Original Article

# Does increased nitric oxide production and oxidative stress due to high fat diet affect cardiac function after myocardial infarction?

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## Abstract

**Background:** High fat (HF) diet by affecting the oxidative stress and nitric oxide (NO) production may lead to different effects on the function of the heart after myocardial infarction (MI). In the present study, we aimed to address the hypothesis that high release of NO by activated macrophages affects left ventricular function after myocardial infarction.

**Materials and Methods:** The animals were randomly divided into four groups comprising each of 10 rats: 1) Sham; 2) MI; 3) Sham+ HF diet; 4) MI+ HF diet. Animals fed with HF diet 30 days before sham and MI surgery. MI was induced by permanent ligation of the left anterior descending coronary artery (LAD). Nitric oxide (NO) production of peritoneal macrophages, the concentrations of MDA in the heart and the infarct size were measured.

**Results:** HF group had adverse effects on myocardium; also, HF group had increased level of NO production as well as oxidative stress, associated with augmentation of infarct size.

**Conclusion:** Our results add to our knowledge that HF diet was associated with overproduction of NO by peritoneal macrophages and reactive oxygen species that lead to the development of infarct size and adverse remodeling.

**Keywords:** High fat diet, myocardial infarction, Nitric oxide, oxidative stress, peritoneal macrophages

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## Introduction

Myocardial infarction (MI) cannot just be considered as a simple contractile disorder or a disease of the heart (1). It afflicts a large number of people every year and is a leading cause of one in every four deaths (2). Although post–MI survival has increased in recent years, adverse left ventricular (LV) remodeling and its progression toward congestive heart failure (CHF) is remained as the main complication in the clinic (3, 4). MI occurrence affects different biological pathways that all are involved in LV remodeling including the changes of LV shape, size and function (5).

Role of nitric oxide (NO) in determination of MI prognosis has been studied; it is believed that NO modulates many processes after MI and improves cardiac function as it prevents the heart against adverse LV remodeling (6). However, high levels of NO, especially when it is accompanied with high levels of reactive oxygen species (ROS), promote detrimental effects on the heart and cause poor

prognosis of MI (7, 8). In this regard it is asserted on the role of high fat (HF) consumption diet as it may impair NO synthesis and stability, resulting in deficiency of cellular turnover and tissue regeneration (9, 10). Due to the role of macrophages in several wound healing events such as scar formation and angiogenesis (11) as well as their role in NO production (11, 12), in the present study, an animal model of MI was applied in the rat to address this hypothesis that high release of NO by activated macrophages affects LV function after MI.

# Methods

**Animal model and experimental protocol**

Adult male rats (250-300 grams) were used in this study. The animals housed in an air-conditioned colony room on a light/dark cycle at 21–23°C with free access to food and water. All experiments were conducted in accordance with the institutional guidelines of Tehran University of Medical Sciences (Tehran, Iran) and the National Institutes of Health guidelines for the care and use of laboratory animals. Before the study, the rats were assimilated into the new home for 2 weeks with no limitation for food (rodent laboratory chow (Daam and Toyur Food Co.) containing by weight: 23.4% protein, 4.5% fat,5% fiber, 7.3% ash, 50% utilizable carbohydrate, and vitamins) and water. The animals were randomly divided into four groups comprising each of 10 rats: 1) Sham; 2) MI; 3) Sham+ HF diet; 4) MI+ HF diet. After these 2 weeks animals of Sham+ HF diet group and MI+ HF diet group ate HF diet instead of rodent laboratory chow for 30 days before Sham and MI surgery. This HF diet contained 20 g of fat/100 g of diet (19 g of butter oil and 1 g of soybean oil to provide essential fatty acids) and provided 19.34 kJ/g of diet, including 7.74 kJ/g as fat (13).

**Rat model of Myocardial Infarction (MI)**

MI was induced by ligation of the left anterior descending coronary artery (LAD). After induction of anesthesia (ketamine (50 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.)), we placed the animals in the supine position and by means of thermal pad and heating lamp we kept the body temperature as close as possible to 37ºC. Then, we intubated and ventilated them using a rodent ventilator (tidal volume 2–3 mL, respiratory rate 65–70 perming, and Harvard rodent ventilator model 683, Holliston, MA, USA). Thereafter we performed an intercostal thoracotomy in the left fourth intercostal space under sterile conditions. We exposed the heart, incised the pericardium and produced MI by permanent ligation of the LAD coronary artery with 6–0 polypropylene suture approximately 1–2 mm distal from its origin. Immediately after ligation we confirmed successful constriction of LAD by ECG changes consisting of ST segment. After completion of all surgical procedures, we closed the chest in layers, inflated the lungs by increasing positive end expiratory pressure, removed the animals from the ventilator and allowed to recover. The sham operated rats underwent the same procedure of thoracotomy, without the ligation of the coronary artery. We used appropriate post-surgery analgesia and post-operative antibiotic (14). Blood samples were collected from retro-orbital plexus at day 1 after surgery to measure serum levels of CK-MB calorimetrically with specific CK-MB kits (Pars Azmun Co, INC, Karaj, Iran), using an auto analyzer (Roche Hitachi Modular DP Systems; Mannheim, Germany).

**Macrophage cell isolation and culture**

After 7 days, we anesthetized the rats deeply and carefully dissected the skin of chest and abdomen of mice without opening the peritoneum. We obtained peritoneal exudate cells from each mouse using lavage method in which we injected 40 ml of cold normal saline twice intraperitoneally. Thereafter, we massaged the abdomen and recovered 90–95% of the injected volume. We centrifuged, washed and resuspended the cells in Roswell Park Memorial Institute medium 1640 (RPMI1640; Gibco, New Jersey, USA) medium supplemented with 10% fetal calf serum (Gibco) and counted these cells using trypan blue dye to detect dead cells which were less than 3% all the times. Then we cultured $8\times10^6$ cell/well in 96-well microplates (Falcon, New Jersey, USA) and incubated at 37°C and 5% $CO_2$ for 2h. We removed the non-adherent cells by washing the plate with normal saline (37°C) and incubated the adherent cells for 24h with or without stimulator bacterial lipopolysaccharides (LPS) (10µg/ml). We conducted all procedures under aseptic condition and the normal saline was sterile and LPS free (injectable grade) (15, 16).

**NO production of peritoneal macrophages**

NO production of peritoneal macrophages was assayed by measuring nitrite in supernatant of cultured macrophages (Griess method) after 18h. Briefly, 50µl of supernatant from each well of microplate was transferred to a 96-well flat-bottom microtiter plate and 50µl of 1% sulfanilamide (Fluka) solution and 50µl of 0.1% N-(1-naphthyl) ethylenediaminedihydrochloride (Merck, Darmstadt, Germany) solution (both in 5% phosphoric acid) were added to each sample and all standards. The absorbencies were read at 492nm and the amount of nitrite was calculated as µM (15).

**Assessment of oxidative stress by TBARS estimation**

After macrophage cell isolation and culture, the hearts of 6 animals per group were excised quickly to evaluate the oxidative damage. MDA level was assessed by reaction with thiobarbituric acid (TBA) at 90–100ºC. A pink pigment with maximum absorption at 532nm is produced by the reaction of MDA or MDA-like substances with TBA. In brief, 50 µl of serum, 1 ml of TBA: 1 ml of trichloroaceticacid (TCA) [0.75% TBA: 30% TCA] were mixed and placed in boiling water bath for 90 min, cooled and centrifuged at 4ºC for10 min at 3000 rpm. The absorbance of the supernatant was measured against a reference blank at 532nm by spectrophotometer. 1,1,3,3-Tetramethoxypropane (Sigma Chemicals, USA) was used as a standard. The results were expressed as nmol/ml of serum (14).

**Assessment of infarct size**

The hearts of 4 animals per group were excised quickly, frozen at −20ºC for 24h and then sliced into 2 mm transverse sections. Slices were then incubated in 1% 2,3,5 triphenyltetrazolium chloride (TTC in 0.1 Mphosphate buffer, pH 7.4 Sigma) solution for 15–20 min at 37°C. TTC reacts with the viable tissue, producing a red formazan derivative, which is distinct from the white necrotic area when placed in 10% formalin for 24–48h. to measure the size of infarcted area at day 7 post-MI, the ratio of infarct size to total LV area (IS/LV%) was quantified using the NIH ImageJ analysis program (NIH, Bethesda, MD, USA) (14).

**Statistical Analysis**

Statistical analyses were performed using the SPSS software version 18 (SPSS Inc, Chicago, IL, USA). Analyses included one-way ANOVA test followed by the post hoc Tukey's test for multiple comparisons. A significance level of $p<0.05$ was used in all cases. Data are presented in the text and in all figures as mean±SEM.

# Results

**The effect of HF on NO production of peritoneal macrophages after MI**

We evaluated NO production of stimulated (LPS) and non-stimulated peritoneal macrophages 21 days after surgery. Figure 1 shows that the use of LPS stimulator significantly increased NO production by peritoneal macrophages in sham group (without stimulator; mean ± SEM: 48.15±5.89 µM and with stimulator; mean ± SEM: 85.36±9.61 µM; p=0.023). Comparison of experimental subjects showed that NO was significantly produced at the presence of LPS stimulator in animals which consumed HF diet (sham+HF diet group: 122.19±13.87 µM, MI+ HF diet group: 205.07±9.28 µM; p<0.001) as compared to Sham subjects and this elevation was higher in animals which underwent MI (p=0.031). Moreover, peritoneal macrophages in MI group produced NO (151.55±15.91 µM; p=0.023) nearly the same as MI+HF diet group; there was no significant difference between MI subjects and Sham+HF group (p=0.083).

**The effect of HF on MDA production by the heart after MI**

We evaluated MDA (a biomarker of cardiac oxidative injury) production by the heart after MI and our results showed (Figure 2) that induction of MI significantly increased MDA levels in comparison with sham group (Sham group: 10.12±2.19 nmol/ml, MI group: 27.08±3.84 nmol/ml; p= 0.041). We didn't see any significant difference between Sham+HF diet group (15.01±5.97 nmol/ml; p=0.12) and sham group; however, there was an obvious increase in MDA production by the hearts of MI+HF rats (31.62±4.52 nmol/ml; p=0.088) as compared to MI subjects. Also, induction of MI significantly increased serum levels of CK-MB as compared to sham group.

**The effect of HF on Infarct size**

As shown in Figure 3, the size of tissue damage after crossing the suture beneath the LAD in sham (10.25±1.06%) and sham+HF diet

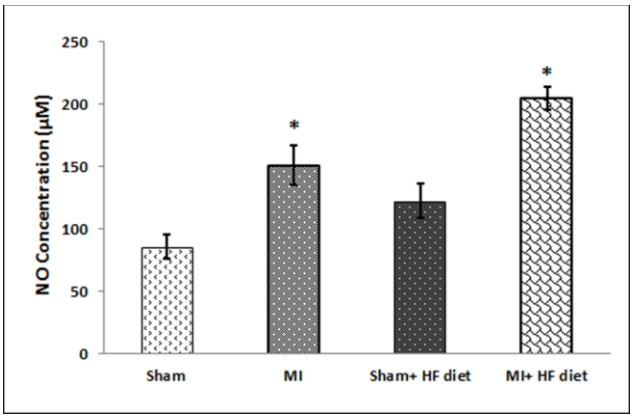


**Figure 1. The effect of high fat (HF) diet on the nitric oxide (NO) production by rat peritoneal macrophages after myocardial infarction (MI)**

*p<0.05: significant differences with sham group at presence LPS (Values are means ± SEM; n=10 animals in each group).

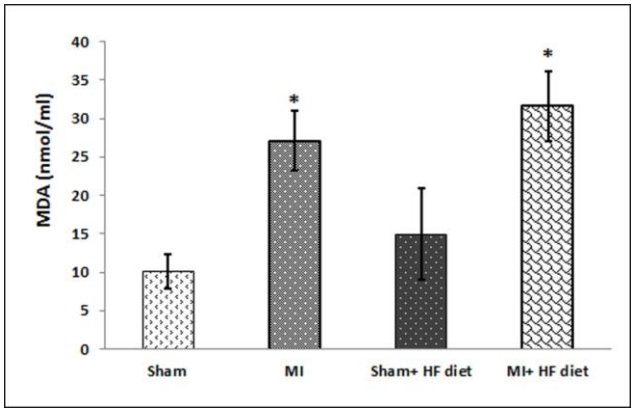


**Figure 2. The effect of high fat (HF) diet on the MDA production by the heart after myocardial infarction (MI)**

*p<0.05: significant differences with sham group at presence of LPS (Values are means ± SEM; n=6 animals in each group).

(15.42±3.95%) groups was not statistically different. However, the size of infarcted area was significantly increased in MI (33.62±2.97%) and MI+HF diet (61.28±2.64%) animals comparing to all experimental subjects which underwent Sham surgery (p<0.05). This increase was more obvious in MI+HF diet groups as compared to MI group (p=0.021).

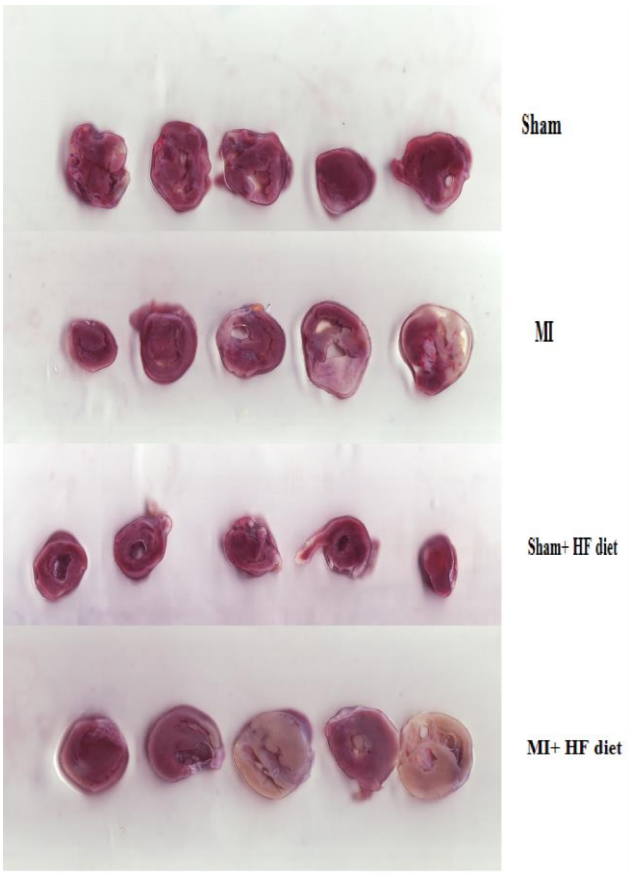


**Figure 3. Morphological Evaluation of infarct size after HF diet 7 days after MI**

## Discussion

In the current study, we aimed to evaluate the effect of HF diet prior MI on NO production by the peritoneal macrophages as well as MDA production by the heart. Our study indicated that HF has adverse effects on myocardium and it may increase NO production as well as oxidative stress, resulting in augmentation of infarct size. Moreover, induction of MI significantly increased serum levels of CK-MB as compared to sham operated animals (data is not shown).

Obesity is considered a major health problem of today's society that causes many health problems

especially cardiovascular disease. Due to genetic factors as well as industrialization of countries the number of obese individuals is increased in developed and developing countries. In this regard environmental factors, especially HF diet, play an important role in progression of heart failure (13). Regardless of obesity, such HF diets cause different changes in the tissues, highlighting its crucial effects in cardiovascular disease (17), as most studies suggest that increased dietary fat defects NO activity and cause it to not protect the heart against adverse remodeling (10). The heart uses saturated fats as the main metabolic fuel and, conversely, accumulation of excess lipid seems to be accompanied with mitochondrial overload that can activate molecular mechanisms related to cardiac remodeling (17), i.e. it may activate oxidative stress and increase free radicals that finally leads to lipid peroxidation, overproduction of MDA and the onset of cardiac remodeling (18). Oxidative stress is considered as an imbalance between ROS production and antioxidant mechanisms that is involved in cardiomyocytes death, enervation of cardiac function and progression of heart failure (19).

Overproduction of free radicals leads to DNA and proteins' oxidative damage and affects many processes that regulate myocardial remodeling (20). ROS production can inactivate the pathway of NO signaling, reduce its bioavailability and affect heart disease pathophysiology (21). A HF diet enhances the recruitment of macrophages into peritoneum and altered levels of macrophages (22). High release of NO by activated macrophages especially when it is coupled with increased ROS production induces cardiac dysfunction and pump failure (12, 23) that are connected with the negative influence of infarct size (24). Accordingly, we found here that NO production by peritoneal macrophages and oxidative stress state as well as the size of infarction in MI+HF diet group was higher. These results suggest that consumption of a HF diet has an additive effect on the infarct area after MI that may occur due to simultaneous increase in NO and MDA levels; however, since we did not evaluate the heart's function by echocardiography and hemodynamic instruments, we could not verdict about observed changes in infarct area due to HF diet and further studies are needed to confirm the exact mechanisms related to HF diet in aggregating left ventricular function.

## Conclusion

This study added to our knowledge that HF diet was associated with overproduction of NO by peritoneal macrophages and ROS; which in turn, lead to development of infarct size and adverse remodeling; however, we should evaluate their effects in expression of hypertrophy-associated genes to conclude about these morphological changes after MI.

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## Conflicts of Interest

The authors declare that they have no conflict of interest.

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