Cardio Protective Effect of Vitamin A against Isoproterenol-Induced Myocardial Infarction

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Summary Vitamin A is beneficial in countering free radical damage, therefore the present study is designed to investigate the effect of vitamin A against isoproterenol-induced myocardial infarction in rats. Male Wistar rats were divided into three groups, namely a normal control group, an isoproterenol group (150 mg/kg, s.c.), and a vitamin A treatment (4, 500 IU/kg/d, orally) group. Vitamin A-treated rats demonstrated significant reduction in ST-segment (p<0.001) and infarct sizes (p<0.05) when compared with the isoproterenol group of rats, suggesting that vitamin A markedly attenuated myocardial injury. Vitamin A treatment resulted in a significant decrease in the serum level of troponin I (p<0.001), creatinine kinase-MB (p<0.01), creatine kinase (p<0.05), lactate dehydrogenase (p<0.05), aspartate aminotransferase (p<0.01) and alanine aminotransferase (p<0.01). Vitamin A treatment resulted in a significant decrease in malondialdehyde (p<0.001), and significant increases were observed in reduced glutathione (p<0.01), superoxide dismutase (p<0.05) and catalase (p<0.001). Vitamin A treatment resulted in a significantly increased level of Na+K+ ATPase (p<0.05) and Mg2+-ATPase (p<0.01) and a significant reduction of Ca2+-ATPase (p<0.01). Vitamin A treatment also demonstrated a significantly decreased level of C-reactive protein (p<0.05) and myeloperoxidase activity (p<0.01). In conclusion, vitamin A attenuated the myocardial infarction and prevention was shown via membrane stabilization, reduction in oxidative stress, and prevention of neutrophil infiltration.

Key Words vitamin A, myocardial infarction, infarct size, ATPase, oxidative stress

Ischemic heart disease is the leading cause of death worldwide (1). According to the third monitoring report of the World Health Organization, 12 million deaths happen by myocardial infarction (MI) throughout the world each year. MI is death of the myocardium tissue; the extent and location of the infarction depend upon the degree of ischemic burden, the availability of coronary collateral blood flow, the rapidity of reperfusion and the location of the afflicted coronary artery.

Isoproterenol (ISO), a β-adrenergic agonist, has been found to cause severe stress in the myocardium, resulting in infarct-like necrosis of the heart muscle. Some of the mechanisms proposed to explain ISO-induced damage to cardiac myocytes includes hypoxia due to myocardial hyperactivity, coronary hypotension, calcium overload, depletion of energy reserve and excessive production of free radicals resulting from the oxidative metabolism of catecholamine (2).

Vitamin A has been shown to have antioxidant characteristics (3). The antioxidant activity of vitamin A and carotenoids is conferred by the hydrophobic chain of polyene units that can quench singlet oxygen, neutralize thyl radicals and stabilize peroxy radicals. Vitamin A and carotenoids can autoxidize when O2 tension increases, and thus are the most effective antioxidants at low oxygen tensions (3). Vitamin A and carotenoids are important dietary factors for reducing the incidence of heart disease (4). However, the role of vitamin A on electrocardiograms, infarct size and membrane-bound enzymes in MI has not been reported to date. Addressing this shortfall the present study aimed to investigate the effect of vitamin A on ISO-induced MI in rats.

MATERIALS AND METHODS

Chemicals. Superoxide dismutase (SOD), crystalline beef liver catalase (CAT), 1,1,3,3-tetrahydroxy-propane, glutathione (GSH), epinephrine hydrochloride, and triphenyl tetrazolium chloride (TTC) were purchased from Sigma Aldrich, USA. Tris buffers, thiobarbituric acid, sucrose, Evans blue, and trichloroacetic acid were purchased from Himedia Lab., Mumbai, India. A diagnostic kit for creatine kinase (CK) and CK-MB was purchased from Reckon Diagnostics, Baroda. All other chemicals used in the study were of laboratory grade.

Experimental design. All experiments and protocols described in the present study were approved by the Institutional Animal Ethics Committee of Babaria Institute of Pharmacy and with permission from the Committee for the Purpose of Control and Supervision of Experiments on Animals (No. 1029/a/07/CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Wistar rats of either sex (200–250 g) were randomly divided into the three groups (n=6).
Table 1. Effect of vitamin A on body weight, heart weight, electrocardiography, cardiac injury markers and on bio-markers of oxidative stress.

<table>
<thead>
<tr>
<th>Groups</th>
<th>NC</th>
<th>ISO</th>
<th>ISO+VIT A</th>
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<tbody>
<tr>
<td><strong>Effect of vitamin A on body weight, heart weight and on electrocardiography</strong></td>
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<tr>
<td>Body weight (g)</td>
<td>227.6±2.126</td>
<td>218.7±2.394</td>
<td>222.4±2.478</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.96±0.009</td>
<td>1.18±0.015*</td>
<td>1.07±0.004**</td>
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<tr>
<td>ST segment (s)</td>
<td>0.03±0.003</td>
<td>0.11±0.008*</td>
<td>0.03±0.0016**</td>
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<tr>
<td><strong>Effect of vitamin A on cardiac injury markers</strong></td>
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<tr>
<td>Troponin I (mcg/L)</td>
<td>0.006±0.0006</td>
<td>7.88±0.41*</td>
<td>5.01±0.33**</td>
</tr>
<tr>
<td>CK (IU/L)</td>
<td>297±4.35</td>
<td>625.7±4.77*</td>
<td>608.1±3.64*</td>
</tr>
<tr>
<td>CK-MB (IU/L)</td>
<td>43.53±2.2</td>
<td>120.5±1.42*</td>
<td>102.4±5.65**</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>923.8±11.28</td>
<td>1.413±8.75*</td>
<td>1.37±6.82*</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>35.28±0.95</td>
<td>164.8±7.73*</td>
<td>113.9±11.14*</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>87.68±2.32</td>
<td>301.6±2.43*</td>
<td>284.6±3.77**</td>
</tr>
<tr>
<td><strong>Effect of vitamin A on bio-markers of oxidative stress</strong></td>
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<tr>
<td>MDA (nmol/g of tissue)</td>
<td>22.0±0.51</td>
<td>27.84±0.35*</td>
<td>24.01±0.51**</td>
</tr>
<tr>
<td>GSH (µg/g of tissue)</td>
<td>27.9±2.4</td>
<td>12.2±0.87*</td>
<td>24.3±2.15</td>
</tr>
<tr>
<td>SOD (U/g of tissue)</td>
<td>27.20±2.38</td>
<td>12.84±1.03*</td>
<td>20.17±1.95*</td>
</tr>
<tr>
<td>CAT (µmol H₂O₂ consumed/min/g of tissue)</td>
<td>232.1±5.08</td>
<td>135.9±3.42*</td>
<td>169.1±2.69**</td>
</tr>
</tbody>
</table>

Values are mean±SE (n=6), analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison tests. *p<0.001 for chance differences vs. NC, $p<0.05$, **p<0.01, $$$p<0.001$ for chance differences vs. ISO.

Group 1: Animals received standard a laboratory diet and normal saline (NC); Group 2: Animals were injected with isoproterenol (150 mg/kg/d, s.c.) at an interval of 24 h for 2 d (ISO) (5); Group 3: Animals received vitamin A treatment (4,500 IU/kg/d, orally) for 7 d and isoproterenol (150 mg/kg/d, s.c.) was injected on the 6th and 7th day (ISO+VIT A).

**Electrocardiography.** Rats were anaesthetized under light ether anesthesia 48 h after the first dose of isoproterenol and electrodes were inserted under the skin in lead II position. ECG recordings were made using a Student physiograph. At the end of the experiments animals were euthanized and blood and tissues samples were collected for various biochemical analyses.

**Determination of myocardial infarct size and histopathology.** The animals were sacrificed and the heart was quickly removed and stained with Evans blue solution (2% in PBS) and TTC (2% in PBS). The area free from blue stain was the area at risk. The portion seen red or pink was salvaged myocardium and the unstained whitish portion was the infarct zone. Infarct size (IS) was expressed as a percentage of the AAR (% AAR/AAR) (6). For histopathological evaluations the heart was fixed in 10% buffered formalin. The fixed tissues were stained with hematoxylin and eosin. The sections were examined under a light microscope (Olympus BX10, Tokyo, Japan) for histopathological changes and photomicrographs (Olympus DP12 camera, Japan) were taken.

**Biochemical estimation in serum.** The collected serum was used for the estimation of troponin I, creatine kinase-MB (CK-MB), creatine kinase, lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) using commercially available standard kits.

**Measurements of tissue defense markers and lipid peroxidation.** The excised heart tissue was homogenized in chilled Tris-HCl buffer (0.1 m, pH 7.4). The homogenate was then centrifuged at 10,000 × g at 0°C using the Remi C-12 high speed cooling centrifuge. The clear supernatant obtained was used for the assay of superoxide dismutase (SOD) (7), catalase (CAT) (8), reduced glutathione (GSH) (9), and lipid peroxidation (MDA) (10).

**Measurements of membrane bound ATPases.** The sediment after centrifugation was resuspended in ice cold Tris-HCl buffer (0.1 m, pH 7.4) and used for the estimations of membrane-bound enzymes. The activity of membrane bound enzymes such as Na⁺-K⁺-ATPase (11), Ca²⁺-ATPase (12), and Mg²⁺-ATPase (13) were assayed as per previously reported methods.

**Myeloperoxidase assay.** To measure MPO activity, samples were mixed on ice and homogenized in 10 mL of ice-cold 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (HETAB). The homogenates were then sonicated and centrifuged for 20 min at 12,000 × g. MPO activity was measured spectrophotometrically as follows: 0.1 mL of supernatant was combined with 2.9 mL of 50 mM phosphate buffer in 0.0005% H₂O₂. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO activity is defined as the change in absorbance per minute at room temperature, in the final reaction (14).

**Statistical analysis.** All the values are expressed as mean±SE. Statistical significance between more than two groups was tested using one-way ANOVA followed by Bonferroni multiple comparisons test using a computer-based fitting program (Prism, Graph pad 5). Differences were considered to be statistically significant when p<0.05.
RESULTS

Effect of vitamin A on general parameters and on electrocardiography

Rats pretreated with vitamin A demonstrated significant (p<0.001) reduction in the heart weight when compared with the ISO group. Vitamin A pretreatment in isoproterenol-treated rats showed significant decrease (p<0.001) in the ST-segment when compared with isoproterenol-alone-treated rats (Table 1).

Effect of vitamin A on myocardial infarct size and on histopathology

Vitamin A-treated rats demonstrated significant (p<0.05) reduction in infarct size when compared with the isoproterenol group of rats, suggesting that vitamin A markedly attenuated myocardial injury (Fig. 1A). The myocardium of the normal control showed adequate cellularity and normal morphology. Histopathologic sections of the heart injected with isoproterenol displayed necrosis of muscle fibers with inflammatory cell infiltration, edema and fragmentation of muscle fibers, which indicates involvement of oxidative stress and inflammatory processes in isoproterenol-induced myocardial injury. Vitamin A-treated rats preserved morphology compared to the diseased group of rats (Fig. 1B–D).

Effect of vitamin A on cardiac injury markers

Vitamin A pretreatment in isoproterenol-treated animals showed significant reduction in the serum level of troponin I (p<0.001), CK-MB (p<0.01), CK (p<0.05), LDH (p<0.05), AST (p<0.01), and ALT (p<0.01) when compared with ISO-alone-treated rats (Table 1).

Effect of vitamin A on biomarkers of oxidative stress

Vitamin A treatment demonstrated a significant decrease (p<0.001) in MDA level when compared with the isoproterenol group. A significant restoration was observed in the level of GSH (p<0.01), SOD (p<0.05) and CAT (p<0.001) in the vitamin A + isoproterenol-treated group when compared with the ISO-alone-treated group (Table 1).

Effect of vitamin A on membrane bound enzymes

Vitamin A+ISO-treated animals demonstrated significantly increased Na⁺-K⁺-ATPase and Mg²⁺-ATPase when compared with isoproterenol-alone-treated rats (p<0.05, p<0.01, respectively). The vitamin A+ISO treated group demonstrated a significantly decreased (p<0.01) level of Ca²⁺-ATPase when compared with isoproterenol-alone-treated rats (Fig. 2A–C).

Effect of vitamin A on inflammatory markers

Serum concentrations of CRP, which was used as a marker of cardiac injury, was significantly reduced (p<0.05) subsequent to pretreatment with vitamin A.
when compared with isoproterenol-alone-treated rats. Myeloperoxidase activity, an accepted indicator of neutrophil infiltration, in vitamin A-pretreated rats demonstrated significantly \( p < 0.01 \) lowered MPO activity when compared with the ISO-alone-treated group (Fig. 2D and E).

**DISCUSSION**

Catecholamine plays an important role as a regulator of myocardial contractility and metabolism. Various mechanisms of isoproterenol-induced myocardial infarction have been reported. Isoproterenol acts both on \( \beta_1 \) and \( \beta_2 \) adrenoceptors, activation of which leads to positive inotropic and chronotropic effects. Thus, isoproterenol produces relative ischemia due to myocardial hyperactivity and coronary hypotension (15). Other probable mechanisms include increased cyclic adenosine monophosphate (16), increased intracellular Ca\(^{2+}\) overload (17), depletion of high-energy phosphate stores and oxidative stress (18, 19). Increased generation of cytotoxic free radicals, due to the autoxidation metabolic products of isoproterenol, is one of the well recognized mechanisms of isoproterenol-induced myocardial necrosis. Following isoproterenol administration, the heart weight increased significantly, with relatively unchanged body weight. The increase in heart weight might be attributed to increased water content, edematous intramuscular space and increased protein content (20). These results are in consistent with a previous report (21), which has observed extensive edematous intramuscular space, accumulation of mucopolysaccharides and cellular infiltration after 4 h of induction of myocardial infarction. It has been proposed that a 1% increase in myocardial water content could be expected to result in possibly a 10% reduction in myocardial function (22). Pretreatment with vitamin A brings down the heart weight, indicative of its protection of the myocardium against infiltration and it also could be due to the decrease in water content of the myocardium. Isoproterenol administration also resulted in an increased ST segment, which is consistent with the observations of earlier reports. Vitamin A pretreatment in isoproterenol-treated rats prevented the pathological alterations in the ECG, suggestive of its cell membrane protective effect. We observed a reduction in infract size with vitamin treatment that further strengthened the ECG analysis and might be due to the radical-scavenging properties of vitamin A. Histopathologic sections of the heart injected with isoproterenol displayed necrosis of muscle fibers with inflammatory cell infiltration, edema and fragmentation of muscle fibers, which indicates involvement of oxidative stress and inflammatory processes in isoproterenol-induced myocardial injury. Vitamin A treatment in ISO-injected rats resulted in moderate changes in histopathologic alterations, confirmed by reduction in infract size, which we observed.

Cytosolic enzymes like troponin-I, CK-MB, LDH, AST and ALT which serve as diagnostic markers, leak out from the damaged tissue to the bloodstream when the cell membrane becomes permeable or ruptured. The amount of these cellular enzymes in serum reflects the alterations in plasma membrane integrity and permeability. In the present study isoproterenol-injected rats showed a significant elevation in the levels of these
marker enzymes in serum that indicated isoproterenol-induced necrotic damage of the myocardium and leakiness of the plasma membrane (23). Vitamin A pretreatment resulted in the lowered activity of the marker enzymes in serum, confirming that vitamin A restored membrane integrity, thereby restricting the leakage of these enzymes.

Free radical-scavenging enzymes such as SOD, catalase and GSH are the first line of cellular defense against oxidative stress, eliminating reactive oxygen radicals such as superoxide and hydrogen peroxide and preventing the formation of more deteriorating hydroxyl radicals. The second line of defense comprises nonenzymatic scavengers, viz. ascorbic acid, α-tocopherol, ceruloplasmin, and sulphhydryl-containing compounds, which scavenge residual free radicals escaping decomposition by the antioxidant enzymes (24). The equilibrium between antioxidants and free radicals is an important process for the effective removal of oxidative stress in intracellular organelles. However, in pathological conditions like myocardial infarction, the generation of reactive oxygen species can dramatically disturb this balance with an increased demand on the antioxidant defense system. Isoproterenol autoxidation leads to generation of enormous amounts of reactive oxygen species. These reactive oxygen species may attack polyunsaturated fatty acids (PUFAs) within membranes, forming peroxyl radicals. These radicals then attack adjacent fatty acids within membranes causing a chain reaction of lipid peroxidation (25). Lipid peroxidation is an important pathogenic event in myocardial necrosis and accumulation of lipid hydroperoxides reflects damage of the cardiac constituents. MDA is a major lipid peroxidation end product; increased MDA content may contribute to increased generation of free radicals and decreased activities of the antioxidant defense system (26). In the present study isoproterenol administration resulted in marked elevation in LPO, expressed as MDA content, which indicated generation of oxidative stress. Vitamin A pretreatment showed a significant decrease in the level of myocardial MDA content, which can be attributed to the potent antioxidant activity of vitamin A against isoproterenol autoxidation-generated free radicals (27). The decrease in the activities of GSH, SOD and CAT in isoproterenol-induced myocardial infarction may be due to increased generation of reactive oxygen species, such as superoxide and hydrogen peroxide, which in turn leads to inhibition of these enzymes. Furthermore, decreased activities of these antiperoxidative enzymes result in decreased removal of superoxide radicals and hydrogen peroxide radicals. It is well known that superoxide anions can participate in a metal ion-mediated Haber-Weiss reaction that converts hydrogen peroxide to very toxic hydroxyl radicals (28, 29). Vitamin A treatment restored the levels of GSH, SOD and CAT in cardiomyocytes. This could be due to the direct free radical-scavenging effect of vitamin A or an indirect effect through its ability to augment the activities of antioxidant enzymes (30) or its ability to protect antioxidant enzymes from oxidative damage (31).

ATPases are intimately associated with the plasma membrane and participate in the energy requiring translocation of sodium, potassium, calcium and magnesium (32). The present study showed decreased activities of Na⁺-K⁺-ATPase and Mg²⁺-ATPase and increased activity of Ca²⁺-ATPase in isoproterenol injected rats, which is in line with the previous report. Na⁺-K⁺-ATPase is a lipid-dependant enzyme containing an -SH group, and increased lipid peroxidation leads to oxidation of the protein and in turn inactivates the enzymes (33). The inhibition of Na⁺-K⁺-ATPase activates the Na⁺ and Ca²⁺ ion exchange mechanism in the myocardium. The increased activity of Ca²⁺-ATPase may be due to activation of adenylyl cyclase. Vitamin A pretreatment showed increased activities of Na⁺-K⁺-ATPase and Mg²⁺-ATPase and decreased activity of Ca²⁺-ATPase which may be attributed to the direct antioxidant activity and antioxidant enzyme-stimulatory effect of vitamin A which thereby protects the -SH group from oxidative damage (33).

Myeloperoxidase, an enzyme that is associated with the azurophilic granules of neutrophils, has been measured to form an index of the infiltration of neutrophils into inflamed tissue. Myeloperoxidase, a green hemoprotein enzyme, can use H₂O₂ generated by NADPH oxidase to oxidize halides (Cl⁻, Br⁻ and I⁻) to their corresponding hypohalous acids (an additional class of active oxygen metabolite) (34). In the present study isoproterenol administration showed a significant increase in myocardial MPO levels and C-reactive proteins, indicative of necrosis-induced inflammation of myocardial tissue and neutrophil infiltration. In the vitamin A-pretreated group, significantly decreased levels of inflammatory markers indicated that the vitamin A pretreatment suppressed the neutrophil infiltration to the injured myocardium and the release of inflammatory cytokines.

CONCLUSION

In conclusion, vitamin A attenuated the myocardial infarction and prevention was shown via a membrane stabilization effect (via restricting the leakage of cardiac injury markers from the membrane and maintaining the integrity of the membrane), reducing the oxidative stress, and preventing neutrophil infiltration.

Acknowledgments

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