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Research Article
Cardioprotective Efficacy of Naringenin Against Isoproterenol Induced Chronic Heart Failure in a Rat Model

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Abstract

Background and Objective: Heart attack or Myocardial infarct (MI) is one of the serious health issue as it claims many lives than any other heart disease. The present experiment was framed to explore the cardioprotective efficacy of naringenin (NG) against isoproterenol (ISO) induced heart failure (cardiotoxicity) in a rat model. **Materials and Methods:** A total of 32 male albino rats were procured and segregated into 4 groups as control-rats treated with saline (14 days), NG alone group- rats were treated orally with 50 mg kg^{-1} of NG for 14 days, ISO induced-MI group-rats were injected intraperitoneally (i.p.) with ISO (85 mg kg^{-1}) for 2 days, NG+ISO induced group-rats orally treated with NG for 14 days followed by 2 days of ISO induction. **Results:** Rats pre-treated with NG for 14 days before ISO induction showed a significantly improvement in antioxidant status (Catalase, CAT, superoxide dismutase, SOD) and hemodynamic parameters (Systolic/diastolic arterial blood pressure). However, the levels of inflammatory markers (Nuclear factor kappa bp65 subunit, NF-κb p65 subunit, Tumor necrosis factor alpha, TNF-α, Interleukin 1 beta/six, IL-1β/6), cardiac markers (troponin C, cTn C, creatine kinase, CPK, lactate dehydrogenase, LDH), heart to body weight ratio and lipid peroxidation products (MDA) were considerably abolished upon NG supplemented rats as compared with ISO-induced rats. Moreover, the protein expression of NF-κb p65 subunit and IL-1β were substantially down regulated in rats that supplemented with NG. **Conclusion:** Taking together, that pretreatment with NG could significantly improve the antioxidant status and attenuate inflammatory response via down regulating NF-κb signaling pathway.

Key words: Heart failure, hemodynamic parameters, naringenin, heart attack, isoproterenol, myocardial infarct, cardiac markers, tumor necrosis factor

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Myocardial infarct (MI) or heart attack (major ischemic heart disease) is ranked as one of the dreadful diseases. It has been predicted that by the year 2030 almost 23.3 million people would die by myocardial infarction. The percentage of people suffering from MI has been considerably increasing especially in Asian countries like India, Malaysia and China[1,2]. The major root cause of MI is atherosclerosis (especially block in coronary artery), which results in an imbalance between blood supply and demand to myocardium, which end up in myocardial hypoxia (lack of oxygen) and accumulation of cellular waste (especially carbon-dioxide) and eventually leads to myocardial cell death (lack of blood supply) owing to ischemia derived excessive free radical generation[3]. Nevertheless, the exact pathophysiology of MI is still fully uncovered. But, numerous scientist has revealed that oxidative stress, inflammation, apoptosis, necrosis, mitochondrial dysfunction and hypoxia are the main contributors for MI[4,5].

Isoproterenol (ISO) is a catecholamine/non-selective β-adrenergic receptor agonist. The IF ISO was injected to experimental animal at a supra maximal dose (more than 80 mg kg^{-1} via i.p.) it triggers excessive free radical generation (oxidative stress) in myocardium owing to auto-oxidation of catecholamine. Those free radical eventually results in a cascade of the inflammatory reaction (response-myocardial hyperactivity) which leads to severe myocardial cell death (necrosis) and ultimately end up in ISO-induced MI[6,7]. Ample amount of studies has indicated that MI or cardiotoxicity induced by ISO is one of the well establish MI model in animals as its pathophysiology and human MI pathophysiology are more are less similar[8,9]. Recently, researchers are trying to develop a novel natural cardioprotective agent against MI as the present treatment regimen were not that effective and it's too expensive and hence most of them cannot afford those medication for MI. Hence, the need of new effective cheap and potent cardioprotective agent with less or no adverse effect are in high demand to reduce the mortality and morbidity related to MI[10,11].

Naringenin (NG, 4',5,7-trihydroxy flavanone) is a aglycone citrus flavanone (flavonoid) commonly found in grapefruit, oranges, lemon. The NG is one of the active metabolites of naringin (citrus flavonoid-glycoside) which are metabolized by gastrointestinal bacteria[12]. The NG shows multiple biological activities including anti-inflammatory, anti-oxidant, anti-diabetic, anti-hyperlipidemic, anti-apoptotic as well as neuroprotective, cardioprotective and nephroprotective[13,14]. Hence, NG has labeled as a popular nutraceutical (functional food) owing to the above mentioned beneficial properties. Naringenin is reported to exhibit cardioprotective property owing to its antioxidant and anti-inflammatory property against hypercholesterolemia induced cardiotoxicity in a rat model[15,16]. Previously, the naringin (citrus flavonoid), which would be metabolized to naringenin has been reported to exhibit cardioprotective activity by attenuating lipid peroxidation and oxidative stress in ISO-induced cardiac damage[8]. However, tilldate, no experiments have been conducted with naringenin against ISO-induced MI model. Hence, the current animal experiment was framed to examine the cardioprotective effect of naringenin on ISO-induced MI animal model.

MATERIALS AND METHODS

Experimental rats: A total of 32 healthy male albino Wistar strain rats were procured and kept in a polycarbonated cage and maintained at 23-24°C with 55-60% humidity under 12/12 light and dark condition with free access to normal rat pellet and water (*ad libitum*). The experimental protocols and procedure used in this study were approved by an ethical committee member board of Capital Medical University (CMU12/890-2018) and by following the guidelines proposed by the NSAG of China for the care and use of experimental animals to make sure less suffering for all experimental rats. This animal study was at the Department of Cardiac Surgery, Beijing Luhe Hospital, Capital Medical University, Beijing, China from 2nd-25th October, 2018.

Experimental grouping: After a week of assimilation period all the 32 healthy male albino Wistar strain rats were randomly divided into 4 groups with 8 rats in each group:

- **Group I (control):** Rats were treated with only saline for all 14 days
- **Group II (NG alone group):** Rats were treated orally (via gastric intubation tube) with 50 mg kg^{-1} of NG for 14 days
- **Group III (ISO induced-MI model):** Rats were injected intraperitoneally (i.p) with ISO at a dose of 85 mg kg^{-1} for 2 days (15th and 16th day-No mortality was found)
- **Group IV (NG+ISO induced):** Rats orally administered (pre-treated) with NG for 14 days followed by 2 days of ISO induction (85 mg kg^{-1}, i.p.)

Sample preparation: All the rats were weighed (after the experimental period) using standard Laboratory Animal

Weighing Scale and the values are noted. All the rats were anesthetized using ethyl ether and killed by cervical decapitation. The blood sample was then collected and serum samples were separated by centrifuging at 2500 rpm for 20 min at 4°C and were stored at -80°C until analysis. Heart (cardiac tissue) were excised immediately from all sacrificed rats and rinsed in chilled saline solution and weighed using a standard weighing scale. The excised cardiac tissue was nicely chopped and homogenized with tris-HCl buffer solution. The resultant homogenate was centrifuged at 10000 rpm for 12 min at 4°C and the resultant supernatant was utilized for all the biochemical and molecular analysis.

Hemodynamic parameters: On the last day of ISO induction (16th day, 2 h after ISO induction), the hemodynamic parameters including Diastolic/Systolic arterial blood pressure (DAP/SAP) and mean arterial blood pressure (MAP) were measured using LE 5002-tail cuff non-invasive blood pressure meter-V25 (Panlab-Harvard Apparatus, MA, USA).

Cardiac lipid peroxidation and antioxidants: The levels of lipid peroxidation products like malondialdehyde (MDA) as well as the activity of cardiac antioxidants like catalase (CAT), superoxide dismutase (SOD) were measured using a commercial kit (Shanghai Yantuo Biotechnology, Shanghai, China) based on manufacturer's specification.

Serum cardiac marker enzymes: The activity of serum cardiac marker enzymes like cardiac troponin C (cTnC), lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB isoform) were assessed to check the cardiac function or integrity. The CK-MB (ELISA kit) and LDH (Colorimetric assay kit) were both assayed using commercial kits bought from Abcam (Cambridge, UK). Whereas, the activity of cTnC were assayed using commercial 24-strip-well ELISA kit purchased from MyBiosource, Inc., (CA. the USA).

Cardiac inflammatory markers: The nuclear/cytosolic fractionation kit (Cell Biolabs Inc.,CA, USA) was used to extract the cytosolic and nuclear fraction from the cardiac tissue supernatant. Then the levels of various cardiac inflammatory markers like Interleukin one beta (IL-1β), tumor necrosis factor alpha (TNF-α) and Interleukin six (IL-6) were measured in cytosolic fraction using commercial ELISA kit provided by Thermo Fisher Scientific, Inc., (MA, USA). Whereas, the concentration of nuclear factor kappa bp65 subunit (NF-κb p65) an active NF-κb subunit was measure in cerebral nuclear fraction using from ELISA NF-κb p65 transcription factor assay kit from Abcam (Cambridge, UK).

Immunoblot: The protein expression of NF-κb p65 and IL-1β were quantified by western blot technique based on the method of Kumar *et al.*[3]. The protein levels were estimated using Pierce BCA protein assay kit from Thermo Fisher Scientific, Inc., (MA, USA) after treating with lytic RIPA buffered solution. A 40 µg of protein from each fraction/sample was uniformly separated using 10% SDS-PAGE apparatus and electro-transferred onto polyvinylidene difluoride (PVDF) membrane. Followed by incubation of membrane with primary antibodies for overnight at 4°C. Primary antibodies: Rabbit polyclonal anti-NF-κb p65 (1:1200 dilution, Santa Cruz Biotechnology, Inc., TX, USA), rabbit monoclonal anti- IL-1β (1:1000 dilution, Santa Cruz Biotechnology, Inc., TX, USA) and rabbit monoclonal anti-β actin (1:1200 dilution, Santa Cruz Biotechnology, Inc., TX, USA) a standard/control. Then followed by the addition of secondary antibody-rabbit polyclonal anti-horseradish peroxidase (HRP) antibody (1:10000 dilution from Santa Cruz Biotechnology, Inc., TX, USA) and incubated for 1 h at 37°C. The protein bands in the PVDF membrane was developed using enhanced Chemiluminescence kit and quantified using image analyzing software.

Data analysis: Data are represented as the mean±standard deviation (SD) for 8 rats (n = 8) in each group. The probability value (p-value) was given based on a comparison between control vs ISO and ISO vs NG+ISO group by one-way analysis of variance (ANOVA) followed by Duncan's multi-range test (post hoc) using GraphPad Prism (ver: 5, Graphpad Software Inc., CA, USA). A $p<0.05$ is deemed as the statistical difference.

RESULTS

Effect of NG on body weight, heart weight and heart to body weight ratio in experimental animals: The data in Table 1 represented the change in heart weight, body weight and heart to body weight ratio in experimental animals. A considerable change ($p<0.01$) in heart weight and heart to body weight ratio was observed in ISO-induced group as equivalent to a control group. However, pretreatment with NG (NG+ISO) for 14 days would significantly reduce ($p<0.01$) the values of heart weight and heart to body weight ratio than ISO-induced group. Whereas, no significant alteration in the levels of body weight between any experimental groups.

Effect of NG on arterial blood pressure in experimental animals: The Fig. 1 depicts the change in arterial blood pressure in experimental animals. The average value of

Table 1: Change in heart weight, body weight and heart to body weight ratio in experimental animals after NG treatment and ISO induction

Indications	Control	NG	ISO	NG+ISO
Heart weight (g)	0.567±0.07	0.571±0.08	0.807±0.09$^{a\#}$	0.660±0.07$^{b\#}$
Body weight (g)	254.200±5.00	252.960±4.50	254.000±5.50	253.800±6.20
Heart to body weight ratio (%)	0.223±0.01	0.225±0.01	0.317±0.01$^{a\#}$	0.260±0.01$^{b\#}$

Data are represented as the Mean±Standard Deviation (SD) for 8 rats (n = 8) in each group. Probability value (p-value, $^{\#}$p<0.01): Where "a" exemplify the comparison between control vs ISO, while "b" exemplify the comparison between ISO vs. NG+ISO

Table 2: change in cardiac marker enzymes (serum) in experimental animals after NG treatment and ISO induction

Indications	Control	NG	ISO	NG+ISO
LDH (ng mL^{-1})	77.28±7.07	76.89±8.10	140.24±12.06$^{a\#}$	94.68± 10.50$^{b\#}$
CK-MB (IU L^{-1})	59.03±6.00	56.90±7.90	139.72±15.80$^{a\#}$	73.45±8.10$^{b\#}$
cTn C (ng mL^{-1})	0.55±0.06	0.53±0.07	1.65±0.16$^{a\#}$	0.65±0.07$^{b\#}$

Data are represented as the Mean±Standard Deviation (SD) for 8 rats (n = 8) in each group. Probability value (p-value, $^{\#}$p<0.01): Where "a" exemplify the comparison between control vs ISO, while "b" exemplify the comparison between ISO vs. NG+ISO

Table 3: change in cardiac antioxidant enzymes and lipid peroxidation products in experimental animals after NG treatment and ISO induction

Indications	Control	NG	ISO	NG+ISO
CAT (U mg^{-1} pro)	17.92±1.34	17.90±1.80	8.54±10.00$^{a\#}$	14.02± 15.72$^{b\#}$
SOD (U mg^{-1} pro)	5.73±8.20	5.90±7.00	3.56±4.96$^{a\#}$	4.58±6.82$^{b\$}$
MDA (nmol mg^{-1} pro)	0.58±0.07	0.59±0.08	1.12±0.11$^{a\#}$	0.72±0.08$^{b\#}$

Data are represented as the Mean±Standard Deviation (SD) for 8 rats (n = 8) in each group. Probability value (p-value, $^{\#}$p<0.01, $^{\$}$p<0.05): Where "a" exemplify the comparison between control vs ISO, while "b" exemplify the comparison between ISO vs. NG+ISO

hemodynamic parameters (arterial blood pressure) like MAP, SAP and DAP were substantially decreased (p<0.01) in ISO injected rats (MI model). A pronounced increase (p<0.01) in the mean levels of arterial blood pressure (MAP, SAP and DAP) were noted in the NG+ISO group as compared to the ISO group.

Effect of NG on serum cardiac marker enzymes in experimental animals: The results in Table 2 show the effect of NG on serum cardiac marker enzymes in experimental animals after NG treatment and ISO induction. The activity of serum cardiac marker enzymes including LDH, CK-MB and cTnC were dramatically elevated (p<0.01) in the MI model vs. control group. About 14 days of pre-supplementation with NG+ISO could concomitantly lower (p<0.01) the activity of serum cardiac marker enzymes including LDH, CK-MB and cTnC as compared to ISO-induced group.

Effect of NG in cardiac antioxidant enzymes and lipid peroxidation products in experimental animals: As indicated in Table 3 the activity of cardiac anti-oxidant enzymes like CAT and SOD were significantly declined (p<0.01) with considerable elevation (p<0.01) in the levels of lipid peroxidation products (MDA) in ISO injected rats than saline-treated control rats. In contrary, pre-treated rats with 50 mg kg^{-1} of NG (NG+ISO) for 14 days could markedly improve (p<0.01) the activity of cardiac anti-oxidant enzymes like CAT and SOD as well as significantly suppress (p<0.01) the levels of lipid peroxidation products (MDA) as compared to ISO-induced group.

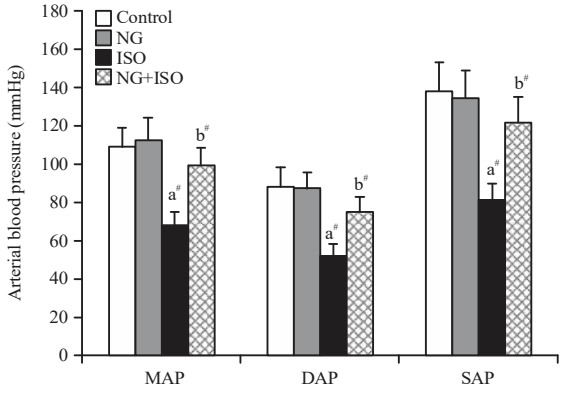

Fig. 1: Change in arterial blood pressure, MAP: Mean arterial pressure, DAP: Diastolic arterial pressure, SAP: Systolic arterial pressure, in experimental animals after NG treatment and ISO induction

Data are represented as the mean±standard deviation (SD) for 8 rats (n = 8) in each group. Probability value (p-value, $^{\#}$p<0.01): Where "a" exemplify the comparison between control vs. ISO, while "b" exemplify the comparison between ISO vs. NG+ISO

Effect of NG on various inflammatory markers in experimental animals: As shown in Fig. 2 the concentration of various cardiac inflammatory markers like IL-1β, IL-6,TNF-α, NF-κb p65 subunit were considerably increased (p<0.01) in

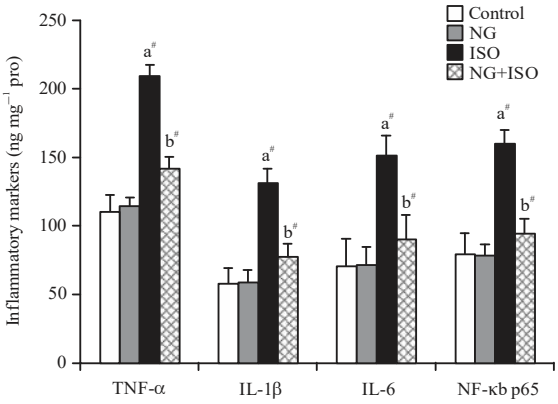

Fig. 2: Change in inflammatory markers, TNF-α: Tumor necrosis factor alpha, IL-1β: Interleukin 1 beta, IL-6: Interleukin six, NF-κb p65 subunit: Nuclear factor kappa bp65 subunit, in experimental animals after NG treatment and ISO induction.

Data are represented as the mean±standard deviation (SD) for 8 rats (n = 8) in each group. Probability value (p-value, #p<0.01): Where "a" exemplify the comparison between control vs ISO, while "b" exemplify the comparison between ISO vs. NG+ISO. Pro: Protein

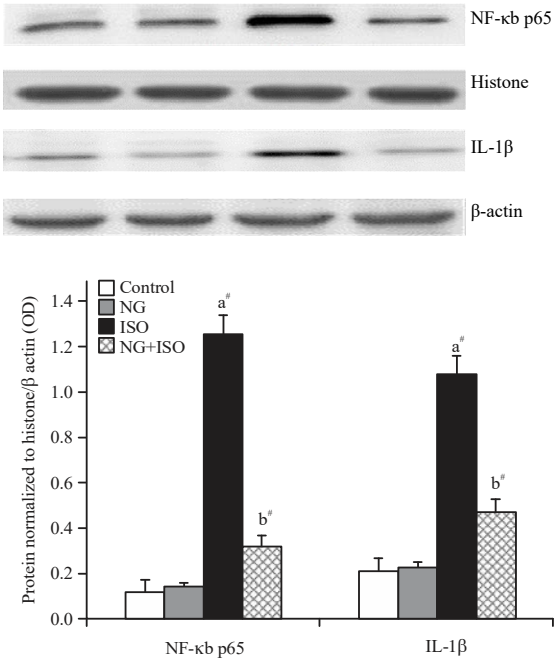

Fig. 3: Change in the protein expression of inflammatory markers like nuclear factor kappa bp65 subunit, NF-κb p65 subunit and Interleukin 1 beta, IL-1β in experimental animals after NG treatment and ISO induction

Data are represented as the mean±standard deviation (SD) for 8 rats (n = 8) in each group. Probability value (p-value, #p<0.01): Where "a" exemplify the comparison between control vs ISO, while "b" exemplify the comparison between ISO vs. NG+ISO, OD: Optical density

ISO administered rats as compared with saline administered rats. While, NG+ISO group rats showed significant decrease (p<0.01) in the concentration of these inflammatory markers as compared with the ISO-induced group.

Effect of NG on the protein expression of NF-κb p65 subunit and IL-1β in experimental animals: The Fig. 3 portraits the change in the protein expression of inflammatory markers like NF-κb p65 subunit and IL-1β in experimental animals. In comparison with the control group, the protein expression of NF-κb p65 subunit and IL-1β were notably up regulated (p<0.01) in the MI model group. Whereas, animals pre-treated with NG (NG+ISO) for 14 days with a dose of 50 mg kg^{-1} showed significant down-regulation (p<0.01) in the protein expression of NF-κ bp65 subunit and IL-1β as compared to ISO injected rats.

DISCUSSION

The data obtained from this animal study showed the potent cardioprotective activity of NG by improving antioxidant status as well as attenuate the inflammatory response via down regulating NF-κb signaling pathway. As indicated by many researchers that animals exposed to supra-maximal dose of ISO could trigger oxidative stress and inflammatory response which results in cardiac hypertrophy as well as disrupt/rupture the myocytes, which leads to increase in water movement (edema) and ultimately results in heart weight in ISO-induced group[6,7]. In consistency with the above statement, the levels of heart weight as well as heart to body weight ratio were significantly increased in ISO-induced group as compared to control. Upon pre-treatment with NG for 14 days considerably protected cardiomyocytes from ISO-induced cardiac damage by lowering oxidative stress (maintain the rigidity of myocytes) and thus suppress the water movement and regulate myocyte homeostasis which subsequently results in decreased heart weight as well as heart to body weight ratio. A copious number of evidence highlighted that ISO-induction could considerably modify the cardiac output and blood pressure and eventually end up in ventricle contractile dysfunction[8,17].

Likewise, the rats injected with ISO showed a significant decrease in the values of hemodynamic parameters like MAP, SAP and DAP. Interestingly, upon supplementation with NG for 14 days prior ISO-induction would considerably improve the mean levels of MAP, SAP and DAP owing to its cardioprotective and antioxidant activity. A similar impression (result) was also observed by Zhang *et al.*[18], who also

demonstrated that treatment with naringenin can markedly alter the cardiac output (blood pressure) and abolish cardiac hypertrophy. Myocytes are highly susceptible to free radical damage as it is rich in polyunsaturated fatty acids as well as lesser cardiac antioxidant capacity[19]. To concord the above statement, present study also showed a significant decrease in the activity of CAT and SOD with increased lipid peroxidation. However, rats administered with NG (NG+ISO group) prior ISO induction showed dramatical improvement in the activity of cardiac antioxidant enzymes like CAT and SOD with reversal of increased MDA levels attributing to its anti-oxidant and free radical scavenging activity mainly because of the presence of 3 free hydroxyl group[20]. Moreover, high dose of ISO injection would trigger overproduction of free radicals (oxidative stress), which attack the PUFA of the plasma membrane of myocytes (rupture) and results in the leakage of cardiac enzymes from myocytes (cytosol) into the blood[21]. Hence, the levels of serum cardiac marker enzymes such as LDH, CK-MB and cTnC are elevated in ISO-induced rats. Nevertheless, pre-treatment with 14 days of NG considerably reverted the increased serum cardiac marker enzymes such as LDH, CK-MB and cTnC to almost near normal due to anti-lipid peroxidation and cardioprotective activity[15].

Inflammation and oxidative stress are the two-pivotal factors that contribute to ISO-induced MI. The concentration of various inflammatory markers such as IL-1β, IL-6, TNF-α, NF-κb p65 subunit was substantially increased in the MI model (ISO-induced) group. The concentration of cardiac inflammatory markers like IL-1β, IL-6, TNF-α, NF-κb p65 subunit was significantly decreased in rats pre-treated with NG for 14 days (NG+ISO) prior to ISO induction due to anti-inflammatory activity. Previously, Chtourou *et al.*[16] reported that treatment with naringenin can significantly decrease the production of pro-inflammatory cytokines like IL-1β, TNF-α, IL-6 by inhibiting the activation of the NF-κb signaling pathway. To confirm the pro-cytokines and NF-κb inhibitory activity by NG, the protein expression of NF-κb p65 subunit and IL-1β were quantified using western blot. After 2 days of ISO induction, the protein expression of NF-κb p65 subunit and IL-1β were markedly up regulated due to increased oxidative stress and inflammatory response. In contrary, the protein expression NF-κb p65 subunit and IL-1β were considerably down regulated in NG pre-treated group due to its potent antioxidant and anti-inflammatory activity[13]. According to Subburaman *et al.*[22], naringenin could significantly down-regulate the expression of IL-1β, TNF-α, IL-6 by inhibiting NF-κb signaling pathway and thus exhibit cardioprotective activity against doxorubicin-induced cardiotoxicity in a rat model. The above statement endorses the cardioprotective active of naringenin by improving antioxidant status as well as by abolishing inflammatory response.

CONCLUSION

Pre-treatment with naringenin for 14 days prior to ISO-induced MI could significantly improve hemodynamic parameters and cardiac antioxidant activity with concomitant decrease in cardiac marker enzymes, lipid peroxidation products and inflammatory markers (pro-inflammatory cytokines) as well as marked down regulation in the levels of protein expressions like NF-κb p65 subunit (activated NF-κb), IL-1β and thus confirming that naringenin exhibit its anti-inflammatory and cardioprotective activity by inhibiting NF-κb signaling pathway. However, more experiments are needed to check the in-depth mechanism behind the cardioprotective activity of NG against ISO-induced cardiotoxicity.

SIGNIFICANCE STATEMENT

The above results show that the cardioprotective active of naringenin is mainly due to enhanced antioxidant activity as well as suppressed inflammatory response. This experiment outcome would contribute for the discovery of novel cardioprotective agent and could be recommended along with common standard MI treatment regimen to treat MI patients.

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