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RESEARCH ARTICLE

VANILLIC ACID PREVENTS LYSOSOMAL MEMBRANE DAMAGE IN ISOPROTERENOL INDUCED MYOCARDIAL INFARCTION IN RATS

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ABSTRACT

This manuscript reveals the preventive potential of vanillic acid on lysosomal damage in isoproterenol induced myocardial infarction in rats. Wistar rats were pretreated with vanillic acid (10mg/kg) daily for a period of 10 days. After the pretreatment period isoproterenol (100mg/kg body weight) was subcutaneously injected to rats at an interval of 24h for two days to induce MI (11th and 12th days). The levels of cardiac markers were increased significantly in the serum of ISO-induced MI rats. The levels of lipid peroxides TBARS was significantly increased in the heart lysosomal fraction of the heart of ISO-induced MI rats. In addition, the activities of lysosomal enzymes in the serum and heart of ISO- induced MI rats were increased significantly. Pretreatment with vanillic acid prevented the changes in the levels of serum cardiac markers, lipid peroxidation product and the activities of lysosomal enzymes in isoproterenol induced MI rats.

INTRODUCTION

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. Isoproterenol is a synthetic catecholamine and β-adrenergic agonist, which has been found to cause severe stress in the myocardium resulting in infarct like necrosis of heart muscles (Wexler, 1978). Isoproterenol is known to generate free radicals as well as accumulation of lipid peroxides has been recognized as one of the possible biochemical mechanisms of myocardial damage (Sushmakumari *et al*, 1989). Experimental induction of MI by ISO in animals is a well established and common model to study the protective role of different cardioprotective agents, since it mimics the clinical conditions of MI due to ischemia in humans (Anandan *et al*, 2007).

Pharmacological induction of MI by subcutaneous administration of ISO in animals like rats has been found to be convenient because of relatively smaller size of coronary arteries. The advantages of ISO induced MI that occur as a result of intense inotropic and chronotropic actions of ISO compared to physical occlusion of the coronary artery as a less invasion accomplished without the complicating factors of general anesthesia and lack of foreign body remaining in the heart. Lysosomal enzymes are important mediators of acute MI. Their release into the cytoplasm stimulates the formation of inflammatory mediators, such as oxygen radicals and prostaglandins (Prabhu, 2009). Lysosomal destabilization may be prevented either by inhibition of cellular peroxidation or by prevention of iron catalyzed oxidative reactions (Ganesan, 2009).

Cathepsins are lysosomal proteases possibly involved in autophagic digestion of discrete areas of cytoplasm and myofibrillary and mitochondrial proteins. There is a report showing that the leakage of hydrolytic enzymes from lysosomes after coronary occlusion may be a causative factor in the development of myocardial cellular injury (Ravens and Gudbjarnason, 1969). Cathepsin-D is a lysosomal aspartic protease present in all animal cells. The release of β-glucuronidase is the best indicator of lysosomal membrane integrity (Ravichandran *et al*, 1990). ISO induced MI causes increased lysosomal hydrolase activities and declined activities of lysosomal enzymes in lysosomal fraction of ischemic heart homogenate (Sathish *et al*, 2003).

Diet and nutrition have substantial impact on reducing the incidence of CVDs. The most active principles having antioxidant property found in botanical products are not only vitamins but also polyphenols, organosulfur compounds and flavonoids. Most of these phenolic compounds are antioxidants *in vitro* and antioxidants may protect against CVDs. Vanillic acid (4-hydroxy-3-methoxybenzoic acid) is a phenolic derivative of edible plants and fruits. The highest amount of

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vanillic acid in plants is found in the root of *Angelica sinensis* (Stanely Mainzen Prince *et al*, 2011a). Vanillic acid possesses antibacterial (Rai and Maurya, 1996), antifilarial (Varma *et al*, 1993) and antimicrobial (Delaquis *et al*, 2005) properties.

Previously, we reported preventive effects of vanillic acid on serum lipid peroxidation, serum and heart antioxidants, serum lipids and pro inflammatory markers in myocardial infracted rats were reported earlier (Stanely Mainzen Prince *et al*, 2011b). In continuation of our research work on vanillic acid, the present investigation was undertaken to study the preventive effects of vanillic acid in lysosomal damage in isoproterenol induced myocardial infarcted rats.

MATERIALS AND METHODS

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 (3rats/cage) in polypropylene cages (47x34x20 cm) lined with husk, renewed every 24 h under a 12:12 h light and dark cycle at around 22^{o}C. The rats had free access to tap water and food. They were fed on a standard pellet 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: 865).

Chemicals

Vanillic acid, isoproterenol hydrochloride, α-N-benzoyl–DL-arginine-p-nitroanilide hydrochloride, Folin's phenol reagent, thiobarbituric acid reactive substances, phenazine methosulphate, nicotinamide adenine dinucleotide, sodium azide, and oxaloacetate were purchased from Sigma Chemical Co., St, and Louis, MO, USA. All the other chemicals and solvents used were of analytical grade.

Preparation of myocardial infarcted rats

Isoproterenol (100 mg/kg body weight) dissolved in saline was subcutaneously injected into rats at an interval of 24 h for 2 days.

Experimental design

The animals were grouped into four groups of six rats each. Group I: normal control rats; Group II :rats were orally treated with vanillic acid (10mg/kg body weight) daily for a period of 10 days by an intragastric tube; Group III: rats were subcutaneously injected with ISO (100 mg/kg body weight) at an interval of 24 h for 2 days (on 11th and 12th day); Group IV: rats were orally pretreated with vanillic acid (10mg / kg body weight) daily for a period of 10days by an intragastric tube and were subcutaneously injected with ISO at an interval of 24 h for 2 days (11th and 12th day). Vanillic acid was dissolved in saline and administered to rats orally by an intragastric tube daily for a period of 10 days. Normal control and ISO control rats were given saline alone orally daily for a period of 10 days by an intragastric tube. Twelve hours after the second dose of ISO injection (on 12th day), all the rats were anesthetized by pentobarbital sodium (60 mg / kg body weight) and then sacrificed by cervical decapitation. Blood was collected serum and plasma were separated by centrifugation and used for the estimation of various biochemical parameters.

Estimation of serum cardiac diagnostic markers and inflammatory marker

Serum LDH-isoenzymes were separated by the method of (McKenzie and Henderson, 1983) HsCRP was estimated by turbiodometry (Roche Diagnostics, USA).

Estimation of lipid peroxidation products in the heart lysosomal fraction

The levels of thiobarbituric acid reactive substances (TBARS) in the heart lysosomal fraction were estimated by the methods of Fraga *et al*, 1988.

Separation of subcellular fractions

The heart tissue samples were cut open and placed in isotonic saline to remove the blood. Then, the heart tissues were rinsed in ice cold 0.25 M sucrose at 4 °C. A portion of this preparation was used to determine the total activity. Another portion of the homogenate was subjected to differential centrifugation and the different fractions were separated as follows: structural proteins, nucleus, and cell debris at 600×g for 10 min; mitochondria at 5000×g for 10 min; lysosomes at 15,000×g for 10 min; microsomes at 120,000×g for 30 min and supernatant, the cytosol. Myocardial subfractions were treated with Triton X-100 (final concentration 0.2% v/v) in ice for 15 min prior to the determination of enzymic activities (Sathish *et al*, 2003).

Assay of lysosomal enzymes in the serum, heart and sub cellular fractions of the heart and sub cellular fractions

The activity of β-glucuronidase in the serum, total heart homogenate and subcellular fractions were assayed by the method of Kawai and Anno 1971. The activity of β-galactosidase in the serum and total heart homogentate were assayed by the method of Morris, 1982. The activity of cathepsin-B in the serum and total heart homogenate were assayed by the method of Barrett, 1972. The activity of cathepsin-D in the serum, total heart homogenate and subcellular fractions were determined by the method of Sapolsky *et al*, 1973.

In vitro study

The reducing power of vanillic acid was determined in vitro by the method of (Oyaizu, 1986).

Statistical Analysis

Statistical analysis was performed by One-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) using Statistical Package for the Social Science software package version 12.00. Results were expressed as mean ± standard deviation for six rats in each group. P values <0.05 were considered significant.

RESULT

Figure1 Shows the agarose gel electrophoretic separation of serum LDH-isoenzyme patterns of normal and ISO-induced myocardial infracted rats. ISO induction caused an increased

intensity of LDH-1 and LDH-2 isoenzyme bands compared to normal control rats. Pretreatment with vanillic acid (10mg/kg body weight) daily for a period of 10 days moderately decreased intensity of LDH-1 and LDH-2 isoenzyme bands in isoproterenol induced myocardial infarcted rats.

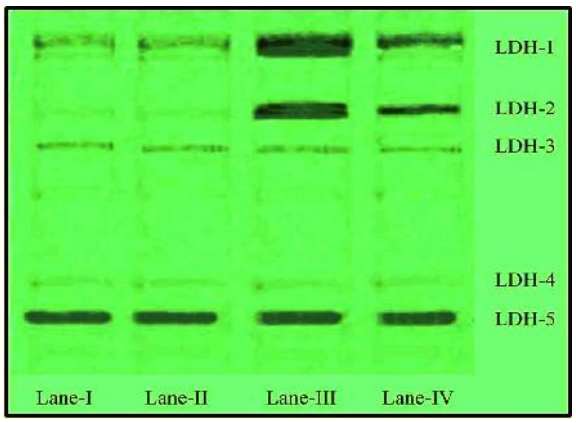

Figure 1 Levels of serum LDH-isoenzymes

Lane I: Normal control, Lane II: Vanillic acid (10mg/kg body weight), Lane III: ISO control (100mg/kg body weight), Lane IV: Vanillic acid (10mg/kg body weight) + ISO (100mg/kg body weight)

Figure 2 shows the effect of vanillic acid on the levels of serum HsCRP in normal and isoproterenol induced myocardial infarcted rats. Isoproterenol induced myocardial infracted rats showed significant ($p<0.05$) increase in the levels of inflammatory marker, HsCRP in the serum of isoproterenol induced myocardial infracted rats compared with isoproterenol alone induced myocardial infracted rats.

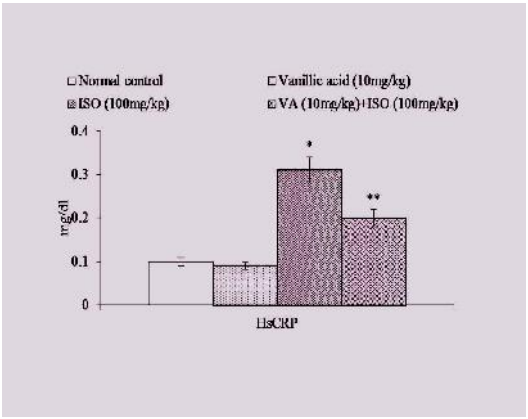

Figure 2 Levels of high sensitive C-reactive protein

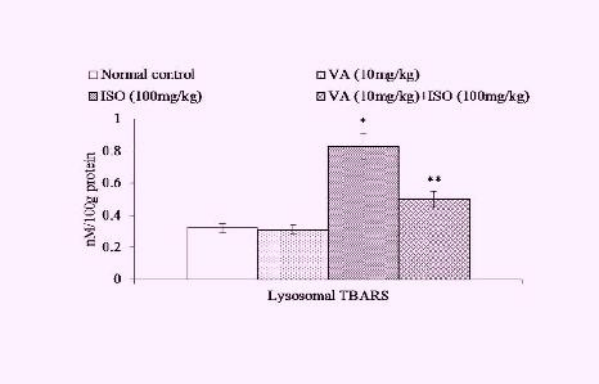

Figure 3 Levels of heart lysosomal TBARS

Each column is mean ± standard deviation for six rats in each group; *p < 0.05 as compared to normal control; ** p < 0.05 as compared to ISO control; Duncan's Multiple Range Test.

Each column is mean ± standard deviation for six rats in each group; *p < 0.05 as compared to normal control; ** p < 0.05 as compared to ISO control; Duncan's Multiple Range Test.Figure 3 reveals the concentrations of lipid peroxidation products such as thiobarbituric acid reactive substances in the heart lysosomal fraction of normal and experimental rats. Isoproterenol induced rats showed significantly increase in the levels of TBARS compared to normal control rats. Pretreatment with vanillic acid showed significantly ($P<0.05$) decreased concentration of TBARS in the isoproterenol induced rats compared with isoproterenol alone induced rats.

Figure 4 reveals the activity of β-glucuronidase in the lysosomal fraction of the heart in normal and experimental rats. Isoproterenol induced myocardial infracted rats showed significant ($P<0.05$) decrease in the activity of β-glucuronidase in the lysosomal fraction of the heart compared to normal control rats. Pretreatment with vanillic acid significantly ($P<0.05$) increased the activity of β-glucuronidase in the lysosomal fraction of the heart in the isoproterenol induced myocardial infracted rats compared with isoproterenol alone induced myocardial infarcted rats.

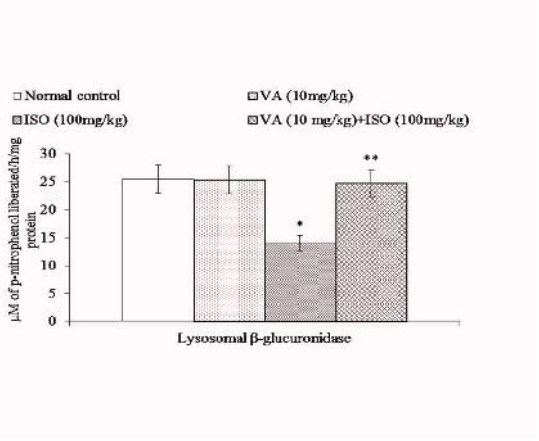

Figure 4 Activity of heart lysosomal β-glucuronidase

Each column is mean ± standard deviation for six rats in each group; *p < 0.05 as compared to normal control; ** p < 0.05 as compared to ISO control; Duncan's Multiple Range Test.

*Unit: β-glucuronidase - µmol of p-nitrophenol liberated / h /mg protein

Figure 5 reveals the activity of cathepsin-D in the lysosomal fraction of the heart in normal and experimental rats. Isoproterenol induced myocardial infracted rats showed significant ($P<0.05$) decrease in the activity of cathepsin-D in the lysosomal fraction of the heart compared to normal control rats. Pretreatment with vanillic acid significantly ($P<0.05$) increased the activity of β-glucuronidase in the lysosomal fraction of the heart in the isoproterenol induced myocardial infracted rats compared with isoproterenol alone induced myocardial infarcted rats. Each column is mean ± standard deviation for six rats in each group; *p < 0.05 as compared to normal control; ** p < 0.05 as compared to ISO control; Duncan's Multiple Range Test.

*Unit: Cathepsin-D: µmol of tyrosine liberated / h /100mg protein.

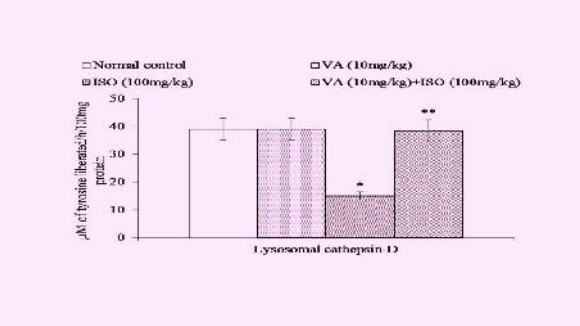

Figure 5 Activity of heart lysosomal cathepsin-D

Figure 6 reveals the in-vitro reducing power of vanillic acid at various concentrations. From the results of this study, the reducing power of vanillic acid increases with increasing concentrations of (10, 20, 30, 40, 50 & 60 µM). Vanillic acid at the concentration of 60 µM shows the highest reducing power compared to other five concentrations (10, 20, 30, 40 & 50). Thus, vanillic acid exhibits potent reducing activity.

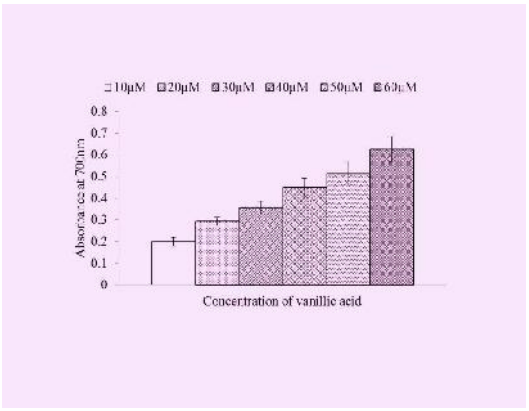

Figure 6 the *in vitro* Reducing power of vanillic acid

Columns are the average of triplicate experiments.

Table: 1 Isoproterenol treated rats showed a significant (P<0.05) increase in the activities of β-glucuronidase, β-galactosidase, cathepsin-B and D in the serum compared to normal control rats. Pretreatment with VA (10 mg/kg) (P<0.05) decreased the activities of these enzymes in the serum compared to isoproterenol control rats (Table 2).

Each value is mean ± SD for six rats in each group; Values not sharing a common superscript (a,b,c) differ significantly with each other (P<0.05;DMRT)

Each column is mean ± standard deviation for six rats in each group; *p < 0.05 as compared to normal control; ** p < 0.05 as compared to ISO control; Duncan's Multiple Range Test.

*Units: β-glucuronidase - µmol of p-nitrophenol liberated / h /mL; β-galactosidase- µmol of p-nitrophenol liberated/ h / mL. *Units: Cathepsin-B: µmol of p-nitrophenol liberated / h /mL; Cathepsin-D: µmol of tyrosine liberated / h /mL.

Table: 2 Isoproterenol treated rats showed a significant (P<0.05) increase in the activities of β-glucuronidase, β-galactosidase and cathepsin-B and cathepsin D in the heart compared to normal control rats. Pretreatment with VA (10 mg/kg) decreased (P<0.05) the activities of these enzymes in the heart compared to isoproterenol alone treated rats (Table 4).

Each value is mean ± SD for six rats in each group; Values not sharing a common superscript (a,b,c) differ significantly with each other (P<0.05;DMRT).

Table 2 Activities of heart β-glucuronidase, β-galactosidase, cathepsin-B and D

Each column is mean ± standard deviation for six rats in each group; *p < 0.05 as compared to normal control; ** p < 0.05 as compared to ISO control; Duncan's Multiple Range Test.

Table 1 Activities of serum β –Glucuronidase, β –Galactosidase, Cathepsin-B, and Cathepsin-D in normal and isoproterenol induced myocardial infarcted rats

Groups	Normal control	Normal+ Vanillic acid (10mg/kg)	ISO Control (100mg/kg)	Vanillic acid (10mg/kg) +ISO
β –Glucuronidase (µmol of p-nitrophenol liberated/h/mg protein)	12.5 ± 1.2 [a]	12.8 ± 1.1 [a]	22.7 ± 2.1 [b]	18.5 ± 1.7 [c]
β –Galactosidase (µmol of p-nitrophenol liberated/h/mg protein)	27.0 ± 2.5 [a]	27.3 ± 2.5 [a]	37.7 ± 3.7 [b]	32.4 ± 3.2 [c]
Cathepsin-B (µmol of p-nitrophenol liberated/h/mg protein)	18.6± 1.8 [a]	19.1 ± 1.9 [a]	35.0± 3.5 [b]	25.7± 2.5 [c]
Cathepsin-D (µmol of tyrosine liberated/h/mg protein)	25.1 ± 2.5 [a]	25.6 ± 2.4 [a]	37.5 ± 3.7 [b]	31.7 ± 3.1 [c]

Table 2 Activities of heart β –Glucuronidase, β –Galactosidase, Cathepsin-B, and Cathepsin-D in normal and isoproterenol induced myocardial infarcted rats

Groups	Normal control	Normal+ Vanillic acid (10mg/kg)	ISO Control (100mg/kg)	Vanillic acid (10mg/kg) +ISO
β –Glucuronidase (µmol of p-nitrophenol liberated/h/mg protein)	39.0 ± 3.7[a]	39.3 ± 3.7[a]	51.6 ± 5.1[b]	46.1 ± 4.5[c]
β –Galactosidase (µmol of p-nitrophenol liberated/h/mg protein)	46.3 ± 4.6 [a]	46.5 ±4.6 [a]	63.1 ± 6.2 [b]	56.6 ± 5.4 [c]
Cathepsin-B (µmol of p-nitrophenol liberated/h/mg protein)	29.5 ± 2.9 [a]	29.9 ± 2.9 [a]	42.4± 4.0 [b]	35.8± 3.5 [c]
Cathepsin-D (µmol of tyrosine liberated/h/mg protein)	31.3 ± 3.1 [a]	31.9 ± 3.1 [a]	51.4± 5.1 [b]	41.7 ± 4.0 [c]

*Units: β-glucuronidase - µmol of p-nitrophenol liberated / h /mg protein; β-galactosidase - µmol of p-nitrophenol liberated/ h / mg protein. *Units: Cathepsin-B: µmol of p-nitrophenol liberated / h /100mg protein; Cathepsin-D: µmol of tyrosine liberated / h /100mg protein.

Oral treatment with VA daily for a period of 10 days to normal control rats did not show any significant change in all the biochemical parameters studied.

DISCUSSION

Cardiac markers or cardiac enzymes are proteins from cardiac tissue found in the blood. These proteins are released into the blood stream when damage to the heart occurs, as in the case of MI. Jaffe *et al*, 1996 have suggested that MI can be differentiated from other types of tissue damage because the LDH-isoenzymes begin to rise in 12-24 h after MI, peaks after 2-3 days, and gradually dissipates over 5-14 days. An increase in serum LDH1 and LDH2 could have been released into the circulation because of necrosis caused by ISO. Vanillic acid ameliorated ISO induced myocardial damage by inhibiting the release of these isoenzymes from the heart into the serum.

The association between elevated C-reactive protein levels and cardiovascular events may be related to the degree of coronary plaque inflammation and instability (Pai *et al*, 2004, Zhang Xing-wei 2006). Hs-CRP, a marker of inflammation, is a strong predictor of future cardiovascular events in individuals both with and without overt cardiovascular diseases. The increased level of serum Hs-CRP, an important marker of inflammation observed in ISO-induced myocardial infracted rats reflects the extent of myocardial necrosis. The oral pretreatment with vanillic acid significantly reduced the elevated levels of serum Hs-CRP in the ISO-induced myocardial infracted rats. This effect revealed the anti-inflammatory property of vanillic acid.

ISO metabolism produces quinones, which react with oxygen to produce reactive oxygen species such as $O_2^{\bullet-}$ and $OH^{\bullet}$ thereby damaging myocardial cells (Rathore *et al*, 2000). Hence, we have addressed the question of how VA scavenged these free radicals *in -vitro*. Lipid peroxidation is one of the main manifestations of oxidative damage initiated by reactive oxygen species (ROS) and has been linked to the altered membrane structure and enzyme inactivation (Yogeeta *et al*, 2006). Increased levels of lysosomal lipid peroxidation is indicated by increased levels of lysosomal TBARS, which might be due to increased production of free radicals in ISO induced MI. Increased free radicals react with the lipid bilayer of intracellular organelles including lysosomal membrane integrity compromise may lead to an undesirable elevation of enzymes in both intra and extra-cellular space, and hence pave the way for cellular and tissue disorders, including apoptosis (Zhao and Xu 2000).

Lysosomal enzymes play an important role in the inflammatory process. In myocardial ischemia, the damage caused by the enzymes of lysosomal and mitochondrial origin and modification of tissue constituents by these enzymes play an important role in MI. ISO-induced MI results in increased lysosomal hydrolyses activity that may be responsible for tissue damage and infarcted heart (Ravichandran *et al*, 1990). Lysosomal enzymes are involved in the pathogenesis of MI. ROS are implicated as mediators of tissue injury in MI. Cytotoxic effects of ROS are related in its reaction with membrane lipids with subsequent membrane damage (Burton *et al*, 1990). ROS, in addition to the myocardial damaging effect, is also responsible for the release of lysosomal enzymes. Furthermore, the localization of acid hydrolases in cardiac myocytes is in the lysosome and the release of these enzymes from the lysosome to the cytosol extends to myocardial cellular injury and death in the ischemic state of the heart (Decker and Wildenthal 1978).

β- Glucuronidase release is an index of lysosomal membrane integrity (Michihara *et al*, 2005). Decreased stability of lysosomal membranes is the reason for the declined activities of β-Glucuronidase and cathepsin-D in the lysosomal fraction observed in ISO induced myocardial infarcted rats (Sathish *et al*, 2003). Vanillic acid pretreatment normalized the activities of β-Glucuronidase and cathepsin-D in the lysosomal fraction as well as normalized the activities of total lysosomal hydrolases, thereby enhancing the stability of lysosomes which might be due to the membrane stabilizing property of vanillic acid.

Furthermore, we investigated the in vitro reducing power of vanillic acid to know the mechanism of action. Previously, we reported the cardio protective effects of vanillic acid on oxidative stress, proinflammatory markers, lipids and lipoproteins in ISO- induced myocardial infarcted Wistar rats (Stanely Mainzen Prince *et al*, 2011b, Duke, 2002). Our study reveals that vanillic acid is a potent reductant and the reducing power reveals its antioxidant capacity. In conclusion, the in vivo and in vitro findings obtained from our study indicate that vanillic acid (10 mg / kg body weight) offers protection to the myocardium against ISO-induced oxidative stress in rats. This could be due to inhibition of lipid peroxidation system by its potent antioxidant effect.

Pretreatment with vanillic acid (10 mg/kg body weight) exhibits preventive effects in isoproterenol induced myocardial infarcted rats by modulating inflammatory marker, lipid peroxidation, lysosomal enzymes and cardiac markers. The intensities of serum LDH-isoenzymes (LDH-1 and LDH-2) were increased in ISO-induced myocardial infarcted rats. Vanillic acid pretreatment decreased the intensities of serum LDH-1 and LDH-2 isoenzymes in ISO-induced myocardial infarcted rats. These effects revealed the cardioprotective effects of vanillic acid. The level of serum HsCRP was increased in ISO-induced myocardial infarcted rats. Vanillic acid pretreatment (10mg / kg body weight) daily for a period of ten days significantly lowered the level of serum HsCRP in ISO-induced myocardial infarcted rats. Increased ROS causing tissue damage and lysosomal inactivation due to increased lipid peroxidation products is reduced by the antioxidant effect of VA by hindering the ROS generation due to ISO-induction. Pretreatment with VA decreased the activities of lysosomal enzymes by inhibiting lipid peroxidation in ISO treated rats.

The observed effects in this study might be due to the antihyperlipidaemic, antilipoperoxidative and membrane stabilizing properties of vanillic acid. Thus, vanillic acid protected the myocardium against ISO-induced MI in the rats. The activities of β–glucuronidase and cathepsin-D in the heart lysosomal fraction were lowered in ISO-induced myocardial infarcted rats. Vanillic acid pretreatment increased the activities

of these enzymes in the heart lysosomal fraction of ISO – induced myocardial infarcted rats. In this study, VA 60 µmol in vitro exhibits 72.6% of scavenging activity. The *in-vitro* study revealed the potent reducing property of vanillic acid.

CONCLUSION

Our study revealed that pretreatment with vanillic acid prevents the lysosomal damage in ISO induced myocardial infarcted rats. The *in vitro* study revealed the potent antioxidant property of vanillic acid. Administration of vanillic acid (10 mg/kg body weight) to normal control rats had no effect on the measured biochemical parameters. This study further strengthens the cardioprotective effects of vanillic acid. Also, vanillic acid was safe and highly effective in preventing cardiac dysfunction in rats, possibly due to its membrane stabilizing property. This study may be useful for the prevention of myocardial infarction.

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