Protective Efficacy of *Nardostachys jatamansi* (Rhizomes) on Mitochondrial Respiration and Lysosomal Hydrolases during Doxorubicin Induced Myocardial Injury in Rats

Rajakannu Subashini, Arunachalam Gnanapragasam, Subramanian Senthilkumar, Surinder Kumar Yogeeta, and Thiruvenkadam Devaki*

Department of Biochemistry, University of Madras, Guindy Campus, Chennai 600 025, Tamil Nadu, India

(Received August 12, 2006; Accepted October 24, 2006)

Doxorubicin is highly effective in treatment for several forms of cancer. However, doxorubicin induces a cumulative and dose dependent cardiomyopathy that has been ascribed to redox-cycling of the molecule on the mitochondrial complex 1 generating in the process of increased oxidative stress. In the search of new potential cardioprotective agents, the present study is directed to explore the effect of ethanolic extract of *Nardostachys jatamansi* on the mitochondrial and lysosomal damage induced by doxorubicin in rats. Heart mitochondria were isolated from rats treated with doxorubicin (15 mg/kg, ip) a single dose, exhibited depressed rates of state 3 respiration, low respiratory control ratio (RCR), decreased Oxidative Phosphorylation ratio, Adenosine Triphosphate content and cytochromes (c, c1, b, a3). In addition the doxorubicin given rats showed significant changes in the lysosomal enzymes (Cathepsin-D, Acid phosphatase, β-D-glucoronidase, β-D-galactosidase and β-N-acetyl glucosaminidase) and membrane bound phosphatases. Also myocardial damage, as assessed by ultrastructural changes showed loss of myofibrils, mitochondrial swelling, and cytoplasmic vacuolization. Pretreatment with *Nardostachys jatamansi* (500 mg/kg body weight orally) for seven days ameliorated the observed abnormalities and significantly prevented mitochondrial respiration, lysosomal integrity, membrane bound phosphatases and ultrastructural studies in doxorubicin induced rats. These findings suggest that the cardioprotective efficacy of *Nardostachys jatamansi* could be mediated possibly through its antioxidant effect as well as by the attenuation of the oxidative stress.

**Key words** —— *Nardostachys jatamansi*, doxorubicin, myocardial injury, oxidative phosphorylation, lysosomal enzymes, membrane bound phosphatase

**INTRODUCTION**

The anthracycline quinone doxorubicin (DOX) has been widely prescribed in the treatment of several human tumors and leukemias. The cytostatic effect of DOX is attributed to intercalation of the planar anthracycline ring structure into the double helix of nuclear DNA to interfere with DNA replication and transcription, especially in rapidly dividing cells. Unfortunately, the therapeutic success is limited by the development of a dose-dependent and irreversible cardiac toxicity that typically present as a dilated cardiomyopathy leading to congestive heart failure.\(^1\) DOX undergoes redox cycling on mitochondrial complex 1 to liberate highly reactive oxygen free radicals.\(^2\) Oxidative damage to the cardiac mitochondria and to the cardiomyocyte has been widely implicated as a primary cause for doxorubicin-induced cardiac toxicity.\(^3,4\) Cytofluorescence microscopy of heart tissue reveals nuclei and mitochondria as exclusive sites of DOX localization,\(^5\) and early stages of DOX cardiomyopathy are characterized by changes in both the morphology and function of heart mitochondria,\(^6,7\) including interference with mitochondrial calcium homeostasis at subclinical cumulative doses.\(^8\)

Lysosomes are membrane bound structures that contain hydrolytic enzymes capable of degrading most of the cellular constituents. In addition, lysosomes play a major role in secretion and transport processes. In myocardial ischaemia, the damage caused by the enzymes of lysosomal and mitochondrial origin and the modification of tissue con-
constituents by these enzymes play an important role. Gebbia et al., reported a marked change in the activity of the lysosomal enzymes in the hearts of mice treated with DOX, wherein the leakage of enzymes from the lysosomes accounts for doxorubicin cardiotoxicity.

The biochemical mechanism underlying the cardiotoxicity caused by DOX has not been fully elucidated. The evidence of evidence indicates that DOX cardiotoxicity is mediated by oxygen free radicals, and that the mitochondrial inner membrane is the primary site of free radical generation and tissue damage. In view of the implication of oxygen free radicals in the pathogenic process, one of the approaches to minimize DOX cardiotoxicity has been through the use of free radical scavengers and other antioxidants.

In recent times, many medicinal plants continue to provide valuable therapeutic agents for the treatment of cardiac diseases both in modern medicine and by the traditional system throughout the world. Since chemical compounds are known to have undesirable side-effects, the present study focused on natural products.

Nardostachys jatamansi (N. jatamansi) Jones DC (commonly called as Jatamansi) belongs to the family Valerianaceae of plant taxa. Various sesquiterpenes (such as Jatamansic acid and Jatamansone), lignans, and neolignans have been reported to be present in the roots of the plant. The decoction of the root is used in mental disorders, insomnia, and disorders of blood and the circulatory system, have demonstrated the protective effect of alcoholic extract of Jatamansi on the thiacetamide-induced liver damage in rats. The rhizomes of Jatamansi are used as a bitter tonic, stimulant, and antispasmodic, as well as to treat epilepsy, hysteric, cornea, palpitations, and convulsions. It has also been reported as anti-lipid peroxidative property of Jatamansi in iron induced lipid peroxidation. In the Unani system of medicine, N. jatamansi has been mentioned as a hepatotonic, cardiotonic, diuretic and analgesic.

The phytochemical analysis of the plant showed the presence of alkaloid, coumarins, lignan, neolignans, and sesquiterpenes. In addition, volatile oils like jatamansic oil and other chemical substances have been isolated from various fractions of roots and rhizomes of the herb. Experimental studies have demonstrated that oxygen generated free radicals derived from mitochondrial cells and enhanced lipid peroxidation play important roles in the pathogenesis of acute cardiac damage induced by DOX. Substances that are able to hinder their formation or capture the free oxygen radicals formed are thus potential cardioprotective agents. We have previously reported the effect of N. jatamansi on the antioxidant defense system during DOX induced cardiotoxicity. Thus the study of mitochondrial and lysosomal status and function during cardiac injury is of particular interest. As any in vivo study on the effect of N. jatamansi on mitochondrial and lysosomal function has not been previously carried out, we investigated the effect of ethanolic extract of N. jatamansi on mitochondrial and lysosomal function during DOX-induced myocardial damage.

MATERIALS AND METHODS

Plant Materials and Chemicals —— Roots of Nardostachys jatamansi De Jones (Valerianaceae) were purchased from an recognized and licensed ayurvedic shop in Chennai (India) and were identified and authenticated by Dr. Sasikala Ethirajulu, (Research Officer, Botany) in Central Institute for Siddha (CRIS), Arumbakkam, Chennai–600 101.

Doxorubicin was procured from Dabur pharmaceuticals (Doxorubicin hydrochloride-Adrim), New Delhi, India. All other chemicals used were of analytical grade.

Preparation of the Extract —— Clean roots were air dried and powdered to prepare the alcoholic extract as earlier described. One kilogram of moderately powdered roots of Jatamansi was extracted by refluxing with 95% ethyl alcohol in Soxhlet extractor for 6–8 hr. The extract was evaporated to dryness under reduced pressure and temperature using rotary vacuum evaporator, and dried residue was stored at 4°C. The yield of dry extract from crude powder of Jatamansi was 10%. The dried ethanolic extract was suspended in distilled water which was then administrated to rats orally at an optimum dosage of 500 mg/kg body wt. This particular dosage was fixed after trying out different doses for different days in the same set of rats.

Animals —— Experimental animals were handled according to the University and institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justices and Empowerment, Government of India (Institutional Animal Ethics Committee No.01/007/06). Male
Wistar albino rats weighing 120–130 g was purchased from Tamil Nadu University of Veterinary and Animal Sciences, India. The animals were housed in polypropylene cages maintained in controlled temperature and light cycle. The animals were fed with food pellets and water ad libitum.

**Experimental Protocols**—— The following groups of animals were used. The rats were divided into four groups (n = 6 in each group): Group I served as control; Group II and IV comprised of rats that were administered a single dose of doxorubicin (15 mg/kg, i.p.). While group II was left untreated, group IV was subjected to *N. jatamansi* treatment (500 mg/kg) for seven days orally prior to the administration of doxorubicin; Group III rats received only *N. jatamansi* extract (500 mg/kg) orally for seven days, serving as drug control; As ethanolic extract of *N. jatamansi* was administered orally for seven days at the dose of 500 mg/kg prior to the induction of DOX showed maximum cardioprotective efficacy, this particular dosage was used as the optimal dosage for the study.21 Doxorubicin was given at the dose of 15 mg/kg i.p. as described by previous study.23 On day 7 and the animals were sacrificed after 48 hr of experimental period.

**Isolation of Cardiac Mitochondria**—— After the experimental period, the animals were sacrificed by cervical decapitation. The heart was excised and washed in ice-cold isotonic saline. Blotted with filter paper, weighed. A portion of heart tissue was weighed and homogenized with 0.25 M sucrose and filter paper, weighed. A portion of heart tissue was washed in ice-cold isotonic saline. Blotted with filter paper, weighed. A portion of heart tissue was excised and the experimental period, the animals were sacrificed by cervical decapitation. The heart was excised and washed in ice-cold isotonic saline. Blotted with filter paper, weighed. A portion of heart tissue was homogenized in 0.1 M Tris-Hcl buffers (pH 7.4). The homogenate was used for the assays of Na⁺-K⁺ ATPase,28 Ca⁺²⁺-ATPase,29 and Mg⁺²⁺-ATPase.30 The activities of Lysosomal enzymes cathepsin-D,31 Acid phosphatase,32 β-D-glucuronidase,33 β-D-galactosidase,34 and β-N-acetyl glucosaminidase35 were also studied.

**Electron Microscopic Studies**—— Small pieces of heart were taken and rinsed in 0.1 M phosphate buffer, pH 7.2. Approximately 1 mm heart pieces were trimmed and immediately fixed into 3% ice-cold glutaraldehyde in 0.1 M phosphate buffer and kept overnight at 4°C. Then tissue processing for electron microscopic studies was carried out.36 The grids containing sections were stained with 2% uranyl acetate and 0.2% lead acetate. The sections were examined on a transmission electron microscope.

**Statistical Analysis**—— All the grouped data were statistically evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. All of these results were expressed as Mean ± S.D. for six animals in each group.

### RESULTS

**Respiration and Oxidative Phosphorylation of Heart Mitochondria**

Mitochondrial respiration and ATP synthesis are the major pathways in the energetics of the mitochondrial system. Maintenance of proper mitochondrial transmembrane potential is essential for the survival of the cell as it drives the synthesis of ATP and maintains oxidative phosphorylation. Table 1 represents the oxidation of succinate in state 4 Adenosine diphosphate and state 4 (−ADP), respiratory control ratio (RCR), ADP/Oxidative Phos-

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>DOX</th>
<th><em>N. jatamansi</em></th>
<th>DOX + <em>N. jatamansi</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3 (+ADP)</td>
<td>38.34 ± 2.86</td>
<td>20.01 ± 1.82a</td>
<td>38.00 ± 5.11</td>
<td>31.15 ± 1.89b</td>
</tr>
<tr>
<td>State 4 (−ADP)</td>
<td>9.77 ± 0.21</td>
<td>6.42 ± 0.23a</td>
<td>9.80 ± 0.16</td>
<td>8.28 ± 0.23b</td>
</tr>
<tr>
<td>RCR</td>
<td>3.93 ± 0.34</td>
<td>3.11 ± 0.16a</td>
<td>3.87 ± 0.09</td>
<td>3.76 ± 0.40a</td>
</tr>
<tr>
<td>ADP/O ratio</td>
<td>1.68 ± 0.11</td>
<td>1.26 ± 0.12a</td>
<td>1.66 ± 0.20</td>
<td>1.63 ± 0.23a</td>
</tr>
<tr>
<td>ATP</td>
<td>4.47 ± 0.95</td>
<td>3.18 ± 0.91a</td>
<td>4.55 ± 1.08</td>
<td>4.18 ± 0.88b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. for six animals in each group. The symbols represent statistical significance: a p < 0.01, b p < 0.05. Comparisons are made between DOX and Control group; DOX and *N. jatamansi* + DOX group. Units: nmol/mg of protein for ATP, nmoles of O₂ utilized/min/mg of protein for RCR (state 3/state 4).
phorylation ratio and ATP content in the cardiac mitochondria of control and experimental group of rats. The state 3, state 4, RCR, ADP/O ratio and ATP concentration in the cardiac mitochondria of control and experimental group of rats are presented in Table 2 and Table 3. In group II, all the parameters showed a significant reduction in comparison with control. The group III rats pretreated with N. jatamansi extract significantly retained the decreased levels of α-TP and ATP content in the cardiac mitochondria of control and experimental group of rats. The cytochrome content decreased statistically significant when compared to control (group I). Group IV animals pretreated with N. jatamansi extract significantly retained the decreased levels when compared to DOX administered group II animals. Group III rats revealed no significant changes when compared to control.

Lysosomal Enzymes Study

In order to examine the membrane stabilizing potential of the drug the activities of lysosomal enzymes were assayed. Tables 2 and 3 indicate the activities of the lysosomal hydrolases in the serum and heart tissue of control and experimental groups of rats respectively. A significant rise in the activities of cathepsin-D, Acid phosphatase, β-D-glucuronidase, β-D-galactosidase, β-N-acetyl glucosaminidase was observed in DOX administered group II rats when compared to control rats. While group IV rats pretreated with N. jatamansi extract, a significant (p < 0.001) decrement in the activity of lysosomal hydrolases in both serum and heart was noticed when compared to rats injected with DOX alone (group II). In group III N. jatamansi only given animals, there was no significant changes in these parameters.

Assay of Mitochondrial Cytochrome Content

Variations in the cytochrome content may affect the electron transport chain and thereby alter the energy production. Figure 1 depicts the cytochromes c, c1, b, a and c2 in the cardiac mitochondria of control and experimental group of rats. The cytochrome content decreased statistically significant (p < 0.001, b p < 0.01, a p < 0.05) in DOX injected group of rats, as compared to control. Prior oral administration of N. jatamansi extract (group IV) showed its protective role by elevating the levels of cytochrome content in comparison with group II. Group III animals showed no significant changes in the cytochrome content.

Transmission Electron Microscopy

Electron microscopic sections of heart in the control and experimental group of rats are presented.
Fig. 1. Cytochrome c, c₁, b, aa₃ in Cardiac Mitochondria of Control and Experimental Group of Rats

Values are expressed as mean ± S.D. for six animals in each group. The symbols represent statistical significance: b \( p < 0.01 \), c \( p < 0.001 \), a \( p < 0.05 \). Comparisons are made between DOX and Control group; DOX and N. jatamansi + DOX group. Units: nmoles/mg of protein.

Fig. 2. Transmission Electron Photomicrograph of Rat Hearts

(a) Control rat showing normal architecture. (b) Doxorubicin treated rat showing myofibril loss, swelling of mitochondria, vacuolization of cytoplasm. (c) N. jatamansi (drug alone) treated rat showing normal architecture. (d) N. jatamansi plus DOX treated rat extenuated the abnormalities and restored near normal morphology of heart.

Assay of Membrane Bound Phosphatases

Altered membrane bound ATPases represents the function of membrane integrity. Therefore the activities of membrane bound ATPases were assayed. Figure 3 illustrates the activities of Na⁺-K⁺ ATPase, Ca²⁺ and Mg²⁺ ATPases in the cardiac mitochondria of control and experimental group of animals. The activity of Na⁺-K⁺ ATPase was significantly depressed (c \( p < 0.001 \)) and Ca²⁺, Mg²⁺ ATPase activity was significantly increased (c \( p < 0.001 \), a \( p < 0.05 \)) in DOX administered group II animals when compared to control (group I). The activities of these enzymes were significantly retained in Fig. 2. Figure 2 (a) showed normal architecture of cardiac mitochondria in the control rats. In Fig. 2 (b), DOX induced rats showed abnormal architecture of mitochondria like swelling, cytoplasmic vacuolization, myofibril loss. In Fig. 2 (c), N. jatamansi (drug alone) treated rats showed normal architecture. In Fig. 2 (d), pretreatment with N. jatamansi rats extenuated the mitochondrial abnormalities and myofibril loss and restored near normal morphology of heart.
Fig. 3. Activity of Membrane Bound Phosphatase in the Heart of Control and Experimental Groups of Rats

Values are expressed as mean ± S.D. for six animals in each group. The symbols represent statistical significance: *p < 0.05, **p < 0.001. Comparisons are made between DOX and Control group; DOX and N. jatamansi + DOX group. Activity is expressed as µmol of Pi liberated/min/mg of protein.

(c * p < 0.001, a * p < 0.05) the normal levels in N. jatamansi extract pretreated rats. Pretreatment with N. jatamansi alone did not register any significant change in the activities of ATPases, when compared to control.

DISCUSSION

It is very well established that DOX, by stimulating free radical generation on the electron transport chain, induces mitochondrial dysfunction, particularly in the myocardium. Mitochondria isolated from DOX treated rats exhibited a marked reduction of oxygen consumption, ATP and oxidative phosphorylation (ADP/O) ratio. Inhibition of State 3 respiration in cardiac mitochondria isolated from rats treated with DOX agrees with previous reports concerning the acute toxicity of DOX. The decrease in RCR suggests that DOX interferes with the fundamental regulation of oxidative phosphorylation in cardiac mitochondria. N. jatamansi pretreatment resulted in a reversal of these alterations induced by DOX and maintained the oxidation of succinate, ADP/O ratio, ATP content at near normal levels. The fact that the N. jatamansi prevented the DOX induced interference with mitochondrial bioenergetics suggests that, the protective effect of N. jatamansi may be at the level of preserving the redox status of critical mitochondrial elements.

Respiratory process involves the transport of electrons via cytochromes the molecular oxygen. Variation in the cytochrome content may affect the transport of electrons via electron transport chain and thereby alter energy production. DOX induction showed a significant decrease in the cytochrome content (c, c1, b, a3) of cardiac mitochondria. Enhanced lipid peroxidation has been reported to decrease complex enzyme activities and easily inhibit the energy transduction in mitochondria. Cytochrome a3 is the terminal cytochrome in the electron transport chain and a decrease in its concentration will lead to a decrease in the uptake of oxygen, resulting in low respiratory ratio. A close correlation between the decline of mitochondrial respiration and the activity of complex-3 strongly suggests that, an impairment of the respiratory chain in the b-c1 region represents one of the functional events in the casual sequence of peroxidative reactions. Thus decrease in mitochondrial cytochrome content, results in a loss of oxidative phosphorylation capacity. Prior oral administration of N. jatamansi extract retains the cytochrome content at significantly near normal condition. The antioxidant activity as well as the free radical scavenging activity of the phytochemical constituents present in N. jatamansi extract might have been responsible for the maintenance of electron transport chain.

ATPases of the cardiac cells are known to be
the most important enzymes to maintain the vital reaction of hydrolysis of terminal high energy phosphates of ATP. The inhibition of Na\(^+\)-K\(^+\) ATPase can activate the Na\(^+\)-Ca\(^2+\) exchange mechanism in the myocardium. This Na\(^+\)-Ca\(^2+\) exchange mechanism may play a role in regulating the cellular calcium level.\(^{43}\) The significant decrease in Na\(^+\)-K\(^+\) ATPase and in Ca\(^2+\) and Mg\(^2+\) ATPase in our DOX administered rats were concurred with the previous findings. The increased activity of Na\(^+\)-K\(^+\) ATPase upon administration of *N. jatamansi* might regulate the intracellular Ca\(^2+\) level there by protecting the myocardium from excess damage by maintaining the membrane integrity. Elevation of intracellular sodium concentration (Na\(^+\)) will operate to depress Ca\(^2+\) effect and augment Ca\(^2+\) influx.\(^{44}\) Lipid peroxides are presumptive markers of free radical generation and development of oxidative stress. In DOX administered group peroxidation of membrane lipids could inactivate Na\(^+\)-K\(^+\) ATPase because of the oxidation of ‘SH’ groups present in its active site leading to the conformational alteration in the enzymes.\(^{45}\) May be in the cardiac cells the administration of *N. jatamansi* could lead to the increase in Na\(^+\)-K\(^+\) ATPase activity due to its antilipid peroxidative property by altering the ‘SH’ group present in its active site leading to restored conformational alterations. The negative inotropic and chronotropic properties as well as the antioxidant potential\(^{46}\) of some of the constituents of *N. jatamansi* could have partially prevented the decrease in Na\(^+\)-K\(^+\) ATPase and increase in Ca\(^2+\), Mg\(^2+\) ATPases activity on DOX administration.

Lysosomes are a group of cytoplasmic organelles present in numerous animal tissues and are characterized by their acid hydrolases content. Hypoxia or ischaemia induced alterations in the membrane integrity of individual lysosomes might result in the hydrolysis of multiple mitochondria. Thus, these alterations may be primary structural lesions responsible for the genesis of the process of ischemic myocardial injury.\(^{47}\) A decrease in lysosomal stability is generally paralleled by increased lysosomal enzyme activity in extracellular fluid.\(^{48}\) Lysosomal enzymes are important mediators of acute and chronic inflammatory diseases and involved in damage to connective tissue.\(^{49}\) Doxorubicin induced myocardial injury results in increased activities of the lysosomal enzymes in both serum and heart might be responsible for myocardial cellular injury and death in the ischemic state of the heart. The increase in lysosomal enzymes produces a reduction in membrane integrity and the leakage of the enzymes from the enclosed sacs lead to intracellular dysfunction and disruption of potential substrates, and organelles such as mitochondria, sarclemma etc.\(^{50, 51}\)

A marked change in the activity of the lysosomal enzyme Cathepsin-D in the heart of rats treated with DOX, wherein the leakage of enzymes from the lysosomes accounts for DOX cardiotoxicity.\(^9\) The increased release of acid hydrolases from lysosomes alters the metabolism of glycoproteins and glycosaminoglycans. These enzymes are involved in the destruction of structural macromolecules in connective tissue due to the enzymatic destruction of proteoglycans.\(^{52}\) These release of enzymes also stimulate the inflammatory mediators like oxygen radicals, prosta glandins etc. which stimulate tissue disruption. It has been suggested that oxygen free radicals generated during ischemia in addition to direct myocardial damaging effect may also be responsible for the cardiac damage through the release of lysosomal enzymes.\(^{53}\)

Pretreatment with *N. jatamansi* extract is able to decrease the release of enzymes which could be due to the membrane stabilizing effect of *N. jatamansi* on the lysosomal membrane. *N. jatamansi* has been reported to possess higher anti-inflammatory activity.\(^{16}\) The antioxidant property of *N. jatamansi* scavenges the oxygen free radicals with the resultant preservation of cellular viability serving secondarily to preserve lysosomes, thereby retaining near normal functioning of the lysosomes. Further, pretreatment with *N. jatamansi* extract significantly attenuated the ultrastructural pathological changes in the cardiac mitochondria induced by doxorubicin as evident from the transmission electron microscopic studies.

*N. jatamansi* has been indicated in ayurvedic text to be a potent cardiotonic agent. A number of active components were also isolated from this plant\(^{54}\) which includes lignans, sesquiterpenes and terpenoids. It has been reported that these compounds possess the cardioprotective effect through antioxidant defense mechanism. The antioxidant property of *N. jatamansi* against DOX induced oxidative stress has earlier been reported by us.\(^{21}\)

The results of the study demonstrate that the *N. jatamansi* possesses cardioprotective property as evidenced by its significant inhibition in the formation of mitochondrial damage, membrane liability induced by doxorubicin and as a potent cytoprotective agent in reducing the extent of lysosomal
damage. It can be concluded from the above observations that *N. jatamansi* prevents myocardial injury by reduction of lipid peroxidation levels which might be through inactivation of free radical and also by increasing the antioxidant levels which limits the toxicity associated with free radicals, formed during DOX induced mitochondrial injury and lysosomal damage.

REFERENCES


50) Kennett, F. F. and Weglicki, W. B. (1978) Effects of well-defined ischemia on myocardial lysosomal and...


