

Increased Inflammatory Response in Myocardial Ischemia-Reperfusion in Normal and STZ Induced Type I Diabetic Rats and Modulation of Inflammatory Response by Quercetin and Rutin

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Summary

Rapidly growing importance of revascularization procedures in the management of myocardial infarction in normal as well as diabetic patients has thrown the ischemia-reperfusion injury into the limelight. Myocardial infarction was produced by occlusion of the left anterior descending coronary artery for 30 min followed by 4 h of reperfusion. Myeloperoxidase activity in heart tissue and interleukin-12 levels in serum was estimated to ascertain the involvement of inflammatory mediators and to evaluate the effect of Quercetin and Rutin on these mediators. Results indicated that Myeloperoxidase and interleukin-12 levels were increased in myocardial ischemia reperfusion injury in both normal and diabetic rats. Furthermore, Quercetin and Rutin ameliorated the inflammatory response by reducing the activity of Myeloperoxidase and interleukin 12 levels. This is the first study to report the involvement of interleukin 12 levels in cardiac ischemia reperfusion injury in both normal and diabetic rats.

Key words: Ischemia –reperfusion Injury, Type-1 diabetes, Quercetin, Rutin

Introduction

Ischemia-reperfusion injury is a common phenomenon in the natural course of ischemic heart disease as well as in the clinical settings like coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA) or thrombolytic therapies. These are the important therapeutic options for acute myocardial infarction to reestablish the blood flow to the ischemic myocardium. Emerging importance of revascularization procedures in the management of myocardial infarction has thrown the ischemia-reperfusion injury into the surgical limelight. Since diabetic patients represents high risk group for the development of ischemic heart disease, diabetic patients constitute a growing segment of the population undergoing coronary revascularization surgical procedures [1-3].

Reperfusion injury is broadly defined as cell death induced by reperfusion of myocytes that were still viable before reperfusion [4]. Reperfusion is a potent stimulus for neutrophil activation and accumulation [5], which in turn serves as potent stimuli for reactive oxygen species production and in turn produces degree of reperfusion injury. There is growing evidence that myocardial cell injury follows by recruitment and activation of polymorphnuclear neutrophils (PMNs), which have been shown to increasingly undergo degranulation within the circulation in myocardial infarction [6]. Previous study reported the implication of several inflammatory mediators in causing neutrophil infiltration following myocardial ischemia and reperfusion [7]. Furthermore, the amount of neutrophil infiltration and the mass of infarcted tissue appear to be correlated [8].

Previous results also indicate that the expression of inflammatory cytokines increases in the ischemic-reperfused myocardium and that the inhibition of the increased expression of cytokines effectively reduces myocardial ischemia-reperfusion injury [9]. Myocardial infarction leads to a systemic as well as to a loco-regional inflammatory response. This inflammation triggers a physiological complex myocardial healing process. Several reports suggest that the inflammation itself may paradoxically have deleterious effects on myocardial cells, especially in the case of an exuberant inflammatory response [10].

It was suggested that inflammation is also an important marker in the post-myocardial infarction setting [11]. For instance, cardiopulmonary bypass activates a proinflammatory cascade, releasing several inflammatory mediators include tumor necrosis factor (TNF)- α and interleukins IL-1, IL-2, IL-6, IL-8 and IL-10 [12, 13]. Inflammation is a cornerstone of the post-myocardial infarction healing process. However, an exuberant systemic as well as loco-regional inflammatory response may have a direct deleterious effect on myocytes extending myocardial necrosis. Interleukin-12 (IL-12) contributes to the induction of inflammatory processes has long been known [14] and interleukin (IL-12) exerts a potent proinflammatory effect by stimulating T-helper (Th) 1 responses.

Recently interleukin-12 was implicated in the pathophysiology of hepatic ischemia-reperfusion injury [15]. There was no report on the involvement of interleukin-12 in ischemia-reperfusion induced myocardial infarction in rat model.

Flavonoids have effects on a variety of inflammatory responses and immune function [16]. Several flavonoids have been demonstrated to have the anti-inflammatory activity [17-19] by suppressing pro-inflammatory cytokines. Considering the potential anti-inflammatory nature of bioflavonoids, the present study was aimed to explore the involvement of inflammatory mediators, myeloperoxidase (MPO) and interleukin-12 (IL-12) in the cardioprotection offered by the Quercetin and Rutin in ischemia reperfusion induced myocardial infarction in normal and diabetic rats.

Our objective is to study the involvement of interleukin-12 in myocardial ischemia-reperfusion injury in both normal and diabetic rats and also to evaluate the anti-inflammatory effect of Quercetin and Rutin against ischemia-reperfusion injury.

Materials and Methods

Drugs and Chemicals

Streptozotocin (STZ), Quercetin, Rutin was purchased from Sigma Chemical Company (St. Louis, USA). Rat IL-12 p70 ELISA Kit (Code No: 065101) was purchased from Biosource International Pvt Ltd (USA). Thiopentone sodium was supplied by Abbott Lab Ltd. (Ankleshwar, India). All other chemicals and reagents were used of analytical grade.

Animals

Albino wistar rats (National Institute of Nutrition, Hyderabad, India) of either sex weighing 200 – 250 g were selected. Animals were maintained under standard laboratory conditions at $25 \pm 2^\circ\text{C}$ relative humidity $50 \pm 15\%$ and normal photoperiod (12 h dark/12 h light and were used for the experiment. Commercial pellet diet (Rayon's Biotechnology Pvt Ltd, India) and water were provided ad libitum. The experimental protocol has been approved by the Institutional Animal Ethics Committee and by the Animal Regulatory Body of the Government (Regd. No. 516/01/A/CPCSEA).

Experimental design

The rats were randomly divided into fourteen groups, of which, seven normal groups and seven diabetic groups. Usage pattern of number of rats for the determination of biochemical parameters as follows. n=5 for myeloperoxidase, n=6 for interleukin-12 levels. Group 1, sham control group; Group 2, Control I/R group treated with saline (0.2 ml). Group 3, Vehicle Control I/R group treated with 0.2 ml of 50% dimethyl sulfoxide (DMSO). Group 4 and 5 were treated with Quercetin 5 and 10 mg/kg respectively.

Group 6 and 7 were treated with Rutin 5 and 10 mg/kg respectively. Group 8 is the diabetic sham control group. Group 9 is the diabetic control I/R group treated with saline. Group 10, Diabetic vehicle control group treated with DMSO. Group 11 and 12, Diabetic control I/R treated with Quercetin at doses 5, 10 mg/kg respectively. Group 13 and 14, are Diabetic control I/R treated with Rutin at doses 5, 10 mg/kg respectively. Quercetin and Rutin were dissolved in 50% DMSO and administered intraperitoneally (i.p) 10 min before reperfusion.

Induction of diabetes

Diabetes was induced by a single i.v. injection of streptozotocin (STZ) 45 mg/kg of body weight, dissolved in citrate buffer (p^H 4.5) into the tail vein of animals lightly anaesthetized with ether. Age-matched rats were injected with citrate buffer only. Diabetes was confirmed by estimations made after third day of STZ injection for serum glucose by semi auto analyzer (Screen Master 3000, USA). Following two weeks of diabetes induction, rats were subjected to surgical procedure. About 20% of mortality was observed in diabetic rats even before being subjected to ischemia-reperfusion injury. Diabetic rats showing more than 350 mg/dl were used for the experiment.

Surgical procedure

Rats were anaesthetized with thiopentone sodium (30 mg/kg, i.p.), tracheotomized and ventilated with room air by a Techno positive pressure mechanical respirator (Animal respirator, Crompton Parkinson Ltd., UK). The right jugular vein was cannulated in order to inject drugs. A left thoracotomy and pericardiotomy were performed, and the left coronary artery was dissected free above the first diagonal branch and was ligated just below the origin of left circumflex artery with the help of a silk thread (6-0). The artery was occluded for 30min by a knot. The silk thread was removed after 30 min with the help of two-knot releasers to allow reperfusion of the heart for succeeding 4 h. At the end of 4 h reperfusion, blood sample was collected by cardiac puncture. Serum was separated, stored at -20°C and used for the biochemical analysis of interleukin-12 (IL-12). Following blood sample collection, the heart was excised from the thorax rapidly and the same was subjected to the estimation of Myeloperoxidase activity.

Determination of Myeloperoxidase (MPO) activity

The cardiac myeloperoxidase activity was assessed using the method modified from that of Mullane *et al.*, (1985) [20]. The myocardial tissue was homogenized in phosphate buffer containing 0.5% hexadecyl trimethylammonium bromide using a Polytron homogenizer to produce a 10 % w/v homogenate. After freeze-thawing for three times, the samples were centrifuged at 15000 rpm for 30 min at 40C and the resulting supernatant assayed spectrophotometrically for MPO. To 40 µl of the sample, 960 µl of phosphate buffer containing o-dianisidine dihydrochloride and hydrogen peroxide was

mixed and shaken vigorously. The change in the absorbance was measured at 460 nm for 3 min at an interval of 60 sec. One unit of enzyme activity was defined as the amount of MPO that causes a change in absorbance measured at 460 nm for 3 min. Myeloperoxidase activity was expressed as U/g tissue.

Determination of serum Interleukin-12

Interleukin-12 concentrations in the serum were determined using ELISA kit that is specific against rat IL-12. Levels IL-12 (p70) was measured using ELISA kit purchased from Biosource International Pvt Ltd (USA). Plates were read at 450 nm by a Spectramax 250 microplate reader from Molecular Devices (Sunnyvale, Calif.). Detection limits were 5pg/ml for IL-12 (p70). Interleukin-12 assay was performed according to the manufacturer's instructions.

Statistical analysis

The results were expressed as (Mean \pm S.D) and factorial One-way ANOVA was used for the statistical analysis. Individual groups were compared using Tukey's test. Statistical analysis was performed using Sigma plot soft ware (Version 10).

Results

Effect of Quercetin and Rutin on Myeloperoxidase activity:

Results of myeloperoxidase (MPO) activity were presented in Table 1 & Figure 1. Inflammatory response was increased in normal control group animals subjected to ischemia reperfusion injury when compared to sham operated group. It was evidenced by the significant ($p < 0.05$) increase in tissue myeloperoxidase (MPO) activity in normal control group animals when compared to sham operated group.

Inflammatory response was exacerbated in diabetic control animals with ischemia reperfusion when compared to normal control group animals with ischemia reperfusion. This was evidenced by the significant ($p < 0.05$) increase in tissue myeloperoxidase (MPO) activity in diabetic control group animals when compared to normal control group animals.

Quercetin and Rutin treatment significantly ($p < 0.05$) diminished the inflammation in terms of significant ($p < 0.05$) reduction in tissue myeloperoxidase (MPO) activity in normal and diabetic group animals in the present study. However, dose dependent reduction of myeloperoxidase (MPO) activity was observed with the treatment of Quercetin and Rutin in both normal and diabetic rats. Furthermore, same degree of reduction of myeloperoxidase (MPO) activity was observed with both Quercetin and Rutin in normal as well as diabetic rats.

Table 1: Myeloperoxidase in heart tissue of sham control, control and groups treated with Quercetin and Rutin in both normal and diabetic animals

S.No	Groups	Tissue Myeloperoxidase activity (U/g tissue)	
		Normal Rats	Diabetic Rats
1	Sham Operated Group	1.99 ± 0.29 ^a	2.28 ± 0.47 ^c
2	Control Group With I-R	10.27 ± 0.38	15.10 ± 0.22
3	Vehicle Control Group with I-R	10.21 ± 0.93	14.88 ± 0.62
4	Quercetin (5 mg/kg)	7.83 ± 0.85 ^{a,b}	9.52 ± 0.39 ^{c,d}
5	Quercetin (10 mg/kg)	3.73 ± 0.52 ^{a,b}	5.19 ± 0.41 ^{c,d}
6	Rutin (5 mg/kg)	7.32 ± 0.68 ^{a,b}	8.37 ± 0.58 ^{c,d}
7	Rutin (10 mg/kg)	3.15 ± 0.34 ^{a,b}	3.70 ± 0.62 ^{c,d}

All values are expressed as Mean ± S.D (n =5), ^a p <0.05, Vs control I/R; ^b p<0.05, Vs vehicle control I/R; ^c p<0.05, Vs diabetic control I/R; ^d p<0.05, Vs vehicle diabetic control I/R.

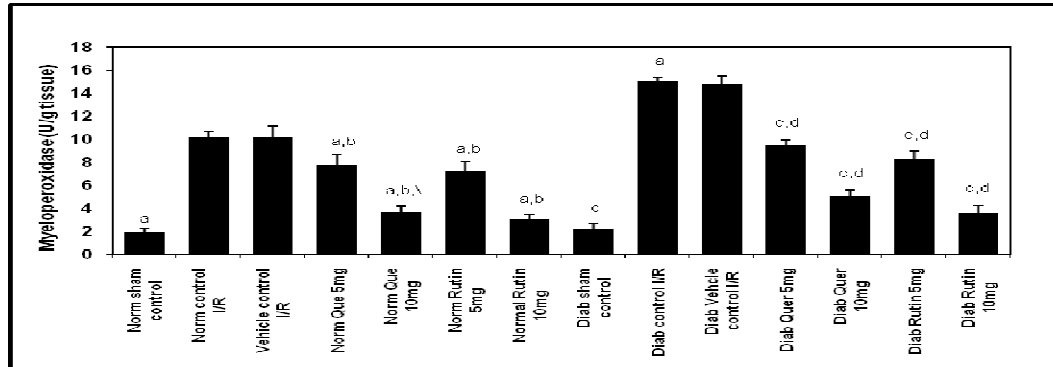
Effect of Quercetin and Rutin on serum interleukin-12 levels:

Results of interleukin-12 (IL-12) levels were presented in Table 2 & Figure 2. Inflammatory response was increased in normal control group animals subjected to ischemia reperfusion injury when compared to sham operated group. It was evidenced by the significant (p<0.05) increase in serum interleukin-12 levels in normal control group animals when compared to sham operated group.

Inflammatory response was exacerbated in diabetic control animals with ischemia reperfusion when compared to normal control group animals with ischemia reperfusion. This was evidenced by the significant (p<0.05) increase in serum interleukin-12 levels in diabetic control group animals when compared to normal control group animals.

Quercetin and Rutin treatment significantly (p<0.05) diminished the inflammation in terms of significant (p<0.05) reduction in serum interleukin-12 levels in normal and diabetic group animals in the present study. However, dose dependent reduction of serum interleukin-12 levels were observed with the treatment of Quercetin and Rutin in both normal and diabetic rats. Furthermore, the same degree of reduction of serum interleukin-12 levels was observed with both Quercetin and Rutin in normal as well as diabetic rats.

Figure 1 : Myeloperoxidase in heart tissue of sham control, control and groups treated with Quercetin and Rutin in both normal and diabetic animals



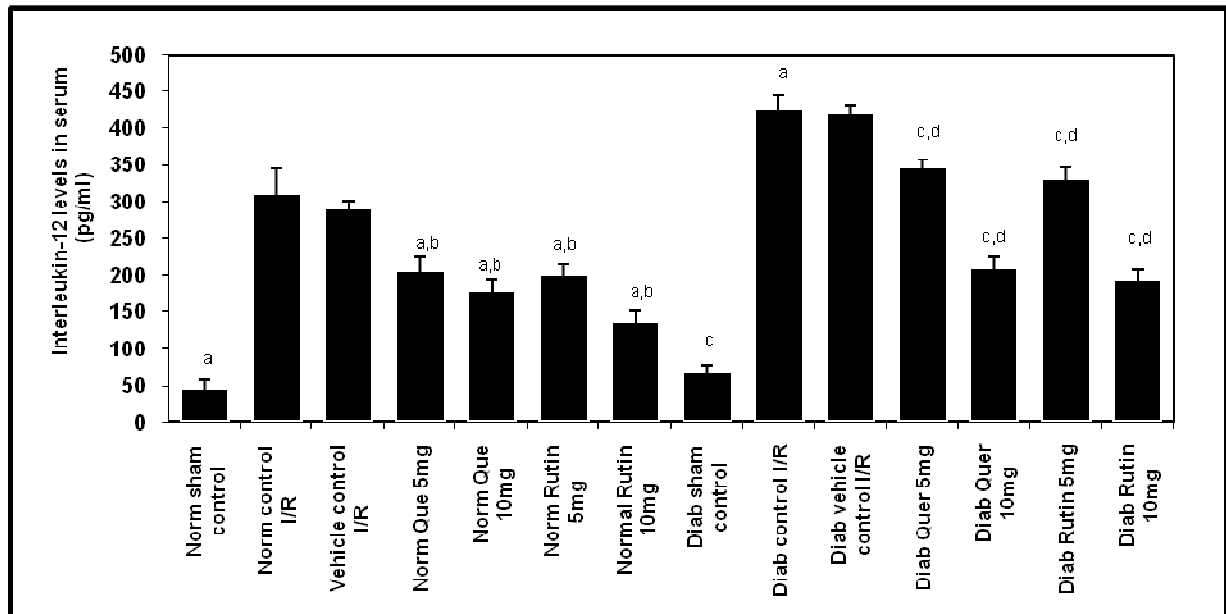
Myeloperoxidase activity was expressed as U/g tissue. All values are expressed as Mean \pm S.D (n =5), ^a p <0.05, Vs control I/R; ^b p <0.05, Vs vehicle control I/R; ^c p <0.05, Vs diabetic control I/R; ^d p <0.05, Vs vehicle diabetic control I/R.

Table 2: Serum interleukin-12 levels of sham control, control and groups treated with Quercetin and Rutin in both normal and diabetic animals

S.No	Groups	Serum Interleukin-12 levels (pg/ml of serum)	
		Normal Rats	Diabetic Rats
1	Sham Operated Group	46.54 \pm 11.92 ^a	68.83 \pm 8.46 ^c
2	Control Group With I-R	310.50 \pm 34.68	426.54 \pm 18.05
3	Vehicle Control Group with I-R	290.91 \pm 10.74	420.08 \pm 12.28
4	Quercetin (5 mg/kg)	205.70 \pm 18.32 ^{a,b}	346.54 \pm 11.05 ^{c,d}
5	Quercetin (10 mg/kg)	178.41 \pm 16.09 ^{a,b}	209.45 \pm 14.45 ^{c,d}
6	Rutin (5 mg/kg)	201.12 \pm 12.96 ^{a,b}	331.75 \pm 15.55 ^{c,d}
7	Rutin (10 mg/kg)	136.12 \pm 15.94 ^{a,b}	194.46 \pm 13.88 ^{c,d}

All values are expressed as Mean \pm S.D (n =5), ^a p <0.05, Vs control I/R; ^b p <0.05, Vs vehicle control I/R; ^c p <0.05, Vs diabetic control I/R; ^d p <0.05, Vs vehicle diabetic control I/R

Figure 2: Interleukin-12 (IL-12) in serum of sham control, control and groups treated with Quercetin and Rutin in both normal and diabetic animals



Interleukin levels were expressed as pg/ml of serum. All values are expressed as Mean \pm S.D (n =5), ^a p <0.05, Vs control I/R; ^b p <0.05, Vs vehicle control I/R; ^c p <0.05, Vs diabetic control I/R; ^d p <0.05, Vs vehicle diabetic control I/R

Discussion

Reperfusion injury triggers an acute inflammatory response in which polymorphonuclear neutrophils infiltrate the myocardium, under the influence of chemotactic attraction and activation of the complement cascade [5, 11]. Although essential in wound healing, these neutrophils may have detrimental effects by producing additional “reactive oxygen species” and proteolytic enzymes [5, 21, 22]. Activated neutrophils generate substantial amounts of HOCl via the myeloperoxidase-catalyzed oxidation of chloride ions by hydrogen peroxide [23]. It is well known that the magnitude of myeloperoxidase activity is directly proportional to neutrophil concentration on the inflamed tissue [24], by which measurement of enzyme activity has been considered a quantitative and sensitive marker of chemotaxis and neutrophil infiltration in the inflammation process [25]. Myeloperoxidase (MPO) activity was used as a marker for neutrophil content in the heart, since it correlates closely with the number of neutrophils.

The results clearly indicated that the activation and infiltration of neutrophils was markedly increased during the ischemia-reperfusion and thereby increased myeloperoxidase activity was observed. It is well recognized that ischemia and reperfusion-induced myocardial injury represents an acute inflammatory response in which leukocytes are intimately involved [26, 27]. There is accumulating evidence that local inflammatory responses with infiltration of leukocytes in the capillary circulation and release of oxygen-free radicals play a key role in this reperfusion-related tissue injury [28, 29]. Indeed, Neutrophils' infiltration into the myocardial ischemic regions may increase the infarct size by promoting inflammation and by their direct entrapment in the capillary micro-vessels, both leading to a reduced myocardial local perfusion [30]. It was reported that infarct size could be reduced by suppressing leukocyte infiltration into the ischemic myocardium with anti-leukocyte antibodies [31].

Exaggerated neutrophil accumulation in the diabetic hearts probably attributed to the enhanced activity of myeloperoxidase in diabetic hearts subjected to ischemia-reperfusion injury when compared to normal rats. Increased leukocyte accumulation in the diabetic heart coupled with enhanced diabetic PMN oxygen free radical production will likely cause an enhanced inflammatory response. This could be the reason for the poor recovery of cardiac contractile function in diabetic hearts early in reperfusion following ischemia. Diabetes enhances leukocyte accumulation in the coronary microcirculation early in reperfusion following ischemia [32].

Quercetin and Rutin treatment significantly diminished the inflammation in terms of significant reduction in tissue myeloperoxidase (MPO) activity. The results are in accordance with the previous reports that Quercetin and Rutin were shown to impact the production of hypochlorous acid (HOCl) by PMNs with ability of Quercetin to effectively inhibit myeloperoxidase (MPO) activity [33]. Furthermore, earlier investigation demonstrated that Flavonoid, 7-Monohydroxy Ethyl Rutinoside reduced the neutrofil influx, infarct size and cardiac contractility in the experimental setting of ischemia reperfusion [34]. This study also further supports the results of the present investigation. Flavonoids are known to inhibit the release and activity of myeloperoxidase [33, 35]. In addition, Rutin *in-vitro* and *in vivo* demonstrated an inhibition of rat PMN-evoked and luminol-enhanced chemiluminescence.

Interleukin-12 (IL-12) contributes to the induction of inflammatory processes has long been known [36, 37] and recently this cytokine was implicated in the pathophysiology of hepatic ischemia-reperfusion injury [38]. The present study demonstrates for the first time elevated serum interleukin-12 levels in ischemia-reperfusion induced myocardial injury in both normal and diabetic rats. It was speculated that ischemia-reperfusion triggers a release of oxygen free radicals immediately following reperfusion.

The generation of oxidants during reperfusion phase is also thought to activate redox-sensitive transcription factors, such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1), which control the expression of proinflammatory mediators, such as interleukin (IL)-12 and tumor necrosis factor (TNF)- α [39-42].

It is well reported that diabetes exacerbates inflammatory responses to ischemia-reperfusion [43]. Probably, exaggerated inflammatory response in myocardial ischemia reperfusion injury partly attributed to increased IL-12 levels in diabetic rats when compared to normal rats.

Quercetin and Rutin significantly ($p < 0.05$) reduced the serum IL-12 levels that were raised during myocardial ischemia reperfusion in both normal and diabetic rats. The result is in agreement with a more recent study in which Quercetin has been reported to be an inhibitor of IL-12 signaling through the JAK-STAT pathway in T lymphocyte (19). Other studies also reported that flavonoids isolated from *Waltheria indica* dose-dependently inhibited the production of the inflammatory mediators TNF- α and IL-12 in activated macrophages (18). Our results are also in agreement with these findings. In addition, Quercetin is suggested to modulate a variety of inflammatory responses of macrophages and T lymphocytes. Moreover, Quercetin ameliorates experimental autoimmune encephalomyelitis, which is associated with Th1-mediated immune responses. Like Quercetin inhibits macrophage-induced cytokine production, it also blocks IL-12-dependent JAK-STAT signaling in Th cells [44].

In conclusion, increased interleukin-12 levels and myeloperoxidase activity clearly indicates the role of inflammatory mediators in cardiac ischemia-reperfusion injury and reduction of these inflammatory mediators with Quercetin and Rutin treatment suggest that modulation of inflammatory response by these drugs.

Limitations

For the technical reasons, we could not estimate the cyclooxygenase (COX) activity and other inflammatory cytokines to provide the rational evidence for the role of inflammation in this investigation.

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