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ORIGINAL ARTICLE

Chitosan treatment abrogates hypercholesterolemia-induced erythrocyte's arginase activation

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Abstract This study aimed to evaluate the protective effect of chitosan (CS) against hypercholesterolemia (HC) induced arginase activation and disruption of nitric oxide (NO) biosynthesis using erythrocytes as cellular model. Human erythrocytes were isolated and classified into eight groups. Next, cells were treated with L-arginine (L-ARG), N^{ω}-nitro-L-arginine methyl ester (L-NAME), CS or CS + L-ARG in the presence of normal plasma or cholesterol enriches plasma. Then, erythrocytes were incubated at 37 °C for 24 h. The present results revealed that, HC induced significant increase of cholesterol inclusion into erythrocytes membrane compared to control. Moreover, HC caused significant decrease in nitric oxide synthase (NOS) activity similar to L-NAME; however, arginase activity and arginase/NOS ratio significantly increased compared to control. On contrast, treatment of HC with, L-arginine, CS or CS plus L-arginine prevents HC induced cholesterol loading into erythrocytes membrane, NOS inhibition and arginase activation. This study suggested that CS could be protective agent against HC induced disruption of erythrocyte's oxidative status and arginase activation.

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1. Introduction

Arginase is an important regulator of nitric oxide (NO) production, and an increase of arginase activity reduces the availability of L-arginine for NO synthase, thus reducing NO production, and leading to endothelial dysfunction (Pernow and Jung, 2013). Increased activity of arginase has been demonstrated in several pathological conditions including cardiovascular dysfunction and other vascular diseases (Pernow and Jung, 2013). Hypercholesterolemia (HC) induces lipemic stress due to extra cholesterol deposition into the membranes of vascular cells and erythrocytes, and this triggers reactive oxygen species (ROS) production, and membrane aberration (Uydu et al., 2012). Furthermore, lipemic stress is associated with disruption of L-arginine transport into cells and inactivation of nitric oxide synthase (NOS), while arginase activity is increased. Additionally, L-arginine analogues increase, particularly asymmetric dimethylarginine and symmetric dimethylarginine (Eligini et al., 2013; Yang et al., 2013; Porro et al., 2014). The net result of these events is the decrease of NO levels as a key player in the regulation of homeostasis, vasodilation, neurotransmission, free radicals scavenging and erythrocytes function (Eligini et al., 2013).

In past decades, several studies reported that vascular NO is mostly produced from endothelial cells by endothelial NO synthase (eNOS); however nowadays, erythrocytes were listed as another major source of NO in vascular lumen (Eligini et al., 2013; Ramírez-Zamora et al., 2013; Porro et al., 2014). For NO biosynthesis, NOS utilizes L-arginine as substrate; flavoproteins and tetrahydrobiopterin were used as coenzymes (Eligini et al., 2013; Porro et al., 2014). Conversely, arginase competes with NOS on L-arginine as common substrate; therefore, it NO production (Yang et al., 2013, Li and Förstermann, 2013). The proper balance between NOS and arginase is essential for maintenance of NO homeostasis (Porro et al., 2014; Yang et al., 2013). Functional erythrocytes have antioxidant machinery that neutralizes ROS generated in the vasculature; however, malfunctioned erythrocytes can act as a source of ROS (Minetti et al., 2007). Moreover, such erythrocytes release arginase that limits NO production (Porro et al., 2014; Yang et al., 2013). Therefore, oxidized erythrocytes act as prooxidant bombs to vascular endothelium. Although, several studies reported that erythrocyte's arginase activity was augmented by oxidative stress (Yang et al., 2013; Porro et al., 2014; Li and Förstermann, 2013), no enough published data address this topic, and further research are necessary to address this issue.

Most of lipid-lowering agents have many therapeutic problems with severe side effects, while dietary fibers as lipid lowering therapy are safer. Chitosan (CS) is a dietary fiber biodegradable, biocompatible and has many health benefits including wound healing, antiinflammatory, anti-cancers, immune-modulator, hemostatic agent, lipid-lowering agent and antioxidants (Xia et al., 2011; Luo and Wang, 2013; Anandan et al., 2013). The lipid-lowering effect of CS is attributed to its binding to fatty acid, cholesterol, and bile salts; this resulted in delaying the digestion and absorption of fat (Xia et al., 2011). Additionally, CS augments lipoprotein lipase activities and influences plasma adipocytokines, which significantly reduce adiposity index. Therefore, CS can regulate the level of circulating triacylglycerol and ameliorates metabolic alterations (Luo and Wang, 2013). CS can help the body maintain the antioxidant activity, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and other antioxidants, which play important roles in counteraction of oxidative stress (Xia et al., 2011; Luo and Wang, 2013).

Despite the beneficial effects of CS in hyperlipidemic conditions have been extensively studied, yet no data available about the effect of CS on arginase activity in HC condition have been explored. Therefore, the main goal of this study was to test the hypothesis that CS as innovative therapeutic agent approaches for treatment of HC induced disruption of erythrocyte NO biosynthetic pathway. NOS activity and arginase activities were investigated under effect of HC exposure in the presence and absence of CS.

2. Materials and methods

2.1. Chemicals

Low molecular weight chitosan, L-Arginine (L-ARG), N^{ω}-nitro-L-arginine methyl ester (L-NAME), and water-soluble cholesterol were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used were of reagent grade.

2.2. Isolation of erythrocytes and experimental design

Blood samples were drawn from healthy human volunteers by venipuncture into heparinized test tubes. Plasma sample was separated by centrifugation at 3000 rpm for 5 min; buffy coat was removed by aspiration. Then, erythrocytes were gently washed 3 times with an equal volume of phosphate buffered saline pH 7.5. Next, supernatant was discarded and erythrocytes were separated. Plasma sample was mixed with water-soluble cholesterol, after this referred to cholesterol-enriched plasma (HC) (Kanakaraj and Singh, 1989). HC was confirmed by measuring cholesterol level using cholesterol oxidase method by commercially available kit (Randox Laboratories, Crumlin, UK). The protocol for this study conformed to the guidelines of the Institutional Ethical Committee.

Washed erythrocytes were classified into eight groups as follows:

Group 1: Normal control (NC), in this group washed erythrocytes were incubated with normal plasma (cholesterol = 150 mg/dl).
Group 2: CS treated group (CS), herein, washed erythrocytes were incubated with *normal* plasma plus CS solution (1 mg/mL) (Fernandes et al., 2008).
Group 3: In this group cells were treated with L-NAME as reference group for NO inhibition, in which erythrocytes were incubated with *normal* plasma plus (10 mM) of L-NAME (Kuwai and Hayashi, 2006).
Group 4: L-arginine treated group as reference samples for NO production, in this group erythrocytes were suspended in normal plasma plus (10 mM) of L-ARG (Kuwai and Hayashi, 2006).
Group 5: HC incubated group, in this set erythrocytes were exposed to HC plasma (cholesterol 450 mg/dl) (Kanakaraj and Singh, 1989).
Group 6: HC + L-ARG, samples of this group, erythrocytes were exposed to HC and treated with (10 μM) L-arginine.

Group 7: HC + CS treated group, in the set erythrocytes were incubated with HC and treated with 1 mg/mL CS.
Group 8: HC + L-ARG + CS treated group, herein erythrocytes were suspended in HC and treated with combination of L-ARG and CS group.

All groups were incubated at 37 °C for 24 h, and afterward samples were utilized for the following investigations.

2.3. Percent of cholesterol inclusion

After 24 h incubation time of erythrocytes with plasma samples in different groups, plasma samples were separated from erythrocytes by centrifugation, and the percent of cholesterol inclusion on erythrocytes was calculated as follows:

$$\text{Cholesterol (Ch) inclusion } \% = \frac{\text{Ch.Conc.}_{\text{Intial}} - \text{Ch.Conc.}_{\text{Final}}}{\text{Ch.Conc.}_{\text{Intial}}} \times 100$$

2.4. Assay of total antioxidant capacity

Plasma total antioxidant capacity (TAC) was determined using TAC Assay Kit (Cell Biolabs OxiSelect™, San Diego, California, USA). This assay is based on the reduction of copper (II) to copper (I) by uric acid. Upon reduction, the copper (I) ion further reacts with a coupling chromogenic reagent that produces a color that measured at 490 nm. Absorbance values were compared with a known uric acid standard curve. The assay processes were achieved following manufacturers assay techniques. TAC was expressed as mM.

2.5. Determination of antioxidant enzymes

Erythrocyte catalase (CAT) activity was determined in erythrocyte lysate using the OxiSelect™ catalase activity assay (Cell Biolabs, San Diego, California, USA). CAT degrades H_2O_2 to water and molecular oxygen, and the amount of degraded H_2O_2 is proportional to the enzyme activity. The color change of the reaction mixture was measured spectrophotometrically at 520 nm.

GPx activity was measured in hemolysate by RANSEL™ assay Kit (Randox Laboratories, Crumlin, UK). GPx activity was assessed by oxidation of NADPH to $NADP^+$; the absorbance was measured at 340 nm. Glutathione reductase (GR) activity was measured by using Randox kit (Randox Laboratories, Crumlin, UK). This method is based on the reduction of oxidized glutathione (GSSG) in the presence of NADPH, which is oxidized to $NADP^+$ and catalyzed by GR. The decrease in absorbance is measured at 340 nm.

SOD activity in the diluted lysate was measured using commercial RANSOD™ assay (Randox Laboratories, Crumlin, UK). This assay employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red dye. The absorbance was determined spectrophotometrically at a wavelength of 505 nm. The assay procedures of were achieved following manufacturers assay procedures and expressed as U/g Hb. Hemoglobin was measured in the whole blood sample by standard laboratory method.

2.6. Assay of glutathione

Erythrocytes reduced glutathione (GSH)/Oxidized glutathione (GSSG) contents of erythrocytes were measured using the method of Hissin and Hilf (1976), using O-phthalaldehyde (OPT), which forms a fluorescent derivative with GSH. Briefly, 0.2 mL erythrocyte pellet was diluted with 0.8 mL distilled water and proteins were precipitated by addition of 1 mL of 10% trichloroacetic acid (TCA). The supernatant was used for GSH and GSSG assays. For GSH assay, 4 mL of reaction mixture consisted of 3.25 mL phosphate–EDTA buffer (0.1 M, pH 8.0), 0.25 mL O-OPT (1 mg/mL methanol) and 0.5 mL supernatant. The fluorescence of the GSH-OPT adduct was measured after 15 min using excitation and emission wavelengths of 350 and 450 nm, respectively.

For GSSG assay, N-ethyl maleimide (NEM) was used to inhibit the oxidation of GSH to GSSG during estimation procedures, allowing the formation of a stable complex with GSH. After incubation of 0.5 mL of supernatant with 0.2 mL NEM (0.04 M) for 30 min, GSSG was measured using the same steps as the GSH assay, but using 0.1 N NaOH as a diluent instead of phosphate–EDTA buffer. The quantifications of GSH and GSSG were calculated using standard GSH and GSSG fluorescence and expressed as μM/g hemoglobin.

2.7. Assay of protein carbonyl

The total protein carbonyl content (PCC) in erythrocytes was determined in hemolysate by a spectrophotometric method described by Levine et al. (1994). The proteins were precipitated from hemolysate by addition of 10% TCA. The precipitate was resuspended in 1.0 mL of 2 M HCl for the blank control, or in 2 M HCl containing 2% 2,4-dinitrophenyl hydrazine for the test samples. After incubation for 1 h at 37 °C, protein samples washed with alcohol /ethyl acetate mixture, and precipitated again by the addition of 10% TCA. The precipitated protein was dissolved in 6 M guanidine hydrochloride solution and the absorbance was measured at 370 nm. The molar extinction coefficient of $22 \times 103\ M^{-1}\ cm^{-1}$ was used for calculating the PCC level and expressed as nM of carbonyls formed per mg protein. The total protein content was determined according to Lowry et al. (1951), using bovine serum albumin as standard.

2.8. Assay of malondialdehyde

Concentration of malondialdehyde (MDA) in hemolysate, an index of lipid peroxidation, was determined spectrophotometrically (Ohkawa et al., 1979). A mixture of 200 μL of 8% sodium dodecyl sulfate, 200 μL of 0.9% thiobarbituric acid, and 1.5 mL of 20% acetic acid were added to a 200 μL supernatant of hemolysate and 1.9 mL of distilled water was used to make the volume up to 4 mL. After boiling for 1 h, the mixture was cooled and 5 mL of an *n*-butanol and pyridine (15:1) solution was added. The mixture was centrifuged at 5000 rpm for 15 min and the absorbance was measured at 532 nm. MDA levels were calculated using tetraethoxypropane as standard.

2.9. Assay of sialic acid

Sialic Acid (SA) content of erythrocyte membranes is based on oxidation of SA to formylpyruvic acid, which reacts with thiobarbituric acid to form a pink colored product (Denny et al., 1983). Briefly, membranes (1 mg membrane proteins/mL) were hydrolyzed in 0.05 M H_2SO_4 to a final volume of 100 μL for 1 h at 80 °C to release SA. Then, the samples were incubated with 250 μL periodate solution (0.025 M periodic acid in 0.25 M HCl) at 37 °C for 30 min. A 250 μL 0.32 M sodium thiosulphate were added, and the reaction was completed by addition of 1250 μL 0.1 M thiobarbituric acid. The samples were heated at 100 °C for 15 min and cooled to room temperature with tap water. Finally, 2200 μL acid butanol was added, mixed, and centrifuged at 1500 rpms in order to extract the product. The top butanol layer was then collected and assayed spectrophotometrically at 549 nm. SA standards (N-acetyl neuraminic *acid)* ranging from 0 to 50 ng/mL were prepared and similarly treated to a volume of 100 μL with 0.05 M H_2SO_4.

2.10. Determination of NOS activity

Erythrocytes NOS activity assay was performed by monitoring the rate of conversion of L-ARG into citrulline as described by Mckee et al. (1994). Erythrocytes hemolysate was incubated with 0.2 mM L-arginine, 10 mM HEPES, 0.425 mM ethylenediaminetetraacetic acid (EDTA), 0.45 mM $CaCl_2$, 80 units of calmodulin, 1 μM tetrahydrobiopterin, 4 μM flavin adenine dinucleotide (FAD), 4 μM flavin mononucleotide (FMN), 0.5 mM dithiothreitol (DTT), 0.16 M sucrose and 1 mM NADPH. One unit of NOS was defined as the amount of enzyme required to catalyze the conversion of 1 μM of L-ARG into citrulline/min/mg of protein under assay conditions used.

2.11. Determination of arginase activity

Erythrocytes arginase activity assessment based on the conversion of L-arginine into urea and ornithine (Kosenko et al., 2012). The level of urea was determined spectrophotometrically using (Randox Laboratories, Crumlin, UK). The assay was performed following the manufacturer's assay procedure. Erythrocyte's arginase was expressed as U/g Hb.

2.12. Determination of nitrite levels

Erythrocytes nitrite levels were determined by using Griess reagent kit (Cayman, Ann Arbor, USA). Firstly, nitrate was reduced to nitrite by nitrate reductase as described by Green et al. (1982). Erythrocytes were hemolyzed by addition of ice-cold distilled water, and hemoglobin was precipitated by addition of cold ethanol and chloroform. After vortex, the mixture was centrifuged at 10,000 rpm for 10 min. One hundred μL of lysate was deproteinized by adding 600 μL of 75 mM $ZnSO_4$ solution. The mixture was stirred, and centrifuged at 10,000 rpm for 5 min at ambient temperature. Then 100 μL of supernatants were added to 40 μL of nitrate reductase (20 mU), 50 μL of FAD (5 mM), 10 μL of NADPH (0.6 mM), and 250 μL of phosphate buffer (50 mM). The mixtures were incubated for 1 h at 37 °C, and then 150 μL of the mixture was added to 450 μL of Griess reagent and incubated in the dark place for 30 min at room temperature. Absorbance was measured at 545 nm; nitrite levels were calculated from sodium nitrite standard curve and expressed in μM.

2.13. Statistical analysis

The statistical differences between groups were analyzed by one way ANOVA followed by Tukey–Kramer multiple comparison test, using GraphPad Prism Software version 5.01. The values at $P \leqslant 0.05$ were chosen as statistically significant.

3. Results

In the present study, exposure of erythrocytes to HC plasma induced a significant increase of cholesterol inclusion into erythrocytes membrane compared to control ones ($P \leqslant 0.01$). On the other hand, treatment of HC group with CS or CS plus L-arginine significantly decreased cholesterol loading into cell membranes compared to untreated HC group. Fig. 1 displays these results. In respect of SOD, GPx, and CAT, these results indicated that, treatment of control erythrocytes with CS, L-NAME and L-arginine preserves activity of these enzymes and their ratios (SOD/CAT and SOD/GPx) at values near that of control one. However, a significant ($P \leqslant 0.05$) decrease in SOD, GPx, and CAT activities was observed in erythrocytes incubated with HC compared to control. Conversely, treatment of HC group with CS, or CS plus L-arginine preserves activity of measured enzymes and their ratios compared to HC group. Table 1 represents these results.

In the current work, HC exposure induced marked decrease of GSH/GSSG ratio; however, MDA and PCC were significantly increased compared to control erythrocytes ($P \leqslant 0.05$). On the other side, treatment of HC with CS or CS plus L-arginine keeps GSH/GSSG, MDA and PCC at values similar to control group, see Table 2. Erythrocytes SA contents were significantly decreased by incubation of cell to HC plasma. However, treatment L-Arginine, CS or CS plus L-arginine prevent HC induces SA loss. Fig. 2 represents these results.

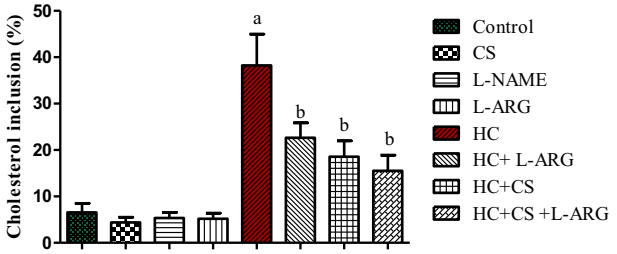

Figure 1 Effect of CS, L-NAME, L-ARG, HC and CS + L-ARG on percent of cholesterol inclusion into erythrocytes membrane compared to normal control. One-way analysis of variance used for data analysis; Tukey's posttest was used to determine the statistical differences between groups. Results were represented as M ± SD ($N = 6$). Superscript letters indicated when significant differences observed from either control group or HC group. [a] Significantly increase from control. [b] Significantly decrease from HC. $P \leqslant 0.05$.

Table 1 Effect of HC with or without different treatment on erythrocyte SOD, GPx and CAT and their ratios after compared to control erythrocytes.

Groups	SOD	GPx	CAT	SOD/GPx	SOD/CAT
NC	1270 ± 94.6	43.17 ± 4.21	74.80 ± 7.28	29.42 ± 3.83	16.97 ± 1.88
NC + CS	1374 ± 107	51.98 ± 7.50	79.9 ± 7.30	26.43 ± 4.51	17.20 ± 1.33
NC + L-NAME	1368 ± 124	50.87 ± 5.59	80.50 ± 6.01	26.89 ± 2.72	16.99 ± 1.84[a]
NC + L-ARG	1388 ± 154	55.77 ± 6.39	82.50 ± 7.22	24.89 ± 2.35	16.82 ± 1.72
HC	938.5 ± 81.8[a]	19.69 ± 3.20[a]	28..58 ± 3.25[a]	47.66 ± 5.46[b]	32.38 ± 3.24[b]
HC + L-ARG	1171 ± 125[c]	37.15 ± 3.79[c]	64.33 ± 6.28[c]	31.52 ± 3.34[d]	18.20 ± 1.55[d]
HC + CS	1143 ± 145 [c]	34.10 ± 5.93[c]	62.10 ± 5.22[c]	33.52 ± 2.93[d]	18.41 ± 1.92[d]
HC + L-ARG + CS	1207 ± 98.6[c]	41.10 ± 4.91[c]	68.10 ± 5.22[c]	29.36 ± 3.10[d]	17.72 ± 1.67[d]

One-way analysis of variance used for data analysis; Tukey's posttest was used to determine the statistical differences between groups. Results were represented as M ± SD ($N = 6$). Superscript letters indicated when significant differences observed from either control group or HC group.
$P \leqslant 0.05$. Units; SOD, GPx and CAT were expressed as U/g Hb.
[a] Significantly decrease from control.
[b] Significantly increase from control.
[c] Significantly higher than HC.
[d] Significantly lower than HC.

Table 2 Effect of HC with or without different treatment on erythrocyte GSH/GSSG, MDA and PCO content compared to control erythrocytes.

Groups	GSH/GSSG	MDA	PCO
NC	44.65 ± 4.41	11.24 ± 1.20	1.79 ± 0. 41
NC + CS	49.51 ± 0.01	10.91 ± 1.27	1.45 ± 0.23
NC + L-NAME	50.56 ± 5.33	11.23 ± 0.98	1.52 ± 0.26
NC + L-ARG	55.39 ± 8.01	10.45 ± 1.10	1.23 ± 0.21
HC	12.17 ± 1.12[a]	29.20 ± 1.89[b]	5.24 ± 0.84[b]
HC + L-ARG	27.97 ± 3.02[c]	17.54 ± 1.44[d]	3.92 ± 0.52[d]
HC + CS	29.82 ± 2.54[c]	15.98 ± 1.15[d]	2.81 ± 0.45[d]
HC + L-ARG + CS	35.92 ± 3.41[c]	12.71 ± 1.73[d]	2.14 ± .0.55[d]

One-way analysis of variance used for data analysis; Tukey's posttest was used to determine the statistical differences between groups. Results were represented as M ± SD ($N = 6$). Superscript letters indicated when significant differences observed from either control group or HC group.
$P \leqslant 0.05$. Units; GSH and GSSG expressed as μM/g Hb MDA, μM; PCO, nM mg/protein and nitrite, μM.
[a] Significantly decrease from control.
[b] Significantly increase from control.
[c] Significantly increase from HC group.
[d] Significantly decreased from HC group.

Plasma TAC significantly decreased in HC group compared to control. However, no significant difference was noted between control and control treated with CS or CS + L-arginine. Treatment of HC group with CS or Cs + L-arginine prevents HC-induced TAC depletion. Fig. 3 annotates these results.

In respect of NO variables, the exposure of erythrocytes to L-NAME caused a significant decrease in NOS activity and NO level compared to control group. A similar result was observed in HC treated group. On the other side, L-arginine treatment induced a significant increase in NOS activity compared to control; however, CS induces a marked but non-

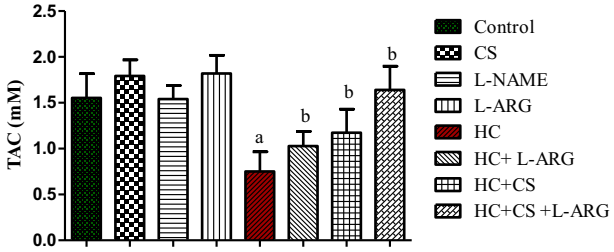

Figure 2 Effect of CS, L-NAME, L-ARG, HC and CS + L-ARG on sialic acid content of erythrocyte membrane compared to control erythrocytes. One-way analysis of variance used for data analysis; Tukey's posttest was used to determine the statistical differences between groups. Results were represented as M ± SD ($N = 6$). Superscript letters indicated when significant differences observed from either control group or HC group. [a] Significantly decrease from control. [b] Significantly increase from HC. $P \leqslant 0.05$.

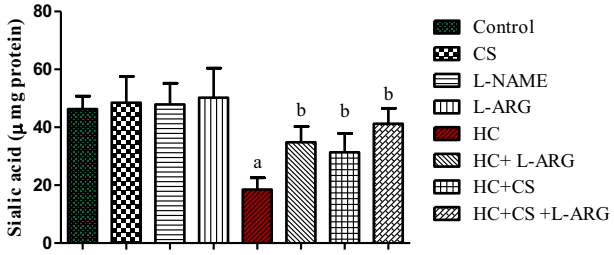

Figure 3 Effect of CS, L-NAME, L-ARG, HC and CS + L-ARG on plasma total antioxidant status compared to control plasma. One-way analysis of variance used for data analysis; Tukey's posttest was used to determine the statistical differences between groups. Results were represented as M ± SD ($N = 6$). Superscript letters indicated when significant differences observed from either control group or HC group. [a] significantly decrease from control. [b] significantly increase from HC. $P \leqslant 0.05$.

significant increase of NOS compared to control group. L-arginine treatment alone fails to prevent HC induced NOS inactivation. However, treatment of HC with L-arginine + CS prevented HC-induced decrease in NOS activity. By contrast, arginase activity was increased by HC exposure; however, CS or CS plus L-arginine treatment prevents HC induced arginase activation. Similar results were observed for arginase/NOS ratio. Table 3 explains these data.

4. Discussion

Previous studies reported that an increase in arginase activity plays a crucial role in the cardiovascular pathobiology, so that the inhibition of arginase may protect against vascular diseases (Rabelo et al., 2015). Therefore, this study was conducted to explore the protective role of CS against HC induced erythrocyte's arginase activation. HC disrupts erythrocytes cholesterol homeostasis, triggers ROS production and depletes antioxidant capability of cells (Devrim et al., 2008). However, cholesterol-lowering therapy prevents HC induced erythrocytes dysfunction (Franiak-Pietryga et al., 2009; Uydu et al., 2012). Herein, incubation of erythrocytes with cholesterol enriches plasma increased cholesterol inclusion into erythrocytes. Similar results were documented in several previous studies (Uydu et al., 2012; Harisa and Badran, 2015). In contrary, CS prevents cholesterol deposition into erythrocyte membranes; similarly, it has been reported that cholesterol lowering therapy decreases erythrocytes membrane cholesterol (Uydu et al., 2012). The attraction between CS and cholesterol decreases cholesterol inclusion into erythrocytes membrane.

In the current study, SA was assessed to confirm the safety of CS addition to erythrocytes SA as well as SA elicits antioxidant action. It has been reported that, ROS induced desialylation by depletion of SA content from cell surfaces (Pawluczyk et al., 2014; Harisa, 2015). Therefore, significant decrease in SA content was noticed in cells incubated with high cholesterol. The decreases of erythrocytes SA content increase their friction among themselves and endothelial cells; this may trigger vascular dysfunction (Fan et al., 2012). By contrast, treatment with CS keeps SA levels at values near that of control. This is in agreement with many studies which demonstrated that CS preserves antioxidants capacity (Anandan et al., 2013). In the present study, plasma TAC levels were significantly decreased by HC incubation. Likewise, several studies reported that oxidative stress exposure induced decline of TAC levels. The decrease in plasma TAC was demonstrated in HC induced oxidative stress (Devrim et al., 2008). Oxidative stress environment causes a plethora of changes in erythrocytes including loss of antioxidant power and enhances proteins and lipids oxidation.

Ramírez-Zamora et al. (2013), reported that erythrocyte membranes are the most targets for deleterious actions of ROS. The equilibrium between SOD as hydrogen peroxide producing agent as well as CAT, and GPx as hydrogen peroxide degrading agents is essential for the removal of ROS. The activity ratios were calculated to better assess antioxidant defenses. Herein, oxidative stress in erythrocytes was increased as confirmed by increase of SOD/CAT and SOD/GPx ratios, GSSG, PCC and MDA in erythrocytes exposed to HC. Similarly, several studies demonstrated that exposure of erythrocytes to HC induced a decrease of antioxidant power; however, biomolecular damage was increased (Devrim et al., 2008; Franiak-Pietryga et al., 2009; Uydu et al., 2012). This attributed to elevation of ROS that consumes antioxidant machinery. Consequently, oxidized GSH, MDA and PCC levels were increased. CS treatment ameliorates these oxidative alterations in erythrocytes by its antioxidant activity. Antioxidant properties of CS are not only confirmed by in vitro studies, but also demonstrated in many in vivo studies using animal models as well as clinical trials. Treatment with CS preserves antioxidant enzymes, particularly glutathione-dependent system, increases in antioxidant potential, and restores redox balance. Therefore, CS decreases lipids and proteins oxidation and attenuates cellular damage (Luo and Wang, 2013).

HC disrupts NO homeostasis through increase of ROS formation, depletion of NOS substrate, and oxidation of cofac-

Table 3 Effect of HC with or without different treatment on erythrocyte NOS, arginase activity, arginase/NOS ratio and nitrite content compared to control erythrocytes.

Groups	NOS	Arginase	Arginase/NOS	Nitrite
NC	1.83 ± 0.24	20.82 ± 1.52	12.47 ± 1.11	586.8 ± 94.14
NC + CS	1.94 ± 0.54	18.24 ± 1.21	9.40 ± 0.94	604.6 ± 89.47
NC + L-NAME	0.71 ± 0.22[a]	21.27 ± 2.11	29.96 ± 1.21	407.0 ± 76.89[a]
NC + L-ARG	2.20 ± 0.41	27.92 ± 1.71	12.96 ± 1.87	720.1 ± 98.47[c]
HC	0.87 ± 0.11[a]	39.78 ± 3.14[c]	45.72 ± 4.52[c]	278.3 ± 44.56[a]
HC + L-ARG	1.04 ± 0.23[b]	27.92 ± 2.43[d]	26.84 ± 1.75[d]	322.4 ± 66.85
HC + CS	1.43 ± 0.26[b]	24.87 ± 2.84[d]	17.27 ± 1.81[d]	422.7 ± 74.58[b]
HC + L-ARG + CS	1.74 ± 0.44[b]	21.92 ± 1.97[d]	12.60 ± 1.43[d]	543.6 ± 91.77[b]

One-way analysis of variance used for data analysis; Tukey's posttest was used to determine the statistical differences between groups. Results were represented as M ± SD ($N = 6$). Superscript letters indicated when significant differences observed from either control group or HC group.

$P \leqslant 0.05$. Units; NOS expressed as U/mg protein, arginase U/g Hb and nitrite μM.

[a] Significantly decrease from control.

[b] Significantly increase from HC.

[c] Significantly increase from NC group.

[d] Significantly decreased from HC group.

tors (Li and Förstermann, 2013). Furthermore, several previous studies have demonstrated that inhibition of arginase increases NO level, and decreases risk of ischemic heart diseases (Pernow and Jung, 2013). The finding of the present study confirmed that HC induced NOS inactivation compared to control cells. These results are in agreement with the finding of Devrim et al. (2008) who reported that HC decreases NOS activity and NO production. Likewise, Ramírez-Zamora et al. (2013) reported ROS inactivates NOS. The increase of cholesterol inclusion into cell membrane impairs entry of L-Arginine as NOS substrate into the cells (Eligini et al., 2013). Conversely, CS restores NOS activity and nitrite level compared to HC treated cells. These results were concurred with several studies which demonstrated that CS improves NO level (Ozcelik et al., 2014; Zhang et al., 2014). Moreover, numerous studies reported that cholesterol lowering therapy improves NO bioavailability (Harisa et al., 2012; Li and Förstermann, 2013).

The current investigations revealed that arginase activity and arginase/NOS ratio were increased by HC exposure. Similarly, Ramírez-Zamora et al. (2013) reported that oxidative stress induced arginase activation. Furthermore, Yang et al. (2013) indicated that ROS augments arginase activity. In such cases, L-arginine availability was limited; therefore, NO production decreases. On the contrary, treatment of HC with CS or CS plus L-arginine prevents HC-induced arginase activation. Similar observation was indicated by Schnorr et al. (2008), who suggested antioxidants treatment diminishes arginase activity in erythrocytes with an increase of NO bioavailability.

5. Conclusion

In conclusion, this study confirmed HC increased cholesterol inclusion into erythrocytes membrane, and this accelerates oxidative stress in erythrocytes. CS could be effective therapeutic agent against HC induced erythrocytes dysfunction by its hypocholesterolemic effect, antioxidant activities as well as normalization of arginase activity and restoration of NO homeostasis. Further in vivo studies are required to confirm this matter.

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