# *Dietary Chitosan Supplementation Ameliorates Isoproterenol-Induced Aberrations in Membrane-Bound ATPases and Mineral Status of Rat Myocardium*

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# Dietary Chitosan Supplementation Ameliorates Isoproterenol-Induced Aberrations in Membrane-Bound ATPases and Mineral Status of Rat Myocardium

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**Abstract** Myocardial infarction is one of the major public concerns in both developed and developing countries. Recently, there is growing interest in potential healthcare applications of marine natural products in the field of cardiovascular research. In the present study, we have examined the membrane-stabilizing potential of marine mucopolysaccharide-chitosan in modulating the aberrations of thiol-dependent membrane-bound ATPases activities, mineral status, and cardiac diagnostic markers in isoproterenol-induced myocardial infarction condition in rats. Dietary intake of chitosan significantly ($p<0.05$) counteracted the isoproterenol-induced lipid peroxidation and maintained the levels of thiol contents and cardiac biomarkers at concentrations analogous to that of normal controls in the rat myocardium. Chitosan administration also significantly mitigated isoproterenol-induced aberrations in the membrane-bound ATPase activities in the heart tissue and preserved the myocardial mineral status in serum and heart tissue of experimental rats at near normal value. The results of the present study have indicated that the salubrious effect of dietary chitosan supplementation in attenuating the experimentally induced myocardial infarction condition is probably ascribable to its antioxidant defense and membrane-stabilizing properties.

**Keywords** Chitosan · Myocardial infarction · Isoproterenol · Membrane-bound ATPases · Mineral status · Cardiac biomarkers

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## Introduction

Myocardial infarction is the major public health concern and the leading cause of mortality and morbidity among the cardiovascular diseases all over the world. Both developed and developing countries are finding difficult to control the sway of myocardial disorders along with the growing saddle of stroke, Type II diabetes, obesity, and hypertension [1]. In recent decade, a growing number of young people are succumbing to myocardial infarction due to abnormal risk factors characterized by high triglycerides, low high-density lipoproteins (HDL), glucosein tolerance, insulin resistance, abdominal obesity, and increased lipoprotein (a) levels [2]. About 16.7 million people around the globe die of myocardial infarction annually, which forms about one third of the total global deaths [3]. Present-day projections propose a highly notorious, but a real fact that by the year 2020, India will have the largest cardiovascular disease lumber in the world [4]. A considerable body of clinical and experimental evidence is now emerging which suggests that catecholamine-mediated oxidative stress, free radical-induced disintegration of cellular and subcellular membrane integrity, and the altered mineral homeostasis are playing important roles in the pathogenesis of myocardial infarction [5]. Developing nutritional and pharmacological interventions to reduce myocardial infarction-related cardiovascular diseases mortality rate is of utmost importance.

Recently, there is a lot of attention in biomolecules derived from marine resources for human healthcare applications. Chitosan (Fig. 1) is an abundant marine polysaccharide with repeated α-(1-4)-D-glucosamine residues present in the exoskeleton of shellfish, clams, krill, oysters, squid, fungi, and insects [6]. This marine biopolymer has intense solicitations

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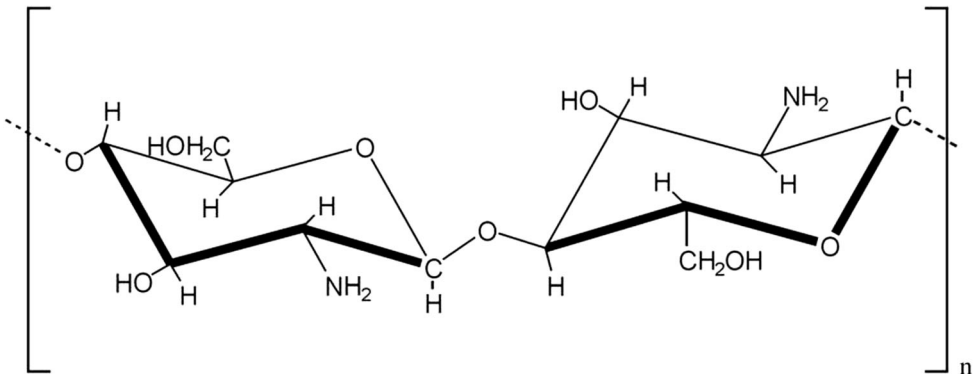


**Fig. 1** Structure of chitosan

in the fields of chromatography, bioremediation, food and nutrition, paper and textiles, pharmaceutics, agriculture, photography, medicine, cosmetics, etc. [7]. Chitosan has been reported to possess immunological, antibacterial, and wound-healing properties. It is also well known to be an effective hemostatic biomolecule even under the most severe conditions of anticoagulation. Its antioxidant defense [8] and membrane-stabilizing activities [9, 10] have been reported in detail. Earlier study of Anandan et al. [11] observed the mucosal membrane protective effect of chitosan against HCl–ethanol-induced peptic ulcer in rats. Previously, we have reported the hypolipidemic and antioxidant properties of chitosan in attenuating experimentally induced myocardial infarction in male albino rats [12–14]. Though the protective effects of chitosan have been extensively studied, the membrane-stabilizing potential of chitosan in modulating the aberrations in membrane-bound ATPases activities and mineral status in experimentally induced myocardial infarction condition has not yet been explored.

Myocardial dysfunction induced by isoproterenol [L-1-(3, 4-dihydroxyphenyl)-2–isopropylaminoethanol hydrochloride] (Fig. 2), a synthetic catecholamine and β-adrenergic agonist, has been reported to show many metabolic and morphologic aberrations in the cardiac tissue of experimental rats similar to those seen in human myocardial infarction [5]. Hence, isoproterenol-induced myocardial ischemic alterations are generally used for examining the beneficial actions of cardioprotective agents.

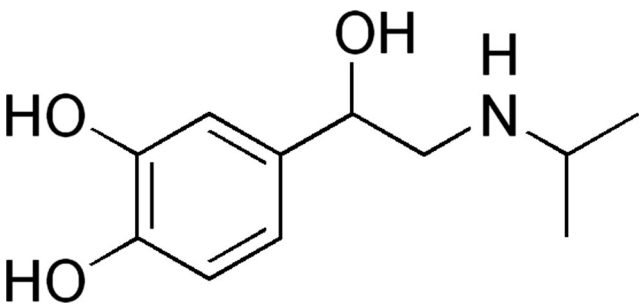


**Fig. 2** Structure of isoproterenol

In the present study, an attempt has been made to assess the cardioprotective effect of chitosan on membrane-bound ATPases, mineral status, and cardiac biomarkers in experimentally induced myocardial infarction condition (isoproterenol-induced myocardial infarction) in male albino rats by virtue of its antioxidant and membrane-stabilizing properties.

## Materials and Methods

### Chemicals

The fine chemicals such as isoproterenol, ATP, tetraethoxypropane, and cysteine were procured from M/s. Sigma Chemical Company, St. Louis., MO, USA. The mineral standard reference materials (Sodium: BWB06NA; Potassium: BWB04K; Calcium: BWB02CA) were obtained from M/s. Aquanet International Limited, Berkshire, UK. The chitosan molecule (molecular weight 750,000 Da; deacetylation rate 85–87 %; viscosity 8 cps; purity 98.6 %) used in investigation was a generous gift from Dr. T.K. Thankkappan, Principal Scientist, Central Institute of Fisheries Technology, Cochin, India. All the other chemicals/reagents used were of analytical grade or better.

### Animals

Male Wistar strain albino rats, weighing 120–150 g were selected for the study. The normal and experimental animals were housed individually in polypropylene cages under hygienic environmental conditions (28±2 °C, humidity 60–70 %, 12-h light/dark cycle). The animals were provided a standard diet (Table 1) with metabolizable energy of 3600 kcal [M/s Sai Feeds, Bangalore, India] and water ad libitum. The entire experiment was carried out strictly as per the guidelines prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

**Table 1** Nutrient composition of the animal feed

| Ingredients | g/100-g diet |
|---|---|
| Carbohydrate (nitrogen-free) | 56.2 |
| Crude protein | 22.0 |
| Ash | 7.5 |
| Crude oil | 4.2 |
| Crude fiber | 3.0 |
| Glucose | 2.5 |
| Vitamins | 1.8 |
| Sand silica | 1.4 |
| Calcium | 0.8 |
| Phosphorus | 0.6 |

### Induction of Myocardial Infarction

The myocardial infarction was induced in experimental rats by intraperitoneally (i.p.) injecting isoproterenol (11 mg/100 g body weight/day) for 2 days [13].

### Experimental Protocol

Seven days after acclimatization, the normal and experimental animals were divided into four groups of six rats each. Group I rats were normal controls given only standard diet. Group I and group III animals were fed on standard diet with cornstarch at 2 % level for 60 days and group II and group IV animals were fed on standard diet with added chitosan at 2 % level for the same period. After 60 days feeding, the group III and group IV animals were intraperitoneally (i.p.) injected with isoproterenol [11 mg (dissolved in physiological saline)/ 100 g body weight per day for 2 days] for the induction of myocardial infarction [13].Control animals (group I and group II) were i.p. injected with physiological saline alone for 2 days.

At the end of the experimental period, i.e., 24 h after last injection of isoproterenol, the experimental animals were sacrificed and blood samples were collected for the separation of serum. The heart tissue was excised immediately, and one portion of tissue was used for mineral analysis. Another portion of accurately weighed heart tissue was homogenized in ice-cold 0.1 M Tris-HCl buffer, pH 7.2 and centrifuged. The serum and tissue homogenate were used for biochemical analyses.

### Biochemical Analyses

Lipid peroxidation level in the heart tissue was determined according to the method of Ohkawa et al. [15]. Total protein and nonprotein sulfhydryl contents were estimated according to the method of Sedlak and Lindsay [16]. This method is based on the development of a yellow color when DTNB is added to compounds contained sulfhydryl groups to form 2-nitro-5-mercaptobenzoic acid. The activities of $Na^+/K^+$-dependent ATPase, $Mg^{2+}$ -dependent ATPase, and $Ca^{2+}$ -dependent ATPase were measured from the amount of Pi released according to the method of Bonting [17], Ohnishi et al. [18], and Hjerten and Pan [19], respectively. The enzyme activity was expressed as micromoles Pi liberated per minutes per milligram protein.

Inorganic phosphorus was estimated by the method of Fiske and Subbarow [20]. The method is based on the formation of phosphomolybdic acid by the reaction between a phosphate and molybdic acid and its subsequent reduction to a dark blue phosphomolybdic acid, the intensity of which is proportional to the phosphate ion concentration. Minerals (sodium, potassium, and calcium) in serum and heart tissue were estimated using Varian Spectra-220 AA, Australia, Atomic Absorption Spectrophotometer after microwave digestion (Anton Paar Closed Vessel Microwave Digestion System) according to the method of the AOAC [21]. Serum troponin T content was measured by electrochemiluminescence immunoassay (Troponin T; Roche Diagnostics) using an Elecsys E170 analyzer (Roche Diagnostics) and enzyme-linked immunosorbent assay (ELISA). Homocysteine (tHcy) concentration in serum was assayed by Microtiter Plate Assay package (Diazyme Laboratories) [22].

## Statistical Analysis

Results are expressed as mean±SD. Multiple comparisons of the significant ANOVA were performed by Duncan's multiple comparison tests. A $P$ value of $<0.05$ was considered as statistically significant. All data were analyzed with the aid of statistical package program SPSS 10.0 for Windows.

## Results and Discussion

Significant ($p<0.05$) rise in the level of lipid peroxidation was recorded in the heart tissue of group III animals as compared to Group I normal control ones, indicating the severity of isoproterenol-mediated oxidative stress (Fig. 3). Lipid peroxidation of membrane phospholipids and oxidation of membrane-bound thiol groups [23] are considered to modify cell membrane permeability and configuration in addition to producing functional modification of lipid-dependent as well as SH-dependent membrane-bound ATPases [24]. In the present study, the levels of sulfhydryl groups (total, nonprotein, and protein-bound sulfydryl contents) and the activities of the membrane-bound ATPases ($Na^+$, $K^+$-ATPase, and $Ca^{2+}$-ATPase) were significantly ($p<0.05$) lowered in the heart tissue of group III isoproterenol-induced myocardial infarcted rats compared with group I normal rats (Tables 2 and 3),

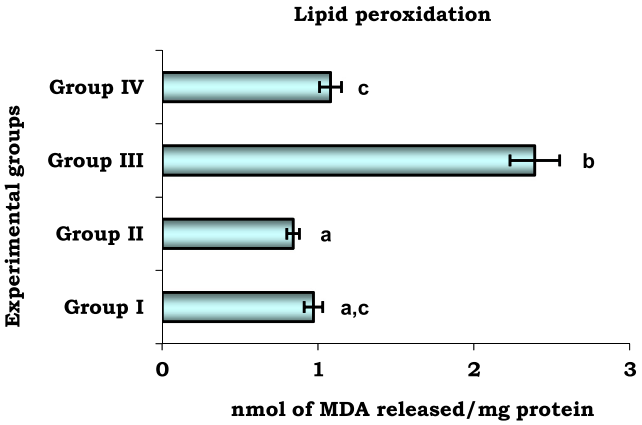


**Fig. 3** Level of lipid peroxides (LPO) in heart tissue of control and experimental groups of rats. Group I and group III animals, fed standard diet with cornstarch at 2 % level for 60 days. Group II and group IV, fed standard diet with added chitosan at 2 % level for 60 days. Group III and group IV animals, intraperitoneally (i.p.) injected with isoproterenol [11 mg (dissolved in physiological saline)/100 g body weight per day for 2 days] for the induction of myocardial infarction after 60 days feeding. Values expressed: nanomoles of MDA released per milligrams protein. Results are mean±SD for six animals; one-way ANOVA; Duncan's multiple comparison test. Values that have a *different superscript letter* (*a*, *b*, and *c*) differ significantly ($p<0.05$) with each other

demonstrating relentless derangement of cellular processes and structural modifications in cardiac cell membrane. Reduction in the activity of $Na^+$ and $K^+$-ATPase may lead to a decline in sodium efflux and thereby alter the membrane permeability [25]. Since $Ca^{2+}$-ATPase modulates the calcium pump function, a diminished $Ca^{2+}$-ATPase activity may lead to increased oxidative stress in cardiac myocytes [26]. Also, the intracellular concentration of calcium influences the activity of the $Mg^{2+}$ and $Na^+$, $K^+$-ATPases. Previously, Zhang et al. [27] have demonstrated that the appearance of isoproterenol-induced myocardial infarction is dependent on intracellular calcium concentrations, as observed in the present study.

**Table 2** Levels of total sulfhydryl groups (TSH), non-protein-bound sulfhydryl content (NPSH), and protein-bound sulfydryl content (PSH) of heart tissue of normal and experimental groups of rats

| Parameters | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| TSH | $2.86\pm0.96^{a}$ | $3.12\pm0.12^{a}$ | $1.38\pm0.10^{b}$ | $2.42\pm0.14^{c}$ |
| NPSH | $0.57\pm0.01^{a}$ | $0.65\pm0.03^{a}$ | $0.36\pm0.02^{b}$ | $0.49\pm0.03^{d}$ |
| PSH | $2.27\pm0.18^{a}$ | $2.47\pm0.20^{a}$ | $1.02\pm0.05^{b}$ | $2.93\pm0.14^{d,e}$ |

Group I and group III animals, fed standard diet with cornstarch at 2 % level for 60 days. Group II and group IV, fed standard diet with added chitosan at 2 % level for 60 days. Group III and group IV animals, intraperitoneally (i.p.) injected with isoproterenol [11 mg (dissolved in physiological saline)/100 g body weight per day for 2 days] for the induction of myocardial infarction after 60 days feeding. Values expressed: micromole per gram wet tissue. Results are mean±SD for six animals; one-way ANOVA; Duncan's multiple comparison test. Values that have a different superscript letter (a, b, c, d, and e) differ significantly ($p<0.05$) with each other

**Table 3** Levels of $Na^{2+}$, $K^+$ ATPase, $Mg^{2+}$ ATPase, and $Ca^{2+}$ ATPase in heart tissue of normal and experimental groups of rats

| Parameters | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| $Na^{2+}$, $K^{+}$-ATPase | $1.95\pm0.13^{c}$ | $1.88\pm0.17^{e}$ | $0.89\pm0.05^{b}$ | $1.76\pm0.07^{h}$ |
| $Mg^{2+}$-ATPase | $1.63\pm0.05^{c}$ | $1.49\pm0.06^{c}$ | $0.85\pm0.03^{b}$ | $1.03\pm0.06^{a,d}$ |
| $Ca^{2+}$-ATPase | $0.69\pm0.03^{a}$ | $0.67\pm0.02^{a}$ | $0.38\pm0.04^{b}$ | $0.59\pm0.02^{d}$ |

Group I and group III animals, fed standard diet with cornstarch at 2 % level for 60 days. Group II and group IV, fed standard diet with added chitosan at 2 % level for 60 days. Group III and group IV animals, intraperitoneally (i.p.) injected with isoproterenol [11 mg (dissolved in physiological saline)/100 g body weight per day for 2 days] for the induction of myocardial infarction after 60 days feeding. Values expressed: micromole Pi liberated per minutes per milligram protein. Results are mean± SD for six animals; one-way ANOVA; Duncan's multiple comparison test. Values that have a different superscript letter (a, b, c, d, e, f, g, and h) differ significantly ($p<0.05$) with each other

Reports by Bironaite and Ollinger [28] have shown that peroxidation of membrane-bound thiols can influence the functions of $Ca^{2+}$- and $Mg^{2+}$-ATPases and the activity of membrane-bound $Ca^{2+}$ translocase. Hence, any isoproterenol-induced alterations in the structural and functional integrity of cell membranes, including membranes of the endoplasmic reticulum and mitochondria may lead to necrobiosis and cytolytic damage.

In the present study, dietary of chitosan supplementation significantly ($p<0.05$) maintained the level of lipid peroxidation and the activities of membrane-bound ATPases at near normal values as compared with that of group III animals. It probably did so by preventing the oxidation of membrane phospholipids and membrane-bound protein-SH groups from ROS by its free radical quenching capability [10, 29, 30]. The reaction of singlet oxygen with unsaturated lipids alters the structural and functional integrity of membranes. The quenching reaction of singlet oxygen is known as an electron-transfer from the quencher to singlet oxygen.

Moderate ($p<0.05$) elevation in the level of calcium was detected in the serum and heart tissue of isoproterenol-administered group III rats as compared with that group I control animals (Tables 4 and 5). This concurs with previous reported studies [31, 32], which have shown that the alterations in calcium, magnesium, and water contents are well interrelated to the morphological changes of myocardial fibers. Earlier studies [33, 34] have indicated that the injection of isoproterenol into experimental animals could maximally stimulate $Ca^{2+}$ influx in myocardium.

Since membrane-bound $Ca^{2+}$-ATPase release $Ca^{2+}$ from the cytoplasm of the cells [35], the inhibition in intracellular $Ca^{2+}$-ATPase may lead to fast cellular $Ca^{2+}$ accumulation, as observed in the present study. Sodium and calcium are thought to be competitive at a number of membrane sites and it seems likely that a high concentration of $Ca^{2+}$ in ischemic myocardial cells would compete with sodium-specific sites at the

**Table 4** Levels of sodium, potassium, and calcium in serum of normal and experimental groups of rats

| Parameters | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| Sodium | 95.7±9.35[a] | 94.5±7.38[a] | 162±9.5[b] | 108±8.75[a,c] |
| Potassium | 8.35±0.82[a,d] | 8.12±0.54[a,d] | 4.58±0.35[b] | 7.91±0.36[e] |
| Calcium | 56.3±5.85[a] | 58.2±3.58[a,d] | 113±7.65[b] | 64.5±3.69[c,d] |

Group I and group III animals, fed standard diet with cornstarch at 2 % level for 60 days. Group II and group IV, fed standard diet with added chitosan at 2 % level for 60 days. Group III and group IV animals, intraperitoneally (i.p.) injected with isoproterenol [11 mg (dissolved in physiological saline)/100 g body weight per day for 2 days] for the induction of myocardial infarction after 60 days feeding. Values expressed: micromole per liter. Results are mean±SD for six animals; one-way ANOVA; Duncan's multiple comparison test. Values that have a different superscript letter (a, b, c, d, e) differ significantly ($p<0.05$) with each other

inner surface of the membrane [36]. This may lead to decrease in sodium content in serum, as recorded in the present study (Table 4). Also, failure of sodium pump may result in a depletion of sodium and rise in and potassium concentration in serum.

In the present study, supplementation of chitosan at 2 % level along with feed significantly ($p<0.05$) prevented all these isoproterenol-induced adverse effects on the levels of these minerals and maintained the rats at near normal status. It probably did so by protecting the plasma membrane-bound ATPases from the free radical attack by its free radical scavenging capability. Previous studies have shown that chitosan potentiates free radical scavenging by a nonenzymatic process of electron donation [37, 38]. Chitosan has a much greater efficiency in neutralizing OH° than the endogenous antioxidant GSH [39].

Increase in the level of troponin T in systemic circulation is a presumptive marker of occurrence of necrotic lesions in the cardiac membrane. Homocysteine is well known to induce atherosclerosis by impairing coronary microvascular dilator function [40]. In the present study, there was a significant ($p<0.05$) elevation in the levels of these diagnostic markers recorded in serum of group III animals compared to group I normal rats, demonstrating the rigorousness of isoproterenol-induced necrotic injury to the heart tissue membrane (Table 6). This present finding concurs with earlier reported study [41]. Occurrence of these diagnostic markers in serum of group III isoproterenol-injected rats reveals a nonspecific alteration in the structural and functional integrity of cellular and subcellular membranes of myocardium. In the present study, dietary supplementation of chitosan significantly attenuated the isoproterenol-mediated liberation of troponin T from the heart tissue into the systemic circulation as compared to group I control rats, thereby representing its defensive action on the myocardial membrane. Also, it maintained the level of homocysteine at near normal value. Restoration of cardiac diagnostic markers at normal values accredits chitosan with cytoprotective role in experimentally induced myocardial infarction condition.

**Table 5** Levels of sodium, potassium, and calcium in heart tissue of normal and experimental groups of rats

| Parameters | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| Sodium | 360±27.4[a,d] | 361±30.5[a,d] | 191±12.1[b] | 358±23.5[a,d,c] |
| Potassium | 124±11.50[a] | 126±8.7[a,d] | 217±18.4[b] | 136±7.5[c,d] |
| Calcium | 74.9±5.56[a] | 73.6±4.8[a] | 143±13[b] | 79.1±7.85[a,c] |

Group I and group III animals, fed standard diet with cornstarch at 2 % level for 60 days. Group II and group IV, fed standard diet with added chitosan at 2 % level for 60 days. Group III and group IV animals, intraperitoneally (i.p.) injected with isoproterenol [11 mg (dissolved in physiological saline)/100 g body weight per day for 2 days] for the induction of myocardial infarction after 60 days feeding. Values expressed micrograms per gram wet tissue. Results are mean±SD for six animals; one-way ANOVA; Duncan's multiple comparison test. Values that have a different superscript letter (a, b, c, d) differ significantly ($p<0.05$) with each other

## Conclusions

The present study has shown that the dietary supplementation of chitosan supplementation is capable to attenuate the isoproterenol-induced alterations in the activities of thiol-dependent membrane-bound ATPases and mineral status of myocardium in experimental animals. The cytoprotective action of chitosan is also evident from the level of inhibition on systemic circulation levels of cardiac biomarkers (troponin T and homocysteine) related to myocardial dysfunction in systemic circulation. In conclusion, the overall cardioprotective action of dietary chitosan supplementation is probably related to its antioxidant and membrane-stabilizing properties, which protect myocardial membrane against oxidative stress by decreasing lipid peroxidation reactions.

**Table 6** Levels of troponin T and homocysteine in serum of normal and experimental groups of rats

| Parameters | Group I | Group II | Group III | Group IVb |
|---|---|---|---|---|
| Troponin T | 0.03±0.001[a] | 0.02±0.001[a] | 0.79±0.04[b] | 0.14±0.002[c] |
| Homocysteine | 2.62±0.12[a] | 2.51±0.14[a] | 8.16±0.34[b] | 3.15±0.022[c] |

Group I and group III animals, fed standard diet with cornstarch at 2 % level for 60 days. Group II and group IV, fed standard diet with added chitosan at 2 % level for 60 days. Group III and group IV animals, intraperitoneally (i.p.) injected with isoprenaline [11 mg (dissolved in physiological saline)/100 g body weight per day for 2 days] for the induction of myocardial infarction after 60 days feeding. Values expressed: troponin T, nanogram per milliliter; homocysteine, micromole per liter. Results are mean±SD for six animals; one-way ANOVA; Duncan's multiple comparison test. Values that have a different superscript letter (a, b, c, d, e) differ significantly ($p<0.05$) with each other

## Limitation

Further studies with larger groups of subjects are necessary to verify and confirm the antioxidant defense and membrane stabilization action of dietary chitosan supplementation in ameliorating experimentally myocardial dysfunction. Also, a clinical study with a longer timeframe could establish the long-term benefits of dietary chitosan intake in counteracting cardiovascular diseases.

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