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Protective effects of vanillic acid on electrocardiogram, lipid peroxidation, antioxidants, proinflammatory markers and histopathology in isoproterenol induced cardiotoxic rats

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ABSTRACT

Myocardial infarction affects a large proportion in the world. This study aims to evaluate the protective effects of vanillic acid in isoproterenol induced cardiotoxic rats. Male Wistar rats were pretreated with vanillic acid (5 mg and 10 mg/kg) daily for 10 days. After pretreatment, isoproterenol (100 mg/kg) was subcutaneously injected to rats at an interval of 24 h for 2 days to induce cardiotoxicity. Isoproterenol induced cardiotoxic rats showed significant increase in serum cardiac troponins, heart lipid peroxidation and significant decrease in the heart antioxidants. In addition, isoproterenol induced group electrocardiogram showed an elevation in ST segments and increased expressions of interleukin-1β, interleukin-6 and tumor necrosis factor-α genes in the myocardium. Pretreatment with vanillic acid showed significant protective effects on cardiac troponins, lipid peroxidation, antioxidant system, electrocardiogram and expressions of interleukin-1β, interleukin-6 and tumor necrosis factor-α gene in the heart of isoproterenol induced cardiotoxic rats. Histopathology of myocardium correlated with these biochemical findings. The *in vitro* study also revealed that vanillic acid is a potent free radical scavenger. Thus, vanillic acid exerts protective effects in isoproterenol induced cardiotoxic rats due to its free radical scavenging, antioxidant and anti-inflammatory properties. Our study also showed that pretreatment with vanillic acid at the dose of 10 mg/kg was highly effective than 5 mg/kg.

1. Introduction

Myocardial infarction affects a high proportion of the population. It is a leading cause of mortality and disability of adults in urban and rural India and occurs at younger age than in Western populations (World Health Organization, 2005). Myocardial infarction is the acute condition of necrosis of the myocardium that occurs as a result of imbalance between coronary blood supply and myocardial demand (De Bono and Boon, 1992). Pharmacological induction of myocardial infarction by subcutaneous administration of isoproterenol in animals like rats has been found to be convenient because of relatively smaller size of coronary arteries (Rona et al., 1959). Isoproterenol hydrochloride, a synthetic catecholamine, and β-adrenergic agonist causes severe stress in the myocardium resulting in infarct like necrosis of the heart muscle (Sushamakumari et al., 1989). Furthermore, free radicals could initiate the peroxidation of membrane bound poly unsaturated fatty acids, leading to both functional and structural myocardial injuries (Thompson and Hess, 1986).

Many modern drugs are effective in preventing cardiovascular diseases, but their use is often limited because of their side effects. Phenolic compounds form a substantial part of plant foods. Most of these phenolic compounds are antioxidants *in vitro* and antioxidants may protect against cardiovascular diseases. Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids. Phenolic acids have received much attention because of their role in the prevention of many human diseases, particularly atherosclerosis and cancer due to their antioxidant properties (Mattila and Kumpulainen, 2002). Vanillic acid (4-hydroxy-3-methoxybenzoic acid) is a phenolic derivative of edible plants and fruits. It has antibacterial (Rai and Maurya, 1996) and antimicrobial (Delaquis et al., 2005) properties. Vanillic acid also exhibits chemopreventive effect in experimentally induced carcinogenesis (Tsuda et al., 1994).

Oxidative stress has been implicated in the pathogenesis of cardiovascular diseases. Therapeutic intervention that could improve impaired antioxidant defense mechanisms or diminish free radical production in the ischemic myocardium has been of great interest. Inflammatory responses are involved in the complex repairing process after myocardial infarction (Diwan et al., 2003). In this context, myocardial expression of cytokines may contribute to the pathogenesis of heart failure (Paulus, 2000). Elevated levels of pro-inflammatory cytokines, namely interleukin-1β, interleukin-6 and tumor necrosis factor-α have been reported in human heart failure (Tsutamoto et al., 2000) and the potential role of these cytokines in the development and progression of disease in the failing heart is a

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topic of current interest (Baumgarten et al., 2000). In our previous phase of experiment, we reported the preventive effects of vanillic acid on lipids, bax, bcl-2 and myocardial infarct size in isoproterenol induced rats (Stanely Mainzen Prince et al., 2011). In this phase, we evaluated the protective effects of vanillic acid on troponin-T and I, ECG pattern, antioxidant system, lipid peroxides and histopathology of the heart in isoproterenol induced cardiotoxic rats. Also, the *in vitro* study on the effects of vanillic acid on scavenging superoxide and hydroxyl radicals and the myocardial expressions of pro-inflammatory cytokine genes were studied to know the underlying mechanism of action of vanillic acid.

2. Materials and methods

2.1. Drug and chemicals

Vanillic acid, isoproterenol hydrochloride, nitroblue tetrazolium, phenazine methosulfate, butylated hydroxy toluene, 1-chloro-2, 4-dinitro benzene, p-phenylene diamine, reduced glutathione and oxidized glutathione were purchased from Sigma Chemical Co., St. Louis, MO, USA. Flavin adenine dinucleotide, thiobarbituric acid, ethylene diamine tetra acetic acid and trichloro acetic acid were purchased from Himedia, Mumbai, India. All the other chemicals used were of analytical grade.

2.2. Experimental animals

Male albino Wistar rats (*Rattus norvegicus*) weighing 180–200 g, obtained from The Central Animal House, Rajah Muthiah Institute of Health Sciences, Annamalai University, Tamil Nadu, India were used in this study. They were housed in polypropylene cages (47 × 34 × 20 cm) lined with husk, renewed every 24 h under a 12:12 h light and dark cycle at around 22 °C. The rats had free access to tap water and food. The rats were fed on a standard pelleted diet (Pranav Agro Industries Ltd., Maharashtra, India). The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India and approved by the Animal Ethical Committee of Annamalai University (Proposal No: 699; Approval Date: 11-01-2010).

2.3. Preparation of cardiotoxic rats and experimental design

Isoproterenol (100 mg/kg) was dissolved in saline and subcutaneously injected to rats at an interval of 24 h for 2 days. The development of cardiotoxicity at this dose was confirmed by elevated levels of troponins in Wistar rats. The rats were randomly divided into six groups of ten rats each. Two rats in each group were used for histopathological study and another two rats in each group were used for Reverse Transcription Polymerase Chain Reaction study. Group I: Normal control rats; Group II: Rats were orally treated with vanillic acid (5 mg/kg) daily for 10 days by an intragastric tube; Group III: Rats were orally treated with vanillic acid (10 mg/kg) daily for 10 days by an intragastric tube; Group IV: Rats were subcutaneously injected with isoproterenol (100 mg/kg) at an interval of 24 h for 2 days (on 11th and 12th day); Group V: Rats were pretreated with vanillic acid (5 mg/kg) orally by an intragastric tube daily for 10 days and then subcutaneously injected with isoproterenol (100 mg/kg) at an interval of 24 h for 2 days (on 11th and 12th day); Group VI: Rats were pretreated with vanillic acid (10 mg/kg) orally by an intragastric tube daily for 10 days and then subcutaneously injected with isoproterenol (100 mg/kg) at an interval of 24 h for 2 days (on 11th and 12th day); and Normal control and isoproterenol control rats were administered saline orally by an intragastric tube daily for 10 days of the experimental period. Vanillic acid was dissolved in saline and administered to rats 1 ml orally by an intragastric tube daily for a period of 10 days. The dose and duration of vanillic acid pretreatment were based on our earlier phase of the study (Stanely Mainzen Prince et al., 2011).

At the end of the experimental period, after 12 h of second isoproterenol injection, (*i.e.*, on 13th day) all the rats were anesthetized by pentobarbital sodium (60 mg/kg body weight) and then sacrificed by cervical decapitation. Blood was collected in tubes without anticoagulant and serum was separated by centrifugation. Heart tissue was excised immediately and rinsed in ice-chilled saline and used for various studies.

2.4. Processing of heart tissue

A known weight of the heart tissue was homogenized in 5mL of appropriate buffer for the assays of superoxide dismutase, catalase, glutathione peroxidase and estimations of thiobarbituric acid reactive substances and reduced glutathione. The heart tissue homogenates were centrifuged and the supernatants were used for the assays/estimations of various biochemical parameters. All enzyme activities were assayed immediately.

2.5. Electrocardiogram

The electrocardiogram-patterns were recorded by 3-lead-16 channel polygraph (Biopac Systems Inc., USA). Electrocardiographic recordings were made in anesthetized rats. The types of alterations (ST-segment elevation) in the experimental rats were recorded.

2.6. Measurement of troponins T and I, antioxidant system and lipid peroxidation products

The levels of serum cardiac troponins T and I were measured using standard kits by Chemiluminescence immunoassay (Roche Diagnostics, Switzerland). Superoxide dismutase, catalase and glutathione peroxidase activities in the heart were assayed by the methods of Kakkar et al. (1984), Sinha (1972) and Rotruck et al. (1973) respectively. Estimation of reduced glutathione in the heart was done by the method of Ellman (1959). Thiobarbituric acid reactive substances in the heart were measured by the method of Fraga et al. (1988). The content of protein in the heart tissue homogenate was determined by the method of Lowry et al. (1951).

2.7. Ribonucleic acid isolation and Reverse Transcription Polymerase Chain Reaction analysis of cardiac proinflammatory cytokines interleukin-1β, interleukin-6 and tumor necrosis factor-α gene expressions

Reverse Transcription Polymerase Chain Reaction was performed to validate the expressions of interleukin-1β, interleukin-6 and tumor necrosis factor-α genes in the heart. Heart samples were immediately removed from each group after sacrificing animals and left ventricular area was removed and chopped into small pieces. To measure myocardial gene expression, total ribonucleic acid was extracted from the heart tissues by Medox-Easy TM spin column total minipreps kit purchased from Medox Biotech India Private Limited, Chennai, India and the isolated ribonucleic acid was treated with RNase-free DNase I (Medox) at 37 °C for 30 min for the removal of deoxy ribonucleic acid. Then the samples were incubated at 30 °C for 60 min. Reactions were stopped by heating at 95 °C for 10 min. Reverse transcription was carried out using 2 μg of total ribonucleic acid and 200 units of M-MuLV reverse transcriptase (Medox Biotech India Private Limited, Chennai, India). Amplification was performed using Medox Polymerase Chain Reaction Master Mix in a volume of 25 μL.

Polymerase chain reaction was carried out using a hot start method by adding 2 μL of cDNA product to 18 μL of polymerase chain reaction buffer containing 67 mM Tris (pH 8.8), 1.5 mM magnesium chloride, 16.6 mM ammonium sulfate, mixed dNTPs at 200 μM, 125 U/