Journal of Food and Drug Analysis

Volume 21 | Issue 3 Article 5

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Recommended Citation

Remya, S.; Chikku, A.M.; Renjith, R.S.; Arunima, S.; and Rajamohan, T. (2013) "Coconut kernel protein in diet protects the heart by beneficially modulating endothelial Nitric oxide synthase, tumor necrosis factor-alpha, and nuclear factor-kappaB expressions in experimental myocardial infarction," *Journal of Food and Drug Analysis*: Vol. 21 : Iss. 3 , Article 5.

Available at: https://doi.org/10.1016/j.jfda.2013.07.012

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.jfda-online.com

Original Article

Coconut kernel protein in diet protects the heart by beneficially modulating endothelial nitric oxide synthase, tumor necrosis factor-alpha, and nuclear factor-kappaB expressions in experimental myocardial infarction

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ARTICLE INFO

Article history:
Received 19 November 2012
Received in revised form
26 January 2013
Accepted 16 April 2013
Available online 7 August 2013

Keywords:
Antioxidant enzymes
Coconut kernel protein
Isoproterenol
L-Arginine
Myocardial infarction

ABSTRACT

Previous studies conducted in our laboratory revealed that coconut kernel protein has a significant cardioprotective effect on isoproterenol-induced myocardial infarction in rats. In the present study, we explored the possible protective mechanism of coconut kernel protein during acute myocardial infarction. Coconut kernel protein (50 mg/100 g) was administered to Sprague-Dawley rats orally for 45 days. Isoproterenol (20 mg/100 g) was injected subcutaneously at an interval of 24 hours twice to induce myocardial infarction. Myocardial infarction was confirmed by the abnormal activities of cardiac marker enzymes in serum. Activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase were decreased ($p < 0.05$) in the heart of isoproterenol-treated rats, whereas pretreatment with coconut kernel protein increased ($p < 0.05$) these activities. An improved antioxidant status in these rats was further confirmed by the increased level of reduced glutathione and decreased level of lipid peroxidation products. Nitric oxide synthase (NOS) activity in the heart and nitrite level in blood were increased ($p < 0.05$) in coconut kernel protein-treated rats administered with isoproterenol compared to isoproterenol control rats. Coconut protein pretreatment upregulated the expression of endothelial nitric oxide synthase (eNOS), whereas expressions of nuclear factor-kappaB (NF-κB) and tumor necrosis factor-alpha (TNF-α) were downregulated in isoproterenol-treated rats. These findings suggest that the protective effects of coconut kernel protein may be mediated in part through upregulation of nitric oxide production, antioxidant mechanisms, and its ability to inhibit TNF-α and NF-κB activation.

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1021-9498
http://dx.doi.org/10.1016/j.jfda.2013.07.012

1. Introduction

Myocardial infarction (MI) is one of the leading causes of death worldwide. The pathophysiology of MI begins with plaque formation in the coronary artery, which ruptures eventually, causing thrombus formation and ischemia in the heart. Experimental and clinical studies indicated that the pathology of MI is mainly associated with oxidative stress [1]. The resulting oxidative stress leads to free radical formation and also triggers the inflammatory pathway that ultimately leads to cell damage [2]. Therefore, therapeutic interventions that improve antioxidant status may exert beneficial effects in ischemic heart diseases. Pharmacological augmentation of endogenous myocardial antioxidants has been identified as a promising therapeutic approach in diseases associated with increased oxidative stress such as MI [3]. Several studies have demonstrated that NF-κB, an ubiquitous transcription factor, activated by various stimuli such as reactive oxygen species (ROS), hypoxia, and inflammatory cytokines such as tumor necrosis TNF-α, is substantially involved in the progression of cardiac remodeling [4,5]. Downregulation of TNF-α and NF-κB may also attenuate cardiac remodeling and cardiac failure after MI [6,7].

Recently, plant-based dietary agents that can minimize cardiac damage during ischemia are gaining wide attention. Our previous experiments showed that the major protein fraction (globulins) isolated from coconut kernel protein (CKP) possesses antidiabetic, antiperoxidative, and cardioprotective effects [8,9]. Studies have suggested that L-arginine, the precursor of nitric oxide (NO), a vasodilatory hormone, can have a positive effect during MI through the endothelial nitric oxide synthase (eNOS) pathway [10]. Evidence suggests that reduced NO availability may play an important role in the pathophysiology both after experimental myocardial ischemia and in patients with MI [11]. Inhibition of NO production results in impaired endothelium-dependent vasodilation, reduces myocardial neovascularization, and augments myocardial remodeling [12].

Isoproterenol (ISO)-induced MI in rats is one of the most widely used experimental models to study the beneficial effects of many drugs on cardiac function. Hence, ISO was chosen to induce experimental MI. Based on these facts, the present study investigated the possible role of CKP in modulating the antioxidant status and expressions of eNOS, TNF-α, and NF-κB against ISO-induced acute MI.

2. Methods

2.1. Drugs and chemicals

All chemicals used for the study, including ISO and TRI reagent, were obtained from Sigma-Aldrich (St Louis, MO, USA). Synthesis of cDNA and polymerase chain reaction (PCR) were performed using commercial kits purchased from Fermentas (Thermo Fisher Scientific Inc., Ottawa, Canada). All other chemicals used were of the highest analytical grade.

2.2. Experimental animals

Male albino Sprague-Dawley rats, weighing 150–200 g and bred in our department's animal house, were used for the study. All animal care and procedures were performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India. The entire experimental protocol was approved by the Institutional Animal Ethics Committee of the University of Kerala. The animals were housed individually in polypropylene cages placed in a room maintained at 25 ± 5 °C, with a 12-hour light and 12-hour dark cycle.

2.3. Isolation of CKP

The total globulin fraction was isolated from the kernel protein following a procedure described earlier [8].

2.4. Experimental protocols

The experimental animals were divided into four groups of six rats each: Group I served as the normal control, Group II as the ISO control, Group III as the CKP control, and Group IV as the CKP-fed, ISO-administered experimental group.

All the animals were given standard rat chow (Sai Feeds, Bangalore, India). CKP was administrated orally to rats of Groups III and IV, at a dose of 50 mg/100 g body weight. Calorie intake was adjusted to be the same in all four groups by supplementing casein at the same dose level as CKP to rats of Groups I and II. The animals were fed the above diet and distilled water *ad libitum*. Food consumption of individual rats was recorded daily, and body weights were measured weekly. Experiments were conducted for 45 days. At the end of the experimental period, MI was induced in rats of Groups II and IV by a subcutaneous injection of ISO at a dose of 20 mg/100 g body weight, administered twice at an interval of 24 hours. Rats that survived after the second dose of ISO injection were deprived of food and subjected to euthanasia by means of an intraperitoneal injection of thiopentone sodium (>40 mg/kg body weight). The blood sample was collected and the heart was removed, washed in ice-cold physiological saline, and stored in a cold condition for various estimations.

2.5. Biochemical estimations

Serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were assayed using the enzyme kit from CML Biotech (P) Ltd. (Kochi, India), based on the method of Reitman and Frankel [13]. The activity of creatine phosphokinase (CK-MB) was assayed by the method of Olive modified by Rosalki [14], using an Enzopak CK-MB kit from Reckon Diagnostics Pvt. Ltd. (Baroda, India). Lactate dehydrogenase (LDH) was estimated using an enzyme kit purchased from ERBA Diagnostics, Mumbai, Maharashtra, India [15]. NOS activity in the heart was determined [16] and the nitrite level in blood was estimated following the method described by Green et al [17] SOD and catalase in the heart tissue were assayed using the method described by Kakkar et al [18] and that by Maehly and Chance [19], respectively. The

reduced glutathione level in the heart was determined by the procedure described by Patterson and Lazarow [20]. Thiobarbituric acid reactive substances (TBARS) were estimated by the assay method described by Ohkawa et al [21].

2.6. Isolation of total genomic RNA

Total genomic RNA was isolated from the heart using TRI reagent (Sigma-Aldrich) by the method described by Chomczynski and Macke [22].

2.7. Synthesis of cDNA and reverse transcriptase–PCR

Synthesis of cDNA and PCR were performed as per the manufacturer's instructions using the kit purchased from Fermentas. The following primers were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH): (forward) 5′-CCT TCA TTG ACC TCA ACT AC-3′ and (reverse) 5′-GGA AGG CCA TGC CAG TGA GC-3′; eNOS: (forward) 5′-ATG GCG AAG CGT GTG AAG-3′ and (reverse) 5′-ATT GTG GCT CGG GTG GAT-3′; NF-κB: (forward) 5′-CCT AGC TTT CTC TGA ACT GCA AA-3′ and (reverse) 5′-GGG TCA GAG GCC AAT AGA GA-3′; and TNF-α: (forward) 5′-AGT CTT CCA GCT GGA GAA GG-3′ and (reverse) 5′-GCC ACT ACT TCA GCA TCT CG-3′. The reverse transcriptase-PCR products were electrophoresed on 1.5% agarose gel and visualized by staining with ethidium bromide. The gels were subjected to densitometric scanning (Gel Doc, Bio-Rad Laboratories, Hercules, CA, USA) to determine the optical density of each and then normalized against an internal control, GAPDH, using Quantity One imaging software (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Histopathology

The heart tissues were dissected out rapidly, washed in saline, and fixed by immersion in 10% formalin solution at room temperature. For histological examinations, paraffin-embedded tissue sections of the heart (5 μm) were stained with hematoxylin–eosin [23]. The sections were then examined under light microscope (Axioscope 2 plus, Zeiss, Thornwood, NY, USA) and photographed (Zoom Browser EX, Canon, Tokyo, Japan).

2.9. Statistical analysis

Statistical analysis was performed by one-way analysis of variance followed by Duncan's multiple range test using SPSS Statistics version 17.0.1 (SPSS Inc, Chicago, IL, USA). Results were expressed as mean ± standard deviation for six rats in each group. A p value <0.05 was considered statistically significant.

3. Results

A marked rise in the activities of cardiac marker enzymes such as LDH, CK-MB, SGOT, and SGPT was observed in the serum of ISO-induced rats in comparison with normal control rats. Rats pretreated with CKP showed a decreased activity of these enzymes as compared to ISO-administered control rats (Table 1). Activities of marker enzymes in Group III rats were not significantly different from that in Group I rats. NOS activity was decreased in rats treated with ISO alone compared to normal rats, whereas the activity was increased in CKP-fed rats on ISO administration (Table 2). Nitrite level in blood also showed a similar trend. Activities of antioxidant enzymes such as SOD and catalase increased in CKP-pretreated rats compared to ISO control rats (Table 3). Level of reduced glutathione, an endogenous nonenzymatic antioxidant, was also increased in these rats. Peroxidative damage of lipids, as evidenced by the thiobarbituric acid reactive substances (TBARS), was low in CKP-pretreated rats (Fig. 1).

The expression of eNOS was upregulated in CKP-fed rats administered with ISO in comparison with ISO control rats. CKP-fed normal rats also showed an increased expression of eNOS, but the increase was not significantly different from normal rats (Fig. 2). NF-κB and TNF-α expressions were increased in ISO control rats compared to normal rats. CKP-fed rats, on ISO administration, showed a decrease in expressions of these inflammatory genes compared to ISO control rats.

3.1. Histopathological findings

Histopathological examination of the heart of control rats showed normal morphology and architecture. The heart of rats subjected to an ISO injection showed focal areas of degeneration and necrosis, as compared to control rats. Fragmented myocytes with eosinophilic and vacuolated cytoplasm were also observed in these rats. The heart of ISO-administered rats that were treated with CKP showed regeneration of myoblasts. Inflammation and necrosis were less in

Table 1 – Activities of LDH, creatine kinase, glutamate oxaloacetate transaminase, and glutamate pyruvate transaminase in serum.

Group	LDH (IU/L)	CK-MB (IU/L)	SGOT (IU/L)	SGPT (IU/L)
I	244.49 ± 22.30	480.83 ± 43.87	181.17 ± 16.51	93.97 ± 8.4
II	768.53 ± 70.11[a]	685.94 ± 62.59[a]	233.03 ± 21.25[a]	197.28 ± 17.95[a]
III	221.48 ± 20.20	458.48 ± 41.84	152.21 ± 13.88	85.67 ± 7.79
IV	589.88 ± 53.82[b]	600.11 ± 54.75[b]	213.06 ± 19.43[b]	176.27 ± 16.07[b]

Data are expressed as mean ± standard deviation of six rats. Significance is accepted at $p < 0.05$ between two groups.
LDH = lactate dehydrogenase; CK-MB = creatine phosphokinase; SGOT = serum glutamate oxaloacetate transaminase; SGPT = serum glutamate pyruvate transaminase.
[a] Values are significantly different from Group I.
[b] Values are significantly different from Group II.

Table 2 – Effect of CKP on NOS activity in the heart and nitrite level in blood.

Group	NOS (unit/mg protein)	Nitrite (μM/L)
I	0.093 ± 0.007	12.62 ± 1.15
II	0.073 ± 0.006[a]	10.62 ± 0.97[a]
III	0.096 ± 0.007	12.91 ± 1.18
IV	0.087 ± 0.007[b]	12.16 ± 1.39[b]

Data are expressed as mean ± standard deviation of six rats. Significance is accepted at $p < 0.05$ between two groups.
CKP = coconut kernel protein; NOS = nitric oxide synthase.
[a] Values are significantly different from Group I.
[b] Values are significantly different from Group II.

Table 3 – Effect of CKP on SOD, catalase activity, and reduced GSH level in the heart.

Group	SOD (unit/mg protein)	Catalase ($\times 10^3$ unit/mg protein)	GSH (mM/g tissue)
I	49.86 ± 4.54	8.97 ± 0.80	1.93 ± 0.16
II	21.06 ± 1.90[a]	2.17 ± 0.18[a]	1.25 ± 0.10[a]
III	49.37 ± 4.48	12.06 ± 1.08	1.80 ± 0.15
IV	38.58 ± 3.51[b]	5.03 ± 0.45[b]	1.60 ± 0.14[b]

Data are expressed as mean ± standard deviation of six rats. Significance is accepted at $p < 0.05$ between two groups.
CKP = coconut kernel protein; GSH = glutathione; SOD = superoxide dismutase.
[a] Values are significantly different from Group I.
[b] Values are significantly different from Group II.

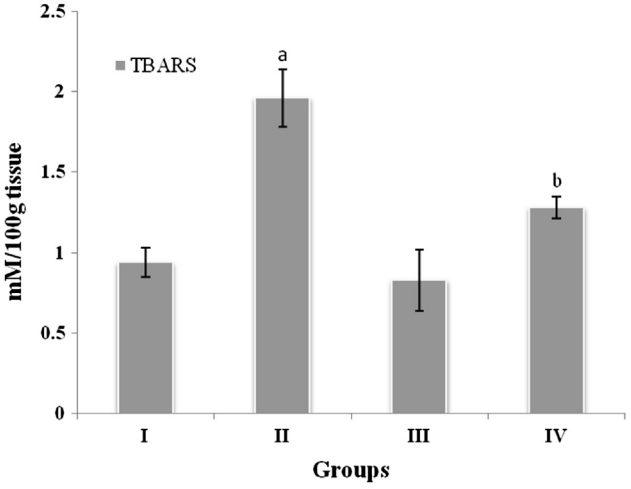

Fig. 1 – Effect of CKP on TBARS level in the heart. The letter 'a' indicates that values are significantly different from Group I and 'b' indicates that values are significantly different from Group II. Data are expressed as mean ± SD of six rats. Significance is accepted at $p < 0.05$ between two groups. CKP = coconut kernel protein; SD = standard deviation; TBARS = thiobarbituric acid reactive substances.

this group compared to the ISO control group (Fig. 3). Histopathological changes were graded and summarized (Table 4).

4. Discussion

ISO is a synthetic catecholamine and β-adrenergic agonist that, in large doses, is documented to induce MI due to the generation of highly cytotoxic free radicals through its autoxidation [12]. Inflammatory response and oxidative stress have been recognized as possible mechanisms of ISO-induced MI. Substantial evidence shows that ischemic tissue generates oxygen-derived free radicals (oxygen molecules containing an odd number of electrons), making them chemically reactive and often leading to chain reactions [24]. Studies carried out in our laboratory indicate that CKP has hypolipidemic and cardioprotective effects [25,26]. We have reported that CKP is rich in L-arginine [8]. The present study is intended to understand the effect of CKP on the antioxidant status, and expressions of eNOS and inflammatory mediators such as TNF-α and NF-κB in rats induced with ISO.

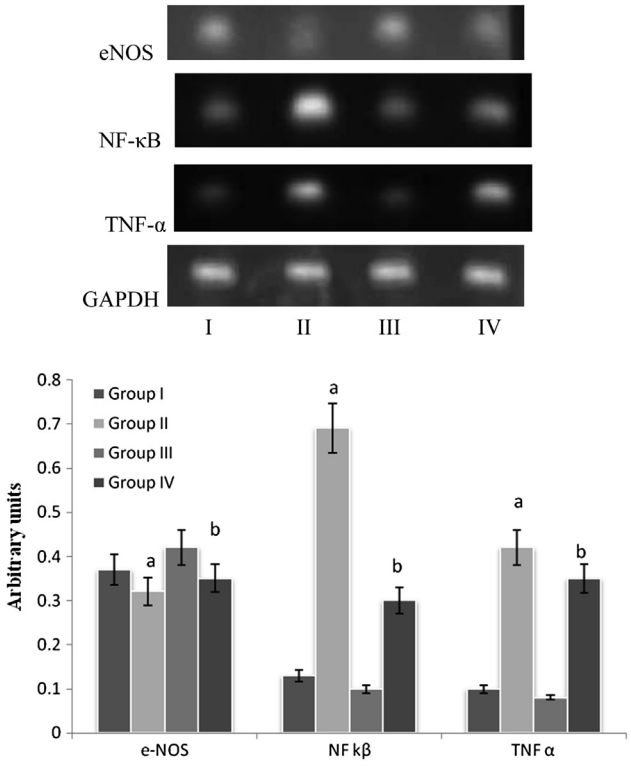

Fig. 2 – Effect of CKP on eNOS, NF-κB, and TNF-α gene expressions on the heart tissue of normal and experimental rats. The letter "a" indicates that are significantly different from Group I and "b" indicates that values are significantly different from Group II. Data are expressed as mean ± SD of six rats. Significance is accepted at $p < 0.05$ between two groups. CKP = coconut kernel protein; eNOS = endothelial nitric oxide synthase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; NF-κB = nuclear factor-kappaB; SD = standard deviation; TNF-α = tumor necrosis factor-alpha.

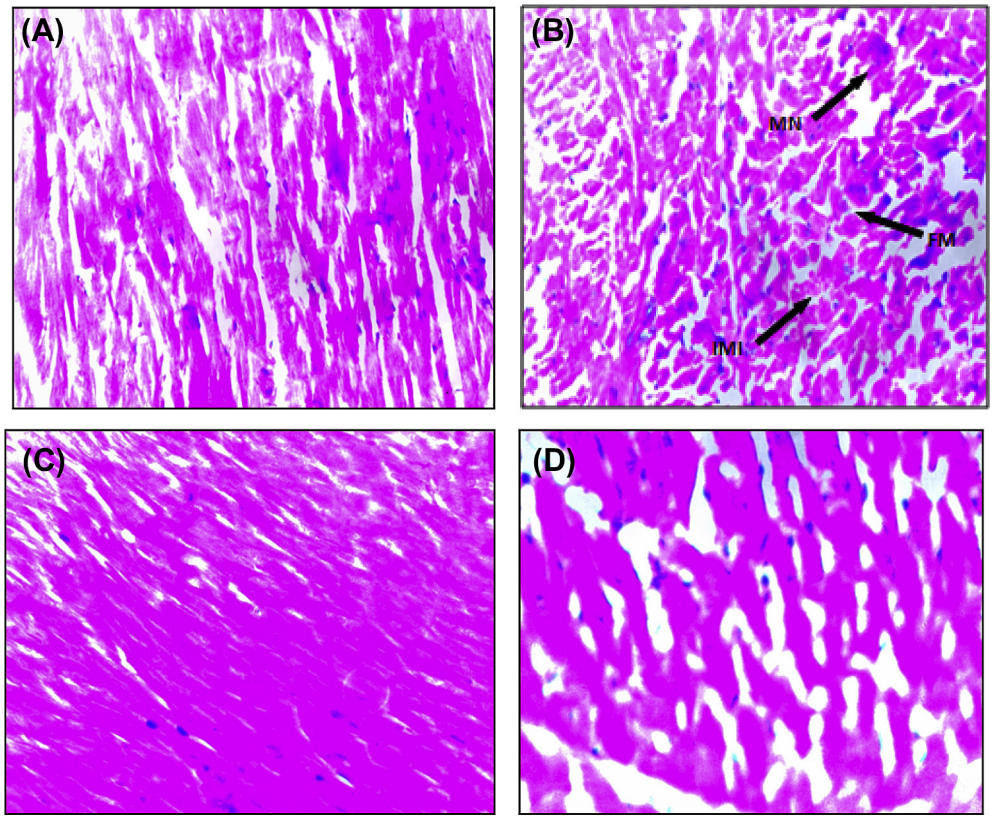

Fig. 3 – Photomicrograph showing histopathology of the heart (H&E 40×). (A) Normal myocardium of control group. (B) ISO-administered group showing diffused inflammation and extensive myocardial damage. (C) Normal myocardium of CKP-treated group. (D) CKP-pretreated group on ISO administration showing almost normal myocardium. CKP = coconut kernel protein; FM = fragmented myocytes; H&E = hematoxylin and eosin; IMI = inflammatory mononuclear infiltration; ISO = isoproterenol; MN = myocardial necrosis.

The myocardium contains an abundant concentration of diagnostic marker enzymes such as CK-MB, LDH, SGOT, and SGPT. Once metabolically damaged, the myocardium releases its contents into the extracellular fluid. Increased activities of these cardiac marker enzymes in serum of ISO-induced rats confirm the onset of myocardial necrosis [27]. Similar results were observed in the present study. An increase in the activities of marker enzymes in serum could be due to the leakage of enzymes from the heart as a result of ISO-induced necrosis, and the amount of enzymes that appear in serum is in proportion to the number of necrotic cells [28]. As evident from the serum marker enzyme activity, pretreatment with CKP attenuated these changes.

Table 4 – Histopathological assessment of myocardium in different experimental groups[a].

Group	Necrosis	Edema	Inflammation
I	–	–	–
II	+++	+++	+++
III	–	–	–
IV	+	+	+

[a] Relative pathological changes were graded as the histopathological score of each slide obtained from rats of four groups used in the study and were ranked as follows: – = no change, + = focal change, ++ = patchy change, and +++ = massive change.

As already indicated, CKP is a good source of L-arginine (17.8%) [8]. Recently, L-arginine supplementation has been identified as a nutritional strategy for preventing and treating MI. L-arginine is acted upon by NOS to produce NO, which is a potent vasodilator [29]. Activity of NOS in the heart was decreased significantly in the ISO-induced group, whereas pretreatment with CKP resulted in a significant increase in the activity of NOS. The higher level of nitrite in blood was due to an increased production of NO, as evident from the increased activity of NOS. Previous studies conducted using different animal models demonstrated that L-arginine can increase NO production, which reduces generation of superoxide anions and prevents reduced expression of eNOS [30,31]. This suggests the protective role of NO and eNOS in heart failure. In a healthy heart, physiological amounts of NO may help sustain cardiac inotropy. In heart failure, both upregulation [32] and downregulation [33] of eNOS have been observed. Loss of eNOS expression and NO production is observed in MI [12]. Overexpression of eNOS in cardiomyocytes was found to improve cardiac function and reduce hypertrophy in heart failure after MI [34]. NO derived from eNOS can decrease the extent of myocardial hypertrophy, fibrosis, and myocyte loss

[35], and increase angiogenesis and myocardial perfusion through vasodilation.

Previous studies have shown that ISO-induced MI generates numerous free radicals and decreases the antioxidant status of the myocardium, causing damage to the cellular membranes through lipid peroxidation [2]. Similar trends were observed in this study. An increased activity of endogenous antioxidant enzymes such as SOD and catalase was observed in CKP-treated rats on ISO administration compared to the ISO control group. Moreover, a decrease in lipid peroxidation and an increase in reduced glutathione content were observed in CKP-treated rats. These results showed that the oxidative stress during ISO administration in rats was alleviated effectively by CKP, possibly due to the presence of L-arginine. Reports suggest that L-arginine supplementation relieves oxidative stress in experimental MI [36]. The protective effect of L-arginine may be due to antioxidant properties of eNOS-derived NO. NO is known to inhibit ROS-mediated reactions, and it has been suggested that the protective effects found in a variety of conditions are due to the ability of NO to detoxify ROS such as O_2^-, $OH^\cdot$, and/or ferryl hemoprotein [37]. Recent studies carried out by us indicate that CKP possesses antioxidant property and the major factor responsible for the effect is L-arginine [8,38].

Histopathological examination of myocardial tissue in control and CKP-treated rats depicted clear integrity of the myocardial cell membrane. No inflammatory cell infiltration was seen in the heart of control and CKP-treated rats. In the ISO-administered group, increased hyalinization, fragmentation of muscle fibers, and myocardial necrosis were observed. Pretreatment with CKP demonstrated reversal of focal lesions, fragmentation of muscle fibers, and retrogressive lesions with hyaline necrosis in the ISO-treated group, confirming further the cardioprotective activity of CKP.

Several studies support the theory that inflammation plays an important role in cardiovascular diseases including MI [39,40]. NF-κB is a prime target for ROS [4]. Activation of NF-κB appears to play a significant role in the pathophysiology of endothelial dysfunction, unstable angina pectoris, acute MI, and heart failure [41]. Thus, suppression of NF-κB activity can be a potential mechanism for regulating inflammatory responses [42]. Upregulation of TNF-α is also observed during ISO-induced MI [43]. Many studies have reported that ISO administration stimulates the proinflammatory cytokine TNF-α [44,45]. Our study showed that CKP treatment downregulates expressions of NF-κB and TNF-α in ISO-induced rats, which may be due to a decrease in oxidative stress stimulus via NO-mediated protection. This observation suggests that CKP is effective in preventing inflammatory responses that are triggered by ISO-induced MI.

The protective effect of CKP seems to be mediated mainly through its ability to modulate the nitric oxide pathway, and inhibit mRNA expressions of NF-κB and TNF-α. In conclusion, these results suggest that dietary supplementation of CKP may have a greater significance in reducing the extent of oxidative stress and inflammatory responses associated with MI. Therefore, CKP may be included as a protein addendum for the development of a cardiotonic nutraceutical or functional food.

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