Modulatory Effect of α-tocopherol in Simvastatin and Gemfibrozil Induced Rhabdomyolysis in Rats

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ABSTRACT

The present study was aimed to evaluate the protective effect of α-tocopherol in simvastatin and gemfibrozil induced rhabdomyolysis in rats. HMG-Coenzyme A reductase inhibitors are the most widely used drug for the treatment of hyperlipidemias but their long term use is associated with rhabdomyolysis. Administration of simvastatin (80 mg/kg, p.o.) and gemfibrozil (600mg/kg, p.o. twice) for 30 days produced significant increase in the level of various serum parameters (creatine Phospho kinase, urea and blood urea nitrogen) indicating development of rhabdomyolysis in rats as well as development of renal complications. Administration of simvastatin + gemfibrozil also caused significant increase in the level of lipid peroxidation and decrease in the antioxidant enzymes (reduced glutathione, superoxide dismutase and catalase) level in rats. α-tocopherol (1gm/kg, p.o) was given to prevent rhabdomyolysis caused by simvastatin + gemfibrozil combination.

Administration of α-tocopherol caused increase in the level of antioxidant enzymes and decrease in the level of lipid peroxidation. α-tocopherol also caused improvement in the serum parameters of rhabdomyolysis in the rats. Histopathology of the kidney from simvastatin + gemfibrozil treated rats showed no significant renal damage. Improvement in the level of biochemical parameters and decrease in the level of oxidative stress indicates that α-tocopherol due to its antioxidant activity modifies the biochemical changes occurred during rhabdomyolysis and thus had a potential protective effect in rhabdomyolysis.

Keywords: Antioxidant, α-tocopherol, Gemfibrozil, Oxidative stress, Rhabdomyolysis, Simvastatin.

INTRODUCTION

Cardiovascular diseases are the major health problem, and leading cause of death. Hypercholesterolemia is the major cause of cardiovascular diseases. Hypercholesterolemia has been linked conclusively with atherosclerosis. Antihyperlipidemic drugs like HMG-CoA reductase inhibitors have emerged as a dominant class for the treatment of hypercholesterolemia. However potentially serious adverse effect of treatment with statin includes the elevation of liver enzymes and the development of skeletal muscle abnormalities including myositis and myopathy[1]. Rhabdomyolysis is defined as a creatine phosphokinase (CPK) value more than 5000 U/L in addition to renal failure and/or electrolyte abnormalities and characterized by massive muscle necrosis and myoglobinurea. Rhabdomyolysis refers to disintegration of striated muscles which results into skeletal muscle injury that alters the integrity of sarcolemma and the release of muscular cell constituents into the extracellular fluid and in circulation[2]. Drug induced myopathy is also reported with fibric acid

derivatives when used along with HMG-CoA reductase inhibitors.

In rhabdomyolysis viscosity of sarcolemma is changed due to activation of phospholipase A_2 as well as various vasoactive molecules and proteases takes place [3]. It causes increased permeability of the sarcolemma, permitting leakage of intracellular contents as well as increased intracellular sodium ions. This leads to an increase in the concentration of intracellular calcium ions[4] which enhances the activity of intracellular proteolytic enzymes and destruction of intracellular structures. In addition, calcium leads to formation of free oxygen radicals which causes damage of the muscles. Damaged muscle is invaded by neutrophils that amplify the damage by releasing proteases and more free radicals. This results in an inflammatory, self-sustaining myolytic reaction, rather than pure necrosis. Severe hypokalemia, increased blood myoglobin and impaired glycogen synthesis along with reduction in energy production in muscles is also associated with rhabdomyolysis.

In patients with rhabdomyolysis high level of oxygen free radicals have been detected before the onset of symptoms. Free radicals are reactive oxygen species which are involved in the pathophysiology of various diseases[5]. Antioxidants are the major cell defense against free radical mediated toxicity. Vitamin E is a fat soluble vitamin also known as α-tocopherol.

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It is major member of Vit.E family, present in the blood as dl-α-tocopherol and is the first line of defense against cell membrane damage. It is the most important free radical scavenger. It gets concentrated in cell membranes and blood lipoproteins and protects them from oxidation. It is also known as competitive antioxidant because it functions to intercept lipid peroxyl radical and so terminate lipid peroxidation chain reaction[6,7].

On the basis of above facts the present study has been aimed to investigate the protective effect of antioxidant α-tocopherol in simvastatin and gemfibrozil induced rhabdomyolysis in rats.

MATERIAL AND METHODS

Drugs and chemicals: Chemicals of highest purity and analytical grade were used for the study and purchased from HIMEDIA, Mumbai. Gemfibrozil was obtained as a gift sample from Sun Pharmaceuticals, Baroda. α-tocopherol was obtained from Troika Pharma Ahmedabad and simvastatin from Carysyon Pharmaceuticals. Span Diagnostic Kits were used for the estimation of serum parameters.

Animals: Wistar albino rats of either sex (150-250 g) were procured from Food and drug Laboratory Baroda, and housed in the departmental animal house. The animals were housed in standard polypropylene cages and maintained under controlled room temperature (22 ± 2^0C) and humidity ($55 \pm 5\%$) with 12:12 hour light and dark cycle. All the animals were provided with commercially available rat normal pellet diet and water ad libitum. The guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA) Govt. of India were followed and prior approval was taken from the institutional animal ethics committee for conducting the animal experimental studies.

Treatment protocol

Animals were divided into five groups of six animals in each. They received the following treatment for 30 days. All the drugs were given as a suspension in distilled water with 0.1% CMC as a suspending agent.

Group I: Normal animals received 0.1% CMC in distilled water (1.0 ml/kg, p.o).

Group II: Normal received simvastatin (80 mg/kg, p.o.) and gemfibrozil (600 mg/kg, p.o,twice)[8].

Group III: Normal animals received α-tocopherol (1gm/kg p.o.) alone to the normal rats.

Group IV: α-tocopherol along with the combination of simvastatin and gemfibrozil.

After the treatment period each blood sample was analyzed for estimating the prooxidant, antioxidant status and various biochemical parameters in order to evaluate the oxidative stress and biochemical abnormalities during the course of rhabdomyolysis. Blood samples were collected from the retro orbital plexus of treated rats and centrifuged by using cooling centrifuge at 2500 RPM and serum was obtained. Serum was used for the estimation various biochemical parameters of rhabdomyolysis by using Span Diagnostic kits on RA chemical analyzer for assessment of rhabdomyolysis.

At the end of experimental period whole blood (5 ml) was collected in EDTA bulb from the treated rats. All blood samples were used for the estimation of thiobarbituric acid reactive substances (lipid peroxidation, LPO) and endogenous antioxidant enzymes reduced glutathione (GSH), superoxide dismutase (SOD) and catalase by using spectrophotometric methods using Hitachi Spectrophotometer.

Evaluation of various biochemical parameters in simvastatin and gemfibrozil induced rhabdomyolysis and treated rats: Creatine kinase was estimated by the colorimetric method [9] at 340 nm using Span Diagnostic Kits. AST and LDH were estimated by UV kinetic method [10] at 340 nm using Span Diagnostic Kits. Creatinine [11] was estimated by picrate method at 500 nm. Urea [12] and blood urea nitrogen (BUN) [12] was estimated at 340 nm by urease method.

Determination of the level of thiobarbituric acid reactive substances (TBARS) in simvastatin and gemfibrozil induced rhabdomyolysis and treated rats: After the treatment period whole blood was collected in EDTA bulb and blood was centrifuged at 3000 rpm in the centrifuged machine (Remi-motors LtD., Mumbai) to obtain plasma. The supernatant (Plasma) was used for the estimation of thiobarbituric acid reactive substances and the settled blood cells were used for the estimation of SOD and catalase. The level of TBARS was estimated by the method of Slater and Sawyer (1971)[13] using Spectrophotometer (UV-250, Shimadzu, Japan).

2 .0 ml of the plasma was added to 2.0 ml of freshly prepared 10% w/v trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 min. After 15 min. the precipitate was separated by centrifugation and 2.0 ml of clear supernatant was mixed with 2.0 ml of freshly prepared 0.67% thiobarbituric acid (TBA). The resulting solution was heated in a boiling water bath for 10 min. It was then immediately cooled in an ice bath for 5 min. The colour developed was measured at 532 nm against reagent blank. Different concentrations of malondialdehyde were taken and processed as above for standard graph. The values are expressed as nM of MDA/mg protein.

2.6.2. Determination of reduced glutathione in simvastatin and gemfibrozil induced rhabdomyolysis and treated rats: Reduced glutathione was determined by the method of Moron et al (1979)[14] using whole blood. Equal volumes of whole blood and 20% trichloroacetic acid were mixed. The precipitated fraction was centrifuged and to 0.25 ml of supernatant, 2 ml 0.6 mM 5,5-dithiobis (2-nitro benzoic acid) reagent was added. The final volume was made upto 3 ml with phosphate buffer (0.2 M, pH 8.0). The colour developed was read at 412 nm against reagent blank. Different concentrations (10-50 μg) of standard glutathione were taken and processed as above for standard graph. The amount of reduced glutathione was expressed as μg of GSH/mg protein.

2.6.3. Determination of superoxide dismutase (SOD) in simvastatin and gemfibrozil induced rhabdomyolysis and treated rats: After the treatment period whole blood was collected in EDTA bulb and blood was centrifuged at 3000 rpm in the centrifuged machine (Remi-motors LtD., Mumbai). The settled blood cells were used for the estimation of SOD and catalase. Superoxide dismutase was estimated by the method of Misra and Fridovich (1972)[15]. 0.5 ml of tissue homogenate was diluted with 0.5 ml of distilled water, to which 0.25 ml of ice cold ethanol and 0.15 ml of ice cold chloroform were added. The mixture was mixed well using cyclo mixer for 5 minutes and centrifuged at 2500 rpm. To 0.5 ml of supernatant, 1.5 ml of carbonate buffer (0.05M, pH 10.2) and 0.5 ml of EDTA solution (0.49M) were added. The reaction was initiated by the addition of 0.4 ml of epinephrine (3 mM) and the change in optical density /minute was measured at 480 nm against reagent blank. SOD activity was expressed as units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.

2.6.4. Determination of catalase (CAT) in simvastatin and gemfibrozil induced rhabdomyolysis and treated rats: Catalase was estimated by the method of Hugo Aebi as given by Colowick *et al* (1984)[16] using settled blood cells. To 2.0 ml of diluted sample 1 ml of hydrogen peroxide (30 mmol/l) was added to initiate the reaction. The blank was prepared by mixing 2 ml of dilute sample (similar dilution) with 1 ml of phosphate buffer (50 mmol/l; pH 7.0). The dilution should be such that the initial absorbance should be approximately 0.500. The decrease in absorbance was measured at 240 nm. Catalase activity was expressed as μ moles of H_2O_2 consumed/min/mg protein.

Histopathology of Kidney: Kidney from the treated rats was isolated and kept in formalin solution. Dehydration and clearing of the tissues were done subsequently. Sections of the 5 μ thickness were cut and they were stained with Hematoxylin and Eosin (H/E) stains. Stained sections were quantitatively viewed under 40X and 100 X magnifications.

Statistical analysis: Results were expressed as Mean ± SEM. The unpaired t-test was used for analyzing the data between two groups. Statistical analysis of data among the groups was performed by using analysis of variance (ANOVA) followed by tukey test of significance.

RESULTS

Effect of simvastatin + gemfibrozil on serum parameters of rhabdomyolysis: Administration of simvastatin + gemfibrozil in rats produces significant increase in all the biochemical parameters used to access rhabdomyolysis compared to control animals (Table 1). Results suggest significant induction of rhabdomyolysis after simvastatin + gemfibrozil treatment.

Evaluation of oxidant-antioxidant status in rhabdomyolysis: Administration of simvastatin + gemfibrozil in rats produced significant increase in lipid peroxidation compared to control animals suggesting formation of free radicals in rhabdomyolysis (Table 2). Blood level of GSH, SOD and catalase reduces significantly after treatment with simvastatin + gemfibrozil compared to respective control. Significant increase in free radical formation and significant decrease in antioxidant enzymes level contributes to the involvement of oxidative stress in rhabdomyolysis.

Modification in biochemical parameter of rhabdomyolysis after treatment with antioxidant α-tocopherol: Results suggests that administration of α-

Table 1: Effect of simvastatin + gemfibrozil on serum parameters in rhabdomyolysis in rats (n= 6)						
Groups + Treatment (p.o.)	CPK (IU/L)	LDH (IU/L)	AST(Units/ml of serum)	Creatinine (mg/dl)	Urea (mg/dl)	BUN (mg/dl)
Control group (D.Water,1 ml/kg)	202.5±2.9	200±1.5	36.6±1.9	0.66 ± 0.3	25.6±0.5	11.9±0.5
Simva + Gem. (80 mg/kg+600 mg/kg, twice)	1116±2.5*	778±3.0*	93.8± 5.2*	5.3±0.38*	145±0.9*	67.6±0.23**

Values are expressed as Mean ± SEM.* <0.01; **<0.001 as compared to control group.
Index: Simva= Simvastatin; Gem= Gemfibrozil

tocopherol to the normal rats produced no significant change in biochemical parameter of rhabdomyolysis but α-tocopherol produced significant improvement in the biochemical parameters of rhabdomyolysis when given along with simvastatin + gemfibrozil (Table 3). Results suggest that α-tocopherol caused improvement in serum parameters and normalizes the elevated level of serum parameters in rhabdomyolysis.

Modification in antioxidant enzymes and lipid peroxidation level after treatment with α-tocopherol in rhabdomyolysis: Administration of α-tocopherol alone produced no change in antioxidant enzymes level in normal rats (Table 4). Administration of α-tocopherol produced significant increase in the level of antioxidant enzymes (reduced glutathione, superoxide dismutase and catalase) in simvastatin + gemfibrozil induced rhabdomyolysis compared to respective simvastatin + gemfibrozil treated group (Table 4). Result suggests significant improvement in antioxidant enzymes level after treatment with α-tocopherol.

Administation of α-tocopherol produced no significant change in lipid peroxidation in normal rats but it caused significant decrease in the level of lipid peroxidation in simvastatin + gemfibrozil induced rhabdomyolysis compared to respective control. Results suggest significant reduction in lipid peroxidation and decrease in free radical formation after treatment with α-tocopherol.

Effect of simvastatin + gemfibrozil on histopathology of kidney: Histopathological study of kidney (figure 2) indicates mild renal damage in simvastatin + gemfibrozil treated group. As it is known that renal damage occurs in severe cases of rhabdomyolysis but duration of treatment (30 days) and dose of the statin in this study may be the reason for mild renal damage.

Table 2: Effect of simvastatin + gemfibrozil on oxidant-antioxidant status in rhabdomyolysis in rats (n= 6)				
Groups + Treatment (p.o.)	LPO (n mole/L)	GSH (µg of GSH / ml of blood)	SOD (EU/L)	Catalase (k/g of Hb)
Control (Water, 1 ml/kg)	13.24 ± .23	113 ± 2.4	100 ± 1.18	403 ± 2.3
Simva + Gem (80 mg/kg + 600 mg/kg, twice)	38.8 ± 1.78*	75.6 ± 0.29*	82.8 ± 2.5*	300 ± 4.43*

Values are expressed as Mean ± SEM., *<0.01 as compared to control group. **Index:** Simva= Simvastatin; Gem= Gemfibrozil

Table 3: Effect of α- tocopherol on serum parameters in simvastatin + gemfibrozil induced rhabdomyolysis in rats (n= 6)						
Groups + Treatment (p.o.)	CPK (IU/L)	LDH (IU/L)	AST(Units/ml of serum)	Creatinine (mg/dl)	Urea (mg/dl)	BUN (mg/dl)
Control group (D.Water,10 ml/kg)	203.5±1.9	210±1.0	35.6±1.6	0.7±0.3	23.6±0.5	12±0.45
Simva. + Gemfibrozil (80mg/kg+600mg/kg)	1118±2.0	776±3.2	94±5.2	5.1±0.3	148±0.7	68±0.3
α-tocopherol (1 gm/kg)	205.0±1.9	215.0±1.8	37.5±0.8	0.65±0 .03	14.0±0.19	13.5±0.19
Simva. + Gem. + α- tocopherol	398±2.3**	357±1.9**	50.6±0.50**	1.15±0.08**	20.5 ±1.5**	21.5±1.2**

Values are expressed as Mean ± SEM.* <0.01; **<0.001 as compared to control group.
Index: Simva= Simvastatin; Gem= Gemfibrozil

| Table 4: Effect of α- tocopherol on oxidant – antioxidant status in simvastatin + gemfibrozil induced rhabdomyolysis in rats (n= 6)				
Groups + Treatment (p.o.)	LPO (n mole/L)	GSH (µg of GSH / ml of blood)	SOD (EU/L)	Catalase (k/g of Hb)
Control group (Distilled Water, 10 ml/kg)	13.64±0.3	115±2.5	105±1.2	400±1.3
Simva. + Gemfibrozil (80mg/kg+600mg/kg)	39±1.2	76±0.3	83±2.5	309±2.43
α- tocopherol (1 gm/kg)	13.5± 0.23	115±1.3	100.9±2.1	403±1.9
Simva+Gem+α- tocopherol	19.5 ± 0.22**	90.6 ± 1.0**	88 ± 0.66**	315 ± 1.1**

Values are expressed as Mean± SEM, * <0.01; **<0.001 as compared to simvastatin and gemfibrozil treated group.
Index: Simva= Simvastatin; Gem= Gemfibrozil,

Fig. 1: Microscopic section of kidney of rats treated orally with simvastatin + gemfibrozil showing normal histology. (H&E X 40)
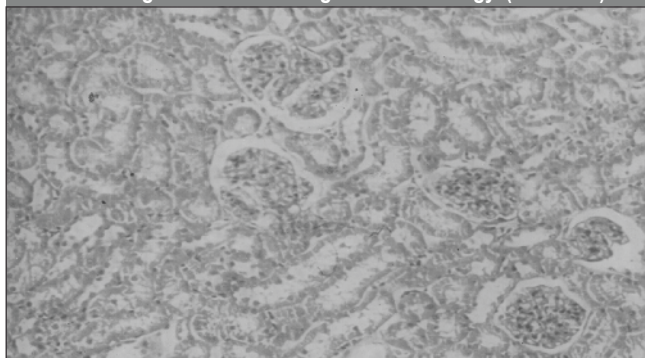
Fig. 2: Microscopic section of kidney of rats treated orally with antioxidant α-tocopherol in simvastatin + gemfibrozil induced rhabdomyolysis. (H&E X 40)
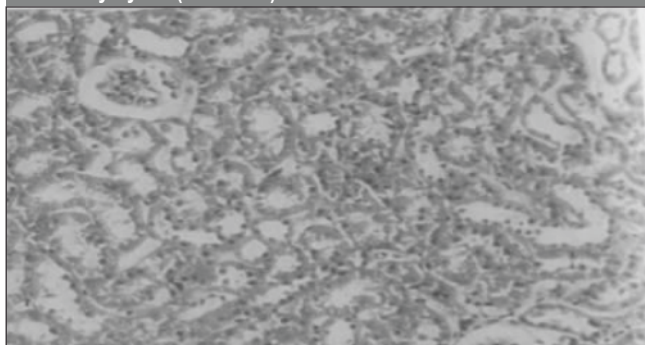
DISCUSSION

Statins and fibric acid derivatives have emerged as a dominant class of drugs for the treatment of hypercholesterolemia, but the use of these drugs is associated with myopathy and rhabdomyolysis.[1] In the present study administration of simvastatin + gemfibrozil produced rhabdomyolysis in rats characterized by increase in CPK, LDH, SGOT, CRTN, UREA and BUN. They also caused increase in the level of oxidative stress, decrease in antioxidant enzymes level and muscle weakness in rats suggesting induction of rhabdomyolysis. There is no correlation of signs and symptoms of rhabdomyolysis in human with such animal models; however above biochemical parameters measured from serum in the animal models suggest induction of rhabdomyolysis. 3-Hydroxy-3-Methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors reduced serum levels of Coenzyme Q A[17]. In patients with rhabdomyolysis high levels of oxygen free radicals were detected before the onset of symptoms. The released myoglobin from muscles may cause free radical generation and damage to kidney. The proposed mechanism for statin induced rhabdomyolysis involves a decrease in the synthesis of ubiquinone and dolichol. Ubiquinone acts as an electron carrier for oxidative phosphorylation, a stabilizer of the cell membranes and an intracellular scavanger of free radicals to prevent lipid peroxidation.

α-tocopherol is an antioxidant and provides major cell defense against free radical toxicity in the body. Antioxidant defense mechanisms operate to detoxify or scavanges reactive oxygen species.

As administration of α-tocopherol caused decrease in the level of all serum parameters to the normal level in rhabdomyolysis. It also caused decrease in the level of lipid peroxidation and increase in the level of antioxidant enzymes (GSH, SOD and catalase) level indicating protective effect of α-tocopherol in rhabdomyolysis. So from the above observation we can assume that α-tocopherol, being a potent antioxidant reduces statin induced increased oxidative stress in the body and normalizes the elevated levels of serum parameters to the normal value in rats with statin induced rhabdomyolysis.

Histopathological study indicates mild renal damage in rats with rhabdomyolysis, duration of treatment may be the reason for this observation.

Biochemical estimations confirmed the induction of rhabdomyolysis and renal failure by chronic administration of simvastatin + gemfibrozil. Pro oxidant-antioxidant status also suggests involvement of oxidative stress in rhabdomyolysis. As statins causes rhabdomyolysis by decreasing antioxidant defense system in the body, α-tocopherol being a potent antioxidant restores the level of antioxidant defense and provides significant protection in statin and gemfibrozil induced rhabdomyolysis.

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