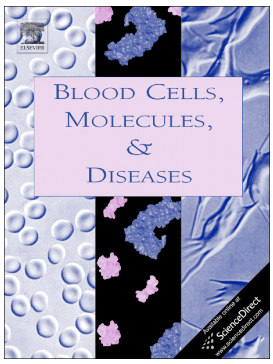

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PII: S1079-9796(18)30449-2
DOI: https://doi.org/10.1016/j.bcmd.2018.12.005
Reference: YBCMD 2297

To appear in: *Blood Cells, Molecules and Diseases*

Received date: 30 November 2018
Accepted date: 25 December 2018

Please cite this article as: Alessandra G. Manzoni, Daniela F. Passos, Jean L.G. da Silva, Viviane M. Bernardes, João M. Bremm, Matheus H. Jantsch, Juliana S. de Oliveira, Thaís R. Mann, Cinthia M. de Andrade, Daniela B.R. Leal , Rutin and curcumin reduce inflammation, triglyceride levels and ADA activity in serum and immune cells in a model of hyperlipidemia. Ybcmd (2018), https://doi.org/10.1016/j.bcmd.2018.12.005

Rutin and curcumin reduce inflammation, triglyceride levels and ADA activity in serum and immune cells in a model of hyperlipidemia

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The authors declare that they have no conflicts of interest.

ABSTRACT

Hyperlipidemia is associated with endothelial dysfunction and inflammatory disorders. Adenosine and adenosine deaminase (ADA) modulate immune responses and lipid metabolism. Curcumin and rutin are polyphenols with antioxidant, anti-inflammatory, and hypolipidemic effects. We evaluated the action of rutin and curcumin in the lipid levels and inflammation, as well as their effect on ADA activity in serum, lymphocyte, platelets, and neutrophils of hyperlipidemic rats. Adult male Wistar rats pretreated with curcumin and/or rutin for 30 days were submitted to Poloxamer-407-induced hyperlipidemia. Biochemical, hematological, oxidative stress parameters, as well as serum and extracellular ADA activity, were performed 36h post-induction. Hyperlipidemia was confirmed by the increase in TC and TG. Hematological alterations, elevated reactive oxygen species (ROS) and myeloperoxidase (MPO) and ADA activity were observed in hyperlipidemic rats. Curcumin and the curcumin/rutin association decreased triglycerides and increased high-density lipids (HDL) levels. The pretreatments reverted the hematological parameters, decreased the activities of MPO in plasma and ADA in serum and cells. Cholesterol and ROS levels were not altered by the pretreatments. Our results show that pretreatments with rutin and/or curcumin prevent the hyperlipidemia-induced inflammation. Pretreatments with curcumin and/or rutin are potential complementary therapies in the prevention of hypertriglyceridemia and inflammation.

Keywords: Hyperlipidemia, rutin, curcumin, adenosine desaminase, oxidative stress.

1. BACKGROUND

Hyperlipidemia is epidemiologically linked to the development of cardiovascular diseases and is an important risk factor and determinant of onset of atherosclerosis. It is defined by the accumulation of the total of cholesterol (TC) accompanied by an increase in the levels of low density (LDL) cholesterol and serum triglycerides [1]. The interplay of lipids, vasculature cells and the immune cells recruited to the site of inflammation drive the onset of atherosclerosis [2,3]. Endothelial and vascular inflammation, which occur in hyperlipidemia and atherosclerosis, are leading causes for the development of cardiovascular diseases [4]

High levels of circulating lipids result in deposition of cholesterol in the arterial walls, oxidation of LDL and subsequent endothelial damage [3]. Excess cholesterol causes deleterious changes in the lipid composition of plasma membranes, which result induce the generation of reactive oxygen species (ROS) and lipid oxidation [5] [6]. This changes caused by hyperlipidemia elicit the innate and adaptive immune responses, accompanied by an expansion of myeloid cells and aggravation of ROS release inflammation, accelerating the progression of atherosclerosis [3]. This process can also cause damage to proteins and DNA, altering their cellular function, leading to tissue damage [7]. Hyperlipidemia may also alter the biological activity of enzymes, which may result in modification of its catalytic activity [8].

Oxidized LDL activates important signaling cascades, such as the purinergic signaling system. Endothelial cells release adenosine triphosphate (ATP) and ROS activating immune cells such as macrophages, lymphocytes, neutrophils, and platelets. Adenosine is produced from ATP via NTPDase (EC 3.6.1.5; CD39), which converts ATP into ADP and AMP; and 5´-nucleotidase (EC 3.1.3.5; CD73) which converts AMP to adenosine [9]. This cascade of events acts as an autoregulatory mechanism to lighten the damage associated with the activation of endothelial and immune cells. Adenosine interacts with four types of P1 purinergic receptors: A1, A2A, A2B, and A3 [10]. Unlike ATP, adenosine downregulates the release of inflammatory mediators, inhibiting the immune response and immune cell function [11].

The metabolism of extracellular adenosine correlates directly with the activity of ecto-adenosine deaminase (E.C 3.5.4.4, ADA) located on the surface

of the cell membrane [12,13] This enzyme is responsible for the deamination of adenosine and generation of inosine, thereby controlling adenosine levels [14]

Lymphocyte function and proliferation is hindered by high cholesterol content [15,16]. In this context, different T cell subsets exert distinct functions, while effector T cells contribute to the onset of atherosclerosis, T regulatory (Treg) cells are atheroprotective. However, hypercholesterolemia increases Treg apoptosis and decreases their function [17,18]. In addition, the function of Tregs is boosted by adenosine, an immunosuppressive and anti-inflammatory molecule. Hence, low levels of adenosine contribute to the formation of atherosclerotic plaques [19].

Platelets are recognized by their role in coagulation through platelet aggregation[20]. Platelet dysfunction during hyperlipidemia has a well-defined role in the progression of atherosclerosis since it affects lipid peroxidation, platelet activation, apoptosis and aggregation [21]. High concentrations of LDL induce platelet hyperactivity, exacerbating the release of inflammatory mediators, promoting thrombus formation [22,23]. In addition, by increasing their adhesion on the walls of blood vessels and releasing more pro-inflammatory cytokines, platelets further contribute to the progression of atherosclerotic plaques, [24] Adenosine, on the other hand, when linked to A2 receptors downregulates platelet activity, showing anti-inflammatory and antithrombotic effects [9].

There has been a growing interest in the role of neutrophils in sterile inflammation [25]. Neutrophil counts were found to be increased in hyperlipidemic rats [26] and patients [27]. Moreover, evidence of hypercholesterolemia-induced activation of neutrophils and artery infiltration indicate their involvement in the onset of atherosclerosis [28]. Activated neutrophils release enzyme myeloperoxidase (MPO) which catalyzes the formation of reactive oxygen intermediates. Increased in MPO activity is related to the spread of cardiovascular diseases.

The metabolism of adenosine and the adenosinergic signaling are relevant in the context of hyperlipidemia because adenosine is involved in both the immune response to augmented lipid levels and the lipid metabolism. The regulation of adenosine levels has a direct impact in its availability to interact with adenosine receptors. The effects of adenosine interaction with its receptors have

an impact on hyperlipidemia, obesity, and diabetes [29]. Adenosine regulates lipolysis, cell foam and atherosclerotic plaque formation, cholesterol efflux and circulating lipid levels [30]. Adenosine signaling via A1 receptors is known to have antilipolytic effects in the adipose tissue, reducing bloodstream levels of free fatty acids and triglycerides [31]. Besides reducing inflammation, A2A receptors are involved in the maintenance of cholesterol homeostasis to regulate cell foam formation [30]. A2B receptors were shown to protect against accumulation of lipids in plasma and atherosclerosis [32].

The use of natural compounds, such as polyphenols, have shown several health applications due to their broad biological activities and their low cost. Curcumin is a natural pigment present in turmeric and has been shown to be an antioxidant and anti-inflammatory compound. Recent studies have explored its effects on inflammatory diseases such as atherosclerosis which is linked to hyperlipidemia [33,34]. Rutin is a bioflavonoid present in leafy vegetables and citrus fruits and has shown a wide range of pharmacological applications. However, current research has highlighted its pharmacological benefits for the treatment of various chronic diseases such as cancer, diabetes, hypertension, and hyperlipidemia [35].

Polyphenols such as curcumin and rutin have demonstrated potent antioxidant, anti-inflammatory, and hypolipidemic actions. The hypolipidemic actions of polyphenols are due several mechanisms including inhibition of lipid synthesis and absorption, reduction of hepatic secretion of apolipoprotein B and foam cell formation, activation of transcription factor PPARγ, upregulation of the LDL receptor, high-density lipoprotein (HDL), fatty acid metabolizing enzymes respectively lipoprotein lipase, enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), hepatic lipase (HL) and cholesterol-7α-hydroxylase (C7αH) enzymes [36].

Though the effects of rutin and curcumin on metabolic and inflammatory diseases have been previously described, the association of these compounds has not been explored. It is also important to investigate the potential effects of these polyphenols on the metabolism of adenosine, as well as their lipid lowering, antioxidant and anti-inflammatory action on rats submitted to hyperlipidemia.

We sought to contribute to the knowledge on the modulation of adenosine deaminase in different immune cell types by hyperlipidemia. In addition, we

pursued to evaluate these compounds, alone and in association, as candidates for new adjuvant therapies that may benefit patients with hyperlipidemia.

METHODS

2.1 Animals

Adult male Wistar rats, weighing approximately 250-300g, heterogenic and conventional, originated from the UFSM Central Vivarium were used in this experiment. The animals were distributed in polypropylene cages (41 x 34 x 16 cm) in a random manner. Each cage contained a maximum of 4 rats. The animals were kept at a constant temperature (23 ± 2 °C) on a 12-h light/dark cycle with food and water freely available. The animals were fed with standard pellet diet (Puro Lab 22PB). All procedures involving the animals were approved by the Ethics Committee on the Use of Animal at the Federal University of Santa Maria (Protocol number: 1006200117).

2.2 Reagents

The bovine serum albumin, Coomassie Brilliant Blue G, rutin hydrate, Poloxamer-407, and adenosine were bought from Sigma-Aldrich (St. Louis, MO, USA). Physiological solution (0.9 g NaCl/100 mL distilled water) was acquired from Fresenius KABI (Brazil) Ficoll-Hypaque (Lymphoprep) was purchased from Nycomed Pharma (Oslo, Norway). The vehicle used was cooking corn oil. The curcumin was bought from Sigma Chemical Co. (St. Louis, MO, USA). Analytical grade and highest purity were ensured for all chemicals used in the experiment.

2.3 Experimental design

This study was designed to evaluate the effects of rutin, curcumin and the rutin/curcumin association in a model of Poloxamer-407 (P407)-induced hyperlipidemia in rats. Rutin and curcumin were orally administrated (gavage), both at a dose of 50 mg/kg, according to previous studies published by our research group [37,38]. Following 30 days of treatment, a single intraperitoneal injection of 500 mg/kg P407 (dissolved in sterile 0.9% NaCl solution) was applied in order to acutely induce hyperlipidemia [39]. The same volume of vehicle (sterile 0.9% NaCl solution) was given to the rats belonging to the non-hyperlipidemic groups. Euthanasia was performed 36 hours after induction. The experiment were performed with eight groups of animals of five or six animals: S: Saline + without induction of hyperlipidemia (n=5); R: Rutin 50mg/kg + without induction

of hyperlipidemia (n=6); C: Curcumin 50mg/kg + without induction of hyperlipidemia (n=6); R + C: Curcumin 50mg/kg + kg Rutin 50mg/kg + without induction of hyperlipidemia (n=6); H+S: Saline + induction of hyperlipidemia (n=5); H+R: Rutin 50mg/kg + induction of hyperlipidemia (n=6); H+C: Curcumin 50mg/kg + induction of hyperlipidemia (n=6); H+RC: Curcumin 50mg/kg + kg Rutin 50mg/kg + induction of hyperlipidemia (n=6).

2.4 Pretreatment with Rutin and/or Curcumin

Animals in R and H+R received 50mg/kg of rutin, C and H+C received 50mg/kg of curcumin, R+C and H +RC received 50mg/kg of association rutin plus curcumin, for a period of 30 days, while the S and H+S received 0.9% saline solution. Rutin and Curcumin were prepared in corn oil. According to previous studies published in our research group, corn oil does not interfere with the activity of the E-ADA [38]. However, this group corn oil was excluded from the study because it did not present a significant difference in the enzymatic activities compared to S.

2.5 Induction of hyperlipidemia by poloxamer 407 (P407)

The animals were randomly divided into hyperlipidemic and control saline rat groups. To induce hyperlipidemia,500 mg/kg of P407 dissolved in sterile 0.9% NaCl solution was administered via intraperitoneal (i.p.) injection [39]. The control saline rats received the same volume of vehicle alone (cold, sterile 0.9% NaCl solution). After 36 h of induction, the animals were anesthetized with isoflurane and euthanized by exsanguination and blood was collected by cardiac puncture.

2.6 Isolation of lymphocytes and neutrophils from blood

Blood was collected in a tube with 7.2 mg dipotassium EDTA (anticoagulant). The whole blood was layered in Ficoll-Histopaque according to Böyum [40]. After centrifugation, a lymphocyte-rich mononuclear cell layer and a polymorphonuclear cell layer were collected and washed with saline. The protocol was carried out according to the manufacturer's instructions. The resulting lymphocyte and neutrophil samples were used immediately for enzymatic assays.

2.7 Isolation of platelets from blood

For the isolation of platelets, platelet-rich plasma was separated from blood collected with anticoagulant Sodium Citrate 3.5% according to the method of Pilla et al. [41].

2.8Separation of blood serum

The blood samples were collected in tubes without anticoagulant and centrifuged at 1400×*g* min at room temperature for 15 min. The resultant serum samples were aliquoted and frozen for analyses.

2.9 Protein determination

Protein was measured by the Coomassie Blue method using serum albumin as a standard [42].

2.10 Hematological and biochemical parameters

Complete blood count was performed in EDTA blood using an automated hematology analyzer (SYSMEX XT-1800i, Roche Diagnostic, USA).

For biochemical parameters, blood was drawn in serum separator tubes, which were spun at 3500 rpm for 15min at room temperature. Serum levels of glucose, total cholesterol (TC), high-density lipoprotein (HDL) and triglycerides (TG) were evaluated in a semi-automatic analyzer (TPAnalyzer Plus®, Thermoplate) using commercial kits (Bioclin/Quibasa).

2.11 E-adenosine deaminase (E-ADA) activity

Giusti and Galanti [43] method was used to quantify E-ADA activity in lymphocytes, neutrophils, and platelets, in addition to ADA in serum, based on the direct quantification of ammonia produced when ADA deaminates the excess of adenosine. Twenty-five (25) µL of cells or serum was added to 21 mM/L of adenosine, pH 6.5, and incubated for 90 min at 37 °C. By adding 106 mM phenol and 167.8 mM sodium nitroprusside and hypochlorite solution, the reaction was stopped. 75 µM of ammonium sulfate was used as the ammonium standard. The protein content was adjusted for lymphocytes (0.1-0.2 mg/mL) and for neutrophils (0.2-0.3 mg/mL) and for platelets (0.4-0.6 mg/mL). All samples were run in triplicate and E-ADA activity was expressed in µmNH3/min/mg of protein.

2.12 Reactive oxygen species (ROS)

Peroxide production of cells and other reactive species were measured using the 2'-7'-dichlorofluorescein fluorescence assay according to Myhre et al. [44]. The aliquots of serum (50µL) are added to a medium containing Tris-HCl buffer (0.01 mM, pH 7.4) and DCFH DA 2'-7'-dichlorofluorescein-diacetate (1 mM). After the addition of DCFH-DA, the medium is incubated in the dark for 1 h until fluorescence measurement (excitation at 488 nm and emission at 525 nm).

Dichloro-oxidized fluorescein is determined using a standard curve of oxidized dichlorofluorescein and the results are expressed as DCFH-DA fluorescence.

2.13 Myeloperoxidase activity (MPO)

MPO activity is measured in EDTA plasma, obtained by centrifugation at 1800×g for 10 min. MPO activity is spectrophotometrically analyzed by a modified peroxidase-coupled assay system involving phenol, 4-aminoantipyrine (AAP) and H_2O_2, following the method previously described by Metcalf et al. [45]. Briefly, 390µL of AAP, 2.5mM of phenol and 20mM of the sample are added in each tube, followed by 450µL of 1.7mM H_2O_2. The reaction product is the quinoneimine and its absorbance was read at 500 nm. The results were expressed in micromolar of the quinoneimine produced in 30 min.

2.14 Statistical analysis

The statistical analysis was performed using two-way ANOVA tests, followed by Tukey´s test since all data were normally distributed. The hyperlipidemic groups were compared to the non-hyperlipidemic and the untreated hyperlipidemic groups. The variables analyzed were hyperlipidemia and pretreatment with compounds. $P < 0.05$ was considered to represent a significant difference in both analyses used. All data were analyzed using GraphPad Prism 6.0 and expressed as mean ± SEM. The correlations were done using the coefficient the correlation of Pearson. For the comparison of cell count ratios, Student's t-test was used.

3 RESULTS

3.1 Biochemical Parameters

We observed a significant increase in TC (P<0.001), TG (P<0.001) and HDL (P<0.01) in hyperlipidemic rats in relation to the control group (S). The pretreatments with curcumin alone (C) (P<0.05) and the rutin/curcumin association (R+C) (P<0.05) prevented the increase in triglyceride levels observed in the untreated hyperlipidemic group (H+S). The pretreatments with curcumin (H + C) (P<0.001) and rutin/curcumin association (H+CR) (P<0.001) also promoted an increase in HDL levels, compared to the control group (S), and show a significant difference (P<0.001) when compared to the untreated hyperlipidemic groups (Table 1). Pretreatment with rutin did not alter total cholesterol, triglyceride and HDL levels compared to untreated hyperlipidemic rats. The pretreatments *per se* did not affect total cholesterol, triglyceride and HDL levels.

3.2 Hematological parameters

The results of hematological parameters are shown in table 2 and 3. Table 2 shows the results for the untreated hyperlipidemic rats against control group where we observed neutrophilia and lymphopenia and increase in the Neutrophil-to-lymphocyte (NLR) (P<0.001) and the Platelets-to-neutrophil ratio (PLR) ratios (P<0.01) in the untreated hyperlipidemic group. Table 3 demonstrates the same parameter as table 2 but compares the results of hyperlipidemic rats pretreated with rutin, curcumin, and rutin-curcumin against untreated hyperlipidemic rats. The treatments were able to prevent neutrophilia and lymphopenia when compared to the hyperlipidemic group, as well as to reduce the NLR and PLR ratios at the basal levels. No difference was observed in the comparison of hematological parameters among pretreated control groups (data not shown).

3.3 ADA activity in serum

The ADA activity in serum (figure 1) was shown to be increased (26.2±0.9) in relation to the control group (16.7±2.2). On the other hand, groups pretreated with rutin (16.9±1.9) and curcumin (16.9±1.0) and rutin/curcumin association (9.4±0.9) prevented the increase in ADA activity when compared to the hyperlipidemic group. The rutin/curcumin association also reduced the adenosine deamination on the pretreated control group (R+C).

3.4 E- ADA activity in lymphocytes

In lymphocytes, the deamination of adenosine by the E-ADA is shown in Figure 2. The group with untreated induced hyperlipidemia (247.0±16.9) showed an increase in E-ADA activity in relation to the control group (137.4±20.3). Pretreatments with rutin (170.4±15.0) curcumin (151.5±23.0) and rutin/curcumin association (161.6±7.8) prevented the increase of ADA activity when compared to the hyperlipidemic group.

3.5 E- ADA activity in platelets

In platelets, the deamination of adenosine by E-ADA is shown in figure 3. The hyperlipidemia-induced group (43.0±0.5) presented an increase in E-ADA activity in relation to the control group (23.0± 1.9). All the pretreatments prevent the increase of ADA activity in relation to the hyperlipidemia group (P< 0.001).

3.6 E- ADA activity in neutrophils

The deamination of the adenosine by the enzyme E-ADA in neutrophils is shown in figure 4. The group with the induction of hyperlipidemia (107.9±4.2) showed an increase in relation to the control group (56.9±3.7). Pretreatments with rutin (26.7±5.7) and curcumin (49.9± 2.5) were able to prevent the increase in E-ADA activity in relation to the untreated hyperlipidemic group. The association was not able to prevent the changes of E-ADA activity levels in these cells.

3.7 Reactive oxygen species in serum

The results for the dosage of reactive oxygen species in serum is demonstrated in figure 5. The untreated hyperlipidemic group (6.3±0.6) exhibited an increase in ROS levels compared to the control group (3.6±0.1). However, the pretreatments were not able to prevent the changes on the levels of ROS compared to the untreated hyperlipidemic group.

3.8 Activity of the myeloperoxidase enzyme in plasma

The activity of the MPO is shown in figure 6. The hyperlipidemic group (15.6±2.7) showed a significant increase compared to the control group (5.4±2.0). Regarding the pretreated hyperlipidemic group, the rutin (7.4±1.5) curcumin (7.2±1.1) and the rutin/curcumin association group (7.2±0.9) has shown a significant power in preventing the increased the MPO activity when compared to the hyperlipidemic group.

3.8 Correlation between the parameters

All the calculated correlations between parameters are shown in table 4. The correlations between serum ADA activity and the production ROS, serum ADA and TC and between neutrophil E-ADA and myeloperoxidase activity were all positive, strong and significant. Also, the production of reactive oxygen species (ROS) correlates positively with total TC and triglyceride TG levels. All the correlations are between the control and untreated hyperlipidemic groups. There was no correlation between MPO activity and ROS levels.

DISCUSSION

We evaluated the effects of the induction of hyperlipidemia and the 30-day pretreatments with curcumin, rutin and the rutin/curcumin association in rats. The results of the biochemical and hematological parameters showed a positive induction of hyperlipidemia and inflammation, respectively. Both curcumin and the rutin/curcumin association presented a hypotriglyceridemic effect. A

significant improvement of hematological parameters was found with all pretreatments. The activities of ADA in serum and E-ADA in the cells were modulated by all pretreatments.

The use of poloxamer-407 for induction of hyperlipidemia in rats is already well-established in the literature [46], causing an increase in TC, TG, and LDL levels [47]. Peak serum levels of these lipids occur after 36 hours of intraperitoneal injection of P407, and continuous administration of this compound leads to the development of atherosclerosis [39]. In this study, the untreated hyperlipidemic group showed an increased in the levels of TC and TG, which confirms the induction of hyperlipidemia by P407. The induction of hyperlipidemia by P407 occurs via inhibition of enzymes involved in triglyceride hydrolysis, cholesterol synthesis, and bile acid synthesis [48].

HDL is responsible for transporting cholesterol from the bloodstream into the liver, thus preventing the development of hyperlipidemia and subsequent atherosclerosis [49]. In this study, the HDL levels were increased in the groups treated with curcumin and the rutin/curcumin association, a result similar to that found by Su et al. (2017) with curcumin in diabetic rats [50].

The lowering of TC and TG by flavonoids is well-described, however, the mechanism involved may vary according to the flavonoid [36,51]. In this study, the pretreatment with rutin and/or curcumin did not revert TC levels to basal levels, however, both curcumin and rutin/curcumin association decreased significantly the TG levels of hyperlipidemic rats. Other studies confirm the potential of curcumin in reducing these indices in models of diabetes mellitus and hypocholesterolemia [50,51]. The lipid-lowering effect of curcumin occurs through downregulation of HMG-CoA reductase and, in the liver, a reduction in the expression of PPARa and LXRa nuclear transcriptional factors [50,52]. Rutin also reduces the HMG-CoA reductase activity as well as increasing fecal sterols [53].

We found a positive correlation between serum ADA activity and TC in untreated hyperlipidemic rats, which corroborates with the findings of Kutryb-Zajac et al. (2016) which found a positive correlation between ADA activity and TC levels, as well as TG levels, in patients with an aortic aneurysm [13]. It

indicates that the increase in cholesterol plays a role in the elevation of serum ADA activity.

In this study, hyperlipidemia was associated with an increase in the neutrophil counts and a decrease in lymphocyte numbers, characteristics of an inflammatory process. Inflammatory conditions are often accompanied by changes in the counts of white blood cells subtype, and endothelial dysfunction is also associated with neutrophilia and lymphopenia [54].

A growing number of studies are reported the use of neutrophil-to-lymphocyte ratio as an inflammation and endothelial damage marker as well as to predictor of the occurrence and the severity of cardiovascular diseases [54–59]. A significant increase in the neutrophil-to-lymphocyte ratio in the hyperlipidemic group was observed in this study, as well as a prevention of this changes by all pretreatments. There were no differences between the hyperlipidemic and the control group on the platelet counts. However, the platelet-to-lymphocyte ratio was significantly increased in the hyperlipidemic group, which returned to basal levels after the pretreatments. Platelet-to-lymphocyte ratio has also been proposed as a predictor and prognostic marker of cardiovascular events [55,58].

In serum, we found that the activity of ADA in hyperlipidemic rats was increased when compared to the control group, which indicates an inflammatory process triggered by hyperlipidemia. In fact, serum ADA has been described as an unspecific marker of cell-mediated immunity and immune cell activation [60,61] and inflammation [62–64]. The activity of serum ADA is also elevated in metabolic diseases such as hyperlipidemia[65], hypertriglyceridemia [66], diabetes [67,68] and metabolic syndrome [69,70]. In hyperlipidemic rats pretreated with rutin or curcumin alone, serum ADA activity was decreased when compared to the untreated hyperlipidemic group, suggesting that the inflammatory process was reduced [68]. A study with diabetic rats found similar results, which suggests that rutin regulates the immune and inflammatory responses to diabetes [68]. Interestingly, the treatment with the association of rutin and curcumin have lowered serum ADA in hyperlipidemic and non-hyperlipidemic rats when compared to the control group, indicating the presence of a synergistic effect capable of modulating serum ADA activity in the absence of an inflammatory process.

The enzymatic activity of ADA has both a pro-inflammatory and a lipolytic effect, antagonizing the effects of adenosine. Increased activity of E-ADA in endothelial cells and macrophages in the vasculature mark the onset of atherosclerosis [13]. Upon an atherogenic stimulus, plasma ADA is also elevated as a result of the increased activity of this enzyme in the surface of these cells. Thus, the inhibition of ADA has been proposed as a therapeutic target for atherosclerosis. The ability of statins and flavonoids to downregulate E-ADA activity from endothelial cells and macrophages has been demonstrated in aortic disease [13]. Little is known about the modulation of E-ADA in lymphocytes, neutrophils, and platelets by polyphenols.

Lymphocyte function is also modulated by adenosine through its interaction with adenosine receptors especially the A2A receptor that inhibits the production of proinflammatory cytokines [71]. In this study, we found an increase in lymphocyte E-ADA activity in the hyperlipidemic rats compared to controls, similar results already were found in previous studies with hyperlipidemic rats [72] and patients with metabolic syndrome [73]. All the pretreatments avoided the increased in lymphocyte E-ADA activity, allowing higher plasmatic levels of adenosine to control inflammation. Similar results were found with treatments of other flavonoids such as quercetin [72].

The involvement of platelets in the formation of thrombi is stimulated by high levels of cholesterol [74]. Hypercholesterolemia activates inflammatory cells which release mediators such as adenosine to modulate the immune response and downregulate the inflammatory process [75]. The results of this study show that the activity of the enzyme E-ADA is increased in platelets of rats with untreated hyperlipidemia and similar results have been found in another study with diabetic rats [76]. All the pretreatments prevented the increase in platelets E-ADA activity, providing protection against atherosclerosis.

Adenosine regulates the chemotaxis and phagocytic activity of neutrophils, as well as the generation of reactive oxygen species (ROS) [4,71]. In this study, the degradation of adenosine by E-ADA anchored to the surface of neutrophils was shown to be increased in hyperlipidemic rats. Neutrophils have a crucial role in the inflammatory process and are related to the progression of various inflammatory diseases such as atherosclerosis [77]. The anti-inflammatory properties of adenosine are controlled by its concentration, thus the

removal of adenosine by ADA supports inflammation and boosts the immune response by neutrophils [78]. Therefore, in this study, the elevated neutrophil E-ADA activity in the untreated hyperlipidemic group, suggests an increase in the inflammatory response by these cells. The pretreatments with rutin and curcumin alone averted the effects of hyperlipidemia in E-ADA of neutrophils.

Activated neutrophils promote the release of MPO and generation of ROS. MPO catalyzes reactions responsible for the formation of ROS, playing an important part of in the innate immune response by promoting inflammation [4]. In this study, we found an increase in the production of ROS and MPO activity in hyperlipidemic rats, thus suggesting that neutrophils responded to the increased levels of lipids. Moreover, the levels of MPO correlate with E-ADA activity in neutrophils in hyperlipidemic rats, showing that E-ADA activity in neutrophils may be an indicator of activation of these cells. Also, our results suggest that increased activity of ADA in the serum activates the neutrophils and stimulate further ROS release. This notion is in accordance with Kalvegren (2010), which found that plasma ADA activity stimulates ROS production by neutrophils [79].

The increase in MPO activity was prevented by all pretreatments, however, the ROS levels remained elevated. This may be explained by the fact MPO activity is not the only mechanism of ROS production [80]. Also, ROS are produced not only by neutrophils but also by other inflammatory cells such as macrophages and endothelial cells. In monocytes, MPO is expressed but when these cells differentiate to macrophages they no longer express this enzyme [81]. The correlation between MPO activity and ROS levels could not be established, corroborating with the fact that not all ROS production is dependent on neutrophil activity. Thus, the phagocytic activity of macrophages contributes to the maintenance of ROS levels [82].

Activated immune cells such as neutrophils, lymphocytes, and macrophages release inflammatory mediators such as inflammatory cytokines and ROS. The increase in ADA activity and ROS levels indicate activation of the immune cells involved in the response against circulating lipids and subsequent endothelial damage. ROS mediates the oxidation of lipids in the membrane of cells forming malondialdehyde (MDA), which increases along with ADA activity in metabolic syndrome [73] and diabetes [83]. In this study, we have found a positive correlation between ADA activity and ROS levels in serum of hyperlipidemic rats.

The pretreatments did not prevent the increase in cholesterol levels and oxidized lipids are phagocyted by macrophages which results in ROS production. Therefore, the elevated levels of ROS may be a result of the increase in circulating lipids as we found a positive correlation between ROS and TC and TG levels. A study using a fruit extract rich in flavonoids including rutin found similar results since the compound prevented the increase in ADA activity in lymphocytes but did not revert lipid peroxidation [84].

The properties of rutin and curcumin control the effects of inflammation induced by hyperlipidemia as given by the normalization of leukocyte counts and reduction in serum ADA as well as E-ADA activities in cells.

The pretreatments, except rutin, were only able to reduce TG levels, but not the cholesterol levels. In addition to the classical mechanisms of reduction of lipids by flavonoids, this may be owing to the role of adenosine in lipid metabolism. Adenosine inhibits lipolysis via A1 receptors thereby preventing the release of free fatty acids and triglycerides into the bloodstream [31]. The maintenance of cholesterol levels may be related to the fact that adenosine promotes the efflux of cholesterol via A2A receptors to avoid the formation of foam cells [30].

CONCLUSIONS

We have demonstrated a hypotriglyceridemic effect of curcumin and the rutin/curcumin association pretreatments. In addition, these treatments effectively increased the levels of circulating HDL.

Rutin and curcumin pretreatments, alone and in combination, have been shown to be effective in preventing the immune cell activation and inflammation caused by hyperlipidemia. ADA activity was modulated by all pretreatments showing a protective effect, suggesting that they are valuable adjuvants in the treatment of hyperlipidemia-induced inflammation.

DECLARATIONS:

Ethics approval and consent to participate

This work was approved by the Ethics Committee on the Use of Animal at the Federal University of Santa Maria (Protocol number: 1006200117).

Consent for publication

Not applicable

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

Funding

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. PROEX number (88882.182184/2018-01)

Acknowledgments

The authors thank Bioclin for kindly supplying the biochemistry kits.

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TABLES

Table 1 Lipid profile after induction of hyperlipidemia in rats pretreated with rutin and/or curcumin

Groups	TC	TG	HDL
S	50.4± 3.3 [a]	376.2± 41.0 [a]	50.2±2.9 [a]
R	93.0±33.8 [a]	323.1±48.8 [a]	48.8±3.0 [a]
C	75.1±13.0 [a]	429.0±133.1 [a]	54.0± 4.5 [a]
R+C	65.6±8.1 [a]	300.2± 42.1 [a]	58.0± 5.7 [a]
H+S	330.2±39.0 [b]	3503.3±710.0 [b]	166.2±22.7 [b]
H+R	395.2±56.5 [b]	3495.5±874.7 [b]	134.0±2.4 [b]
H+C	310.8±50.0 [b]	1630.3±193.4 [c]	289.0±32.3 [c]
H+RC	249.8±39.8 [b]	1399.3± 138.1[c]	275.7±34.9 [c]

TC: total cholesterol (mg/dL); HDL-C: high-density lipoprotein-cholesterol (mg/dL); TG: triglycerides (mg/dL); Groups: (S) saline control, saline + (R) rutin 50 mg/kg, saline + (C)curcumin 50 mg/kg, saline + (R+C) rutin and curcumin 50 mg/kg, (H+S) hyperlipidemia, (H+R) hyperlipidemia + rutin 50 mg/kg, (H+C) hyperlipidemia + curcumin 50 mg/kg, (H+RC) hyperlipidemia + rutin, and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA-Tukey multiple comparison tests and expressed as mean ± S.E.M. Values with a different letter (^{a,b,c}) differ significantly from each other.

Table 2 Comparison of laboratory findings between control and hyperlipidemic groups.

	Control (n = 5)	**Hyperlipidemic (n =5)**	***P***
WBC (10^3/µL)	8.1 ± 1.1	6.6 ± 1.1	> 0.05
Neutrophils (10^3/µL)	2.0 ± 0.06	6.2 ± 1.1	< 0.001***
Lymphocytes (10^3/µL)	5.4 ± 0.9	1.6 ± 0.6	< 0.001***
Platelets (10^3/µL)	1118 ± 170	1213 ± 136	> 0.05
NLR	0.37 ± 0.05	4.4 ± 1.3	<0.001***

	Control (n = 5)	Hyperlipidemic (n =5)	*P*
PLR	222.7 ± 55.2	670.6 ± 208	<0.01**

WBC: White blood cell; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelets-to-neutrophil ratio. The results were analyzed using Student t-test expressed as mean ± standard deviation. The values are significantly different when compared to the control group.

Table 3 Comparison of hematological parameters between the untreated hyperlipidemic rats and each of the pretreatments.

	Hyperlipidemic (n =5)	Rutin (n=6)	Curcumin (n=6)	Rutin+ Curcumin (n=6)	*P*
WBC (10^3/µL)	6.2 ± 1.1	6.7 ± 1.0	5.4 ± 1.7	6.4 ± 0.8	> 0.05
Neutrophils (10^3/µL)	6.6 ± 1.1	1.5 ± 0.6	1.3 ± 0.7	1.4 ± 0.3	< 0.001***
Lymphocytes (10^3/µL)	1.6 ± 0.6	4.7 ± 1.0	3.9 ± 0.8	4.6± 1.2	< 0.001***
Platelets (10^3/µL)	1213 ± 136	1090 ±183	1164 ± 229	1239 ± 142	> 0.05
NLR	4.4 ± 1.3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	<0.001***
PLR	670.6 ± 208	237.4± 67.3	315.8 ± 87.4	278.6 ± 59.9	<0.01**

WBC: White blood cell; NLR: Neutrophil-to-lymphocyte ratio; NPR: Neutrophil-to-platelets ratio. The results were analyzed using Student t-test expressed as mean ± standard deviation. The values are significantly different when compared to the hyperlipidemic group.

Table 4: Correlations between the parameters;

	Correlation coefficient (*r*)	*P*
sADAx ROS	0.894	< 0.05
SADAxTC	0.915	< 0.05
nADAxMPO	0.959	< 0.05
ROSxTC	0.986	< 0.05
ROSxTG	0.880	< 0.05
MPOxROS	0.210	> 0.05

sADA: Serum ADA activity; nADA: Neutrophil ADA activity; ROS: Reactive oxygen species levels in serum; MPO: Myeloperoxidase activity in plasma; TC:

Total cholesterol in serum; TG: Triglycerides in serum; the results were analyzed using the correlation of Pearson, expressed as mean ± standard deviation.

LEGENDS

Figure 1 Adenosine deamination in the serum of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. Enzyme activities are reported as (U/L) Groups: saline control, saline + rutin 50mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. ## indicates that the value is significantly different from the control group ($P < 0.01$, $n = 6$). # indicates that the value is significantly different from the control group ($P < 0.05$, $n = 6$) *** indicates that the value is significantly different from the untreated hyperlipidemic group ($P < 0.001$, $n = 6$) ** indicates that the value is significantly different from the untreated hyperlipidemic group ($P < 0.01$, $n = 6$).

Figure 2 Adenosine deamination in lymphocytes of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. Enzyme activities are reported as (µmNH3/min/mg of protein) Groups: saline control, saline + rutin 50mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. ### indicates that the value is significantly different from the control group ($P < 0.001$, $n = 6$). * indicates that the value is significantly different from the untreated hyperlipidemic group ($P < 0.05$, $n = 6$) ** indicates that the value is significantly different from the untreated hyperlipidemic group ($P < 0.01$, $n = 6$).

Figure 3 Adenosine deamination in platelets of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. Enzyme activities are reported as (µmNH3/min/mg of protein) Groups: saline control, saline + rutin 50mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. ### indicates that the value is significantly different from the control group ($P < 0.001$, $n = 6$). *** indicates that the value is significantly different from the hyperlipidemic group ($P < 0.001$, $n = 6$).

Figure 4 Adenosine deamination in neutrophils of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. Enzyme activities are reported as (μmNH3/min/mg of protein) Groups: saline control, saline + rutin 50mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. ##indicates that the value is significantly different from the control group (P=0.01, n = 6). *** indicates that the value is significantly different from the hyperlipidemic group (P < 0.001, n=6).

Figure 5 Reactive oxygen species in the serum of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. Dosage is reported as (DCFH-DA Fluorescence) Groups: saline control, saline + rutin 50mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. ## indicates that the value is significantly different from the control group (P < 0.01, n = 6)

Figure 6 Myeloperoxidase activity in plasma of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. Enzyme activities are reported as (μM/quinoneimina/30min) Groups: saline control, saline + rutin 50mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. ### indicates that the value is significantly different from the control group (P < 0.001, n = 6). ** indicates that the value is significantly different from the hyperlipidemic group (P < 0.01, n = 6)

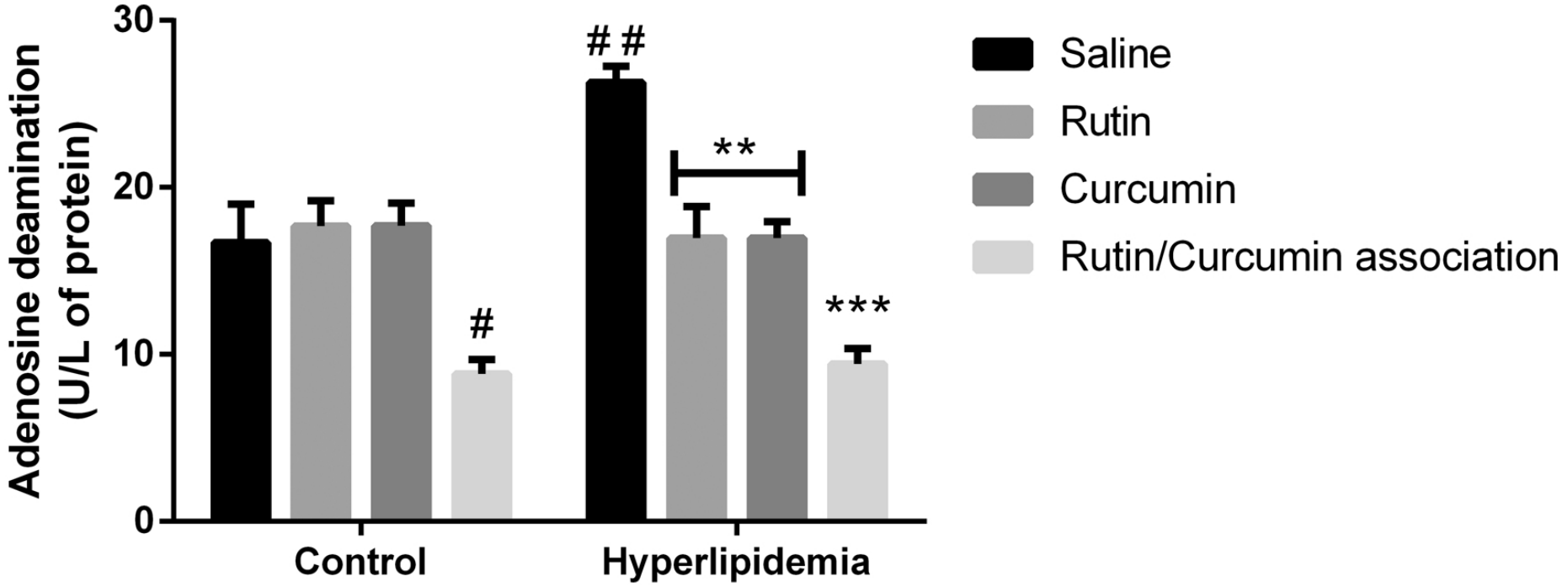
Adenosine deamination (U/L of protein)
30
20
10
0
#
##
**
***
Control
Hyperlipidemia
Saline
Rutin
Curcumin
Rutin/Curcumin association

Figure 1

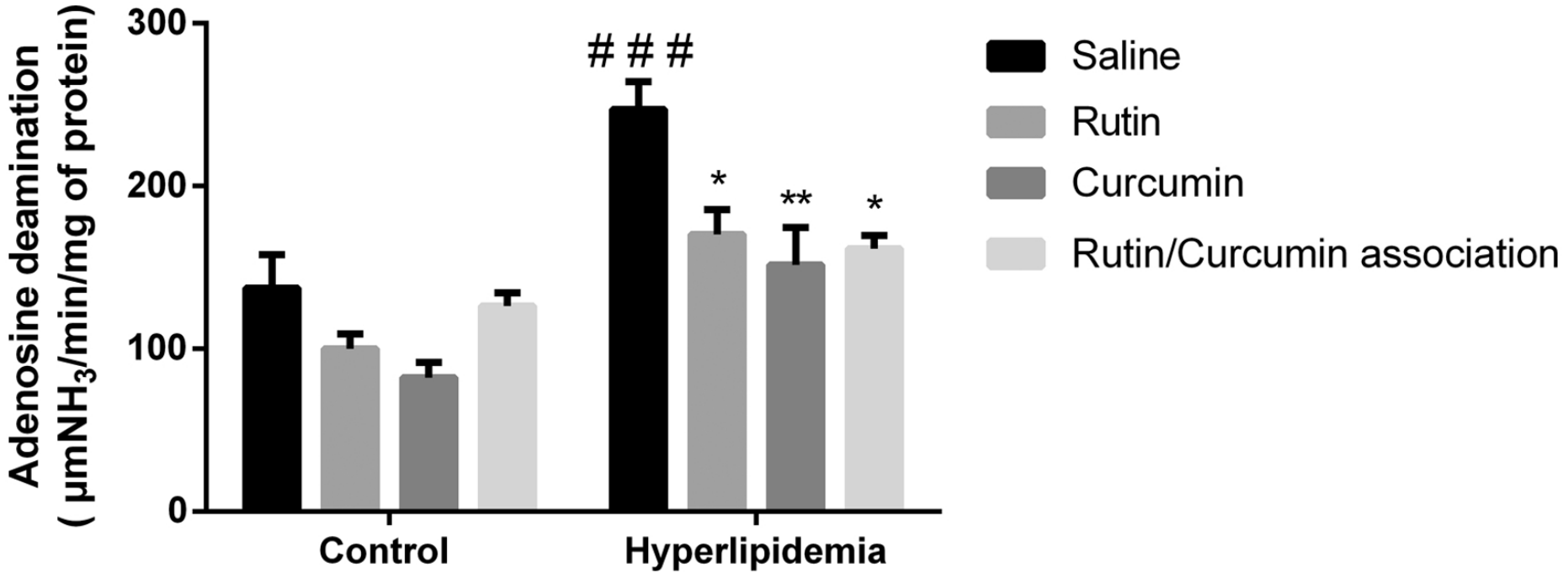
Adenosine deamination
(μmNH3/min/mg of protein)
300
200
100
0
###
*
**
*
Control
Hyperlipidemia
Saline
Rutin
Curcumin
Rutin/Curcumin association

Figure 2

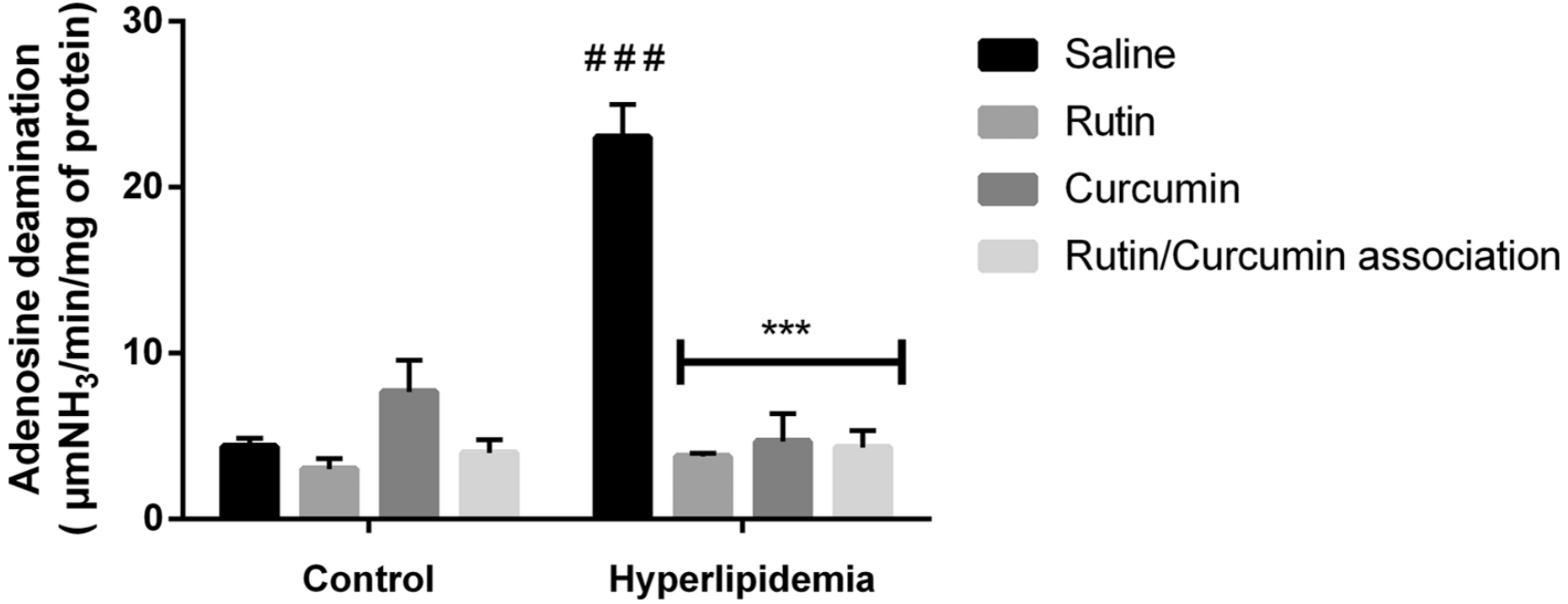
Adenosine deamination
(μmNH3/min/mg of protein)
30
20
10
0
###
***
Control
Hyperlipidemia
Saline
Rutin
Curcumin
Rutin/Curcumin association

Figure 3

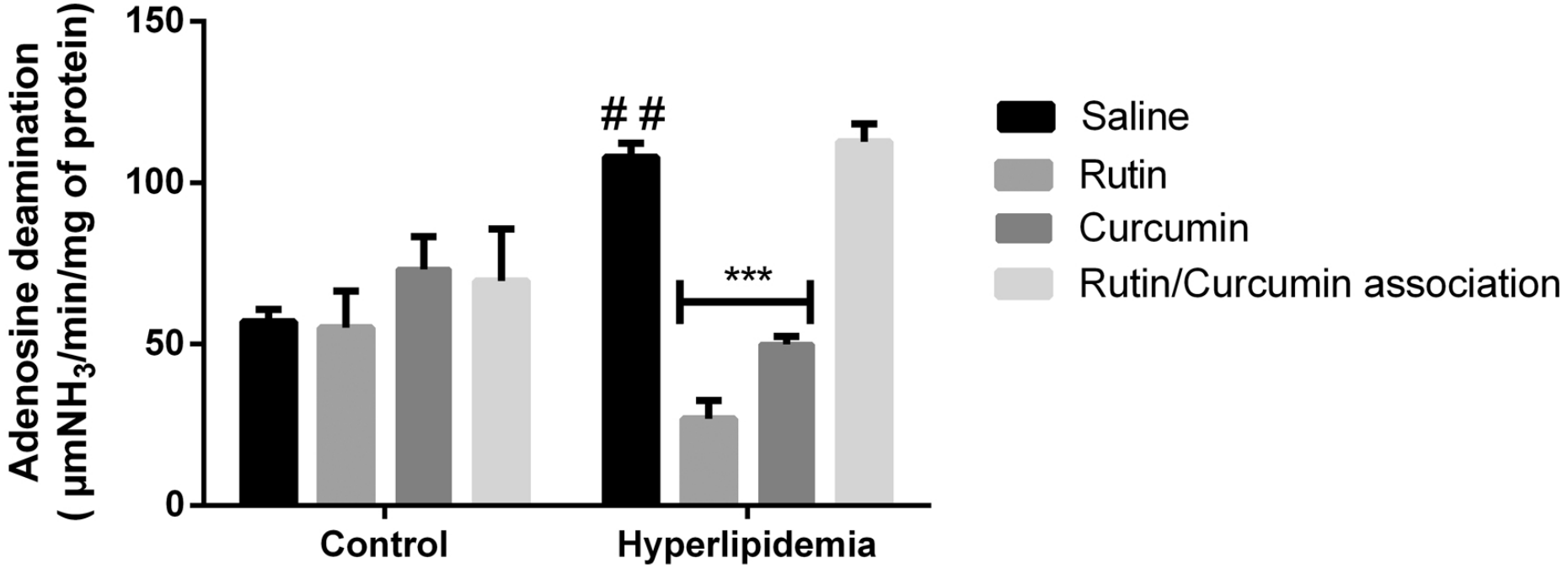
Adenosine deamination
(μmNH3/min/mg of protein)
150
100
50
0
##
***
Control
Hyperlipidemia
Saline
Rutin
Curcumin
Rutin/Curcumin association

Figure 4

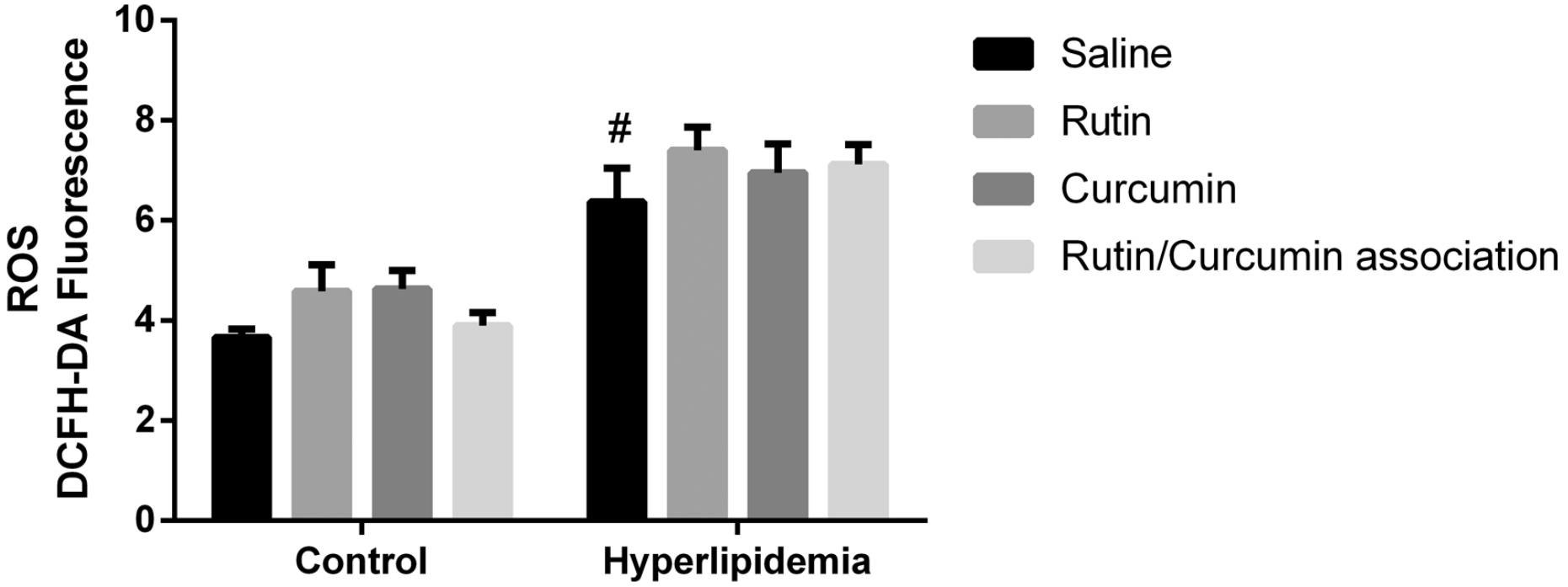
ROS
DCFH-DA Fluorescence
10
8
6
4
2
0
#
Control
Hyperlipidemia
Saline
Rutin
Curcumin
Rutin/Curcumin association

Figure 5

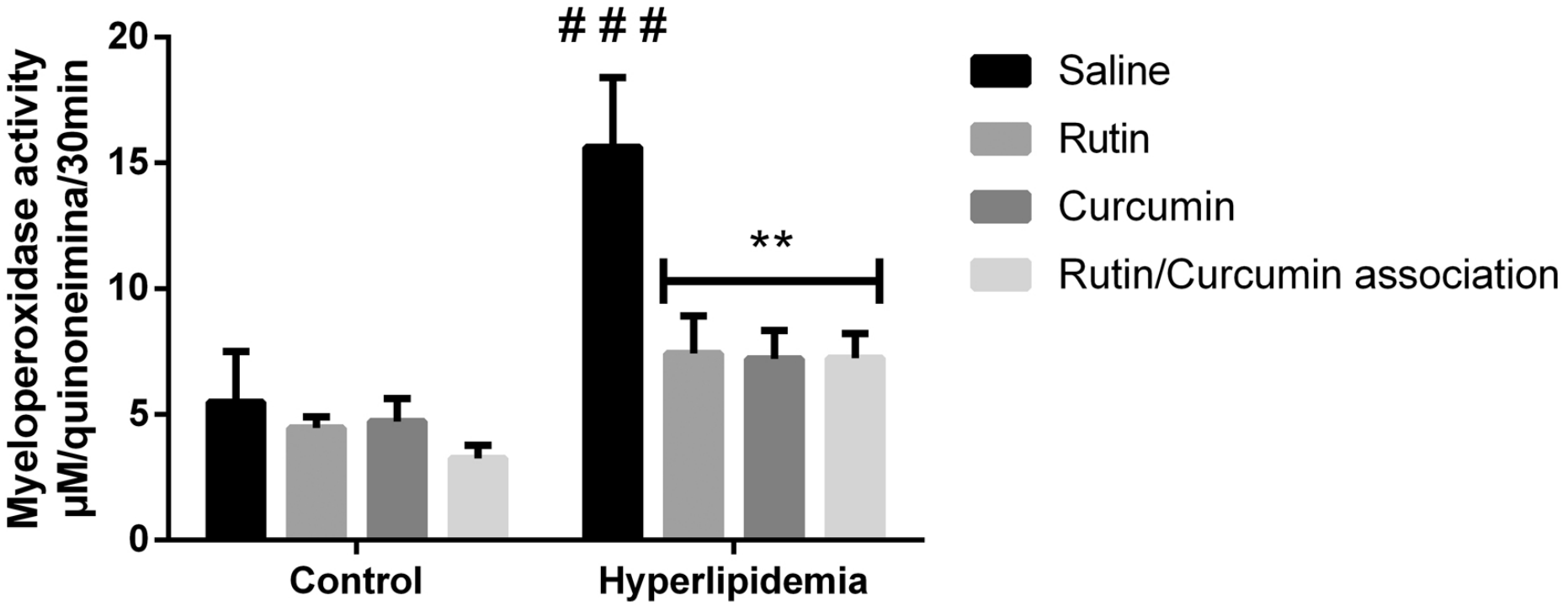
Myeloperoxidase activity
µM/quinoneimina/30min
20
15
10
5
0
###
**
Control
Hyperlipidemia
Saline
Rutin
Curcumin
Rutin/Curcumin association

Figure 6