Influence of anti-hyperlipidemic activity by co-administration of polyamines and rutin with simvastatin

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A R T I C L E I N F O

A B S T R A C T

Introduction: Natural polyphenols, rutin, endogenous polycations, and polyamines (spermine and spermidine) are reported to have beneficial effects on hyperlipidemia, obesity, and cardiovascular disease (CVD). The present study attempts to evaluate the combined effects of polyamines or rutin and simvastatin on hyperlipidemic rats.
Methods: Wistar rats were maintained on a high-fat diet (HFD) for 60 days. The HFD rats were administered from the 31st day onward with polyamines (PAs), rutin, and simvastatin for the next 30 days. The body weight, serum lipid profile, biomarkers, liver cholesterol, triglycerides, hydroxymethylglutaryl-CoA (HMG-CoA) reductase activity, antioxidants, and marker enzymes in HFD rats were estimated along with the liver histoarchitecture of the rats.
Results: The experimental findings demonstrated abnormal alterations in body weight, serum lipid profile, hepatic lipid, and HMG-CoA reductase activity, hepatic antioxidants, serum marker enzymes, blood glucose, total protein, and histoarchitecture of the liver in HFD rats. The aforementioned treatment elicited a significant reduction in the biochemical parameters, biomarkers, and the evaluated enzymes in the present study. Furthermore, an improvement in the histoarchitecture of the liver was observed.
Conclusion: The experimental results point out the positive role of antioxidant polyamines along with rutin on different parameters in HFD rats. Thus the combinatorial effect of polyamines and rutin with simvastatin (5 mg/kg) was found to be greater than simvastatin (10 mg/kg) alone. The enhancement in the anti-hyperlipidemic effect of simvastatin may be due to the intrinsic antioxidant property of polyamines and rutin.

Introduction

Abnormal alterations in the major circulatory lipid profile are the typical characteristics of hyperlipidemia (1). Dyslipidemia or hyperlipidemia is one of the leading lipoprotein metabolic disorders with characteristic advancement in serum level of triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and concomitant decline in high-density lipoprotein (HDL) cholesterol (2). Hyperlipidemia is considered to be the most prevalent positive risk factor for imitation of atherosclerosis, myocardial infarction, coronary heart disease, and stroke, followed by the

progression of cardiovascular diseases (CVDs), a major cause of morbidity/mortality worldwide. Usually, a high-fat diet (HFD) is a threatening issue as it causes accretion and redeployment of fats in the tissue of all visceral organs (3, 4).

Documented reports have revealed that to combat CVD or associated complications in clinical practice, lipid-lowering drugs like simvastatin and other statins are widely used. Statins are getting the attention of many clinicians as well as researchers for the evaluation of new chemical entities in preventing cholesterol synthesis due to their predominant effects on hydroxymethylglutaryl-

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CoA (HMG-CoA) reductase (the rate-limiting enzyme) and having a primary role in lipid biosynthesis (5). Patient tolerability is an important factor for anti-hyperlipidemic drugs since their therapy is likely to be a lifelong process.

Besides therapeutic efficacy, many presently available anti-hyperlipidemic drugs (statins, fibrates, niacin, bile acid sequestrants) are often linked with characteristic side effects like hepatotoxicity, diarrhea, hyperuricemia, severe muscle damage (myopathy), gastric irritation, abnormal liver function, statin-associated muscle symptoms, and central nervous system grievances (6,7). Thus, due to their many limitations in several clinical situations, the need for natural medications having minimal or no detrimental side effects is preferred (8). Therefore, the development of safe as well as effective pharmacological treatment for hyperlipidemia is warranted on an urgent basis.

The polyamines, spermine and spermidine are pervasive, low molecular weight, polycationic aliphatic amines, synthesized and prerequisite in every living cell for growth and differentiation (9). Potential antioxidant activities of both spermine and spermidine have been established in *in vivo* and *in vitro* conditions (10-14). Polyamines have been shown to have positive benefits in hyperlipidemia in numerous trials. Spermidine's protective impact on obesity and CVDs has been demonstrated in epidemiologic and clinical research. Additionally, the epidemiological studies of dietary polyamines have demonstrated that consuming more of these nutrients might significantly lower mortality, particularly from CVDs (15,16). Many *in vivo* and *in vitro* experiments have revealed that increased polyamines metabolism may produce beneficial effects on lipid metabolism progression concerning physiological and dietary-induced obesity. Furthermore, it has been reported that treatment with spermine in mice can improve the lipid oxidation process and reduce adipose mass injury (17). However, despite experimental reports on the individual role of spermine and spermidine, there is no specific experimental study to show/reveal the combined action of spermine and spermidine by oral administration for the treatment of hyperlipidemic rats.

According to reports, naturally occurring polyphenols may have a significant impact on the treatment of dyslipidemia by acting through their hypolipidemic effects (18,19). Moreover, the studies confirm that increased dietary intake of polyphenols (flavonoids) reduces the risk of CVD and improves the dysfunctions related to lipid metabolism (20). According to research findings, flavonoid and polyphenolic compounds such as rutin have a crucial protective role in preventing free radical generations and liver injuries through their antioxidant activity. Rutin is also reported to protect against oxidative stress-induced degenerative illness and lipid peroxidation (LPO) (21-24). Among the existing animal models, a HFD produced hyperlipidemia has been a popular method to induce lipid metabolic diseases and related issues in experimental animals (25).

Keeping this background in mind, the present study was undertaken to find out whether the exogenous PAs (spermine and spermidine) and naturally occurring polyphenol, rutin, combinatorial therapy potentiates the anti-hyperlipidemic effect of simvastatin in HFD-induced hyperlipidemic rat model, and also to investigate the amelioration of associated side effects of long-term treatment of simvastatin.

Materials and Methods
Chemicals and reagents
The test drugs, spermine tetrahydrochloride, spermidine (Sisco Research Lab, Mumbai, India), rutin (Yucca Enterprises, Mumbai, India), simvastatin (Lupin Limited, India), HMG-CoA reductase kit, malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) (Sigma-Aldrich, USA), reduced glutathione (GSH) (Loba Chem, India), rat lipid profile kits (HIMEDIA Laboratories, India) were procured. All the other diagnostic kits and chemicals used in the experimental work were of high quality, pure, and analytical grade and were procured from local suppliers. The normal and HFDs were obtained from Nutrivet Laboratory, Pune, Maharashtra.

Experimental animals
The male Wistar rats (6-7 weeks old, 160-180 g) were procured from NIB, Pune, and kept under standard housing conditions: temperature ($24 \pm 2°C$), relative humidity ($45 \pm 10\%$), and 12 hours light12/ hours dark cycles, feed with standard animal diet food pellets and water *ad libitum* in our animal house. The general care of the experimental animals used for this study was done in compliance with the Animal Welfare Act.

Animal grouping and experimental design
In the present investigation, hyperlipidemia was induced in the rats by keeping them on a HFD for 60 days straight. The following ingredients made up the HFD utilized in the study: gram flour (20%/g), coconut oil (20%/g), corn flour (20%/g), wheat flour (30%), sodium chloride (0.5%/g), cholic acid (0.5%/g), cholesterol (1%/g), egg yolk (8%/g), and corn flour (20%/g) (26). All of the rats (n=6) were randomized into six groups at random on the thirty-first-day experiment, and each group received the following treatment for the next 30 days (27).

Group I: Normal control (NC), normal diet + received 1% w/v CMC, daily orally for 30 days.
I. Group II: HFD control + 1% w/v CMC, daily orally for 30 days.
II. Group III: HFD + PAs (250 µg/kg), daily orally for 30 days.
III. Group IV: HFD+PAs (250 µg/kg), + rutin (100 mg/kg), daily orally for 30 days.
IV. Group V: HFD+PAs (250 µg/kg), rutin (100 mg/kg), simvastatin (5 mg/kg) daily orally for 30 days.
V. Group VI: HFD + simvastatin (10 mg/kg), daily orally

for 30 days.

On the 60th day, at the end of the study, after the administration of the last dose of treatment, rats from all the above groups fasted for 12 hours. Later, the blood samples were collected and centrifuged at 3000 g, the serum was separated for further biochemical estimations, and then the rats were sacrificed humanely. Immediately the liver was removed, washed with normal saline, and stored at -80°C for further studies. A part of liver tissue was preserved (10% formalin) for histopathological studies.

Measurement of body weight

The body weight of rats from each group was measured on the 0th, 30th, 45th, and 60th day of the study period.

Determination of serum lipid profile

The serum lipid profile, i.e., TC, TG, and HDL of the experimental rats was assayed using the commercially available readymade diagnostic kits (HiMedia Laboratories, India) by the methods described earlier (28-30). The very low-density lipoprotein cholesterol (VLDL-C) and LDL-C levels were calculated using the Friedewald formula (31):

LDL-C = Total cholesterol - [HDL-C + VLDL-C].

The atherogenic index was calculated by using the following formula:

Atherogenic index = TC - HDL-C/HDL-C (32).

Determination of liver total cholesterol, triglycerides, and HMG-CoA reductase activity

Using 1 g of liver samples centrifuged at 4000 g for 15 minutes, the liver homogenates 10 % (w/v) were prepared in phosphate buffer (0.1M, pH 7.4) at a controlled temperature (0-4°C). The supernatant was used to determine TC (33) and TG using a commercially available kit.

The activity of HMG-CoA reductase was analyzed in liver homogenates using a commercially available kit and activity was expressed in terms of the HMG-CoA/mevalonate ratio (34). The calculated ratio was used as an indicator of inhibition of HMG-CoA reductase activity, whereby the conversion of HMG to the mevalonate enzyme is catalyzed by the enzyme. Briefly, the liver samples were homogenized (with 1 g/L arsenate solution), and equal volumes of liver tissue homogenate (10%) were added and further diluted with perchloric acid. After standing for 5 minutes, the mixture was centrifuged at 2000 rpm for 10 minutes, filtered, and then 1 mL of filtrate was mixed with 0.5 mL of freshly prepared hydroxyl amine (2 mol/L, pH 5.5) reagent for HMG-CoA and with 0.5 mL of freshly prepared hydroxyl amine (2 mol/L, pH 2.1) reagent for mevalonate. Further, 1.5 mL of ferric chloride reagent was added to each of the above mixtures, and the absorbance was measured against arsenate as blank at 540 nm on Shimadzu 1700 UV spectrophotometer (34).

Estimation of biochemical changes in liver homogenates

The liver tissue from all the above groups was washed and homogenized in phosphate buffer (0.1M, pH 7.4) at a controlled temperature (0-4°C) using a homogenizer to obtain 10% (w/v) liver homogenates. Further, the homogenates were centrifuged at 4000 g for 15 minutes at 4°C and used for the determination of LPO, GSH, CAT, and SOD.

The quantitative assay of LPO was performed by determining the formation of MDA formation in the liver by a previously described method (35). The amount of MDA formed was quantified by the reaction with TBA and used as an index of LPO, and the result was expressed as nmol/mg of protein.

The reduced GSH in liver homogenates was determined by the method of Beutler et al (36), using a DTNB reagent, and the results were expressed in terms of µmol of GSH/mg of protein.

The CAT activity in liver homogenates was determined by the method of Aebi (37), and the activity was expressed in terms of units of H_2O_2 formed/mg of protein.

The SOD activity in liver homogenates was assayed by the method of Misra and Fridovich (38), whereby the SOD activity was determined spectrophotometrically at 320 nm and expressed as Units of SOD/mg of protein.

Further, the estimation of nitric oxide (NO) was performed as per the procedure described by Green et al (39). Briefly, an equal quantity of the supernatant of liver homogenates and Griess reagents was mixed and kept in darkness for 10 minutes. The absorbance was recorded at 540 nm on a UV spectrophotometer. By plotting a standard curve, the nitrite content of samples was determined and expressed as µg/mL of tissue.

Estimation of biochemical changes in serum

The separated serum was analyzed for lysosomal marker enzymes, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (40), and alkaline phosphatases (ALP) as per the procedure described in the manufacturer kit and expressed as IU/L.

The total protein content in serum was determined as per the procedure described in readymade kits. The serum glucose level was determined by GOD/POD method (41).

Histopathological studies

The liver samples from all the animals were collected, fixed in 10% (v/v) formalin (neutral, buffered), and dehydrated with alcohol followed by xylene. Then the fixed samples were prepared by embedding in paraffin wax and sectioned into 4-6 µm thickness on the microtome. Further, the sections were stained using hematoxylin and eosin (H&E); slides were prepared and examined under scanning electron microscopy for histoarchitecture alterations.

Statistical analysis

All the results were expressed as mean ± SEM values for

each group. The statistical assessment among different groups for all the experimental data was performed using the one-way analysis of variance (ANOVA) method followed by Tukey's multiple comparison test using GraphPad Prism version 5.0. P value < 0.05 was considered to be statistically significant.

Results

Measurement of body weight

The changes in body weight of all the experimental rats on the 0th, 30th, 45th, and 60th days of the experimental period are presented in Figure 1. The weight of the rats in the control group increased rapidly and significantly ($P < 0.001$) compared to the normal control group over the experimental period. Compared to normal rats, the weights of all other experimental rats showed progressive growth; the effect can be attributed to the feeding high fat diet. However, the rats treated with rutin, PAs, and simvastatin, per se or in combination, showed a significant ($P < 0.001$) reduction in weights on the 45th and 60th days of the experiment. The maximum reduction in weight was found in rats treated with combinatorial treatment, rutin, PAs, and simvastatin compared to simvastatin alone (Figure 1).

Determination of serum lipid profile

As per the results depicted in Figure 2, it was observed that the levels of TC, TG, LDL-C, and VLDL-C were increased significantly ($P < 0.001$) in the serum of HFD control rats, as compared to the normal diet-fed rats. Notably, the HDL-C levels were decreased significantly ($P < 0.001$), whereas the serum atherogenic index level was increased significantly ($P < 0.001$) in the HFD-control rats, as compared to the normal diet-fed rats. Interestingly, the administration of PAs ($P < 0.05$),

Figure 1. Effect of rutin, polyamines, and simvastatin on body weight in HFD rats. Data are expressed as mean ± SEM (n=6). Statistical significance was determined by using ANOVA followed by Tukey's multiple comparison tests. ###$P < 0.001$ and ns$P > 0.05$ when compared with the normal control group, ***$P < 0.001$ and **$P < 0.01$ when compared with the HFD control group. NC: normal control on normal diet (received 1% w/v CMC), HFD: high-fat diet (received 1% w/v CMC), PAs: high-fat diet rats + polyamines (250 µmol/kg), PAs+RUT: high-fat diet rats + polyamines (250 µmol/kg)+ rutin (100 mg/kg), PAs+RUT+SIM: high-fat diet rats + polyamines (250 µmol/kg)+ rutin (100 mg/kg)+ simvastatin (5 mg/kg), SIM: high-fat diet rats + simvastatin (10 mg/kg).

PAs+rutin ($P < 0.01$), PAs+rutin+simvastatin ($P < 0.001$), and simvastatin (10) ($P < 0.001$) to HFD rats significantly decreased the levels of TC, TG, LDL-C, and VLDL-C and increased the HDL-C and atherogenic index as compared to untreated HFD rats. Importantly, from the results, it was evident that administration of HFD rats with the PAs+rutin+simvastatin combination resulted in remarkable restoration ($P < 0.001$) of serum lipid profile as compared to simvastatin alone (Figure 2).
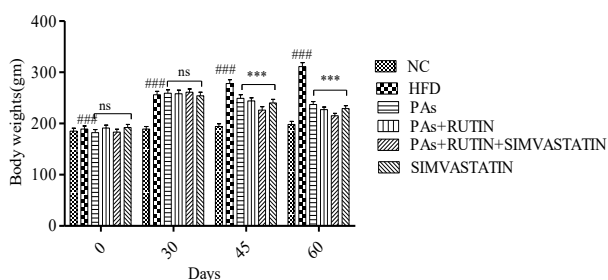
Determination of liver total cholesterol, triglycerides, and HMG-CoA reductase activity

The concentration of hepatic TC and TG in the entire experimental animals is depicted in Table 1. Due to the accumulation of fats, the rats from the vehicle-treated HFD control group showed significant ($P < 0.001$) elevations in TC and TG levels as compared to the normal control rats. The treatment with rutin (100), PAs (250), and simvastatin (5), either per se or in combination with HFD, significantly ($P < 0.001$) prevented the elevation in TC and TG levels as compared to the control HFD rats.

The inhibitory effects of test drugs on the biosynthesis of cholesterol were determined by HMG-CoA reductase activity. For the same, to determine its impact, the index of HMG-CoA reductase, i.e., the ratio of HMG-CoA/mevalonate, was determined in both treated and untreated rats. As the HMG-CoA/mevalonate ratio is in reverse proportional to enzyme activity, the rise in ratio shows a decline in enzyme activity and vice-versa. As depicted in Table 1, rutin, PAs, and simvastatin treatment to hyperlipidemic rats, consuming an HFD, significantly ($P < 0.001$) restored the decreased ratio of HMG-CoA/mevalonate as compared to the control rats. However, the PAs (250), rutin (100), and simvastatin (5) combination showed more improvement in HMG-CoA/mevalonate ratio compared to simvastatin (10) alone, showing the larger inhibitory effect of the combination on HMG-CoA reductase activity.
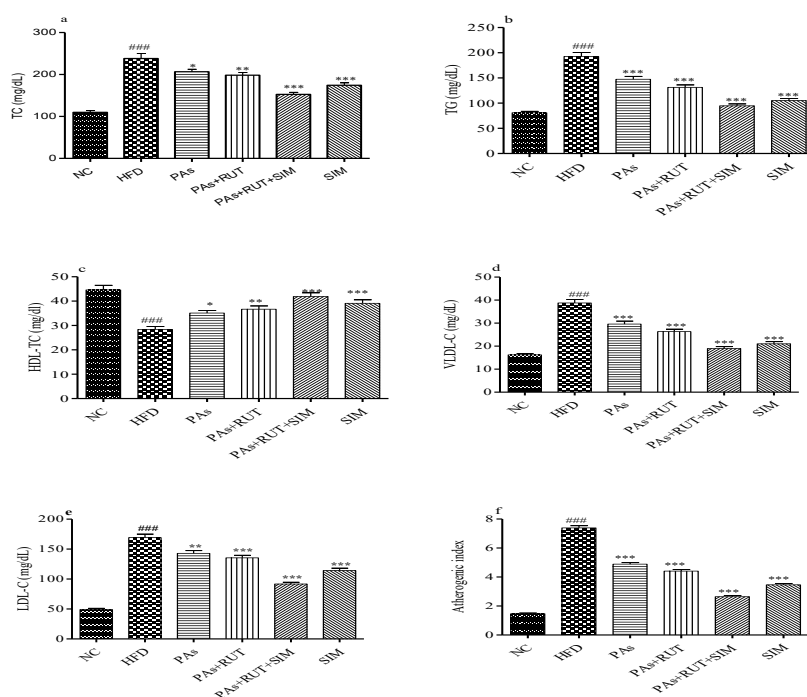
Estimation of antioxidant parameters and NO in liver homogenates

Long-term (60 days) feeding of a HFD significantly ($P < 0.001$) reduced the levels of GSH, SOD, and catalase in liver homogenates of hyperlipidemic control rats compared to their levels in normal control rats, whereas MDA (oxidative stress marker) levels were significantly elevated in HFD control rats compared to normal rats. However, treatment with rutin (100), PAs (250), per se or in combination with simvastatin (5), to HFD rats showed a statistically significant increase ($P < 0.001$) in the levels of GSH, SOD, and catalase and decreased ($P < 0.001$) MDA formation in liver homogenates of HFD control rats compared to untreated HFD rats (Figure 3). The restoration of antioxidant activity was found to be highly significant in HFD rats when treated with the combination of rutin, Pas, and simvastatin compared to the rats treated with above treatment alone.

Figure 2. Effect of rutin, polyamines, and simvastatin on lipid profile in HFD rats.a)TC-total cholesterol, b) triglycerides, c) HDL-C- high-density lipoprotein, d) VLDL-C- very low-density lipoprotein, e) LDL-C- low-density lipoprotein, f) Atherogenic index. Data were expressed as mean ± SEM (n=6). Statistical significance was determined by using ANOVA followed by Tukey's multiple comparison tests. ###$P < 0.001$ when compared with normal control group,***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$, when compared with HFD control group. NC: normal control on normal diet (received 1% w/v CMC), HFD: high-fat diet (received 1% w/v CMC), PAs: high-fat diet rats + polyamines (250 µmol/kg), PAs+RUT: high-fat diet rats + polyamines (250 µmol/kg)+ rutin (100 mg/kg), PAs+RUT+SIM: high-fat diet rats + polyamines (250 µmol/kg)+ rutin (100 mg/kg)+ simvastatin (5 mg/kg), SIM: high-fat diet rats + simvastatin (10 mg/kg).

The NO levels were significantly ($P < 0.001$) diminished in untreated HFD rats. Treatment with polyamines, rutin, and simvastatin, alone or in combination, restored ($P < 0.001$) the diminished NO levels in HFD rats to normalcy (Figure 3).

Biochemical estimations in serum

As liver functions are concerned, it was observed that the liver marker enzymes, AST, ALT, and ALP were found to be significantly higher ($P < 0.001$) in rats fed with a HFD compared to rats with a normal diet. Treatment

with simvastatin (10), rutin (100), and PAs (250), per se or in combination with simvastatin (5), to HFD rats significantly ($P < 0.001$) restored the elevation of these liver enzymes to normalcy as compared to vehicle-treated HFD rats. However, the protection of the liver from these enzymes was found to be larger, when HFD rats were treated with combinatorial treatment rutin, PAs, and simvastatin compared to simvastatin (10 mg/kg) alone treatment (Table 2).

As depicted in Table 2, it was observed that the serum of HFD control rats showed a higher level of glucose and

Table 1. Effect of polyamines, rutin, and simvastatin on liver total cholesterol (TC), triglyceride (TG), and hydroxymethylglutaryl-CoA (HMG-CoA) reductase activity in high-fat diet (HFD) rats

Treatment and dose (p.o.)	TC (mg/g)	TG (mg/g)	HMG-CoA / Mevalonate-ratio
	Mean ± SEM (n=6)		
NC (1% w/v CMC)	11.27 ± 1.4	7.86 ± 0.90	2.88 ± 0.12
HFD(1% w/v CMC)	34.17 ± 2.7###	16.69 ± 1.1###	1.15 ± 0.097###
PAs (250 µmol/kg)	26.12 ± 1.9*	12.56 ± 0.87*	2.24 ± 0.12**
PAs (250 µmol/kg) + RUT (100 mg/kg)	22.79 ± 1.8**	11.97± 0.86*	2.41 ± 0.14***
PAs (250 µmol/kg) + RUT (100 mg/kg) +SIM (5 mg/kg)	14.91 ± 1.2***	9.84 ± 0.81***	2.97 ± 0.23***
SIM (10 mg/kg)	17.28± 1.4***	11.14 ± 0.85**	2.65 ± 0.27***

NC: normal control, HFD: high-fat diet, PA: polyamines, RUT: rutin, SIM: simvastatin, TC: total cholesterol, TG: triglyceride, HMG-CoA: hydroxymethylglutaryl-CoA.
###$P < 0.001$, when compared with normal control group. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$, when compared with diabetic control group.
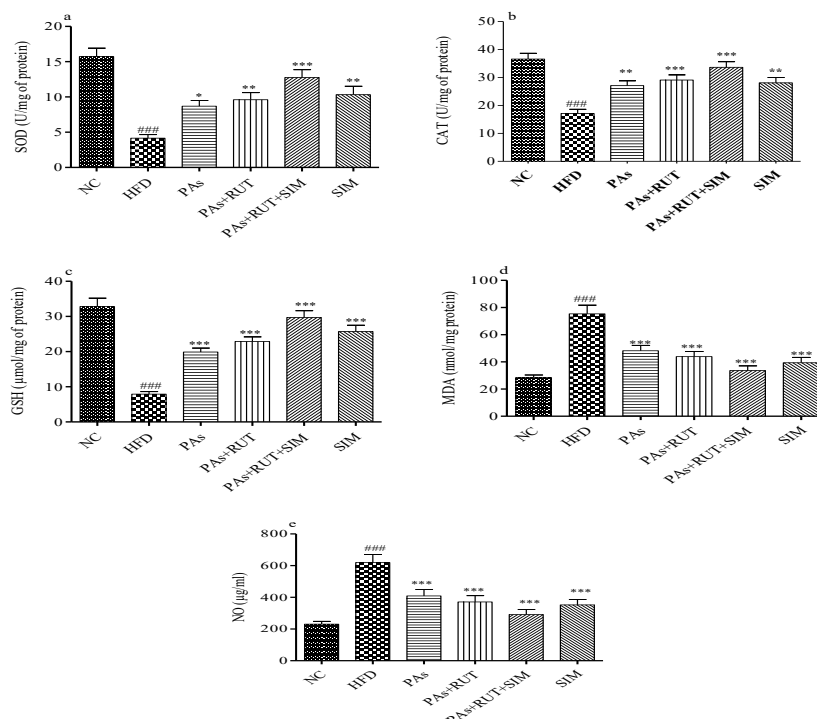
Figure 3. Effect of PA, rutin, and simvastatin on biochemical changes in the liver of HFD rats. (a) SOD: superoxide dismutase, (b) CAT: catalase, (c) GSH: glutathione, d) MDA: malondialdehyde, e) NO: nitric oxide. Data were expressed as mean ± SEM (n=6). Statistical significance was determined by using ANOVA followed by Tukey's multiple comparison tests. ###$P < 0.001$ when compared with normal control group,***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$, when compared with HFD control group. NC: normal control on normal diet (received 1% w/v CMC), HFD: high-fat diet (received 1% w/v CMC), PAs: high-fat diet rats + polyamines (250 µmol/kg), PAs+RUT: high-fat diet rats + polyamines (250 µmol/kg)+ rutin (100 mg/kg), PAs+RUT+SIM: high-fat diet rats + polyamines (250 µmol/kg)+ rutin (100 mg/kg)+ simvastatin (5 mg/kg), SIM: high-fat diet rats + simvastatin (10 mg/kg).

a lower level of total protein as compared to normal rats, showing a state of hyperglycemia. Treatment with PAs ($P < 0.05$), rutin ($P < 0.01$), and simvastatin ($P < 0.05$), alone or in combination ($P < 0.001$) to HFD rats, significantly restored the levels of serum glucose and total protein as compared to untreated HFD rats (Table 2).

Histopathological studies
As depicted in Figure 4, the histopathological examination of liver sections of NC rats showed normal histoarchitecture. The photomicrographs of liver sections from HFD control rats showed marked fatty deposition, swelling of hepatocytes, cellular infiltration with inflammation, and granular degenerative changes

as compared to the normally fed rats. The HFD-fed rats treated with PAs, PAs+rutin, and simvastatin, per se, exhibited the improvement of hepatocyte architecture with a marked decrease in fatty deposition, and cellular infiltration, with mild degeneration of hepatocytes. However, in comparison with simvastatin (10) alone, the combined treatment of PAs (250) and rutin (100) with simvastatin (5) significantly restored the histoarchitecture of hepatocytes to normalcy, showing enhanced hepatic protection (Figure 4).

Discussion
Hyperlipidemia is characterized by a rise in the blood levels of TC, TG, or both, as well as LDL and a decrease in

Table 2. Effect of polyamines, rutin, and simvastatin on biochemical changes in serum of high-fat diet rats

Treatment and dose (p.o.)	AST (U/L)	ALT (U/L)	ALP (U/L)	Total protein (g/dL)	Blood glucose (mg/dL)
NC (1% w/v CMC)	81.7 ± 3.9	45.7 ± 1.7	214 ± 9.7	7.91± 0.18	117.5 ± 9.5
HFD(1% w/v CMC)	133.2 ± 4.3###	95.6± 2.9###	479 ± 22.3###	6.03 ± 0.13###	185.9 ± 10.4###
PAs (250 µmol/kg)	108.1± 3.5***	72.1 ± 2.3***	394 ± 17.1**	6.86 ± 0.14**	148.5± 7.1*
PAs (250 µmol/kg) + RUT (100 mg/kg)	105.3± 3.4***	67.3 ± 2.2***	324 ± 16.2***	7.15 ± 0.15***	136.7± 7.2**
PAs (250 µmol/kg) + RUT (100 mg/kg) +SIM (5 mg/kg)	92.9 ± 3.1***	56.7 ± 2.1***	267 ± 12.7***	7.59 ± 0.15***	121.3± 6.9***
SIM (10 mg/kg)	102.1± 3.4***	73.1 ± 2.5***	307 ± 13.5***	6.94 ± 0.18**	143.7± 7.9*

NC, normal control; HFD, high-fat diet; PA, polyamines; RUT, rutin; SIM, simvastatin; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase.
Data are expressed as Mean ± SEM (n=6).
###$P < 0.001$, when compared with normal control group. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$, when compared with diabetic control group.
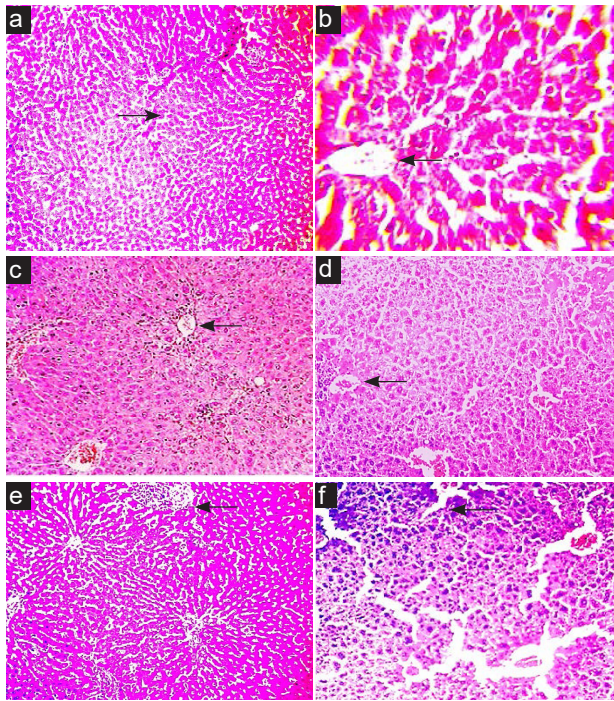
Figure 4. Effect of rutin, polyamines, and simvastatin on histopathology of liver (Photomicrograph stain by H&E) in HFD rats. (a) Normal histoarchitecture of liver cells of rats from the normal group; (b) HFD: Fatty liver, with an accumulation of lipids, granular degeneration, congestion, and infiltration accompanied by inflammation in hepatocytes of HFD rats; (c) PAs: polyamines treated rats showing mild to moderate improvement in histoarchitecture of hepatocytes of HED; (d) PAs+RUT treated rats showing mild to moderate improvement in histoarchitecture of liver cells concerning fatty deposition, infiltration, and inflammation of cells; (e) PAs+RUT+SIM treated rats showing higher improvement in hepatocytes concerning fatty deposition, granular generation, and cellular inflammation; (f) SIM treated rats showing mild to moderate improvement in histoarchitecture of liver cells for fatty deposition, infiltration, and inflammation of cells. NC: normal control on a normal diet (received 1% w/v CMC); HFD: high-fat diet (received 1% w/v CMC); PAs: high-fat diet rats + polyamines (250 μmol/kg); PAs+RUT: high-fat diet rats + polyamines (250 μmol/kg)+ rutin (100 mg/kg); PAs+RUT+SIM: high-fat diet rats + polyamines (250 μmol/kg)+ rutin (100 mg/kg)+ simvastatin (5 mg/kg); SIM: high-fat diet rats + simvastatin (10 mg/kg).

HDL, which commonly progresses to atherosclerosis and, eventually, CVDs, which have become a rising burden around the world, particularly in developing countries. Its domination is expanding in India as well, accounting for approximately 24% of fatalities between the age group of 25 and 70 years (42). Daily feeding of a HFD to rats causes hyperlipidemia and influences many biochemical, cardiovascular, and histological alterations, commonly known as a metabolic disease (43,44), which may lead to an increase in body weight, and accumulation of fat in the liver and visceral tissues (45).

In the management of hyperlipidemia and its associated complications due to increased lipid levels, statins are preferred as a choice of drug. Simvastatin is a known conventional lipid-lowering drug, especially for serum LDL-C and inhibition of HMG-CoA reductase activity. Moreover, it is used as a reference drug for evaluation and comparisons with other lipid-lowering drug entities

(8,46,47).

Rutin, a polyphenolic flavonoid, also called rutoside, is generally found in various dietary food plants with functional properties and with a large spectrum of health benefits. It also elicits many pharmacological activities like antidiabetic, antioxidant, vasoprotective, cardioprotective, anti-hyperlipidemic. It has been shown that treatment with rutin restored the decreased HDL-C, TG, and elevated AST, ALT, ALP, TC, and LDL-C serum levels in HFD rats (23). In addition, rutin also showed significant antioxidant, hypoglycemic, and hypolipidemic activity in HFD rats (24,48,49).

Polyamines, either directly or through dietary-rich food, are known to play an important role in cellular lipid metabolic pathways, protecting from dietary-induced obesity and, thus, reducing the prevalence of cardiovascular abnormalities (16). It has been demonstrated that owing to the anti-inflammatory and antioxidant effect, exogenous administration of spermidine alleviates the increased level of IL-6, augments the autophagy, improving the vascular mitochondria utility and thereby preventing atherogenesis in hyperlipidemia, especially in aged mice (50,51). Further, documented studies in rodents indicated that exogenous spermidine/PAs treatment, results in (a) increased phosphorylation of adenosine monophosphate-activated protein kinase, (b) the overexpression of genes in hepatic and adipose cells, which in turn enhances the glucose and lipid metabolism and decreases the process of lipogenesis in hepatocytes of rodent (52).

Although documented scientific reports are available for the use of individual PAs in hyperlipidemia, no research data is available regarding their combinatorial (spermine and spermidine) effects. Further, rutin, known as a polyphenolic flavonoid, is an effective natural compound in the amelioration of lipid disorders. Considering the limitations of statins, especially simvastatin, it was thought worthwhile to evaluate the combinatorial importance of spermine + spermidine and rutin along with simvastatin in HFD-induced hyperlipidemic rats.

The increased body weight of HFD rats during the experimental study may be due to the accumulation of fats consequent to consumption of the HFD (Figure 1). Treatment of HFD rats for 30 days prevented further increase in body weights of HFD rats in comparison with untreated HFD rats. Prevention of an increase in body weights of HFD rats by PAs, rutin, and simvastatin treatment may be due to reduced fat built or fat accumulation or increased utilization of body fats.

Under normal physiological conditions, when HDL-C gets metabolized to bile juice as a free sterol, it plays an important role in the transfer of TC from different tissues of the body to the liver. Such cellular events increase serum HDL-C, which normally manifests in a decrease in serum and peripheral tissue cholesterol levels (53). The above reports are in line with the outcome of the present study, where HFD rats demonstrated a significant

reduction in serum HDL-C level along with a significant increase in serum and hepatic TC. PAs and simvastatin, either per se or in combination with rutin, significantly restored the alterations of HDL-C and TC, suggesting the supporting role of PAs and rutin with simvastatin to act as atheroprotective since the HDL-C levels are inversely correlated with the development and progression of atherosclerosis (54).

It has been shown that the production of ROS during dyslipidemia causes oxidative stress, resulting in the conversion of LDL-C to the oxidized form of LDL-C, which fascinates macrophages in the arterial walls and leads to the development of arteriosclerosis via atheroma plaque formation. Furthermore, it pertains to the development of various cardiovascular complications like stroke, coronary heart disease, and angina pectoris (55). In the present study, the increased LDL-C, VLDL-C, and atherogenic index in rats due to the HF diet were significantly attenuated by PAs, rutin, and simvastatin administration for 30 days signifying the potentiating antioxidant effects of PAs and rutin.

It has been well established that, for the maintenance of normal metabolism of lipids, triglycerides display a crucial role via lipoprotein interface regulation, and their enhanced serum levels have been linked to an increased risk of coronary artery diseases (56). In the present study, the rats fed with the HFD for 60 days had significantly elevated serum and hepatic levels of TC and TG. However, after oral treatment with test drug regimens, PAs, PAs+rutin, PA+rutin+simvastatin (5 mg/kg), and simvastatin (10 mg/kg) alone, a significant reduction in complete lipid profile was observed. It is important to mention here the order of significance for the prevention of lipid profile alterations in the HFD rats by the aforementioned treatment is PA + rutin + simvastatin (5 mg/kg) ≥simvastatin (10 mg/kg) ≥ Pas + rutin > PAs. Interestingly, the hyperlipidemic effect of PAs and rutin along with simvastatin in HFD rats is greater (highly significant) than that of simvastatin (10 mg/kg) alone. Thus, combinatorial effect of PA + rutin + simvastatin (5 mg/kg) increases the therapeutic efficacy (equal/better) than that of simvastatin (10 mg/kg) treatment. This may help to reduce /ameliorate the long-term deleterious effects of simvastatin in the clinical management of dyslipidemia.

One of the essential enzymes for the synthesis of cholesterol is the NADPH-dependent HMG-CoA reductase, which catalyzes the conversion of HMG-CoA to mevalonate. As a result, using HMG-CoA reductase inhibitors as a kind of treatment is one of the more effective ways to manage dyslipidemia and cardiovascular illnesses by lowering blood cholesterol levels that are too high (57,58). Documented reports strongly claim the anti-hyperlipidemic activity of simvastatin due to the HMG-CoA reductase inhibition, the rate-limiting step in cholesterol biosynthesis (59). The enhanced HMG-CoA reductase activity shown by combined treatment of PAs

with rutin and simvastatin may be due to the metabolic conversion of rutin to quercetin, which causes a reduction in liver cholesterol and the HMG-CoA reductase activity (60). The antioxidant effect shown by PAs and rutin potentiates the inhibitory effect of simvastatin on HMG-CoA, which ultimately may lead to a cholesterol-lowering effect.

The enzymes SOD, CAT, and GSH are known naturally created antioxidants accountable for decreasing cellular oxidative stress. Enhanced oxidative stress induced due to an HFD compromised these antioxidant activities in the control HFD rats.

The present study shows that the long-term intake of an HFD has significantly decreased the serum levels of SOD, CAT, and GSH, and the overproduction of MDA formation in control rats. During the progress of hyperlipidemia, the deficiency or failure of the antioxidant defense system leads to the production of free radicals, increases intracellular oxidative stress, and ultimately leads to enhancement in LPO (61,62). Experimental studies have shown that consumption of saturated fatty acids results in hypocholesteremia, which triggers the induction of oxidative stress due to enhanced LPO. Additionally, it also encourages the generation of superoxide anions and the inactivation of NO (63). When the free radicals attack or alter the cellular membrane lipoprotein and polyunsaturated fatty acids, numerous oxygenated compounds like conjugated dienes and MDA are released. It has been shown that both enzymatic (SOD, GSH) and non-enzymatic antioxidants (CAT) aid in scavenging the free radicals induced tissue/organ damage. It has been shown that antioxidant treatment also ameliorates elevated serum MDA levels in hyperlipidemic animals (64,65). The present study suggests that oral combinatorial treatment can significantly restore depleted antioxidants and decrease the elevated MDA and NO levels in HFD rats. The restoration of NO levels in liver homogenates may signify the protective role of the above combinatorial regimens on hyperlipidemia-induced vascular dysfunctions (66). Noteworthy, the enhanced antioxidant activity observed with combinatorial treatment of PAs, rutin, and simvastatin (5 mg/kg) compared to simvastatin (10 mg/kg) alone, indicates the significant decline of oxidant molecules and thus protecting the hepatocytes from free radical-generated ROS scavenging effect. Thus our findings are in total agreement with the earlier reports of PAs (spermine) in enhancing antioxidant effect (enzymatic and non-enzymatic activities) and rutin in rat liver beneath oxidative stress (67).

The characteristic liver enzymes, AST and ALP, are released into the systemic circulation depending on the degree of liver injury, and thus, higher levels of these enzymes in the blood are the index of pathological conditions such as liver cirrhosis, fatty liver, hepatitis, etc, which reflects the destruction of the structural integrity of hepatocytes (68). In the present study, the increase in AST,

ALT, and ALP enzyme levels in the blood of HFD rats may be due to HFD-induced oxidative damage to hepatocytes leading to seepage of enzymes from the hepatic cell membrane (69,70). However, the combinatorial treatment reduced the elevated levels of liver marker enzymes (AST, ALT, and ALP) in the serum of HFD rats. Thus it is proposed that combinatorial treatment of rutin and PAs with simvastatin may be able to reverse the liver damage/injuries induced by hyperlipidemia. Feeding a HFD to rats demonstrates higher levels of blood glucose, which may be due to the presence of saturated fats in HFD rats, which is in agreement with the earlier reports (69). Combinatorial treatment significantly reduced the blood glucose level indicating the role of PAs (16) and rutin (71) in improving the metabolism of glucose, which may be a beneficial factor in hyperlipidemia treatment. In the evolution of hyperlipidemia, oxidative stress due to ROS may be an early cellular event, which later on leads to the glycation of protein and auto-oxidation of glucose (72). Thus, the prevention of these alterations in the serum of HFD rats with the combinatorial treatment suggests their role in the prevention of protein glycation by scavenging generated ROS induced by a higher level of cholesterol in the blood.

The improvement in histoarchitecture of the liver in oral combinatorial treatment with PAs, rutin, and simvastatin further strengthened the proposal of reversal of liver injury/damage due to hyperlipidemia.

In summary, the experimental findings demonstrated the role of the combinatorial treatment in the reduction of peroxidation rate, restoration of the body's defense mechanism(s), and protection of hyperlipidemia development due to free radicals induced oxidative stress damage caused by the release of lipid affluent environment in HFD rats. Further, it also demonstrated significant hepatic injury protection through the restoration of hepatic biomarker enzymes, AST, ALT, and ALP along with serum and hepatic liver profile (TC, TG, and HDL). The histological findings also suggest and support the improvement in the size and structure of hepatocytes in HFD rats.

Conclusion
The experimental findings in the present study demonstrated that the combination of natural antioxidants (rutin) and endogenous antioxidants (PAs) with simvastatin could provide effective and therapeutic intervention in experimentally induced hyperlipidemic rats. This combination elicited better protective effects concerning lipid and associated pathological anomalies against HFD-induced hyperlipidemia, specifically repairing the antioxidant defense system in the liver. This combination may avert the hepatotoxicity effects of simvastatin, too.

Authors' contributions
RRP performed the work and drafted the manuscript, MKA and SBB designed and reviewed the manuscript, and SRN reviewed and finalise the draft of the manuscript. All the authors have read and further approved the manuscript.

Conflict of interests
The authors declared no conflict of interest.

Ethical considerations
All authors declare that principles of laboratory animal care (National Institute of health guide for care and use of laboratory animals) (NIH Publication No. 85-23 received 1985) were followed. All the experimental procedures and protocols of the study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Sinhgad Institute of Pharmaceutical Sciences, Lonavala Pune (registration number: 962/c/06/CPCSEA), with the protocol approval number SIPS/IAEC/2017-18/01.

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