

A Cardioprotective Role of *Nerium oleander* with the Expression of Hypoxia Inducible Factor 2A mRNA by Increasing Antioxidant Enzymes in Rat Heart Tissue

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ABSTRACT

Background: *Nerium oleander* (NO) distillate is used to either protect heart cells against oxidative stress or reduce the risk of cardiovascular disease by regulating the production of reactive oxygen species. Hypoxia-inducible factors (HIFs) regulate cellular antioxidant defense mechanisms under hypoxic conditions in which heart cells survive; however, the key responsible mechanism of NO distillate for cardioprotection remains elusive. The objective of this study was to evaluate the effects on heart tissue at different time intervals after administering NO distillate intraperitoneally (IP) while considering the transcriptional regulation of HIFs and representative antioxidant enzymes.

Materials, Methods & Results: The NO plant was chopped, and distilled water was added. The mixture was distilled, and the distillate separated and collected into tubes, after which it was lyophilized to obtain dry material. Twenty male Wistar albino rats (2-3 month-old, 250-300 g each) were used in the study. The rats were randomly divided into four groups. The control group (n = 5) received IP injections of saline; the remaining 15 rats received IP injections of a single dose of 7.5 mL NO distillate. The NO distillate injected rats were divided into three groups according to the time from injection to harvest the heart tissue samples. The tissues were collected at 0 h (control; n = 5), 2 h (group 2; n = 5), 4 h (group 3; n = 5), and 8 h (group 4; n = 5) after injection and under general anesthesia (60 mg/kg ketamine, IP + 10 mg/kg xylazine, IP). Quantitative polymerase chain reaction (qPCR) was used to assess the expression profiles of the genes of interest in the heart tissues. Hypoxanthine phosphoribosyltransferase was used as the reference gene. The expression of manganese superoxide dismutase (MnSOD) mRNA was in a steady state level between the control group and group 2 ($P > 0.05$); however, it significantly increased in group 3 and 4 compared with that in the control ($P < 0.05$). Expression of catalase (CAT) mRNA was significantly higher in group 2 than in the control group ($P < 0.05$) although it was lower in group 3 and 4 than in group 2 ($P < 0.05$); however, it appeared to be similar among the control group, group 3, and group 4 ($P > 0.05$). Copper (Cu) SOD mRNA was equally expressed in both the control group and group 2 ($P > 0.05$) but was lower in group 3 and 4 than in group 2 ($P < 0.05$). Expressions of HIF1A, HIF2A, and HIF3A mRNA were detected in the rat heart tissues in the control and 2, 4, and 8 h after administration of NO distillate. Expression of HIF1A mRNA was in a steady state and did not differ among groups 2, 3, and 4 ($P > 0.05$). Similarly, the expression of HIF2A mRNA did not change between the control group and group 2 ($P > 0.05$); however, it was higher in group 3 than in the control ($P < 0.05$) and tended to be higher in group 3 than in group 2 ($P = 0.063$). HIF3A mRNA expression did not change significantly in the heart tissue of any of the groups ($P > 0.05$).

Discussion: The present study using rats determined that MnSOD, CAT, CuSOD, HIF1A, HIF2A, and HIF3A mRNA are expressed in the heart tissues after administration of NO distillate. The increased expression of HIF2A mRNA after 4 h in accordance with a rise in CAT mRNA after 2 h, and MnSOD mRNA after 4 and 8 h might confirm the role of HIF2A mRNA in oxidative stress defense by regulating antioxidant enzymes; consequently, this study may expand our understanding of uses of NO distillate with respect to molecular pathways.

Keywords: *Nerium oleander*, hypoxia inducible factors, antioxidant enzymes, gene expression, cardioprotection.

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INTRODUCTION

Nerium oleander (NO), a member of the Apocyanaceae family, is a poisonous evergreen shrub [19] that grows mainly in northern Africa, the eastern Mediterranean region, and Anatolia. NO distillate has been experimentally shown to serve as a cardioprotective agent by boosting some antioxidant components to combat oxidative stress [7,9]. Ayaz *et al.* [2] reported the preservative effects of NO on the heart of type II diabetes; however, little is known about effects of NO distillate on heart tissue at the molecular level.

Each cell in the heart demands high oxygen levels for contractility and can sense a lack of oxygen (hypoxia) [4]; however, abnormal oxygen homeostasis in heart tissue is often associated with the generation of excessive reactive oxygen species (ROS). The initial defense mechanism against oxidative stress in the heart involves the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) (copper [Cu]SOD and manganese [Mn]SOD), which counterbalance ROS effects [30].

Hypoxia-inducible transcription factors (HIFs), transcription factors, act as heterodimers that comprise oxygen-mediated alpha (HIF- α) and constitutively expressed beta (HIF- β) subunits [14,37]. HIF- α has been reported to be present in three different isoforms (HIF-1 α , HIF-2 α , and HIF-3 α), which control cellular response to oxygen deficiency by gene expression [16,32]. HIF-1 α , specifically, also plays a cardioprotective role during hypoxia [25,29,36,39].

In this study, putative effects of intraperitoneally (IP)-administered NO distillate on heart tissue were investigated at different time intervals, while considering the transcriptional regulation of HIFs and representative antioxidant enzymes.

MATERIALS AND METHODS

Extraction of NO distillate

NO samples were authenticated, washed, chopped, and combined with distilled water. The mixture was then distilled, and the distillate separated and collected into new tubes. The NO distillate was then lyophilized to obtain dry material [3].

Animals

Twenty male Wistar albino rats (2-3-month old, 250-300 g each) were kept at a central location under the appropriate conditions of 55% \pm 5% humid-

ity at 22°C \pm 2°C and a 12-h light/dark cycle in the Experimental Research Centre of Mehmet Akif Ersoy University, Turkey. The rats were housed in standard rat cages and provided with water and a commercial feed *ad libitum*.

Experimental Design

The 20 rats were divided into four groups, one of which was the control group and the others of which were designated according to the time interval after NO injection. The control group (n = 5) received an IP injection of saline; the others received an IP injection of a single dose of 7.5 mL NO distillate. After 2 h (group 2, n = 5), 4 h (group 3, n = 5), and 8 h (group 4, n = 5), heart tissue samples were collected from the rats under general anesthesia [60 mg/kg ketamine (Ketalar, inj sol)¹, IP + 10 mg/kg xylazine (Alfazyme %2 inj sol)², IP] followed by euthanasia by cervical dislocation.

RNA extraction, cDNA synthesis, and quantitative polymerase chain reaction

Total RNA was extracted from the heart tissues using TRIzol^{®3} according to the manufacturer's protocol. RNA integrity with distinct 18s and 28s bands was verified using agarose gel and NanoDrop (Colibri Microvolume Spectrometer)⁴ with optical density measurements (260/280) of 2 \pm 0.1. Total RNA was purified from gDNA contamination by treating with the DNase I kit⁵. One microgram of total RNA was converted to cDNA using the RevertAid[™] First-Strand cDNA Synthesis Kit⁵. Primers were designed using the (Table 1).

A quantitative polymerase chain reaction (qPCR) was conducted to assess the expression profiles of the genes of interest in the heart tissues. qPCR was established as follows: 10 μ l iTaq[™] universal SYBR[®] Green⁶, 5 pMol each primer, 1 μ L cDNA, and ddH₂O to final volume of 20 μ L [11]

The thermal cycling conditions were as follows: initial denaturation, 95°C for 5 min followed by 45 cycles of denaturation, annealing, and amplification at 95°C for 15 s, 60°C for 30 s, and 72°C for 45 s, respectively. The melting curve analysis was conducted as follows: 95°C for 1 min, then fluorescence was measured at 1° increment from 55°C to 95°C using qPCR (LightCycler[®] Nano instrument)⁷. A negative control with no cDNA template was included in each run. To verify reaction specificity, the amplification products were run on 2% agarose gel. Hypoxanthine phosphoribosyltransferase (HPRT) was used as the

reference gene to normalize qPCR data. Real-time (RT)-PCR analysis was conducted in triplicate for each gene investigated.

Statistical Analyses

The quantitative RT-qPCR (threshold cycle or Ct) data were calculated as relative changes in the control and groups 2, 3, and 4. During the relative ex-

pression calculation, mean Ct values from the control samples were used as the reference points, and the Ct values of group 2, 3, and 4 were used to calculate the fold changes from those reference points based on the $2^{-\Delta\Delta Ct}$ method defined by Livak and Schmittgen [22]. Normalized data were analyzed using analysis of variance and Tukey’s posthoc test. $P < 0.05$ was considered as a significant difference.

Table 1. Primers and reference gene used for Real Time PCR.

Gene	Primer sequence (5’-3’)
<i>HPRT</i>	Forward- 5’CTCATGGACTGATTATGGACAGGA ‘3
	Reverse- 5’GCAGGTCAGCAAAGAAGCTTATAGCC ‘3
<i>MnSOD</i>	Forward-5’ACGCGACCTACGTGAACAATCT ‘3
	Reverse-5’CAGTGCAGGCTGAAGAGCAA ‘3
<i>CuSOD</i>	Forward-5’TTCCATCATTGGCCGTACTA 3’
	Reverse-5’AGCGGCTTCCAGCATTT3’
<i>CAT</i>	Forward- 5’ACAACCTCCCAGAAGCCTAAGAATG 3’
	Reverse- 5’GCTTTTCCCTTGGCAGCTATG-3’
<i>HIF1A</i>	Forward- 5’ TGGTGCTGATTTGTGAACCCATTCC ‘3
	Reverse- 5’ CAGGGCATCGGGCTCTTTCTTAAG ‘3
<i>HIF2A</i>	Forward- 5’ GCGACAATGACAGCTGACAAGG 3’
	Reverse- 5’ CCTCCAAGGCTTTCAGGTACAAGT 3’
<i>HIF3A</i>	Forward- 5’ ACCAAGACAGGTGCAACACC ‘3
	Reverse- 5’ TTTTCCACCTGGTTCCACTC ‘3

RESULTS

Expression of antioxidant enzyme profiles of MnSOD, CAT, and CuSOD mRNA

Expressions of MnSOD, CAT, and CuSOD mRNA were detected in rat heart tissues in control, group 2, 3, and 4. Expression profiles of MnSOD, CAT, and CuSOD mRNA are demonstrated in Figure 1. Expression of MnSOD mRNA was in steady state level between the control and group 2 ($P > 0.05$); however, it significantly increased in groups 3 and 4 compared with that in the control ($P < 0.05$; Figure 1A).

Expression of CAT mRNA was significantly higher in group 2 than in the control ($P < 0.05$) but was lower in groups 3 and 4 than in group 2 ($P < 0.05$) and appeared to be similar among the control and groups 3 and 4 ($P > 0.05$; Figure 1B). The expression of CuSOD mRNA was steady between the control group

and group 2 ($P > 0.05$) but was lower in groups 3 and 4 than in group 2 ($P < 0.05$; Figure 1C).

Expression profiles of HIF1A, HIF2A, and HIF3A mRNA

Expression levels of HIF1A, HIF2A, and HIF3A mRNA were detected in rat heart tissues in all groups. The expression profiles of HIF1A, HIF2A, and HIF3A mRNA are demonstrated in Figure 2. The relative gene expression of HIF1A mRNA was at a steady state and did not differ among control, groups 2, 3, and 4 ($P > 0.05$; Figure 2A). Similarly, the expression level of HIF2A mRNA did not change between the control and group 2 ($P > 0.05$); however, it was higher in group 3 than in the control ($P < 0.05$) and tended to be higher in group 3 than in group 2 ($P = 0.063$; Figure 2B). Expression levels HIF3A mRNA did not change significantly in the heart tissue in all groups ($P > 0.05$) after NO distillate administration (Figure 2C).

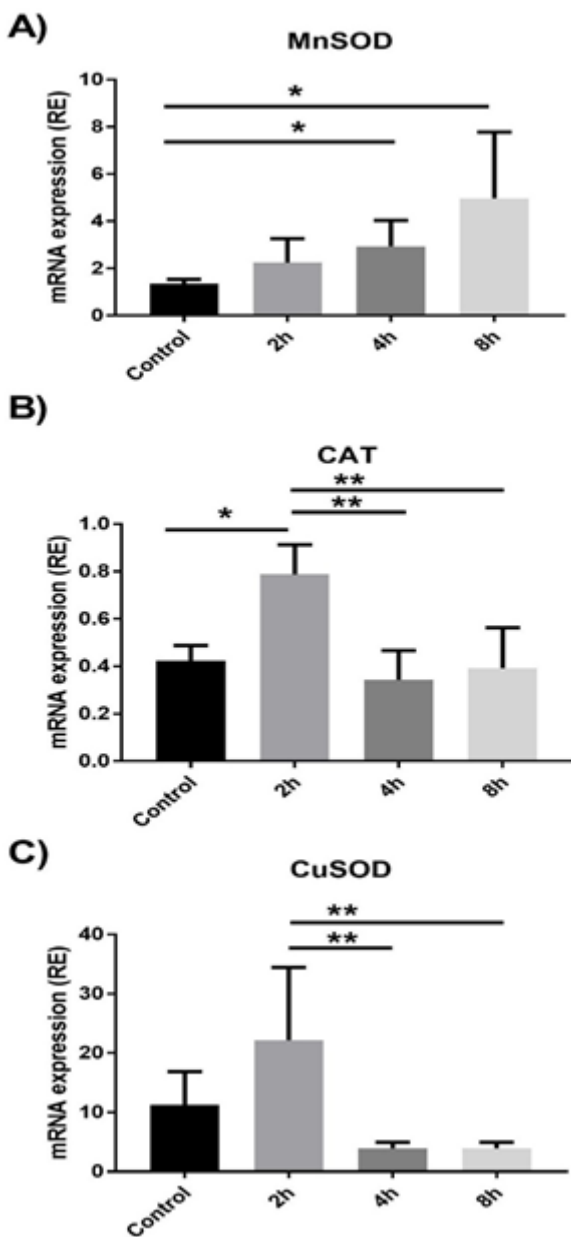


Figure 1. A- Relative mRNA expression of manganese superoxide dismutase A. B- Relative mRNA expression of catalase. C- Relative mRNA expression of copper. The control group received only saline injections; groups 2, 3, and 4 correspond to 2, 4, and 8 h after administration of *Nerium oleander* distillate. [* = $P < 0.05$, ** = $P < 0.01$].

DISCUSSIONS

The present study using rat heart tissue determined that MnSOD, CAT, CuSOD, HIF1A, HIF2A, and HIF3A mRNA are expressed in these tissues after administration of NO distillate. Natural herbs are used to either protect heart cells against oxidative stress or reduce the risk of cardiovascular disease by regulating the production of ROS [28,38]. Of these, the antioxidant properties of NO are used extensively

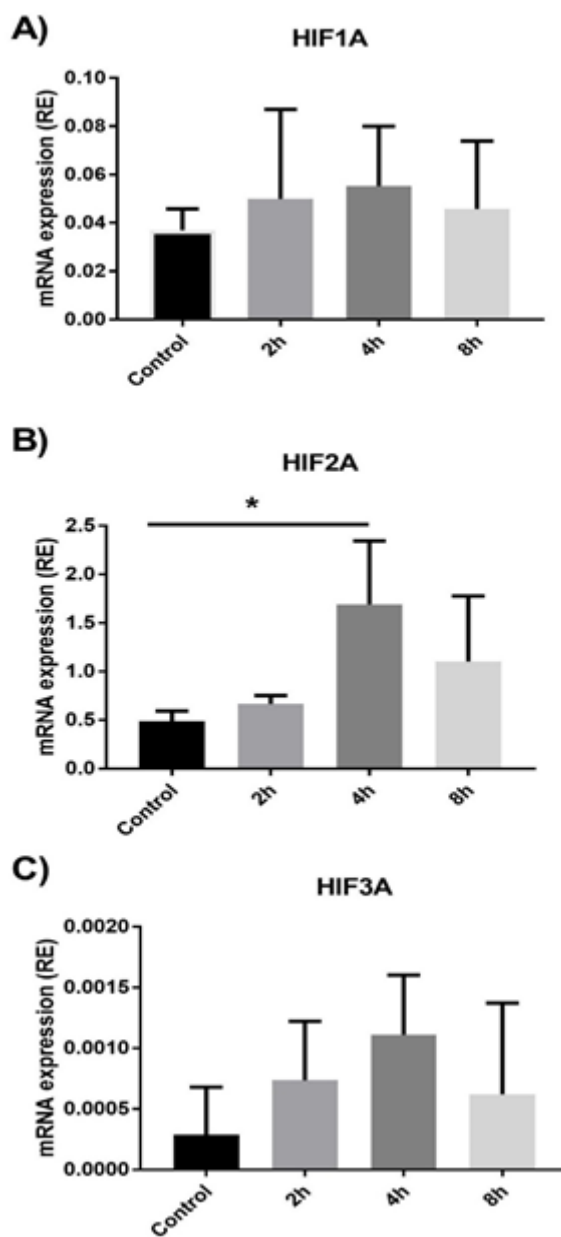


Figure 2. A- Relative mRNA expression of hypoxia inducible factor (HIF)1A. B- Relative mRNA expression of HIF2A. C- Relative mRNA expression of HIF3A. The control group received only saline injections; groups 2, 3, and 4 correspond to 2, 4, and 8 h after administration of *Nerium oleander* distillate. [* = $P < 0.05$, ** = $P < 0.01$].

to protect cells against free radicals by elevating SOD activity [7,24,34].

Antioxidant enzymes (CAT, CuSOD, MnSOD) counteract the production of ROS, which result from oxidative stress; however, gene polymorphisms in these antioxidant enzymes can lead to heart failure related to oxidative stress [1]. Furthermore, polymorphism in the MnSOD allele with reduced SOD activity is associated with heart disease [35]. The MnSOD promoter is sensitive

to hypoxia and notably upregulated by oxidative stress. Studies have shown that animals deficient in the MnSOD allele suffered from cardiac dysfunction [21] and heart-specific MnSOD knockout mice experienced severe heart failure with deterioration in mitochondrial respiration resulting from oxidative stress [26]. In our study, mRNA expression of MnSOD was significantly higher in groups 3 and 4 than in the control but also elevated in group 2. Dieterich *et al.* [6] reported that *CAT* mRNA increased in the failing human heart. Moreover, in concurrence with this study, Sam *et al.* [31] revealed an increased expression of *CAT* and MnSOD in patients with heart failure, which represents an adaptive response to oxidative stress. Specifically, the knockdown of *CuSOD* mRNA in mice predisposed the heart to injury from oxidative stress [45]. Furthermore, compounds with antioxidant properties induced the expression of *CAT* and *CuSOD* in the rat heart [43]. In our study, the expression of *CuSOD* mRNA was lower in groups 3 and 4 than in group 2; however, the expression of *CAT* mRNA was higher in group 2 than in the control but lower in groups 3 and 4 than in group 2. This elevated expression of MnSOD in groups 3 and 4 and *CAT* mRNA in group 2 could result from an antioxidant adaptive response to the NO distillate, which we have also reported in our previous study [7].

Heart hypoxia occurs as a result of an imbalance between the supply and demand of oxygen to the cardiac cells. This condition is a primary factor for cardiac pathophysiology, including congenital heart disease [5,44] and myocardial infarction [8]. Studies conducted on heart tissue related to hypoxia clearly revealed the role of HIF-1 as a key factor [15,23,33]. The functions of HIF-1 in the heart are influenced by hypoxia through modulation of HIF-1 α mRNA and protein levels [15,23]. HIF-1 α expression was reported to mediate cardioprotection with metabolic influences in the mouse heart [41]. In our study, HIF1A expression was at a steady state and did not differ among the control group and groups 2, 3, and 4. It is likely that HIF1A is ubiquitously expressed, in accordance with its evolutionarily conserved structure, in heart tissue as in any tissues; therefore, NO distillate might not exert an effect through HIF1A mRNA. Although crosstalk between HIF-1 α and MnSOD and elimination of ROS-related hypoxia remain elusive, Kaewpila *et al.* [17] showed that MnSOD is critically important in the modulation of HIF-1 α through superoxide; however, in our study, the expression levels of MnSOD and HIF1A mRNA did not appear to correlate with administration of the NO distillate.

The expression level of HIF-2 α was also reported in different cell types, including the kidney, liver, heart, and vascular structures [13,27,40]. A study on whether HIF expression might counterbalance oxidative stress revealed that knockout of HIF-2 α leads to multiple organ pathology, including cardiac hypertrophy, related to impairment of ROS homeostasis. HIF-2 α was also reported to play a protective role in the kidneys by improving oxidative stress [20]. HIFs also regulate cellular antioxidant defense mechanisms under hypoxic environments [12]. In mice exposed to chronic and intermittent hypoxia, the expression of MnSOD mRNA increased in both conditions with the rise of HIF2A mRNA, while the expression *CuSOD* mRNA was upregulated only in chronic hypoxia, which suggested an antioxidant defense mechanism reflected in cardioprotection [18]; therefore, given that HIF family members are sensitive to ROS, it is suggested that NO distillate might play a potential role in cardioprotection by upregulating HIF2A mRNA together with stimulating the antioxidant enzyme pathway.

Expression levels of HIF-3 α in heart tissue were slightly induced after exposure to systemic hypoxia [10]. Moreover, expression levels of the HIF-3 α major isoform, namely neonatal and embryonic PAS, were reported to be highest in mice hearts during the late embryonic and first neonatal stages [42]. In this study, although expression of HIF3A mRNA appeared to increase in groups 2, 3, and 4, this increase was not significant. Accordingly, because the expression of HIF3A mRNA was weaker, it might not be directly affected by NO distillate.

CONCLUSIONS

The increased expression of HIF2A mRNA in group 3 in accordance with rise in *CAT* mRNA in group 2 and MnSOD mRNA in groups 3 and 4 might confirm the role of HIF2A mRNA in the defense against oxidative stress by regulating antioxidant enzymes; consequently, this study might help to increase understanding of the various uses of *Nerium oleander* distillate with respect to molecular pathways.

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Ethical approval. Animal procedures and experiments were approved by Ethics Committee of Experimental Medicine Research and Application Centre of Mehmet Akif Ersoy University (MAKU)

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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