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Morin controls high-cholesterol diet-induced inflammatory cardiac dysfunction through the regulation of nitric oxide synthesized enzymes and P65 NF-ϰB gene

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Summary. Morin is a natural yellow compound that is scientifically proven to have hypoglycemic, anti-inflammatory and anti-oxidant properties. The ameliorative effect of morin on high-cholesterol-diet (HCD) induced cardiac damage has not yet been assessed. Hence, in the present study, we evaluated the ability of the compound to controls HCD induced cardiac inflammation and oxidative damage through the regulation of nitric oxide synthesized enzymes and nuclear factor kappa B (P65 NF-κB) gene. HCD rats exhibited an increased activity of markers of cardiac enzyme in serum such as lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB) and creatine kinase (CK). Administration of HCD to the rats was fond to elevate the serum and cardiac lipids profiles. Moreover, cardiac pro-inflammatory and cardiac oxidative markers were raised and the antioxidant markers were lowered. However, in the morin treated to HCD rats, the above markers for biochemical, inflammatory, antioxidants, and oxidative changes were reverted to near normal food consumed control rats. The myocardial protein expressions of neuronal nitric oxide, inducible nitric oxide, and endothelial nitric oxide synthases, as well as P65 NF-κB were significantly increased in rats supplemented with HCD. When treated with morin, these protein levels were lowered and were comparable to those of normal food consuming rats. Such an alleviated inflammatory myocardial dysfunction upon morin administration has been proven by the improvement of histological features. The results of this study suggest that morin administration combats HCD induced myocardial inflammation and dysfunction through the regulation of nitric oxide synthesized enzymes and P65 NF-κB gene. These results vouch for benefits of dietary morin against HCD induced cardiac dysfunction.

Key words. hypercholesterolemia, morin, oxidative stress, inflammation process

Introduction

Hyperlipidemia associated oxidative inflammation plays an important function in atherosclerosis and cardiovascular diseases (CD). CD, particularly coronary heart disease (CHD), is a well-known growing public health issue worldwide. Hypercholesterolemia is extensively known as a lipoprotein metabolic disorder characterized by accentuated lipid profiles and is the most important risk issue for CD (1-3). Obesity is an established causative factor for the expansion of atherosclerosis and CHD, and is known to produce systemic and cardiac inflammation finally resulting in a failure of cardiac cell function (4). A prolonged consumption of high cholesterol food is the predominant reason for obesity as well as an increase in systemic and cardiac inflammatory markers such as tumor necrosis factor (TNF-α,), interleukin-1 beta (IL-1β), interleu-

kin (IL-6) and caspase-3. Earlier studies have shown that increased levels of proinflammatory markers, including TNF-α, IL-6 and IL-1β occur in heart failure conditions (5-7). Cytokines are known to play a protective role in certain developed and developing diseases, including heart failure (8). Therefore, cytokine-based preventive drugs without prominent side effects are urgently needed for treating high cholesterol food induced heart failure patients.

Chronic hypercholesterolemia has been found to induce oxidative organ damage, which is an important factor for raising the risk of CD (9). Earlier studies have clearly indicated that hypercholesterolemia is causally associated with a significant increase in reactive oxygen species (ROS) and a concomitant lowering of the cardiac tissue antioxidant capacity (10, 11). High circulating cholesterol levels attributed to HCD consumption may activate endothelial cells and lead to increased production of ROS (12, 13). This mechanism induces vascular function impairment, cell proliferation, cell death, and cardiac remodeling (14, 15). Certainly, HCD supplementation leads to oxidative stress, impulsive arterial vasoconstriction, and systemic hypertension (16). Goncalves and his colleagues (17) reported that the Western diet is high in fat and induces biventricular cardiomyocyte hypertrophy, increased stiffness, and impaired relaxation in rats. However, the effects of HCD on bioenergetics and oxidative stress, and impairment of cardiac function are only partially understood and no treatment has demonstrated compelling effectiveness.

Currently, the use of dietary flavonoids, known as polyphenolic compounds, is gaining the attention of researcher. The compound is abundant in plant-derived beverages such as red wine and tea, as well as in many fruits, green vegetables, and traditional medicinal plants. Flavonoid display anti-inflammatory properties as it brings about detoxification of free radical, metal chelation, antioxidant enzyme modulation, and inflammatory cytokine regulation (18, 19). Recently, it has been shown that flavonoids can also improve intracellular bioenergetics (20, 21). Several reports have concluded that these compounds can exert cardio-protective effects due to their ability to attenuate oxidative stress. Morin, a yellow colored bioflavonoid possesses an extensive assortment of pharmaceutical and biological properties including antioxidant, antiviral, anti-carcinogenic, and anti-inflammatory properties (19, 22, 23). Morin also decreases oxidative damage in the fibroblast cells of the lungs (24), cardiovascular cells (25) and hepatocytes (26, 27). The effectual characteristics of morin against high-HCD-induced cardiac damage have not yet been explored. Hence, in this study, we attempted to check whether the compound controls HCD induced inflammatory cardiac dysfunction through the regulation of nitric oxide synthesized enzymes and P65 NF-κB gene.

Materials and Methods

Animals

140-160 g adequate numbers of Wistar albino rats were attained from Pharmacy College Animal Care Center at King Saud University. All received animals were acclimatized for 10 days prior to start the experiments. All rats were sustained in standard conditions such as 22 ± 1 °C temperature, 50-55% humidity, and equal 12 h day/night cycles. All the experimental protocol such as euthanasia procedure, blood sampling and final sacrifice were followed by National Institute of Health guide care policy (NIH, 1996) and this animal study was approved (647-EACC-2017 dated 02-01-2017) by Pharmacy College Animal Care Center Ethical Committee.

Food composition for normal food and high cholesterol food

High cholesterol diet in pellet form was prepared by adding 1% cholesterol + 0.5% cholic acid with normal cholesterol rat chow (NCRC) powder. Six rats were fed on NCRC (content: protein 20%, fat 4%, fiber 3.5%, ash 6%, total energy 2850 Kcal/kg) and twenty-four rats were fed HCD for 6 weeks. Water and food were allowed to free access in this whole experiential duration.

Chemicals

Morin, cholesterol and cholic acid were purchased from Riedel-del Haen, Germany, Alpha Chemika, India and Fluka, Switzerland, respectively. The diagnostic kits of total cholesterol (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C),

high-density lipoprotein-cholesterol (HDL-C), CK-MB, LDH and CK were acquired from Human Diagnostics, Wiesbaden, Germany. The diagnostic kits of IL-1β, TNF-α, caspase-3, IL-6, Superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione peroxidase (Gpx) were acquired from standard R&D company from USA. The diagnostic kit of Glutathione (GSH) and thiobarbituric acid-reactive substance (TBARS) were obtained from Cayman Chemical, USA.

Study design:

Ten days of environmental espoused animals were administered HCD or NCRC for 6 weeks. After 6 weeks the rats were randomly divided in to five groups by taking six rats in each group: 1) NCRC control, 2) HCD control 3) HCD + Morin (25 mg/kg/day), 4) HCD + Morin (50 mg/kg/day) and 5) HCD + Morin (100 mg/kg/day). Morin was treated orally for four consecutive weeks and this period HCD was continued until end of the experiment. The body weight and health conditions of animals were checked carefully by weekly once. Blood were gathered by cardiac puncture under total anesthesia state. The serum was suppurated by 4,000 rpm centrifugation of sample for 10 minutes and stored at -20 ºC prior to analysis. Finally, animals were decapitated and heart tissues were dissected, weighed, immediately small cross section of each heart tissue and dipped into liquid nitrogen for 1 min. This heart section was stored at -80 º C until analysis. Another cross sectioned heart was preserved in 10% formaldehyde for histopathological evaluations.

Estimations of lipid levels in serum

TC, TG, LDL and HDL levels were estimated by commercially existing kits.

Estimations of cardiac enzymes in serum

CK-MB, CK and LDH were estimated by commercially existing diagnostic kits.

Estimations of lipid profile in cardiac tissues

Heart lipids were extorted by standardized Folch et al (28) method and used in chloroform–methanol mixture (2:1 v/v). Briefly, tissues were homogenized with 0.74% potassium chloride (1:1 w/v) and suspended in 2 ml of chloroform andmethanol mixture for 2min and then centrifuged.The chloroform layer was dried and the remaining cardiac lipid contents were used for analysis. The tissue phospholipid (PL) was ascertained by ideal method of Zilversmit and Davis (29). FFA was ascertained by ideal method of Falholt et al. (30). TC and TG were ascertained by kits which are available in commercially. Results were articulated as mg/g of tissue.

Estimations of inflammatory biomarkers in cardiac tissue

IL-1β, TNF-α, IL-6 and caspase-3 were ascertained by ELISA kits which are available in commercially.

Western blot analysis

Myocardial total protein was assayed in each sample by using assay kit (BioRAD, Hercules, CA). The protein bonds were separated by using SDS-PAGE. The obtained protein bonds were transferred from gel to PVDF membrane at 25 volt. The membrane was incubated with primary antibody solution by overnight at 4°C. After the membrane was washed tree to five times with TBST buffer and then incubated with the secondary antibody (HRP-conjugated solution) for one h at room temperature. Finally, the membrane was washed for three to five times by using TBST buffer. The chemiluminescent substrate was used for bonds development and the bands were seen and captured by CCD camera-based ChemiDoc TM imager (Bio-Rad Laboratories, Inc, 2000 Alfred Nobel Drive, Hercules, California 94547, USA). The band intensity of target protein was calculated by image analysis software.

Estimations of oxidative stress parameters in cardiac tissues

TBARS and GSH levels were ascertained by kits which are available in commercially. SOD, CAT, GPx and GST were ascertained by using commercially available ELISA kits.

Histological assessments procedure

A collected portion of a heart tissue from each group was conserved in 10% formalin. Each sample was implanted separately in paraffin blocks and then 5 mm section was removed by using rotary microtome.

The section was stained with haematoxylin and eosin. Finally, the histology was captured by microscope and evaluated the changes of histology.

Statistical Analysis

Result was conveyed as mean ± standard error. Statistical variations from used groups were analysed using one-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparisons test. Different letter and symbol are represents as statistically different if the p value was less than 0.05, 0.01 and 0.001.

Results

The enzymes of CK, CK-MB and LDH are considered the cardiac markers and these were estimated and shown in Figure 1. In HCD administered rats, the serum enzymes of CK, CK-MB and LDH were shown to increases (P<0.001) compared to NCRC control group. 100 g of morin treatment (100 mg/kg/day) markedly inhibited these enzymes changes in HCD fed animals. Moreover, the lower and higher dose of morin (25 and 50 mg/kg/day) also inhibited these activity of enzymes but not statistically significant.

Serum TC, TG and LDL-C were increased significantly (P<0.001) while HDL-C was shown unchanged level in HCD fed animals compared to NCRC control rats. The elevated TC and LDL-C levels were found in markedly decreased (P<0.05 and P<0.01) by morin (50 and 100 mg/kg/day) treatment as compared to HCD fed control group. However, morin treatment significantly and dose dependently inhibited the increased TG levels in serum of HCD fed rats while compared to untreated HCD fed animals. These interesting serum lipid profiles results are shown in Figure 2.

Myocardial lipids including TC, TG, PL and FFA were estimated and presented in Figure 3. Cardiac TC, TG and FFA levels were significantly (P<0.001) increased in HCD fed rats when compared to that of NCRC control group. Cardiac PL was markedly (P<0.01) inhibited in HCD consumed rats when compared to NCRC control rats. Treatment of morin in HCD consumed rats, these TC, TG and FFA levels were reduced and PL level was increased (P<0.05) when compared to HCD consumed control rats. These

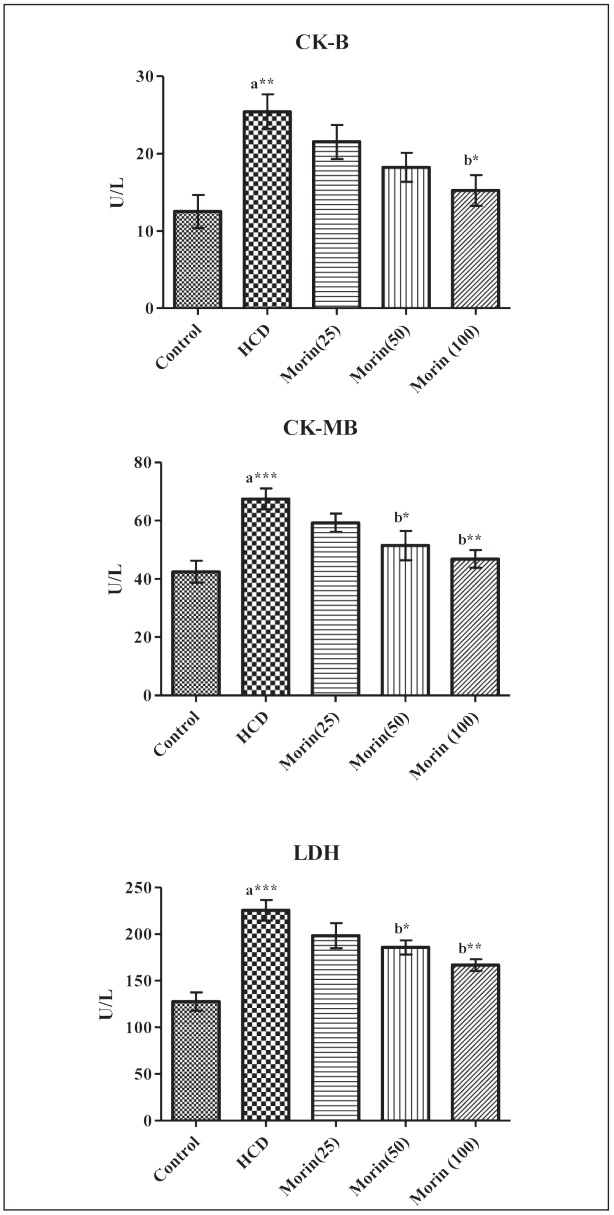

Figure 1. Effect of morin on HCD-induced changes in serum CK, CK-MB and LDH levels.

cardiac lipids results are shown in Figure 3.

TNF-α, IL-1β, IL-6 and caspase-3 are the markers of cellular inflammation and these were found in increases significantly (P<0.001) in cardiac tissue of HCD consumed rats when compared to NCRC rats. Morin treatment to HCD fed rats these cellular inflammatory markers were inhibited significantly when compared to HCD control group. These cardiac cellular inflammatory markers results are shown in Figure 4.

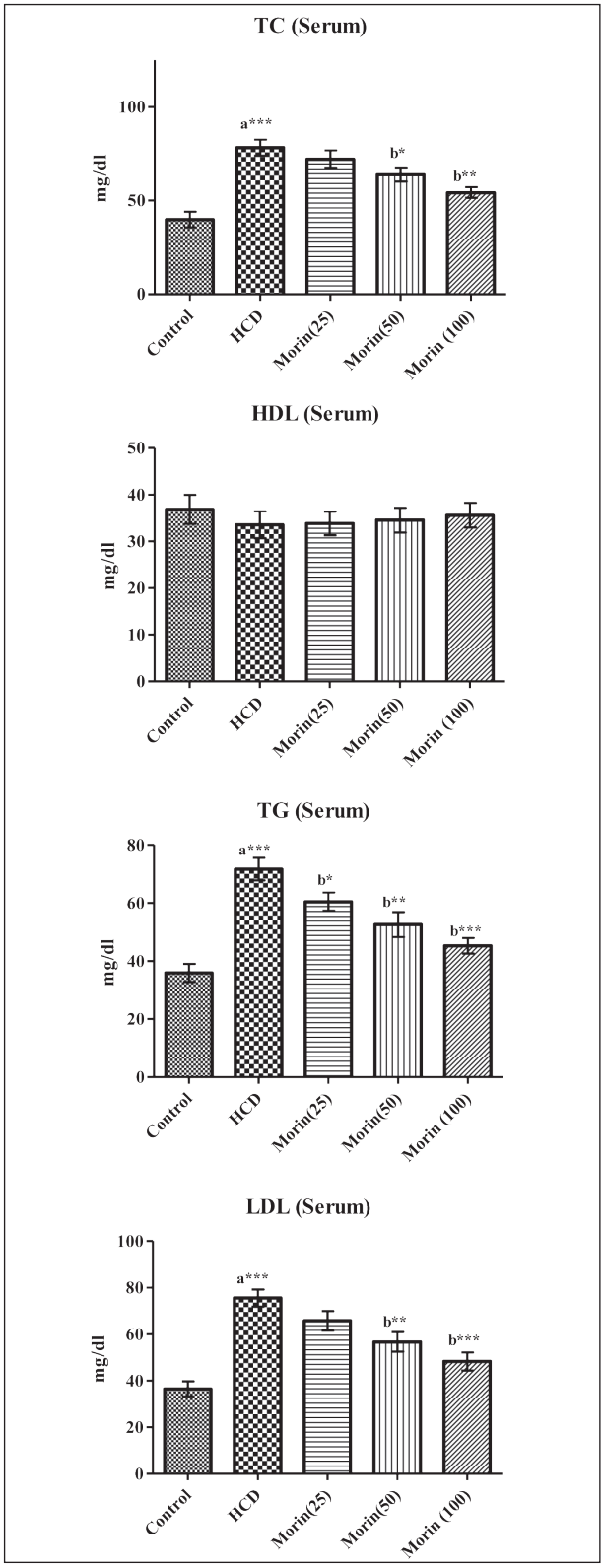

Figure 2. Effect of morin on HCD-induced changes in serum lipid levels of TC, TG, HDL-C and LDL-C in rats.

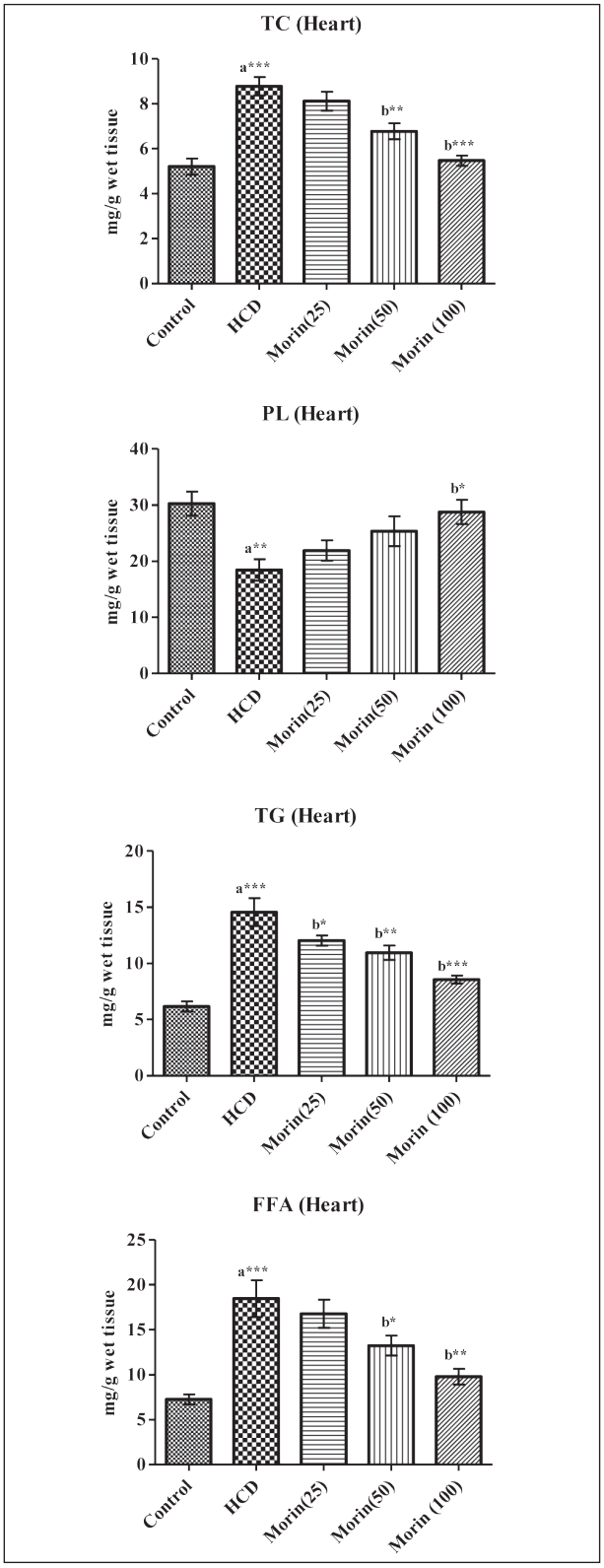

Figure 3. Effect of morin on HCD-induced changes in cardiac lipid levels of TC, TG, PL and FFA in rats.

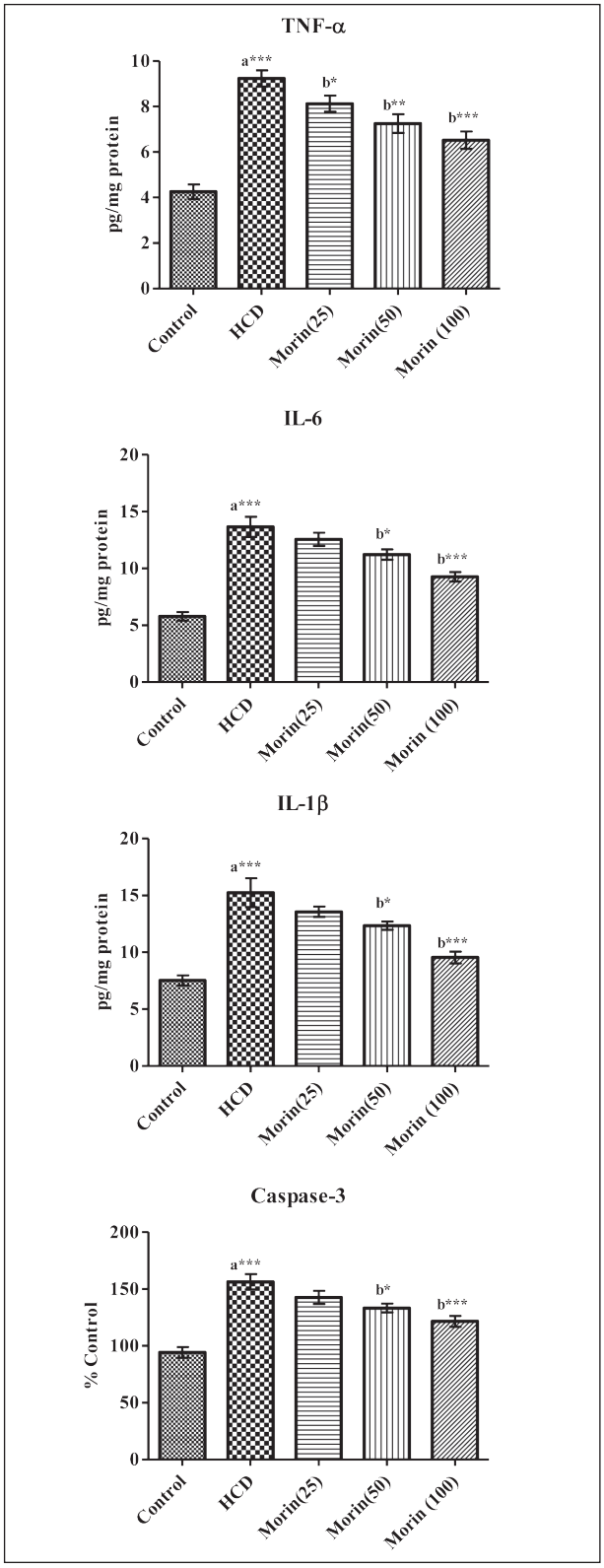

Figure 4. Effect of morin on HCD-induced changes in cardiac pro-inflammatory biomarkers including TNF-α, IL-6, IL-1β, and caspase-3 levels.

Myocardial protein expressions of inducible (iNOS), neuronal (nNOS), and endothelial (eNOS) nitric oxide synthases, and NF-kBp65 were significantly (P<0.001) increased in HCD consumed rats when compared to that of NCRC control animals. Morin treatment markedly decreased these protein expressions in dose dependent manner and these results are shown in Figure 5.

Oxidative stress in cardiac tissue was seen in HCD supplemented rats (Figure 6). TBARS level was in high significantly (P<0.001) while GSH level was reduced (P<0.001) in cardiac cells of HCD fed rats compared to NCRC control animals. Morin treatment (50 and 100 mg/kg/day) for 4 weeks to HCD fed rats, the TBARS was reduced markedly (P<0.05 and P<0.001, respectively) and the GSH was increased (P<0.05 and P<0.01, respectively) when compared to HCD supplemented

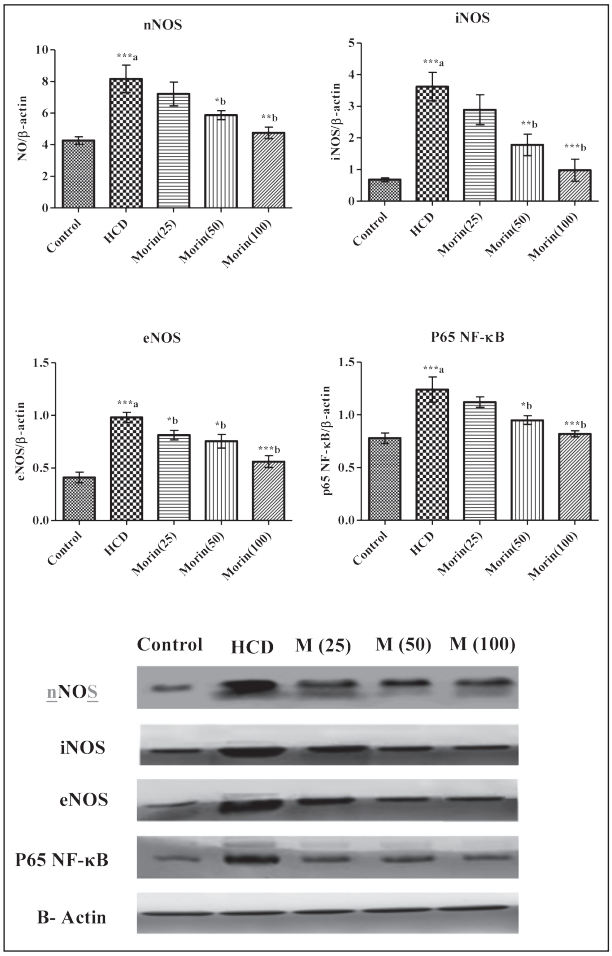

Figure 5. Effect of morin on HCD-induced nitric oxide synthase and P65 NF-κB gene changes in heart.

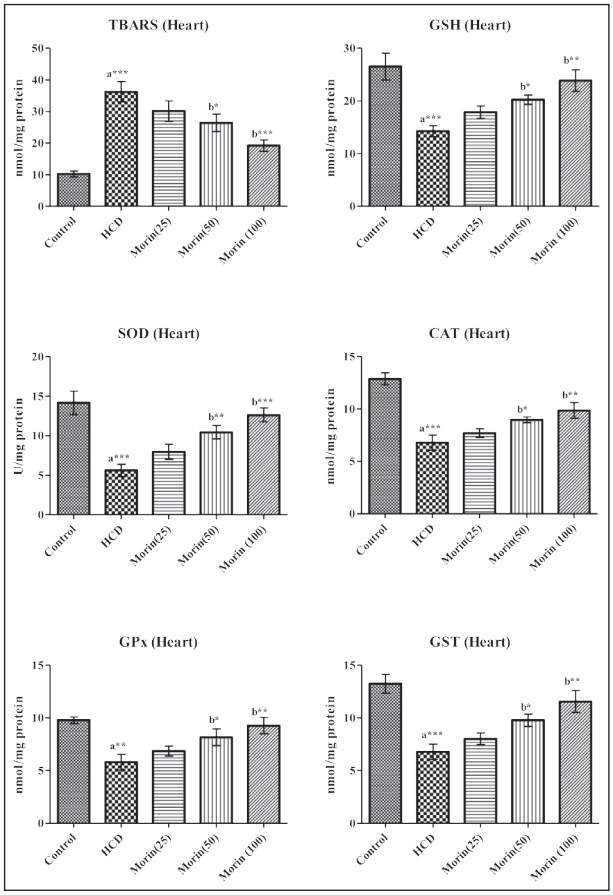

Figure 6. Effect of morin on HCD-induced antioxidant systems markers in heart

control rats. Enzymatic cardiac antioxidants of SOD, CAT, GPx and GST were found to reduces ($P<0.001$) in HCD fed rats compared to NCRC control group. Morin higher dose markedly enhanced the enzymatic cardiac antioxidants when compared to HCD control group and these results are sown in Figure 6.

Histopathological changes are reported in Figure 7. NCRC control rats [Picture A] showed normal myocardial tissue with normal blood vessels. In HCD fed rats [Picture B] revealed blocked vessels due to deposition of cholesterol on inner wall of the vessels (plaque), and then the arthrosclerosis was developed. In the middle dose (50 mg/kg/day) of morin treated group showed reduced cholesterol deposition on inner wall of the vessels [Picture C]. The higher dose (100 mg/kg/day) of morin treated group showed normal blood vessels and the cholesterol deposition was seen in minimal [Picture D].

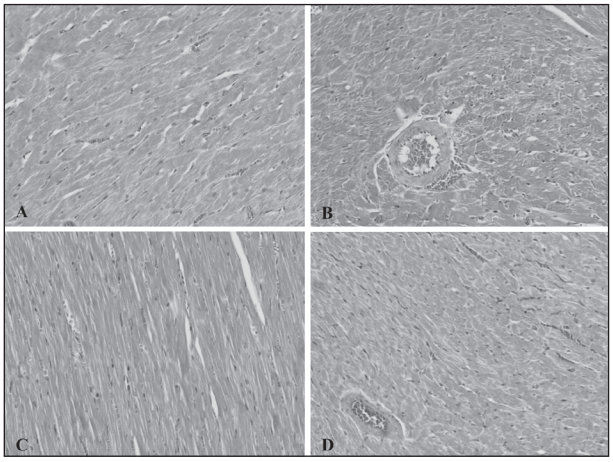

Figure 7. Effect of morin on HCD-induced changes in cardiac tissue where [A] Control [B] HCD, [C] morin (50 mg/kg/day) treated to HCD fed rats and [D] morin (100 mg/kg/day) treated to HCD fed rats

Discussion

Recently, the prevalence of nutrition associated diseases such as over weight, obesity, diabetes and cardiovascular problems have been increased worldwide. Lipids are very energetic source in heart health but, currently, studies revealed that excess lipids in human body associated with much kind of diseases including cardiac cell dysfunction. However, earlier studies also reported that HCD supplementation induces excess lipids in blood and organs including heart that is characterized by increasing in TG, FFA, TC, LDL-C, CK, CK-MB and LDH in blood and heart (31-33). The CK-MB, LDH and CK are definite markers of myocardial injury and these showed peak release at 5 min after the reperfusion (33). Hypercholesterolemia, a foremost threat factors for heart disease development. It was suggested that the LDL peroxidation is foremost factor for the enlargement of atherosclerosis (34-36). Therefore, the cholesterol lowering successful compounds without any harmful effect are urgently needed in current society (37-39). In our study, the HCD supplemented rats showed increased serum TC, TG, LDL-C, CK, LDH and CK-MB levels. Moreover, in this study, the cardiac TC, TG and total FFA increased while the PL decreased in HCD supplemented rats when compared to NCRC control rats. Morin treatment improved these cardiac markers and lipids changes to nearby NCRC control rats which clearly showed that the potential

successful role of morin against HCD-induced cardiac toxicity in rats. Earlier studies proved that many phenolic compounds contains protective role against the metabolic diseases and atherosclerosis due to their mechanism of inhibiting LDL-C, oxidation of lipids, and enhanced cellular inflammatory signaling pathways (40-42). In our study, morin supplementation ameliorated the HCD-induced cardiac toxicity in rats due to lowering of TC, TG, and LDL-C. Earlier study also showed that morin decreases lipid profile against the hypercholesterolemic rats (43). Recently, Naowaboot and his colleagues (44) reported hypolipidemic effect of morin against obese mice.

It is well documented that the inflammatory biomarkers are linked with the cardiovascular threatening factors (45). Earlier studies proved that the improvement of myocardial infarction and atherosclerosis are linked with the peripheral and cardiac tissues inflammation (46-48). TNF-α, IL-6, and IL-1β cytokines are regulated by several biological progressions and participates in inflammation, host defense against organ disorders, and others (49). Similar results were reported for a clinical study, wherein IL-6 and TNF-α levels increased in fatty liver disease patients (50). In an experimental study, HCD-fed rats presented with significant increases in cardiac TNF-α and IL-6 levels (51, 52). It has been proposed that inflammation and immune system anomalies are associated with atherosclerosis. This hypothesis was reinforced by the detection of plaques composed of pro-inflammatory cytokines including TNF-α (53) and IL-1β (54). These were activated by NF-κB. Activated TNF- α /CD95 interacts with at least one cell surface receptor and triggers caspase activation and cytochrome c release. Caspase-3-mediated P21 cleavage and subsequent upregulation of cyclin A/Cdk2 activity are important cell death mechanisms. Our results align with those in a recent report which showed that HCD upregulate caspase-3 expression in cardiac cells. In our study, TNF-α, IL-1β, caspase-3, and IL-6 were found to enhances in circulatory and heart of HCD supplemented rats and upon treated with morin were inhibited these cytokines production to nearby NCRC control rats. It has been reported that morin has several pharmacological functions including protective role of oxidation of cellular lipids (55, 25) and cellular inflammation (56). It has also proved that morin reduces inflammation of liver by downregulating SphK1 activity, blocking NF-kB nuclear translocation, and inhibiting IL-1β, IL-6, and TNF-α secretion by hepatocytes (57). Furthermore, Lee et al., (58) demonstrated that morin pretreatment protected mice from hepatic damage by reducing NF-kB activation.

An earlier study suggested that hypercholesterolemia-induced organ damage is probably associated with ROS accumulation (59). Other studies suggested that the association between the hypercholesterolemia-induced tissue damage and ROS overproduction and the results enhance the lipid peroxidation, damage DNA, degrade proteins, and deplete antioxidant defense systems (60). In this study, HCD supplemented rats showed enhanced levels of oxidative stress biomarker like TBARS and decreased the antioxidant systems biomarkers like SOD, GSH, GPx, CAT and GST compared to NCRC control rats. Montilla et al. was reported similar results in HCD fed animals (61, 62). Administration of morin significantly improved the cellular antioxidant systems due to reducing effect of cellular lipids and inflammation. Flavonoids are natural antioxidants (63). Morin, a yellow colored flavonoid, protects against nephrotoxicity, hepatotoxicity and ischemia-reperfusion through the anti-inflammatory and anti-oxidant properties (24, 25, 64, 65).

Nitric oxide (NO) act as a gaseous cellular messenger and this synthesized from L-arginin by the nitric oxide synthase (NOS) enzyme which is in three isoforms including iNOS, nNOS, and eNOS. NO plays a crucial role for the NF-kB causative effect of cardiac diseases. Enhanced nitric oxide free radical bioavailability has shown in hypercholesterolemia condition and this is plays a decisive role in cardiac cell dysfunction and apoptosis in high cholesterol associated diseases (66, 67). The DNA binding protein of p65 NF-kB is playing a central role for the pathophysiology of cardiac dysfunction by the transcription of pro-inflammatory cytokines. Increased level of reactive oxygen species including nitric oxide may represent an initial step in the signal cascade of NF-kB activation (68, 69). In this study, the protein level of nNOS, iNOS, eNOS, and NF-kB (p65) were increased in HCD supplemented rats and these were reverted by administration of morin. In our experiment, such an

alleviated inflammatory myocardial dysfunction upon morin administration due to the inhibitory effect of nitric oxide synthesized enzymes and p65 NF-kB gene activation. The detailed mechanism should be studied in future. Present study concluded that the morin treatment has improved the cardiac markers and lipids changes which clearly proved that the potential role of morin against HCD-induced cardiac toxicity in rats. This protective role of myocardial inflammatory dysfunction might be due to the regulation of nitric oxide synthesized enzymes and p65 NF-κB gene. Such an alleviated inflammatory myocardial dysfunction upon morin administration has been proven by the improvement of histological features. These results vouch for benefits of dietary morin against HCD induced cardiac dysfunction.

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