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Evaluation of Antihyperlipidemic Activity of HMG-Co A Reductase Inhibitor and Curcumin in Combination on Diet Induced Hyperlipidemic Rats

Aijaz A. Sheikh¹*, Tanwir Z. Khan¹ and Sajid R. Sheikh²

¹Anuradha College of Pharmacy, Chikhli, Maharashtra ²Dr. Vedprakash Patil Pharmacy College, Aurangabad, Maharashtra

ABSTRACT

Hyperlipidemia a well known risk factor for cardiovascular disease, especially atherosclerotic coronary artery disease is one of the major cause of premature death globally. Cholesterol feeding has been often used to elevate serum or tissue cholesterol levels to assess hypercholesterolemia-related metabolic disturbances in different animal models. Hyperlipidemia was induced in rats by giving high cholesterol diet for seven days in standard rat chow diet. High cholesterol fed diet rats exhibited significant increase in total serum cholesterol, triglycerides, low density lipoprotein, very low density lipoprotein, atherogenic index and lipid per-oxidation and significant decrease in high density lipoprotein. The CCl₄-induced hepatotoxicity model is extensively used to evaluate the hepatoprotective effects of drugs and extracts. In this study we diagnosed the different liver function parameter like, total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDLC) in serum by using kits. Also other diagnosed parameters such as low density lipoprotein (LDLC), atherogenic index, very low density lipoprotein (VLDL-C), HDL-C ratio and lipid per-oxidation (LPO). From obtained result it was observed that keeping the animal on atherogenic diet model (AD) significantly increased the TC, TG, LDL-C level in serum as compared to rats on normal diet. When AD was co-administered with curcumin and rosuvastatin in combination, the elevated levels of TC, TG and LDL-C and LPO condition has shown considerable decline. It was noted that TC, TG and LDL-C lowering activity of curcumin (300mg/kg) and rosuvastatin in combination was more significant as compared to curcumin and simvastatin.

Key words: Curcumin, HMG-Co A reductase inhibitor, Antihyperlipidemic, CCL₄ induced hyperlipidemia, Histopathological study

INTRODUCTION

Cholesterol levels have become the source of health concerns, even though cholesterol is one of the most valuable substances in the human body. Over the past few years, the amount of cholesterol information and dietary advice bombarding the public has grown exponentially. Cholesterol plays a major role in the assembly of membranes and performs other important biological functions in human heart health [1]. However, when plasma cholesterol exceeds the level required for these functions, it results in the development of atherosclerotic cardiovascular disease such as coronary heart disease and stroke. Hyperlipidemia may also induce other abnormalities like oxidation of free fatty acids, leading to the formation of ketone- bodies as well as masking liver and muscles resistance to insulin which initiates the progress of diabetes in patients [2].

Hyperlipidemia is a major cause of atherosclerosis and atherosclerosis related conditions like coronary heart disease, ischemic cerebrovascular disease, peripheral vascular disease and pancreatitis. The increase in lipids like low density lipoproteins (LDL), cholesterol (esters derivatives) and triglycerides are mainly responsible for this condition [3]. Simvastatin and rosuvastatin was shown to be a highly effective member of statins for lowering blood cholesterol, acts by stabilizing plaque and preventing strokes through anti-inflammatory and other mechanisms [4]. Among the plant foods that possess hypocholesterolemic property in clinical, as well as experimental studies are curcumin, garlic, gum guggul and capsicum. The aim of present study was the evaluation of antihyperlipidemic activity of HMG-CoA reductase inhibitor and curcumin in combination on diet induced hyperlipidemic rats.

MATERIALS AND METHODS

Male albino wistar rat 6-8 weeks of age, weighing between 180–240 gm of either sex were procured from animal house of Anuradha College of pharmacy, Chikhli, district Buldana (MS) India. Animals were housed in polypropylene cages under the standard conditions of temperature, pressure and humidity. (12 hrs light and dark cycles, at $25\pm3^{\circ}$ C and 35-60% humidity) [5]. The animals were maintained under standard condition as per CPCSEA guideline. Animals were fed with standard normal pellet diet and water *ad* libitum during the course of the experiment. The animals were given standard diet supplied by local market of Chikhli. The composition of the diet are 3615 (Kcal/kg), crude protein 22.05%, crude oil 4.5%, crude fibre 4.10%, ash 11.10%, sand silica 0.75% [6,7]. The experimental design and research plan along with animal handling and disposal procedure were placed before the animal ethics committee. The committee granted approval after careful evaluation of research project. Proposal No. 751/03/abc/CPCSEA) and Ref. No. IAEC/ 2011-2012.

Extract was gifted by Aldrich Pharma, simvastatin and rosuvastatin gifted by Dr. Reddy Hyderabad. Kits (total cholesterol, triglycerides and HDL-C) were purchased from Ashrita Dia Tech Hyderabad.

Induction of Hyperlipidemia

Atherogenic Diet Induced Method

Hyperlipidemia was induced in rats by giving high cholesterol diet for seven days in standard rat chow diet. The drug solution was administered orally. High cholesterol diet was prepared by mixing cholesterol 2%, sodium cholate 1% and coconut oil 2% with standard powdered standard animal food. The diet was placed in the cage carefully and was administered for seven days [8].

CCL₄ Induced Method

Hyperlipidemia was induced in rats by giving CCl4 for seven days orally [9]. Animal were administered with daily dosage of CCl4 as 1:1 dilution with liquid paraffin for every 72 hours.

Atherogenic Diet Model

Healthy wistar albino rats divided into seven groups. Each group contains six rats. Group I was taken as a normal treated with normal diet, group II treated with atherogenic diet was kept as toxicant, group III treated with atherogenic diet and simvastatin(10mg/kg), group IV treated with atherogenic diet and rosuvastatin (10mg/kg), group V treated with atherogenic diet and curcumin (300mg/kg), group VI was treated with atherogenic diet, simvastatin(2.5mg/kg) and curcumin(300mg/kg) and group VII was treated with atherogenic diet, rosuvastatin (2.5 mg/kg) and curcumin (300mg/kg) administered orally for seven days [10]. Blood was collected on 8th day by retro orbital puncture technique under mild anesthesia after 8 hour fasting and allowed to clot for 30 min at room temperature. Blood samples were centrifuged at 3000 rpm for 20 min. Serum was separated and stored at -20°C until biochemical estimation carried out. Serum sample were analyzed spectrophotometrically for total serum cholesterol (TC), triglycerides (TG) and high density lipoprotein cholesterol (HDL-C) was estimated by using diagnostic kit [11]. Very low density lipo-protein (VLDL), High density lipoprotein ratio (HDL-ratio), Atherogenic-index (AI) and low density lipoprotein cholesterol (LDL-C) were calculated by using the formula of Modi and colleague.

Table 1, Experimental desigr	1 for atherogenic diet model
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Group No.	Group Name	Treatment	Observations
Ι	Normal control	Normal diet	
Π	Toxicant control	Atherogenic diet	
III	Standard I	Atherogenic diet+ simvastatin(10mg/kg)	
IV	Standard II	Atherogenic diet+ rosuvastatin(10mg/kg)	Estimation of parameters
V	Curcumin	Atherogenic diet+ curcumin(300mg/kg)	
VI	Curcumin+ Standard I	Atherogenic diet+ simvastatin(2.5mg/kg)+ curcumin(300mg/kg)	
VII	Curcumin + Standard II	Atherogenic diet+ rosuvastatin(2.5mg/kg)+ curcumin(300mg/kg)	

CCL₄ Induced Hyperlipidemia

Healthy wistar albino rats were divided into seven groups. Each group contains six rats. Group I was taken as a normal treated with tween 80, group II treated with CCL_4 (1ml/kg) was kept as toxicant, group III was treated with CCL_4 (1ml/kg) and simvastatin (10mg/kg), group IV was treated CCL_4 (1ml/kg) and rosuvastatin (10mg/kg), group V treated with CCL_4 (1ml/kg) and curcumin (300mg/kg), group VI was treated with CCL_4 (1ml/kg), simvastatin (2.5mg/kg) and curcumin (300mg/kg) and group VII treated with CCL_4 (1ml/kg), rosuvastatin (2.5mg/kg) and curcumin (300mg/kg) and group VII treated with CCL_4 (1ml/kg), rosuvastatin (2.5mg/kg) and curcumin (300mg/kg).

Table 2, Experimental design for CCL₄ model

Group No.	Group Name	Treatment	Observations
Ι	Normal control	Normal diet	
II	Toxicant control	$\text{CCL}_4(1\text{ml/kg})$	
III	Standard I	CCL ₄ (1ml/kg)+ simvastatin 10mg/kg)	
IV	Standard II	$CCL_4(1ml/kg) + rosuvastatin(10mg/kg)$	Estimation of parameters
V	Curcumin	$CCL_4(1ml/kg)$ + curcumin (300mg/kg)	
VI	Curcumin+ Standard I	CCL ₄ (1ml/kg)+ simvastatin (2.5mg/kg)+ curcumin (300mg/kg)	
VII	Curcumin+ Standard II	CCL ₄ (1ml/kg)+ rosuvastatin (2.5mg/kg)+ curcumin (300mg/kg)	

Dosage of CCL_4 was administered as 1:1 dilution with liquid paraffin (Christina et al, 2006) for every 72 hours. The rats were sacrificed after 48 hour of last dose by cervical decapitation. The blood samples were collected separately by cardiac puncture and allow to clots for 30 min at room temp. The clear serum was separated by centrifugation at 2500 rpm for 10 min [14]. The TC, TG and HDL-C were determined using standard kits.

1. Total Cholesterol

It was estimated by CHOD-PAP method using kit. The procedure was given in following Table No.3.

Fable3, Procedure for	r total	cholesterol	determination
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Pipette into tubes marked	Blank	Standard	Test
Serum/plasma	-	-	10µl
Reagent 2	-	10µ1	-
Reagent 1	1000µl	1000µl	1000µl

Mix well and incubate at 37 °C for 10 minutes or at room temperature (15-30 °C) for 30 minutes.

Total cholesterol (mg/dl) =

Absorbance of test

_____× 200 Absorbance of standard

2. HDL-Cholesterol Determination:

CHOD-PAP method was used to estimate the serum HDL- cholesterol. Step A – HDL Cholesterol separation

Table 4, Procedure for HDLC separation

Pipette in centrifuge tube	Quantity
Sample	200µl
Precipitating reagent	200µl

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Mix well and keep at room temperature for 10 minutes and then centrifuge at 2000 rpm to obtain a clear supernatant. Proceed to step- B of procedure.

Step B – Colour development

Table 5, Procedure for HDLC determination

Pipette into tubes marked	Blank solution	Standard sample	Test sample
Supernatant from step A	-	-	10µ1
HDL-cholesterol Standard	-	10µ1	-
Cholesterol reagent	1000µ1	1000µ1	1000µ1

Mix well and incubates at 37° C for 10 minutes. Read absorbance against reagent blank at 505 nm within 60 minutes. HDL cholesterol (mg/dl) = Absorbance of test

Absorbance of standard

3. Triglycerides: It was estimated by GPO-PAP method by using kit.

Table 6, Procedure for triglycerides determination

Pipette into tubes marked	Blank solution	Standard sample	Test sample
Serum/plasma	-	-	10µ1
Reagent 2	-	10µ1	-
Reagent 1	1000µl	1000µl	1000µl

Total triglyceride (mg/dl) =

Absorbance of test

_____ × 200 Absorbance of standard

4. LDL and VLDL cholesterol

LDL and VLDL-cholesterol was calculated by following method of Friedwald *et al.* 1992, Ghube B.V. *et al* 2009. VLDL- Cholesterol = TG/5

LDLC = (Total cholesterol) - (HDLC) - (VLDLC)

5. Atherogenic index

Atherogenic index and LDL-C/HDL-C was calculated by following formula. AI = (Total cholesterol-HDL-C)/HDL-C

6. LDL-C/HDL-C ratio

LDL-C/HDL-C was calculated as the ratio of serum LDL-C to HDL-C level.

7. Estimation of lipid Peroxidation

From each experimental rat 900mg of liver tissue was collected and washed with normal saline then soaked in filter paper. The tissues were then homogenized in 10 ml of 0.15 M Tris-buffer (pH-7.4) and centrifuged at 3000g at 4° C for 30 min [15, 16]. The supernatant collected was taken for lipid peroxidation assay. Malondialdehyde (MDA), produced during the peroxidation of lipids, served as an index of lipid peroxidation. MDA reacts with thiobarbituric acid (TBA) to generate a colored product which absorb at 532nm. Reagents used were normal saline (0.9% w/v), 10% TCA, and 1% thiobarbituric acid.

Lipid per-oxidation (LPO) was assayed according to the method of Okhawa., et al.1979. TCA 10% was added and mixed well. The mixture (3000 g) was then centrifuged at room temperature for 10 min to separate proteins. Supernatant (2 ml) was taken, 0.5ml 1.0% TBA was added followed by heating at 95° C for 60 min to generate the pink colored MDA. The samples were measured at 532 nm using Beckman DU 64 spectrophotometer. The levels of lipid peroxides were expressed as nm of MDA/mg wet tissue using extinction co-efficient of 1.56 x105 M^{-1} cm⁻¹ [17].

% lipids Inhibition = $\{Ao-A1\}/Ao \times 100$

Where; Ao is the absorbance of the control and A1 is the absorbance of the sample extract.

8. Histopathological Examination

On the day of blood withdrawal the two animals from each group were sacrificed and liver were isolated. Tissue samples were immersed in10% formalin for at least 24 hr to fix the tissue. The tissue was embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. The sections were viewed under the light microscope for histopathological changes [18]. The study was conducted in Omega laboratories, Khandala, district Satara.

9. Statistical analysis

The data were presented as Mean \pm SD and the statistical analysis by one way ANOVA followed by Dunnett's multiple comparison tests [19, 20].

RESULTS AND DISCUSSION

Serum total cholesterol level significantly elevated in both models i.e. AD and CCl4, as compare to normal control group. Treatment with curcumin alone and its combination with simvastatin and rosuvastatin for seven days showed marked decrease in concentration of serum cholesterol as compare to toxicant control group [21]. Serum HDL-C level decreased in both models (AD and CCl4) as compare to the normal control group. Treatment with curcumin alone and its combination with simvastatin and rosuvastatin for seven days showed marked increased in concentration of serum HDL-C as compare to toxicant control group. The serum triglyceride significantly elevated in atherogenic diet model and decreased in CCl4 model as compare to normal control group. Treatment with curcumin alone and its combination with simvastatin and rosuvastatin for seven days showed marked decrease in concentration of serum triglyceride in atherogenic diet model and increased in CCl4 model as compare to toxicant control group. Serum low density lipoprotein cholesterol significantly elevated in both models (AD and CCl4) as compare to the normal control group [22]. Treatment with curcumin alone and its combination with simvastatin and rosuvastatin for seven days showed marked decrease in concentration of serum low density lipoprotein as compare to the toxicant control group. Serum very low density lipoprotein cholesterol elevated in atherogenic diet model and decrease in CCl4 model as compare to the normal control group. Treatment with curcumin alone and its combination with simvastatin and rosuvastatin for seven days showed marked decrease in concentration of serum VLDL-C in atherogenic diet and increased in CCl4 model as compare to toxicant control group[23, 24]. Serum atherogenic index increased in both models (AD and CCl4) as compare to normal control group. Treatment with curcumin alone and its combination with simvastatin and rosuvastatin for seven days showed marked decrease in concentration of serum atherogenic index as compare to toxicant control group. Serum LDL-C/HDL-C ratio significantly elevated in both models i.e. AD and CCl4 as compare to normal control group. Treatment with curcumin alone and its combination with simvastatin and rosuvastatin for seven days showed marked decrease in concentration of serum LDL-C/HDL-C ratio as compare to toxicant control group [25].

Serum lipid per oxidation increased in both models i.e. AD and CCl4 as compare to normal control group. Treatment with curcumin alone and its combination with simvastatin and rosuvastatin for seven days showed marked decrease in concentration of serum lipid per oxidation as compare to toxicant control group. Liver of rat from normal group showed normal histological structure of hepatic lobule [26]. Liver of rat from toxicant group showed normal histological structure of hepatic lobule [26]. Liver of rat from toxicant group showed focal hepatic necrosis, hemorrhages, fibroplasias in portal triad associated with chronic cholangitis. Liver of rat from standard I group depicted hydropic degeneration of some hepatocytes [27]. In liver section of standard II group there was congestion of hepatic sinusoid observed. Hepatocellualr vacuolization was seen in liver section of rat from curcumin. Improvement in hepatocytes was noticed in liver of rat belongs to standard I and curcumin group. Finally liver of rat from standard II and curcumin group showed apparently normal hepatocytes [28]. In present study rosuvastatin and curcumin combination was found to be better as compare to other groups.

Sr					Gre	oups		
No	Parameters	Normal	Toxicant	Standard	Standard	Curcumin	Curcumin +	Curcumin +
		control	control	I	П		Standard I	Standard II
1	TC	96.28	244.98	169.41	153.70	208.34	131.29	117.34
1.	(mg/dl)	±1.05	±1.74	±1.91	±1.31	±2.23	±1.50	±1.41
2	TG	81.66	160.62	124.35	114.68	137.36	105.78	94.06
۷.	(mg/dl)	±0.68	±1.64	±1.37	± 1.40	±1.27	±1.67	±1.33
2	HDL-C	64.34	27.52	45.38	50.53	38.92	59.26	62.21
5.	(mg/dl)	± 0.88	± 0.80	± 0.77	± 1.96	±1.38	± 1.59	± 0.57
4	LDL-C	31.27	244.55	145.87	122.36	195.41	86.47	64.58
4.	(mg/dl)	±1.62	±2.16	±1.16	±1.14	±3.65	±2.60	±1.51
5	VLDL-C	16.33	32.12	24.86	22.93	27.47	21.15	18.80
5.	(mg/dl)	±0.13	±0.32	±0.27	±0.28	±0.25	±0.33	±0.26
6	LDL-C/ HDL-C	0.38	9.09	3.015	2.15	5.06	1.32	0.89
0.	ratio	±0.02	±0.23	±0.05	±0.10	±0.27	±0.06	±0.02
7	ΔĪ	0.140	7.92	2.5	1.81	4.40	0.99	0.62
7.	AI	±0.02	±0.20	±0.05	±0.05	±0.26	±0.05	±0.02
0	L DO	27.71	65.70	54.91	50.32	58.11	38.15	33.7
8.	LPO	±0.56	±0.26	±0.24	±0.34	±0.16	±0.81	±0.33

Table 7, Effect of curcumin alone and in combination on liver function parameters in AD induced hyperlipidemic model

Values are mean \pm SEM (n=6), p<0.01 as compared to toxicant control group

The statistical analysis of data was carried out by one way ANOVA followed by Dunnett's multiple comparison test.

Table 8, Effect of curcumin alone and in combination on liver function parameters in CCl4 induced hyperlipidemic model

		Groups						
Sr. No	Parameters	Normal control	Toxicant control	Standard I	Standard II	Curcumin	Curcumin + Standard I	Curcumin + Standard II
1	TC (mg/dl)	77.31	191.11	122.73	135.77	150.07	94.47	84.32
1	i C (iiig/ui)	±1.23	±1.75	±1.75	±1.94	±1.32	±1.43	±1.38
2	TC(mg/dl)	110.19	55.81	80.12	86.14	68.03	93.77	103.76
۷.	TO(ing/ui)	±1.21	±0.64	±0.98	±1.06	±1.61	±1.25	±1.20
2		62.31	30.50	44.10	50.49	38.66	54.21	58.18
5.	HDL-C(IIIg/dl)	± 2.80	± 2.35	± 1.60	±1.47	± 1.19	± 1.81	± 0.93
4		19.38	228.11	123.07	98.39	174.82	65.06	45.59
4.	LDL-C	±1.91	±3.22	±1.35	±2.08	±2.97	±1.99	±1.52
5	VIDL C(ma/dl)	22.03	11.16	16.02	17.13	13.60	18.59	20.74
5.	VLDL-C(IIIg/dI)	±0.24	±0.13	±0.19	±0.26	±0.32	±0.25	±0.24
6	LDL-C/ HDL-C	0.33	5.23	2.01	1.560	3.22	1.00	0.71
0.	ratio	±0.01	±0.31	±0.06	±0.02	±0.21	±0.04	±0.02
-	A T	0.17	7.93	2.50	1.82	4.39	0.93	0.62
7.	Al	±0.01	±0.20	±0.05	±0.05	±0.24	±0.05	±0.02
0	LDO	26.78	68.30	41.19	35.61	52.32	36.55	33.03
ð.	8. LPO	+0.80	+0.60	+0.51	+0.81	+0.72	+0.11	+0.49

Values are mean \pm SEM (n=6), p<0.01 as compared to toxicant control group

The statistical analysis of data was carried out by one way ANOVA followed by Dunnett's multiple comparison test.

Table 9, Scores of histopathological study

Group	Congestion	Hydropic degeneration	Necrosis	Cellular Vacuolization	Fibroplasia
Normal	00	00	00	00	00
Toxicant (CCl4)	++++	++++	++++	++++	++++
Standard-I	++	+++	++	+++	++
Standard –II	+++	+++	++	++	+++
Curcumin	++	+++	++	+++	++
Standard-I+ Curcumin	+	++	++	+	+
Standard-II+Curcumin	+	+	+	++	+

0: No abnormality detected

+: Damage/ active changes up to less than 25%

++: Damage/ active changes up to less than 50%

+++: Damage/ active changes up to less than 75%

++++: Damage/ active changes up to more than 75%



Figure 1, Liver section- Normal



Figure 2, Liver section- Toxicant



Figure 3, Liver section- Standard I



Figure 4, Liver section- Standard II



Figure 5, Liversection- Curcumin



Figure 6, Liver section standard I and Curcumin



Figure 7, Liver section- Standard II and curcumin

CONCLUSION

Two models were used for inducing hyperlipidemia i.e. atherogenic diet and CCl4. Test group having rosuvastatin and curcumin in combination showed better antihyperlipidemic effect as compare to other test groups in both atherogenic diet induced model and CCl4 induced model.

The histopathology scores also revealed that the group receiving CCl4 showed marked liver damage. The group which received the curcumin alone and in combination showed better liver condition as compared to standard drugs i.e., simvastatin and rosuvastatin. It was found that the test group of rosuvastatin and curcumin in combination showed better antihyperlipidemic activity when it compare with other test groups. The results also support the fact that rosuvastatin and curcumin in combination treats hyperlipidemia in a better way.

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