Erythrocyte Membrane Bound ATPase and Antioxidant Enzyme Changes Associated with Vascular Calcification is Reduced by Sodium Thiosulfate

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Abstract Sodium thiosulfate (STS), a cyanide antidote has been reported to possess antioxidant and calcium chelation effects, useful for the treatment of renal failure due to vascular calcification and urolithiasis. The present study investigated the in vivo modulatory effects of STS on erythrocyte calcium, phosphorous levels, lipid peroxidation, antioxidant enzyme and membrane ATPase activities (Ca^{2+} , Na^+K^+ , Mg^{2+} and 5' nucleotidase) in an adenine induced model of vascular calcification in rats. Adenine (0.75%) was supplemented through the diet for 28 days, which resulted in significantly ($P < 0.05$) increased circulating calcium and phosphorous product and oxidative stress within the RBCs, as measured from lipid peroxidation and reduced antioxidant enzymes. The membrane ATPase activities were altered (increased Ca^{2+} , Na^+K^+ ATPase and decreased Mg^{2+} ATPase, 5' nucleotidase) compared to the rats fed on normal diet. STS (400 mg/kg) given orally was effective in establishing a normalcy in the RBC alterations. This effect was more pronounced, when STS was given from day 28 to day 49 after induction of calcification, instead of day 0 to day 28. These findings may benefit to evaluate the effectiveness of STS therapy in patients with chronic renal failure associated with increased circulating calcium and phosphorous product that leads to stiffening of vascular smooth muscles of aorta, due to calcium deposition.

Keywords Vascular calcification · Adenine · Erythrocytes · ATPase activity · Oxidative stress

Abbreviations

STS	Sodium thiosulfate
ET	Early treatment
LT	Late treatment
$\text{Ca} \times \text{P}$	Calcium phosphorous product

Introduction

Calcification is a normal process of accumulation of calcium resulting in hardening of body tissues especially bone and teeth. However, an imbalance in calcium homeostasis may lead to its deposition in blood vessels, lungs, kidney, heart, and brain, leading to associated pathophysiology like vascular calcification, calcified pulmonary nodule, chronic kidney disease (CKD), aortic valve calcification and Fahr's syndrome. Among these, vascular calcification has higher clinical implications as evident from the 50% rise in the number of publications in last 5 years in PubMed alone. Five types of vascular calcification has been defined recently [1] such as: calcific aortic stenosis (CAS), medial artery calcification, atherosclerotic intimal calcification, vascular calcification of chronic kidney disease, and calcific uremic arteriolopathy, which require surgery for a better life expectancy.

Erythrocyte membrane contents were found to be altered in vascular calcified patients, especially those patients undergoing hemodialysis. Furthermore, the previous study has shown that elevated lipid content in erythrocyte membrane adversely affect transmembrane erythrocyte cationic fluxes. Erythrocyte ATPases play a central role in the

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regulation of calcium homeostasis in blood. Serum calcium, phosphorous and magnesium; major players in maintaining the calcium homeostasis are associated with the prevalence and severity of chronic kidney disorders (CKD), through correlation with atherogenic lipids [2]. Maintaining their concentration gradient between cytoplasmic and extracellular spaces in the blood is mediated through erythrocyte ATPase, whose activities are modulated during vascular calcification. In chronic kidney disease, calcium homeostasis is disturbed by increasing calcium in the erythrocyte, thereby induce decreased cell flexibility, elevated osmotic fragility and even hemolysis [3].

Sodium thiosulfate (STS) is well documented and accepted agent to prevent calcification [4] without adverse effects, which made it popular for treating calcification in dialysis patients [5]. Recently, our group provided a mitochondria dependent mode of action for the thiosulfate induced renal protection [6]. The therapeutic efficacy of STS in ameliorating the pathogenesis of vascular calcification specifically in case of erythrocyte membrane pump activities remains unresolved. The present study was, therefore, designed to investigate the influence of STS on erythrocyte membrane bound ATPase pump activity and antioxidant enzyme activities, which influenced the deposition of calcium into the major tissues such as aorta, heart, brain and kidney [7].

Materials and Methods

Induction of Vascular Calcification in Wistar Rats

The institutional animal ethical committee at SASTRA University, Thanjavur, India has approved all the experimental procedures under the approval no. 258/SASTRA/IAEC/RPP. Wistar rats weighing 200–250 g were used in the present study. Calcification was induced using adenine 0.75%, mixed with diet and fed for 28 days as per previous protocol [8]. The rats were divided into four groups of six rats each; normal group fed on regular diet and water ad libitum; vascular calcification control (VC) fed on adenine mixed with normal diet for 28 days; sodium thiosulfate (STS = 400 mg/kg) given orally for 28 days along with adenine diet as an early treatment (STS_ET) and finally STS given after 28 days of adenine diet as a late treatment (STS_LT), up to day 49. At the end of study period, rats were euthanized and blood was collected for biochemical analysis.

Erythrocyte Membrane Isolation

The blood was collected in K₂EDTA tubes after cardiac puncture at sacrifice and centrifuged at 5000 rpm for

10 min at 4 °C. The plasma was removed and the packed RBC were washed twice with 10 mM phosphate buffered saline pH 7.4. The erythrocyte membranes or ghosts were isolated according to the method of Marchesi and Palade [9] and purified by gradient centrifugation. The erythrocyte membrane proteins were quantified using Bradford reagent from BioRad, USA and standard bovine serum albumin.

Calcium × Phosphorous Product

Erythrocyte membrane protein (0.5 mg) was soaked in 0.6 N-HCl for 24 h, followed by neutralization with 3 M NaOH [7]. The samples were vortexed and centrifuged at 5000 rpm for 5 min to collect the supernatant. Calcium and phosphorous content were measured in the supernatant using kit from Span Diagnostics Ltd, India. The calcium x phosphorous product was reported as a measure of degree of calcification. The erythrocyte Ca × P product was normalized with the normal group and compared with other organs such as aorta, heart, kidney and brain for correlation.

Lipid Peroxidation and Antioxidant Enzymes

The oxidative stress induced due to adenine diet was measured in the erythrocytes in terms of lipid peroxidation expressed as thiobarbituric acid reactive species (TBARS) content according to Ohkawa [10] and antioxidant enzymes mainly superoxide dismutase, catalase and glutathione peroxidase as per previous protocols [11–13]. All the experiments were normalized with the protein concentrations and spectrophotometric analysis was done using Synergy H1 multimode reader equipped with Gen 5.0 software from BioTek, USA.

Erythrocyte ATPase Activity

Na⁺K⁺-ATPase activity was estimated by previous method [14], using 0.5 mg/ml of the erythrocyte membrane in a reaction mixture containing (in mM) NaCl: 140, KCl: 14, MgCl₂: 3, Tris-HCl, pH 7.4, Na₂-ATP: 30. Two controls were kept of which one was a blank specimen which contained the heat-inactivated membrane preparation (boiled for 30 min). The second had the inhibitor of Na⁺K⁺-ATPase, ouabain, at a concentration of 10 μM/l. The samples were incubated for 2 h at 37 °C and reaction was terminated using 2 ml of 10% (w/v) trichloroacetic acid. The Inorganic phosphate formed was measured by the method of Fiske and Subba Row [15].

Ca²⁺-ATPase activity was measured according to the previous methods [14]. Briefly 0.5 mg/ml erythrocyte membrane was incubated in a reaction mixture containing (in mM) CaCl₂: 5, Tris-HCl: 20, pH 8.2, NaCl: 10 and Na₂

ATP: 5. Ethacrynic acid, the inhibitor of Ca^{2+} ATPase, was added at a concentration of 5 mM. The samples were incubated for 2 h at 37 °C and reaction was terminated using 2 ml of 10% (w/v) trichloroacetic acid. The Inorganic phosphate formed was measured by the method of Fiske and Subba Row [15].

Mg^{2+} -ATPase was determined by previous method [14], where the membrane was incubated in Tris-HCl buffer (375 mM; pH 7.6), MgCl_2 (25 mM), ATP (10 mM) and the membrane suspension obtained. The content was thoroughly mixed and incubated at room temperature for 15 min. The reaction was arrested by adding 1 ml of 10% TCA. The liberated inorganic phosphate was measured by the method of Fiske and Subba Row [15].

5' nucleotidase activity in erythrocyte membranes was estimated according previous protocol [16]. The protein was incubated at 22 °C in a reaction mixture containing (in mM) Tris-acetate: 100, pH 7.5, AMP: 1.0 and $\text{Mg}(\text{NO}_3)_2$: 1.0. The reaction was stopped using 20% trichloroacetic acid and the inorganic phosphate analyzed using Fiske and Subba Row reagent [15].

Statistical Analysis

The data were represented as mean $\pm$ S.D. One way ANOVA followed by Dunnet's test was used to calculate significance of difference between mean values from the normal and calcification induced groups using PRISM Graph Pad 5.0.

Results

The adenine diet induces renal failure due to the deposition of 2, 8-dihydroxyadenine [8], which was confirmed through histopathology from our previous studies [7] and calcium $\times$ phosphorous product in aorta (Table 1). The resulting elevations in circulating urea, uric acid, creatine and alkaline phosphatase levels in blood is known to induce free radical induced oxidative stress in heart, brain, kidney and blood vessels such as aorta [7]. However its impact on erythrocyte has not been reported previously.

Table 1 showed significantly ($P < 0.05$) elevated deposition of calcium and phosphorus represented as $\text{Ca} \times \text{P}$ product, within the erythrocyte membranes of rats treated with adenine diet, compared to normal. This deposition was minimized by 13% upon thiosulfate treatment in the late treatment group compared to calcified rats. Among various organs, the $\text{Ca} \times \text{P}$ product in erythrocyte was well correlated in the order of aorta ($r = 0.79$) > heart ($r = 0.56$) > brain ($r = 0.48$) > kidney ($r = 0.46$).

In Fig. 1, the lipid peroxidation, measured by TBARS level of erythrocyte membrane was significantly elevated

Table 1 Data represents the calcium, phosphorous and the $\text{Ca} \times \text{P}$ product in erythrocyte membranes, kidney, heart, aorta and brain

Group	Erythrocyte			Kidney			Heart			Aorta			Brain		
	Ca	P	$\text{Ca} \times \text{P}$	Ca	P	$\text{Ca} \times \text{P}$	Ca	P	$\text{Ca} \times \text{P}$	Ca	P	$\text{Ca} \times \text{P}$	Ca	P	$\text{Ca} \times \text{P}$
Normal	10.00 $\pm$ 0.1	5.20 $\pm$ 0.1	52.00 $\pm$ 2.0	0.13 $\pm$ 0.01	11.00 $\pm$ 0.2	1.43 $\pm$ 0.1	0.04 $\pm$ 0.001	0.04 $\pm$ 0.001	0.38 $\pm$ 0.04	0.20 $\pm$ 0.01	1.80 $\pm$ 0.02	0.36 $\pm$ 0.01	0.40 $\pm$ 0.01	11.86 $\pm$ 0.1	4.74 $\pm$ 0.4
VC	9.80 $\pm$ 0.1	6.10 $\pm$ 0.1	59.78* $\pm$ 2.0	0.20 $\pm$ 0.03	6.50 $\pm$ 0.1	1.30* $\pm$ 0.2	0.04 $\pm$ 0.003	0.04 $\pm$ 0.003	0.35 $\pm$ 0.03	0.27 $\pm$ 0.01	2.00 $\pm$ 0.04	0.54* $\pm$ 0.02	1.10 $\pm$ 0.02	10.90 $\pm$ 0.3	11.99* $\pm$ 0.6
STS_ET	10.40 $\pm$ 0.2	5.80 $\pm$ 0.2	60.32* $\pm$ 1.5	0.15 $\pm$ 0.01	9.80 $\pm$ 0.2	1.47 $\pm$ 0.1	0.06 $\pm$ 0.002	0.06 $\pm$ 0.002	0.57* $\pm$ 0.05	0.23 $\pm$ 0.02	1.49 $\pm$ 0.03	0.34* $\pm$ 0.01	0.33 $\pm$ 0.02	10.50 $\pm$ 0.2	3.47* $\pm$ 0.4
STS_LT	8.70 $\pm$ 0.1	5.30 $\pm$ 0.1	46.11* $\pm$ 1.2	0.11 $\pm$ 0.02	11.50 $\pm$ 0.1	1.27* $\pm$ 0.1	0.04 $\pm$ 0.001	0.04 $\pm$ 0.001	0.36* $\pm$ 0.03	0.09 $\pm$ 0.01	1.40 $\pm$ 0.01	0.13* $\pm$ 0.01	0.28 $\pm$ 0.01	9.20 $\pm$ 0.2	2.58* $\pm$ 0.2

The values are represented as mean $\pm$ S.D. (n = 6). * Significant ($P < 0.05$) versus normal. The units are expressed in terms of mg/g (Calcium and phosphorous) and mg^2/g^2 ($\text{Ca} \times \text{P}$ product)

Fig. 1 Data represents the lipid peroxidation and antioxidant enzyme changes observed in the erythrocyte membranes **a** TBARS, **b** GPx, **c** Catalase and **d** SOD. The values are represented as mean $\pm$ S.D. (n = 6). *Significant ($P < 0.05$) versus normal

($P < 0.05$) by 2.76 fold after 28 days of adenine diet, compared to the normal. Sodium thiosulfate reduced the lipid peroxidation close to normal, more so when administered as a late treatment. The antioxidant enzymes (GPx, catalase and SOD) were reduced significantly ($P < 0.05$) in calcification control animals compared to the normal, which was improved slightly after thiosulfate treatment, without significant difference between early and later treatment schedules.

The ATPase activity in erythrocyte membrane represents the status of membrane transport systems, which are usually altered during stress, to maintain ionic balance. After adenine diet for 28 days, in Fig. 2, the Ca^{+2} and $\text{Na}^{+}\text{K}^{+}$ ATPase activities were found significantly ($P < 0.05$) increased by approximately twofold, while the Mg^{+2} ATPase and 5' nucleotidase activities were decreased by 0.34 and 0.56 fold compared to the animals on normal diet. Thiosulfate treatment normalized the ATPase activities close to the normal rat, more so in-case of thiosulfate administered after induction of calcification, from day 29 to day 49.

Discussion

Vascular calcification causes imbalances in the circulating calcium, phosphorous levels and their absorption into various body compartments. Erythrocyte being a major blood cell type, acting as a carrier for calcium and

phosphorous, is affected by the elevated levels of these minerals. This is evident from present study in which we have analyzed the erythrocyte antioxidant and membrane ATPase activity and found an elevated Ca^{+2} ATPase and $\text{Na}^{+}\text{K}^{+}$ ATPase activities in the erythrocyte membranes of vascular calcified animals, but a significant decline in the Mg^{+2} ATPase, indicating the imbalance of electrochemical gradients across the erythrocyte membrane. The erythrocyte membrane calcium ATPases contribute to the maintenance of appropriate cytoplasmic calcium levels by removing calcium from the cell to the extracellular environment and thereby act as the detrimental factor that promotes calcification [17]. It is a known that calcium can deposit anywhere in the body and can cause a serious pathology, if it is not balanced with magnesium. This observation emphasizes the significant role played by erythrocyte ATPase that can reflect the calcium deposition in the soft tissue. Another major observation, we made in this study is the efficacy of STS in normalizing erythrocyte ATPase, where the latter acts as an agent that alleviates the vascular calcification, when it is administered as late treatment.

One of the major reasons for calcium deposition outside the bones is tissue damage and elevated calcium in the blood [17]. In the present study, kidney failure induced by adenine treatment resulted in excessive absorption of calcium from intestine and bones, leading to its deposition in the blood vessels. Evidence from previous studies suggest that higher blood calcium levels can impact loss of

Fig. 2 Data represents the ATPase activity changes observed in the erythrocyte membranes **a** Ca^{+2} ATPase, **b** $\text{Na}^{+}\text{K}^{+}$ ATPase, **c** Mg^{2+} ATPase and **d** $5'$ nucleotidase. The values are represented as mean $\pm$ S.D. (n = 6). *Significant ($P < 0.05$) versus normal

magnesium in the blood [18]. This will aggravate the pathology of calcification as the calcium pump in the membrane is magnesium dependent. In addition, low activity of $5'$ nucleotidase in VC group indicates impaired uptake of adenosine, which probably influences the salvage of purines nucleotides from the extracellular nucleotides. It is noteworthy to mention that $5'$ nucleotidase can influence the O_2 binding capacity of hemoglobin through 2, 3 bis-phosphoglyceric acid [19]. Hence alterations in erythrocyte ATPase activities can be used as an index to identify the abnormalities in the vasculature due to calcification.

Sodium thiosulfate which is being used to treat dialysis patients is beneficial to erythrocytes as well, by impairing the active calcium uptake and recycling of adenosine levels [5, 20]. A defective ionic exchanger activity caused by free radical damage is [21] normalized by STS administration, as observed in the present study (Fig. 1). We found a positive correlation with respect to $\text{Ca} \times \text{P}$ product in erythrocytes, with $\text{Na}^{+}\text{K}^{+}$ ATPase ($r = 0.65$) and Ca^{+2} ATPase ($r = 0.69$) activities. The effectiveness of thiosulfate is due to the antioxidant potential and mild calcium chelation effect as established by numerous studies including ours [4, 6].

Conclusion

In conclusion, erythrocyte ATPase enzyme activities can be used as an index for evaluating the therapy against renal failure and vascular calcification due to its specific changes with respect to erythrocyte membrane contents. Furthermore, STS improved the functional activities of erythrocyte

ATPase and antioxidant enzyme, support the previous findings regarding the efficacy of STS in ameliorating the VC associated dysfunction in kidney.

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Compliance with Ethical Standards

Conflict of interest The authors have declared no conflict of interest.

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