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Original Article

Role of *Hridayarnava Rasa* on Erythrocyte Membrane Stabilization via Na+/K+ ATPase Activity in Atherosclerosis-induced Rabbits

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Abstract

Background and objectives: *Hridayarnava Rasa* is traditionally used cardio tonic in Ayurveda. This drug was selected for the evaluation of stabilization of erythrocyte membrane (EM) in high-fat diet induced atherosclerosis via rabbit model.

Methods: A total of 24 male white New Zealand rabbits were randomly divided into 6 groups (n = 4 each). Rabbits in group I were fed a standard pellet diet, those in group II rabbits a high-fat diet (HFD), those in groups III, IV and V increasing doses of *H. Rasa* and an HFD, and those in group VI an HFD diet plus Atorvastatin.

Results: There was a significant reduction in rabbit sodium/potassium adenosine triphosphatase (Na^+/K^+ ATPase) at 30 (58.51%), 60 (61.40%), and 90 (64.92%) days of an HFD diet compared to the control group. Upon treatment with *H. Rasa,* the activity of Na^+/K^+ ATPase in groups III, IV, and V increased at 30, 60 and 90 days, respectively, compared to HFD induced rabbits. The Na^+ concentration also increased significantly in HFD-administered rabbits at 30, 60 and 90 days as compared to controls. Serum K^+ concentration was reduced at days 30, 60 and 90 in the HFD group and was increased in group V as compared to the control group. These levels improved with *H. Rasa* treatment whereas the atorvastatin-treated group exhibited an improvement only between dose levels 2 and 3.

Conclusions: These results suggest that HFD diminishes EM stabilization in atherosclerosis whereas *H. Rasa* protects EM by maintaining the Na^+/K^+ ATPase activity through a Na^+/K^+ pump. In atherosclerosis, an HFD reduces EM stabilization after administration of *H. Rasa*, which maintains Na^+/K^+ ATPase activity through a Na^+/K^+ pump.

Keywords: Na^+/K^+ ATPase; Atherosclerosis; *Hridayarnava Rasa*; Ayurveda; Na^+/K^+ pump.

Abbreviations: ATP, adenosine triphosphate; EDTA, ethylene diamine tetra acetic acid; EM, erythrocyte membrane; HFD, high-fat diet; *H. Rasa*, *Hridayarnava Rasa*; KCl, potassium chloride; $MgSO_4$, magnesium sulphate; mM, milli molar; NaCl, sodium chloride; Na+/K+ ATPase, sodium/potassium adenosine triphosphatase; OECD, Organisation for Economic Co-operation and Development; RH, relative humidity; SD, standard deviation; TCA, trichloro acetic acid.

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Introduction

Sodium/potassium adenosine triphosphatase (Na^+/K^+ ATPase) is expressed in almost all cells of higher organisms. This protein is heterodimeric and trans-membranal, and regulates ion homeostasis, substrate transport, neuronal signaling and muscle contraction.[1] In addition to its inotropic effects, it acts as a signal transducer, which controls many cellular events.[2] The P-type Na^+/K^+ ATPase is composed of an active α-unit containing 10 trans-membrane segments (*i.e.* αM1-αM10), a sugar-rich auxiliary β-unit and a hydrophobic single membrane crossing protein γ-unit that regulates the entire

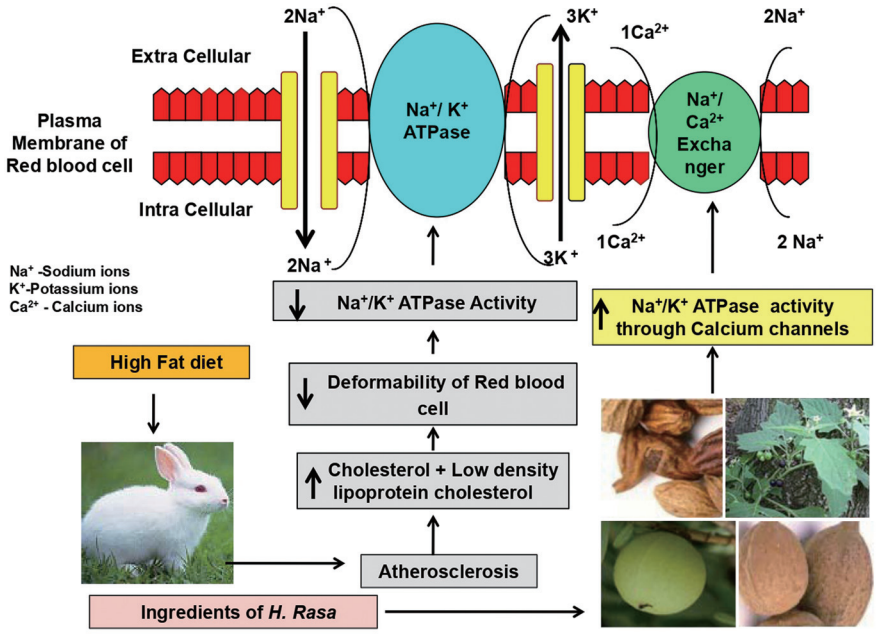

Fig. 1. Possible mechanism of action of *Hridayarnava Rasa* on changes of erythrocyte membrane in experimental rabbits. Na^+/K^+ ATPase, sodium/potassium adenosine triphosphatase.

ionic gradient cell membrane.[3]

The primary catalytic unit present in various tissues has several isoforms of binding units: α1 is present in nerves kidney and lung, α2 in heart and skeletal muscle, α3 in brain and α4 in the testis (especially in spermatozoa).[4] The Na^+/K^+ ATPase in human erythrocytes is composed of the α1, α3, β1 and β3 isoforms,[5] and regulates numerous erythrocyte functions. The physical and biochemical properties of membranes are strongly controlled by the lipid composition and redox status of the environment. Changes in membrane fluidity have been shown to modify the activity of membrane-bound receptors, enzymes and ion-exchangers.[6,7] Na^+/K^+ ATPase activity is controlled by the microenvironment surrounding the lipid, and therefore, modifications in membrane fluidity have an effect on the activity of this enzyme. Membrane fluidity and permeability affect ion transport due to changes in cholesterol and lipid fractions, thereby reducing the functional efficiency of the erythrocyte. These effects cause changes in membrane elasticity and thus hinder the passage of erythrocytes through narrow capillaries. The lateral mobility of Na^+/K^+ ATPase can also be affected, which is important for cell function.[8–11] Cholesterol molecules were recently shown to specifically bind to three different sites in the enzyme, as studied by X-ray crystallography on Na^+/K^+ ATPase.[12,13] The activity of Na^+/K^+ ATPase is controlled by intracellular and extracellular ATP and Na^+ concentrations. The affinity of Na^+/K^+ ATPase for Na^+ and K^+ appears to be modulated by tissue-specific factors, such as the lipid composition of the membrane.[4,14,15] It is estimated that roughly 25% of all cytoplasmic ATP is hydrolyzed by Na^+ pumps in resting humans. In nerve cells, about 70% of ATP is consumed to fuel Na^+ pumps. In erythrocytes, intrinsic K^+ has been demonstrated to behave as a competitive inhibitor of intrinsic Na^+ binding and an activator of maximal pump flux. Importantly, cholesterol deficiency amplifies each of these K^+ effects. In the absence of internal K^+, the reduction of cholesterol no longer has any effect on the enzyme.[16] It has been suggested that biochemical and biophysical abnormalities of cell membranes[17] may actively participate in the pathogenesis of hypertension.[18] Furthermore, such abnormalities may be involved not only in vascular smooth muscle cells, but also in circulating blood cells.[19] Reduced activity of Na^+/K^+ ATPase in erythrocyte membranes (EMs) and its inverse relationship with the lipid peroxidation product also occur in cardiac and vascular smooth muscle cells taken from patients with prehypertension. Increased lipoperoxidation has been proposed as a cause of Na^+/K^+ ATPase reduction in EM.[18] Lipid peroxidation directly alters membrane fluidity, an important feature for maintaining the optimal functioning of erythrocytes. Membrane fluidity affects the homeostatic control of erythrocytes, which in turn affects the passage of oxygen, water and ions such as Na^+, K^+ and Ca^{2+} through the membrane. This in turn facilitates a balance between the intracellular and extracellular media. These changes affect the kinetic parameters of the Na^+/K^+ ATPase and modify the enzyme-substrate affinity.[20] Increased lysosomal fragility can lead to the release of proteolytic enzymes that have been seen in other cells.

Hridayarnava Rasa, an Ayurvedic formulation composed of six constituents, including *Terminalia chebula* Retz., *Terminalia bellerica* (Gaertn) Roxb, *Embelica officinallis* Gaertn (Kasayam Vara), Copper (*Tamra)*, Mercury (*Suta*) and Sulphur (*Gandhaka*). These constituents are processed in a *Solanum nigrum* Linn *(Svarasam Kakamachi Rasa*) decoction. As per Ayurveda, this medicine is used in the treatment of cardiac disorders. *H. Rasa* can also be used to treat several diseases associated with Angina Pectoris (*Hridshoola*).[21] *Tamra bhasma* is an important component of *H. Rasa* that is used in the treatment of various ailments.[22,23] Atherosclerosis and hypertension are directly related to the reduced status of Na+/K+ ATPase activity.[24] The antihyperlipidemic and antioxidant with antiobesity activity of *T. bhasma* has also been reported.[25,26] Data on the role of *H. Rasa*, Na+/K+ ATPase and ion transport in EM have yet to be studied. Therefore, the aim of the present study is to evaluate the correlation between the cholesterol-lowering agent *H. Rasa* on EM Na^+/K^+ ATPase activity in rabbits with high-fat diet (HFD)-induced atherosclerosis[23] (Fig. 1). This study also investigated the dose-and time-dependent activity of *H. Rasa*.

Methods

Male New Zealand white rabbits were purchased from Biogen Laboratory Animal Facility, Bangalore, Karnataka and adapted to laboratory conditions for 7 days before use. The average body weight of rabbits ranged from 1.9 to 2.2 kg, which were fed rabbit pellet feed and reverse osmosis water *ad libitum.* The variation in body weight of animals upon randomization did not exceed ±20% of the mean body weight. Temperature and relative humidity (RH) were maintained at 22±2°C and 40 to 60% RH respectively. Illumination was controlled by a light/dark cycle of approximately 12/12 h. Each rabbit was individually housed in its own rabbit cage. This study was approved by Institutional Animal Ethics Committee (IAEC/CSMRADDI/17/2017). An atherogenic diet[27] consisted of 1% cholesterol, 5% egg yolk, 5% lard and 89% normal diet. *H. Rasa,* an Ayurvedic drug (Batch No. 191248; (MFG. LIC. Number: 1/25D/76; Date of Manufacture: 03/2018 and Date of Expiry: 02/2023) was procured from Arya vaidya sala, Kottakkal, Kerala, India and was kept under a temperature of 25 ± 3°C and humidity of 52 ± 10% RH until the experiments were completed. An acute oral toxicity study on *H. Rasa* was performed as per the OECD 423 guideline. No remarkable toxicity was found.

Experimental design

A total of 24 rabbits were randomly divided into 6 groups of 4 rabbits. Briefly, Group I rabbits were fed with standard pellet diet, Group II rabbits with HFD, Group II rabbits with HFD + *H. Rasa* (10.27 mg/kg.b.wt/p.o.) Group IV rabbits with HFD + *H. Rasa* (20.53 mg/kg.b.wt./p.o), Group V rabbits with HFD + *H. Rasa* (41.07 mg/kg.b.wt/p.o), and Group VI-rabbits with HFD + atorvastatin (0.513 mg/kg.b.wt/p.o). The drug and vehicle were administered daily by oral (gavage) for up to 90 days.

Isolation of erythrocyte membrane and estimation of Na^+/K^+ ATPase

At the end of 30, 60 and 90 days of the diet, blood was collected from the saphenous vein of rabbit under thiopental sodium anesthesia. Blood was collected in heparin tubes, plasma was separated, and red blood cell pellet was subjected to erythrocytes membrane isolation using a standard procedure.[28] Na^+/K^+ ATPase was also estimated using a standard procedure.[29,30] Briefly, two sets of test tubes were marked as test and the other as control and were filled with membrane samples. A total of 1.0 mL of Tris-HCl buffer (90 mM, pH 7.5), 0.2 mL of $MgSO_4$ (500 mM), NaCl (600 mM), KCl (50 mM), EDTA (1 mM), ATP (40 mM) were added to each tube. The tubes were incubated at 37°C for 15 min and the reaction was arrested by adding 1.0 mL of TCA (10%). A total of 0.2 mL of the membrane preparation was added to the control tubes. The phosphorus content in the supernatant was estimated by the method of Fiske and Subbarrow.[31] Membrane proteins were then estimated,[32] with enzyme activity in the erythrocyte membrane expressed as µmoles of Pi liberated/hr/mg protein. Serum was used for the estimation of Na^+ and K^+ using a semi-automated analyzer.

Histopathology

The liver, heart, aorta, kidney and spleen were harvested on the 91st day of diet and were subjected to histopathological evaluation using hematoxylin and eosin staining.

Statistical analysis

Statistical analysis was performed using the Graph Pad Prism software, version 8.4. All values are expressed as the mean ± SD (n = 4). A one-way analysis of variance was used to compare group means with Turkey's test to correct for multiple comparisons. A p-value<0.05 was considered statistically significant.

Results

ATPases are membrane-bound enzymatic proteins that are sensitive to changes in membrane lipid composition. An increase in the amount of cholesterol in plasma membranes leads to a decrease in the activity of ATPases. Erythrocytes are unique among mammalian cells and the red cell membrane has been provided with several receptor activities. The Na^+/K^+ ATPase activity at different time intervals of treatment in the EM of the control and drug treatment groups being fed with different doses of *H. Rasa* is depicted in Figure 2. The activity of Na^+/K^+ ATPase was significantly reduced in group II-IV ($p<0.0001$) at 30, 60 and 90 days and group VI ($p<0.05$) at 90 days compared to group I. The activity of group V was significantly increased at 30 ($p<0.001$), 60 ($p<0.0001$) and 90 ($p<0.0001$) days, as was that of group VI at 30, 60 and 90 ($p<0.001$) days of treatment compared to group II. The level of Na^+/K^+ ATPase was also significantly increased in group V at 30 ($p<0.05$), 60 ($p<0.05$) and 90 ($p<0.01$) days of treatment compared to group III.

The level of Na^+ in the serum of the control and drug-treated groups being treated with different doses of *H. Rasa* and at different time intervals of treatment is shown in Figure 3. The Na^+ level was significantly increased in group II at 30 ($p<0.05$), 60 ($p<0.001$) and 90 ($p<0.0001$) days, and in group III ($p<0.01$) and VI ($p<0.001$) at 90 days compared with group I. The level of Na^+ was also significantly reduced in group III at 90 days ($p<0.01$), group IV at 60 ($p<0.01$) and 90 ($p<0.0001$) days, group V at 30 ($p<0.05$), 60 ($p<0.001$), and 90 days ($p<0.0001$), and group VI ($p<0.01$) compared to group II at 90 days. There was also a significant reduction in group V ($p<0.05$) at 60 of 90 days compared to group III.

The serum potassium level of the control and drug-treated groups at different doses of *H. Rasa* and at different time intervals of treatment is shown in Figure 4. The K^+ level was significantly reduced in group II ($p<0.001$) at 30, 60 and 90 days, in group III and IV at 60 ($p<0.01$) and 90 ($p<0.001$) days, in group IV ($p<0.05$) at 30 and 60 days and in group VI ($p<0.01$) at 30, 60 and 90 days compared to group I. The K^+ level was significantly increased in group IV at days 60 and 90 ($p<0.01$), in group V at 30 ($p<0.01$), 60 and 90 ($p<0.001$) days and in group VI at 90 ($p<0.05$) days compared with group II. There was also a significant increase in group V ($p<0.05$) at 60 of 90 days compared to group III.

Discussion

The EM consists of two domains, a lipid bilayer and a cytoskeleton. The lipid domain exhibits structural similarity in almost all mammalian cells. The erythrocyte carries oxygen and is exposed to a wide range of substances dissolved in blood plasma, and is particularly vulnerable to oxidative damage. The effect of those oxidative stresses depends on the compounds involved, their concentration, and the metabolic capabilities of the erythrocyte.[33] In the present study, Na^+/K^+ ATPase was significantly reduced at 30,

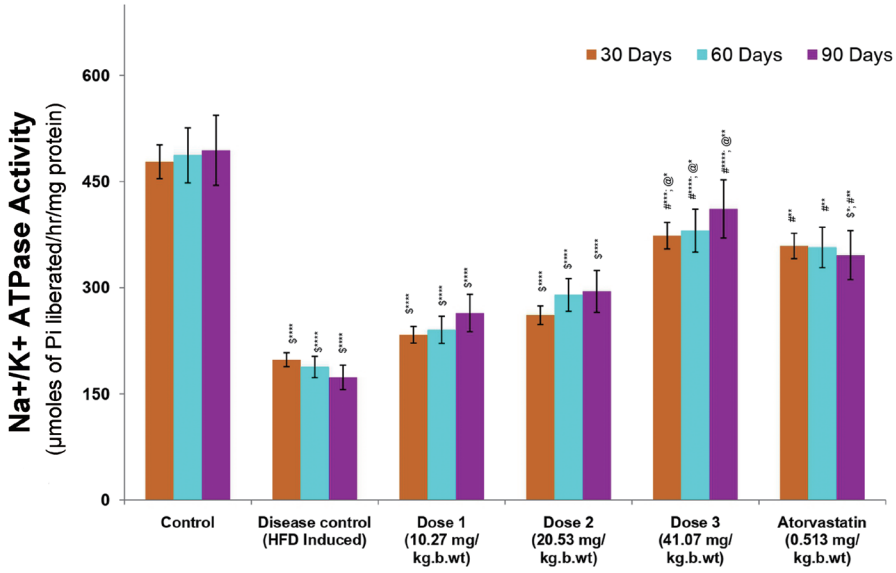

Fig. 2. Status of Na^+/K^+ATPases in erythrocyte membrane of experimental groups. (Gp I-Control; Gp II-Disease Control; Gp III-Dose 1; Gp IV-Dose 2; Gp V-Dose 3; Gp VI-Standard drug). Values are expressed as mean ± SD of 4 rabbits; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$;^{NS}-Non significant. $^{\$}$Statistical analysis of one way ANOVA was used to compare results with group I. $^{\#}$Statistical analysis of one way ANOVA was used to compare results with group II. $^{@}$Statistical analysis of one way ANOVA was used to compare results of group IV and V with group III HFD, high-fat diet; Na^+/K^+ ATPase, sodium/potassium adenosine triphosphatase.

60 and 90 days of HFD-induced rabbits. Hypercholesterolemia can lead to reduced denaturation of red blood cells, which impairs their hemorrhagic behavior and promotes atherosclerosis.[34] The transport of cations and anions through the membrane is regulated by a number of enzymes, including Na^+/K^+ ATPase, Ca^{2+} ATPase, Na^+/Ca^{2+} exchanger, $Na^+/K^+/Cl^-$ co-transporter and H^+ ATPase.[35–37] Na^+/K^+ ATPase is an important protein that regulates the cellular volume of erythrocytes, which in turn protects hemolysis and has a major effect on the deformability of erythrocytes. These tolerate blood pressure and allow passage through narrow vessels and are thus important factors for erythrocyte viability.[38]

The viscosity and stiffness of EMs are elevated in hypertensive rats[18] and in patients with essential hypertension.[39] The EM fluidity depends on Na^+/K^+ ATPase activity[18] and may suggest that early damage in cell membranes leads to further complications, such as decreased erythrocyte Na^+/K^+ ATPase activity and the development of hypertension. In addition, changes in antioxidant status and increased lipoperoxidation have also been proposed to

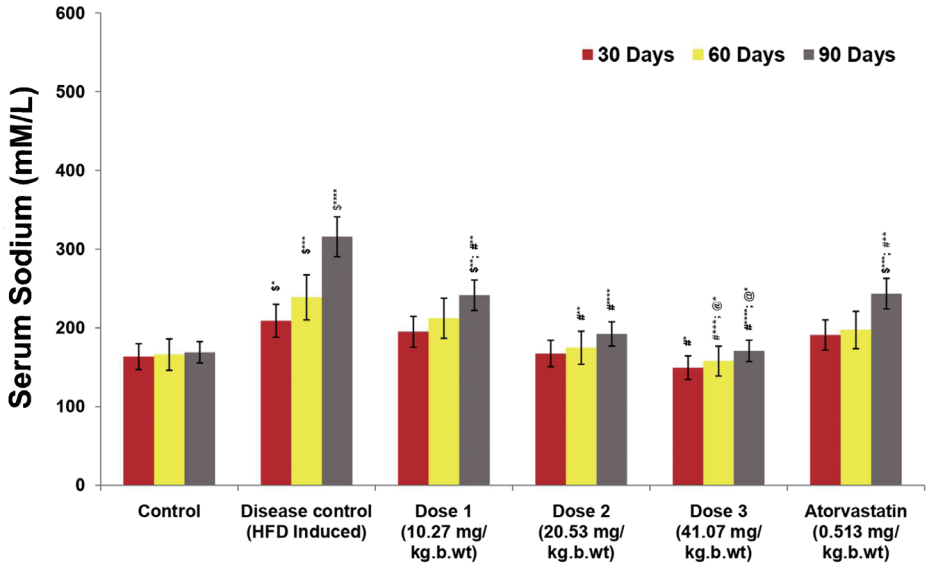

Fig. 3. Status of Sodium in serum of experimental groups. Gp I-Control; Gp II-Disease Control; Gp III-Dose 1; Gp IV-Dose 2; Gp V-Dose 3; Gp VI-Standard drug. Values are expressed as mean ± SD of 4 rabbits; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ^{NS}-Non significant. $^{\$}$Statistical analysis of one way ANOVA was used to compare results with group I. $^{\#}$Statistical analysis of one way ANOVA was used to compare results with group II. $^{@}$Statistical analysis of one way ANOVA was used to compare results of group IV and V with group III.

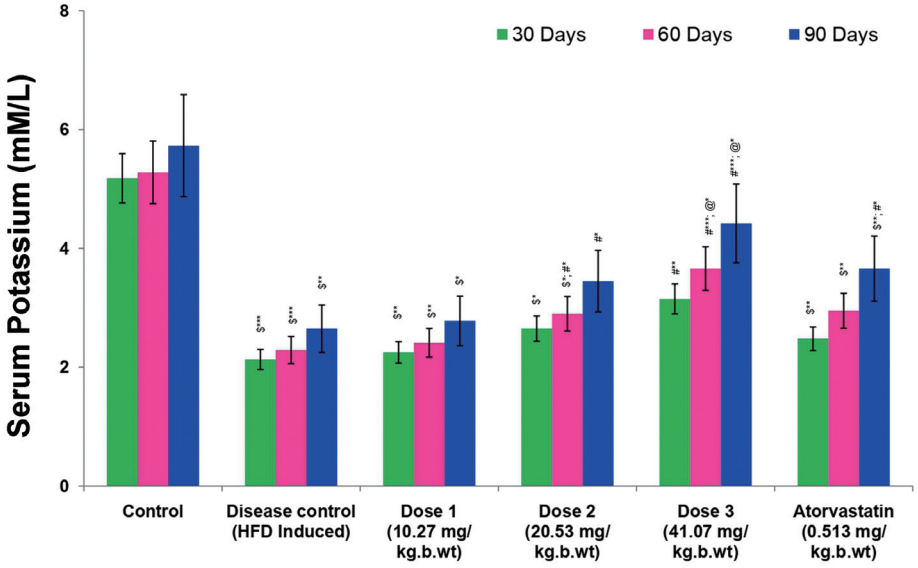

Fig. 4. Status of Potassium in serum of experimental groups. Gp I-Control; Gp II-Disease Control; Gp III-Dose 1; Gp IV-Dose 2; Gp V-Dose 3; Gp VI-Standard drug. Values are expressed as mean ± SD of 4 rabbits; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ^{NS}-Non significant. $^{\$}$Statistical analysis of one way ANOVA was used to compare results with group I. $^{\#}$Statistical analysis of one way ANOVA was used to compare results with group II. $^{@}$Statistical analysis of one way ANOVA was used to compare results of group IV and V with group III

be a reason for the reduction in Na^+/K^+ ATPase activity in EM.[39] The current work suggests that the drastic reduction in Na^+/K^+ ATPase activity at 30 (2.41 fold), 60 (2.59 fold), and 90 (2.85 fold) days in HFD-induced rabbits may be one of the pathophysiological aspects associated with atherosclerotic status. Changes in intracellular Na^+ and K^+ levels were also related to the reduced activity of the erythrocyte Na^+/K^+ ATPase.[40]

There was a significant reduction in Na^+/K^+ ATPase at days 30 (58.51%), 60 (61.40 %), and 90 (64.92 %) in HFD-induced rabbits compared to the control group. Since Na^+/K^+ ATPase is essential for maintaining various cellular functions, its inhibition can result in a variety of pathological conditions. The association between cardiovascular risk factors and Na^+/K^+ ATPase activity in diabetes patients leads to cardiovascular complications. While studies have shown that the concentration of total Na^+/K^+ ATPase is 40% lower in heart failure patients,[41] our present result showed a reduction of 64.92 % at 90 days after atherosclerosis induction. Decreased Na^+/K^+ ATPase activity is strongly associated with a reduction in lecithin cholesterol acetyl transferase.[42] The ATPase of Na^+/K^+ in the EM has been shown to be inhibited by cholesterol *in vitro*,[43] a concept that was related to our previous study.[44] An inverse correlation between EM Na^+/K^+ ATPase activity and polyunsaturated fatty acid levels has also been reported.[45] Na^+/K^+ ATPase is an important scaffolding protein that can interact with signaling proteins such as protein kinase C and phosphoinositide-3-kinase.[46]

The Na^+ concentration was increased by 21.77%; 30.47% and 46.48% in HFD-induced rabbits at 30, 60 and 90 days of the diet, respectively, and may be due to the presence of Na^+ in the serum and extracellular fluids. The concentration of Na^+ is maintained within a narrow range by osmoregulation, and notably, serum Na^+ is positively associated with the risk of coronary heart disease.[47] Increased extracellular Na^+, even within physiological limits, is accompanied by vascular changes that facilitate the development of atherosclerosis.

Serum K^+ levels were reduced in the HFD-induced group at 30 (58.88%), 60 (56.82%) and 90 (53.75%) days compared to the control group, which can be attributed to the Na^+/K^+ pump maintaining intracellular K^+ within the cell. The concentration gradient of Na^+ and K^+ ions mainly depends on the action of membrane-bound enzymes of the cell. Due to peroxidation of membrane lipids, the osmatic stability of electrolytes in the divalent metal Ca^{2+} changes. The risk factors for the shortened existence of electrolytes and the reduced denaturation may be closely related to the inhibition of membrane-bound ATPase. Aging has been shown to cause oxidative damage, balance the antioxidant system and stimulate metabolism of oxidative products. Therefore, *T. chebula* may act as a potent drug to prevent age-related degenerative diseases and improve general health. Atherosclerosis is an age-related disorder and is associated with many oxidative stress factors that are directly linked to the reduced ATPase activity and K^+ transport that can cause membrane changes in red blood cells. These changes can be more damaging to the cell and are more attributable to hemolysis than hemoglobin denaturation. Upon treatment with *H. Rasa,* Na^+/K^+ ATPase activity improved in group III (15.06%; 21.80%; 34.41%), IV (24.08%; 35.13%; 48.23%) and V (46.91%; 52.24%; 61.60%) at 30, 60 and 90 days, respectively, when compared to HFD-induced rabbits. Na^+ concentration was reduced at 30, 60 and 90 days of diet in group III (6.70%; 11.10%; 23.52%), IV (19.86%; 26.81%; 39.11%) and V (28.47%; 33.93%; 45.92%) when compared to the HFD-administered group. The K^+ concentration gradually increased in group III (5.63%; 5.24%; 4.91%), IV (24.41%; 26.64%; 30.19%) and V (47.89%; 59.83%; 66.79%) at 30, 60 and 90 days of diet, respectively. The administration of *H. Rasa* significantly increased the activity of this enzyme, and may be due to the properties of *T. chebula* (a component of *H. Rasa*). *T. chebula* acts as a reducing agent, and in turn helps to maintain the membrane thiol which is essential for the activity of Na^+/K^+ ATPase in the reduced state. These results suggest that *T. chebula* is highly protective against disease. The other ingredient present in *H. Rasa* is *T. bellerica* Roxb, which has been shown in several studies to have anti-hypercholesterolemia activities.[48] The other vital constituent of *H. Rasa* is *E. officinalis*, which is a potent anti-oxidant and also prevents lipoperoxidation.[48] An increased lipoperoxidation and poor antioxidant status are major factors for decreasing

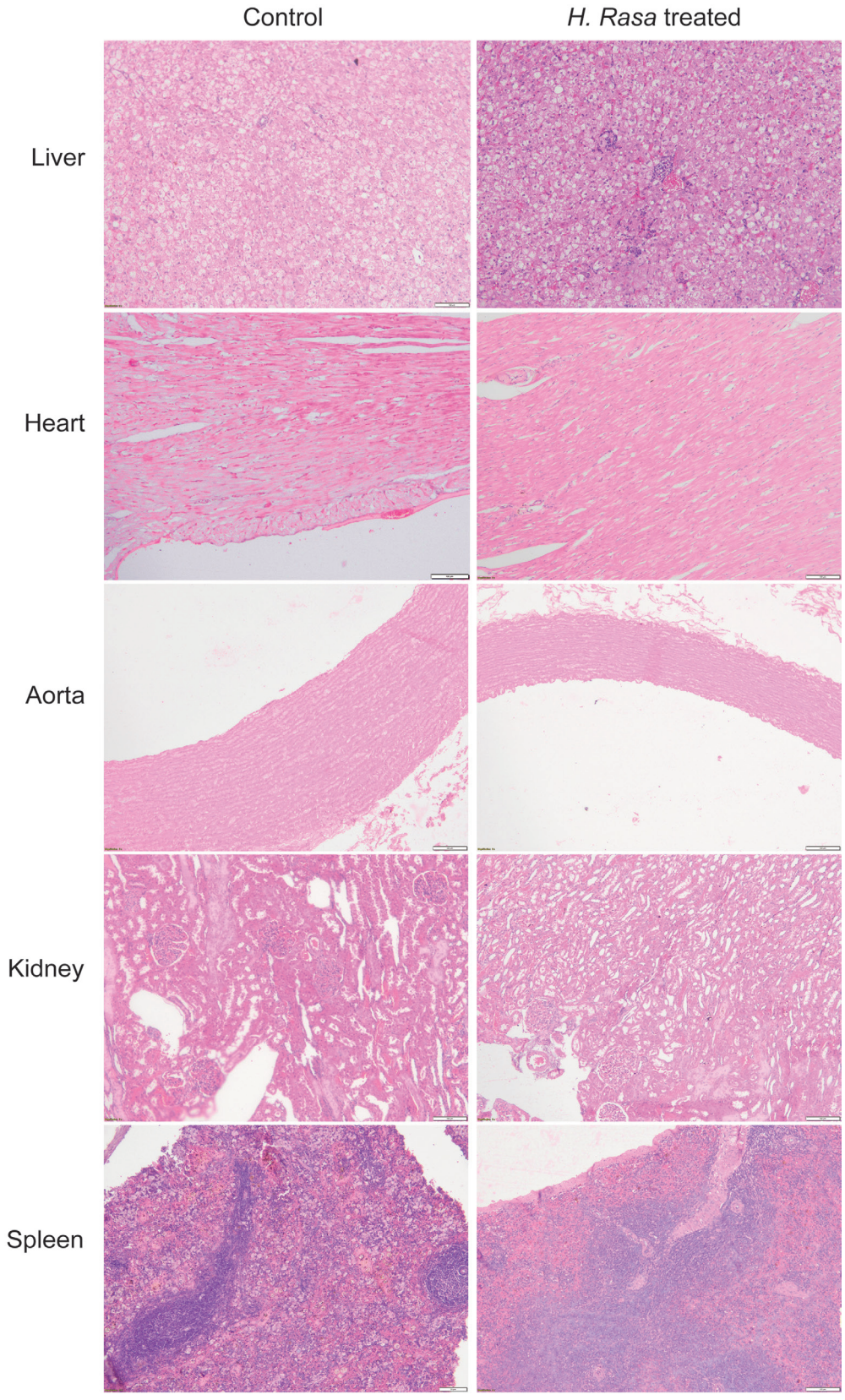

Fig. 5. Histopathological findings of major organs in control and high dose of *H. Rasa* treated groups (10 × 10X).

Na^+/K^+ ATPase activity.[49] Na^+/K^+ ATPase activity was improved in the groups administered with the middle (IV) (10.62%; 17.04%; 21.07%) and highest (V) dose (37.50%; 38.92%; 41.46%) of *H. Rasa* at 30, 60 and 90 days of diet, respectively, when compared to the lowest dose group (III). The Na^+ concentration in the middle (IV) (14.10%; 26.81%; 39.11%) and high dose (V) (23.33%; 25.68%; 29.30%) groups, and the K^+ concentration in the middle (IV) (17.78%; 20.33%; 24.10%) and high (V) (18.87%; 26.21%; 28.12%) dose groups were improved at 30, 60 and 90 days, respectively. The Na^+ concentration was reduced at 30 (6.70%), 60 (6.70%), and 90 (6.70%) days in group VI, and the K^+ concentration was increased at 30 (16.43%), 60 (28.82%), and 90 (38.11%) days in group VI compared to group II. The major phenolic constituents and potent anti-oxidants of *H. Rasa* are gallic acid and its derivatives, chebulagic acid, tannins such as emblicanin A and B, flavonoids such as quercetin, alkaloids and free radical scavengers.[50–52] These phytoconstituents protect erythrocytes from free radical damage and maintain the Na^+/K^+ ATPase activity and Na^+ and K^+ concentrations, resulting in membrane fluidity. We studied the effects of different doses of *H. Rasa* at different time intervals on Na^+/K^+ ATPase activity in rats. We found that erythrocytes were protected in a dose- and time-dependent manner.

The commercially available anti-hypertensive drug, atorvastatin was used as a standard drug candidate to compare with the efficacy of *H. Rasa* in atherosclerosis-induced rabbits. Na^+/K^+ ATPase activity was increased at 30 (44.76%), 60 (47.33%) and 90 (49.90%) days of diet when compared to HFD-induced rabbits. Comparing the levels of Na^+/K^+ ATPase (statin-treated group) with different doses of *H. Rasa*, the activity was found to lie between the middle and high doses of *H. Rasa* in the present study. By contrast, the concentration of Na^+ and K^+ were closest to the lowest dose of *H. Rasa*. This may be due to increased endothelial production of nitric oxide (NO), which is controlled by statin and is involved in the upregulation of endothelial NO synthase activity.[53] This effect may be potentiated by the simultaneous inhibition of the protein with reduced endothelial NO synthase mRNA degradation and, thus, increased NO bioavailability.[54] In addition, NO acts as a powerful free radical scavenger, and statins inhibit the production of reactive oxygen species such as superoxide anion and hydroxyl radicals.[55] Group VI of the present study that Na^+/K^+ ATPase can protect and normalize the electrolyte balance in cells of statins when administered simultaneously with HFD in rabbits. When we analyzed the plasma and tissue concentration of mercury, copper and sulfides by ICP-OES after 90 days of treatment, we found that there was no detectable limit of the above metals in the plasma or various organs such as heart, aorta, spleen, liver, kidney, etc. (Data not shown). To support this statement, we also studied the histopathology of major organs, which showed that normal or near-normal histological architecture was found in the control group and the high-dose of *H. Rasa*-treated group (Fig. 5).

Clinical significance

In atherosclerosis, HFD reduces EM stabilization after being administered with *H. Rasa*, an Ayurvedic polyherbo-metalo-mineral drug. This agent protects EM by maintaining Na^+/K^+ ATPase activity through the Na^+/K^+ pump.

Limitations

Membranes play an important role in the maintenance of cell fluidity and integrity. This study investigated the role of *H. Rasa* on membrane stabilization through Na^+/K^+ ATPases. More research is needed to evaluate the potential uses of *H. Rasa* on the protection of erythrocytes.

Future directions

Hyperlipidemia is closely associated with atherosclerosis and increasing evidence suggests that erythrocytes may participate in atherogenesis. The increased generation of reactive oxygen species occurs in atherosclerosis and may be responsible for the increased oxidative injury to the erythrocyte membrane in the atherosclerotic condition. Therefore, we studied the effects of *H. Rasa*, an ayurvedic formulation, on erythrocyte membrane stabilization through Na+/K+ ATPase activity. Data on the role of *H. Rasa*, Na+/K+ ATPase and ion transport in EM have yet to be studied. However, the aim of the present study was to evaluate the correlation between the cholesterol-lowering agent *H. Rasa* and EM Na^+/K^+ ATPase activity in rabbits with HFD-induced atherosclerosis. This study also focused on the dose- and time-dependent activity of *H. Rasa*. The commercially available anti-hypertensive drug, atorvastatin was used as a standard drug candidate to compare with the efficacy of *H. Rasa* in atherosclerosis-induced rabbits. Based on the preliminary results of this study, we speculate that *H. rasa* is a potential drug candidate for the treatment of atherosclerosis. In addition, to ensure its potential efficacy, additional research is needed to study the mechanism of action of *H. Rasa*.

Conclusions

These results suggest that HFD markedly reduces EM stabilization in atherosclerosis whereas *H. Rasa* protects EM by maintaining Na^+/K^+ ATPase activity through the Na^+/K^+ pump.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

Objective, study design and write-up of the manuscript (CS), rabbit handling, blood collection and maintenance (AR), critical review

and suggestions (SG, IR); drug administration, experimentation and animal maintenance throughout the study (SDV); statistical analysis and editing of the manuscript (MKG).

Ethical statement

All procedures involving animals were reviewed and approved by the Institutional Animal Ethics Committee of Captain Srinivasa Murthy Central Ayurveda Research Institute, Chennai (IAEC/CSMRADDI/17/2017).

Data sharing statement

Any data related to this paper is available with corresponding author at chiresh2006@gmail.com.

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