. When performing One Way ANOVA and comparing all pair of means with Diseased control group (3.37 ± 0.06); significant reduction in MDA level with Standard (1.07 ± 0.08), S1 (1.63 ± 0.05), S2 (1.26 ± 0.03), S3 (0.81 ± 0.04), M1 (2.31 ± 0.10), M2 (1.45 ± 0.09) and M3 (0.85 ± 0.05); where P<0.05, by applying Post Hoc Tukey’s Test. Withnia somnifera at the dose of 300 mg/kg reduced oxidative stress caused by doxorubicin induced cardiac toxicity in rats. Thus it is a useful adjuvant therapy when oxidation of LDL cholesterol acts as proatherogenic stimuli.12 Proatherogenic stimuli like injury, hyperglycemia will increase the expression of Thrombospondin 1 which will lead to endothelial dysfunction and apoptosis and increases the smooth muscle cell proliferation. Thus initiation of atherosclerotic lesion is there. It is also involved in progression and maturation of atherosclerotic plaque.13
Fig. 10: Effect on MDA level

**HISTOPATHOLOGY:**
By use of Hematoxylin and eosin stain histopathology was performed. In control group (Fig. 11) there was no any accumulation of fatty streaks and proliferation of smooth muscle cell observed. Where as in diseased control group (Fig. 12) there was accumulation of fat diposits and disrupted intimal lining. With standard treatment (Fig. 13) with Atorvastatin there is inhibition of adhesion and recruitment of monocytes and other immune cells was observed. In Sirolimus treatment group (Fig. 14, 15 and 16 respectively) there is remote infiltration of immune cells and fatty diposits. In Myphenolate mofetil Treatment group (Fig. 17, 18 and 19 respectively) smooth muscle proliferation and fatty diposits are less predominant. Smooth muscle proliferation and recruitment of T cell and immune cell was inhibited in Sirolimus and Mycophenolate treated groups. Sirolimus and its synthetic derivative everolimus (EVL), which inhibits a kinase, the mammalian target of rapamycin (mTOR), are reported to have a beneficial effect on cardiac allograft vasculopathy (CAV) development. Since mTOR activation signals proliferation of both smooth muscle and endothelial cells, inhibition of mTOR by Sirolimus and its analog everolimus could prevent arterial smooth muscle and endothelial proliferation, and thus, graft atherosclerosis and intimal hyperplasia after vascular injury.\(^\text{10}\) Martinet et al also reported Everolimus-Induced mTOR Inhibition Selectively Depletes Macrophages in Atherosclerotic Plaques by Autophagy. They demonstrated that stent-based delivery of the rapamycin derivative everolimus in atherosclerotic plaques from cholesterol-fed rabbits leads to a marked reduction in macrophage content via autophagic cell death without altering the amount of SMCs. Moreover they concluded that everolimus induced inhibition
of de novo protein synthesis in both cell types, followed by bulk degradation of long-lived proteins, processing of LC3 and cytoplasmic vacuolization in macrophages but not in SMCs in in vitro studies.\textsuperscript{11} Baetta et al reported Everolimus Inhibits Monocyte/Macrophage Migration in Vitro and Their Accumulation in Carotid Lesions of Cholesterol-Fed Rabbits. They proposed that everolimus may possess a significant antimacrophage activity, mediated by at least two potentially independent mechanisms, namely the inhibition of monocyte recruitment into the arterial wall and the induction of macrophage death by autophagy. In particular, inhibition of monocyte recruitment is effective in vitro at drug concentrations that are in the range of the therapeutic levels achieved in kidney and heart transplanted patient.\textsuperscript{12} Martinet et al also reported Everolimus-Induced mTOR Inhibition Selectively Depletes Macrophages in Atherosclerotic Plaques by Autophagy.\textsuperscript{11} Beutner et al investigated effect of everolimus on pre-existing atherosclerosis in LDL-receptor deficient mice. They reported Everolimus (5mg/kg) resulted in an arrest of CD68 positive plaque area and nearly halved CD68 fraction in aortic root lesions but not in brachiocephalic lesions. They also proposed that everolimus might exert more potent anti-atherogenic properties in earlier stages of atherogenesis.\textsuperscript{13} Vietinghoff et al reported that macroscopic and histologic aortic atherosclerotic lesions were significantly decreased in Mycophenolate mofetil treated groups. Moreover the T cell cytokine interleukin (IL)-17 was significantly reduced in plasma of MMF-treated mice and supernatants from their aortas after T cell stimulation. Mycophenolate mofetil treatment decreased aortic a\textsuperscript{b}TCR+ lymphocyte proliferation and cell numbers.
Also, aortic content of CD11b+CD11c+ cells and their proliferation were reduced in Mycophenolate mofetil -treated Apoe-/- mice. They concluded that the lymphocyte-directed immunosuppressant mofetil that curbs IL-17 production was a successful anti-atherosclerotic treatment. Romero et al suggested upon their investigations that Mycophenolate mofetil ameliorates the atherogenic potential of a high cholesterol diet and this effect is associated with a reduction in macrophage and foam cell infiltration and smooth muscle cell proliferation and infiltration. MMF has been shown to reduce the expression of vascular adhesion molecules in atherosclerosis by inhibiting the nuclear factor Nfkb which is required for their transcriptional upregulation. Raisanen et al showed that MMF treatment reduces the appearance and proliferation of smooth muscle cells in the intima, which normally contribute to atherosclerotic plaque formation by recruitment of extracellular matrix and self-proliferation. Thus, MMF has properties that could be considered anti-atherogenic: inhibiting T-cells, blocking leukocyte adhesion and inhibiting proliferation of smooth muscle cells.

CONCLUSION:
There was significant dose dependent reduction in Triglyceride, LDL cholesterol, Total Cholesterol, Atherogenic Index, HMG to mavenolate, C reactive protein and MDA level was observed with treatment of Sirolimus and Mycophenolate mofetil. There was significant elevation in HDL cholesterol level and was observed with treatment of Sirolimus and Mycophenolate mofetil. Sirolimus and Mycophenolate mofetil effectively reduce the risk of atherogenesis.

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