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Effect of resveratrol on the biomarkers of oxidative stress and inflammation in monocyte cultures from PBMC's of patients with myocardial infarction

Shireen[1], Anjum Chugtai[2], Wasil Hasan[1], Abbas Ali Mahdi[3] & Najmul Islam[1]*

[1]Department of Biochemistry, [2]Department of Medicine, Faculty of Medicine, J N Medical College, Aligarh Muslim University, Aligarh-202 002, Uttar Pradesh, India

[3]Department of Biochemistry, King George's University, Lucknow-226 003, Uttar Pradesh, India

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Globally, one of the major causes of mortality and morbidity is ischemic heart disease (IHD). It has been established that in cardiac disorders, there exists a synergistic correlation between the oxidative stress and inflammatory cytokines. The stabilization and regulation of the oxidative stress and inflammatory cytokines in these patients is essential for the better management of the disease. Hence, the aim of this study was to study the effect of natural antioxidant, resveratrol, on the oxidative stress and inflammatory biomarkers in cultures of monocytes isolated from peripheral blood mononuclear cells (PBMCs) from the patients with myocardial infarction. Monocytes isolated from peripheral blood mononuclear cells (PBMC's) of patients with myocardial infarction (MI) and healthy controls were employed in culture studies (with and without resveratrol). The 24 h cultures were subjected to evaluation of cytokine/ interleukins levels *i.e.* TNF-α, IL-1 and IL-6 as well as oxidative stress markers like MDA and Glutathione. The patient's samples exhibited a significantly decreased level of intramonocyte glutathione as compared to samples of healthy subjects**.** On the other hand, significantly increased levels of MDA were observed in culture supernatants of monocytes isolated from PBMC's of patients with MI in comparison to those of healthy controls. A significant degree of amelioration in intramonocyte glutathione levels coupled with decreased MDA levels (culture supernatants) were observed in cultures treated with resveratrol (20 ug/mL). Furthermore, 24 h culture supernatants of untreated patients cells exhibited augmented levels of IL-1, IL-6, and TNF-α. However, co-culturing with resveratrol exhibited a significant decrease in the levels of all the IL-1, IL-6 and TNF-α. Resveratrol—a potent polyphenol from grapes and also a natural antioxidant regulates the oxidative stress and inflammatory biomarkers and may be used as a prophylactic antioxidant in high risk patients.

Keywords: IL-1, IL-6, Ischemia-reperfusion, PBMC, Resveratrol

Ischemic heart disease (IHD) is well known to be the major cause of deaths and disability which occurs when there is an imbalance between blood supply and demand. Blood flow in the coronary artery decreases abruptly when the development of thrombus takes place in the coronary artery at the site of vascular injury which usually results in myocardial infarction (MI). The injury may be due to emotional, metabolic or environmental stress[1]. Later, re-establishment of the blood flow in the occluded artery takes place in an event termed as Ischemia-reperfusion that generates reactive oxygen species (ROS)[2] and activates complement which attracts the neutrophils in the infarcted myocardial tissue[3]. The main characteristic of the inflammatory response in the heart is the interaction between the polymorphonuclear leukocytes (like neutrophils) and the vascular endothelium[4] where adherence of activated neutrophils to myocytes takes place as a result of apoptosis occurring in myocardial infarction[5-8].

As evident, the progress of CVD is associated with oxidative stress, vascular inflammation, and endothelial dysfunction[9] which emphasizes the need for the development of novel pharmaceutical and/or functional foods. Hence, recent advancement and the increasing interest in natural antioxidants which can improve cardiac health have become noteworthy.

Resveratrol (3,4,5-trihydroxystilbene) is a phytoalexin generally present in the grapes (50-100 μg/g of grape skin), coffee, blueberries, peanuts and other nuts[10].

Resveratrol acts as a free radical-scavenger in which the unpaired electron quenched from ROS may be delocalized over its aromatic rings[10]. Various

*Correspondence:
Phone (Mob.): +91-9412976384
E-mail: nxi7@hotmail.com

studies have shown that resveratrol carries out the chelation of transition metal copper, which is responsible for the generation of free radicals and thus lipid peroxidation[10]. Also, it upregulates the endogenous cellular antioxidant systems like superoxide dismutase (Mn-SOD), catalase, thioredoxin, and glutathione peroxidase[10].

Furthermore, heme oxygenase-1 (HO-1) which is a stress-response protein, and may be protective against injury occurring during oxidative stress, is upregulated by resveratrol[11,12]. Resveratrol increases the plasma nitric oxide (NO) levels as it augments the expression of the endothelial nitric oxide synthase (eNOS)[13,14]. *In vivo*, it reacts with superoxide radical (O_2^-) and produces peroxynitrate radicals ($ONOO^-$) as the affinity of NO for O_2^- is quite high[15,16]. Ample amounts of thiol and ascorbate are present in physiological systems like the heart, where the detrimental effect of $ONOO^-$ is lost when it reacts with the –SH groups of either thiol or ascorbate[15]. Thus, the anti-inflammation action of resveratrol/NO pathway plays a crucial role in the removal of notorious O_2^- [15].

The augmentation/upregulation of cytokines (TNF-α, IL-1, and IL-6) due to oxidative stress in patients with cardiac disorders/failures are well documented[16].

The cytokine hypothesis proposes that in heart failure (HF) various endogenous cytokines come into play via their local and systemic actions[16]. Although the injured myocardium releases these cytokines in order to repair the damage but their long-term exposure promotes cardiac dysfunction. Also, in the case of MI due to the sudden death of cardiomyocytes, innate immune pathways are activated which triggers inflammatory reaction[16]. As a result, various inflammatory mediators (cytokines and chemokines) are released that further attracts inflammatory cells like neutrophils and macrophages, thus enhancing the oxidative stress[16].

Thus, our aim in the present study was to probe the beneficial effects of resveratrol in cultures of monocytes isolated from PBMC's from patients with MI, wherein, stabilization/regulation of the oxidative stress and inflammatory biomarkers may occur, which in turn, may help in the better management of cardiac diseases.

Methods

Study subjects: Present study had prior clearance from the Institutional Ethics Committee. Blood samples from healthy volunteers (including both sexes), as well as patients after 6-12 h of myocardial infarction (n =20), were obtained from the patients admitted in J.N. Medical College Hospital of A.M.U. PBMC's were isolated from the above blood samples for monocyte isolation, and in turn, for culture studies.

In the present study, all the experiments with resveratrol with the dose of 20 µg/mL were carried. This dose was selected based on our preliminary studies (data not shown). Furthermore, all evaluations were carried out in culture supernatants except for glutathione, where instead of culture supernatants, cultured monocytes were employed. Thus, glutathione levels were intramonocytic.

Isolation of peripheral blood mononuclear cells (PBMCs) for obtaining monocyte and culture study

As described earlier[17,18] Ficoll-hypaque density gradient method was used to isolate PBMCs from both normal healthy individuals (n =20) as well as from the MI patients (n =20).

In brief, diluted blood was under layered with Ficoll-Paque and the gradient was centrifuged at 1800 rpm for 30 min at room temperature. After the centrifugation, the PBMCs appeared as a dense white band (buffy layer) above the RBCs and granulocytes layer which was removed. Banded PBMCs were diluted with PBS and centrifuged at 1100 rpm for 12 min in order to remove platelets. PBMCs coming in pellets were then combined and resuspended in complete medium. These isolated PBMC's were employed for monocyte isolation and treatment in culture studies.

As already established by us and other workers[18,23,24], the isolated PBMC'S have two type of cells- adherent (around 90%) and non-adherent (around 10%), where the adherent cells are pure monocytes.

Thereafter, for culture studies, one million cells (PBMC's) in complete medium were plated per well onto a 6-well plate. The final volume/well was 1 mL. The culture plates were incubated for 2 h at 37°C/5% CO_2 for complete adherence of monocytes onto the plates. Thereafter, the plates were washed 2X with complete medium to remove non-adherent cells. The cells were replenished with complete medium (1 mL/well) followed by overnight incubation at 37°C/5% CO_2 for complete milieu adaptation. The cells were washed 1X with complete medium and then were treated for 24 h with varying doses of resveratrol as per experimental design, followed by harvesting the cells for further study.

Determination of interleukin levels

The levels of inflammatory biomarkers namely IL-1 and IL-6, in 24 h monocytic culture supernatants were determined by Quantikine Human IL-1 and IL-6 Immunoassay Kits (R&D Systems, Inc., Minneapolis, MN, USA) according to manufacturer's instruction.

Determination of TNF-α levels in cell culture supernatants

Culture supernatants of monocytes isolated from PBMCs from healthy subjects as well as MI patients were subjected to treatment with resveratrol (20 μg/mL), and the levels of TNF-α was assessed by the method as described by us earlier [21-25].

Determination of MDA levels

Determination of MDA level in culture supernatants of monocytes isolated from PBMC's from healthy as well as patients with MI was carried out as described by Kaur[19].

Determination of glutathione

Glutathione level in treated/untreated adhered monocytes isolated from PBMCs and cultured for 24 h as mentioned above, was measured according to the method described by Anderson[20].

Statistical analysis

Paired t-test was used for the analysis of the results and are expressed as the means ± S.E. of ten experiments. P <0.05 was considered statistically significant.

Results

Effect of resveratrol on oxidative stress biomarkers

The levels of glutathione were significantly decreased in adhered monocyte (intramonocytes) of patients with MI (18.83 pg/mL) as compared to those of healthy control (67.11 pg/mL, P <0.001) (Fig. 1A).

Regarding MDA levels, there was a significant increase of MDA in culture supernatants as observed in patient's sample (34.23 nmol/mL) in comparison to healthy controls (5.12 nmol/mL, P <0.001) (Fig. 1B). A significant degree of amelioration in intramonocyte glutathione levels (59.35 pg/mL) and decreased MDA (9.33 nmol/mL) levels in culture supernatants were observed in cultures treated with 20 ug/mL resveratrol.

Effect of resveratrol on inflammatory biomarkers

Untreated control 24 h culture supernatants of monocytes isolated from PBMC's of patients with MI, exhibited augmented levels of IL-1 (49.16 pg/mL) and IL-6 (53.11 pg/mL) (Fig. 2A). On the other hand, co-culturing/treatment with resveratrol exhibited a decreased suppression of the levels of IL-1 (12.32 pg/mL) and IL-6 (9.12 pg/mL, P <0.001). The IL-1 and IL-6 levels in healthy control cells were recorded at 3.22 pg/mL and 4.09 pg/mL, respectively (Fig. 2B).

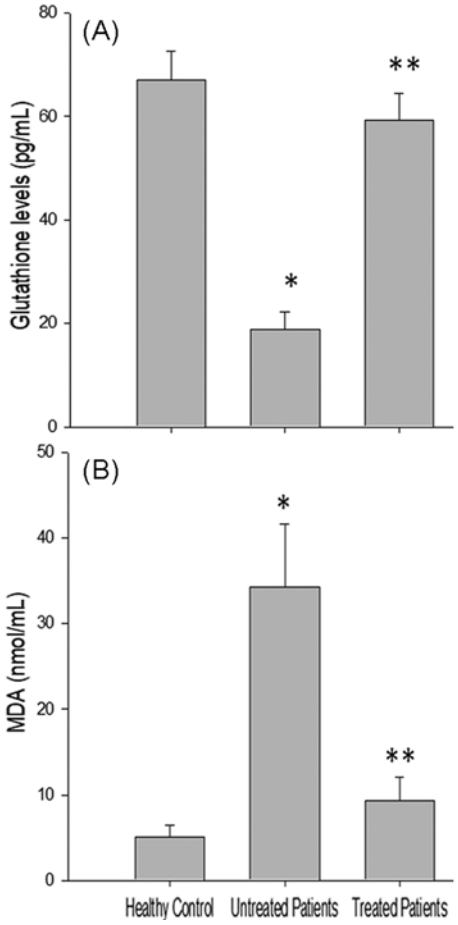

Fig. 1 A&B—Levels of Intramonocyte Glutathione and MDA (in culture supernatants) in 24 cell culture. Monocytes from PBMC's of healthy controls and patients with MI were isolated and cultured for 24 h with or without resveratrol as described in methods. The data represents mean ± S.E.M, n =20;* P <0.001 compared with healthy control group and ** P <0.001 compared with untreated patients group.

Similarly, supernatants of resveratrol treated and untreated PBMCs from patients with MI exhibited a decreased levels of TNF-α expression by around 76% in comparison to TNF-α levels in untreated cell cultures (Fig. 3, P <0.001).

Discussion

The physiological imbalance between free radical generation and the cellular antioxidative defense has been observed to cause damage as a consequence of MI[26]. Myocardial infarction is considered to be the

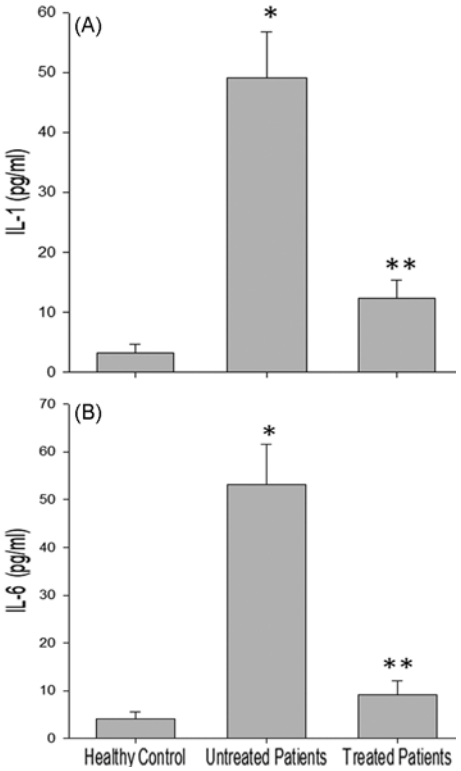

Fig. 2 A&B—Effect of resveratrol on IL-1 and IL-6 expression in 24 h monocyte culture supernatants. Monocytes from PBMC's of healthy controls and patients with MI were isolated and cultured for 24 h with or without resveratrol as described in methods. The data represents mean ± S.E.M, n =20; *P <0.001 compared with healthy control group and ** P <0.001 compared with untreated patients group.

result of cardiac dysfunction due to inflammation, lipid peroxidation, modified expression of cardiac markers, and reduction in cellular antioxidants[27]. In addition, due to the subordinate working of antioxidant defence in the heart tissues, it becomes susceptible to oxidative injury as compared to other tissues[28].

Apart from a wide spectrum of beneficial effects, one of the main properties of resveratrol is anti-inflammatory. Resveratrol interferes with the release of pro-inflammatory mediators like platelet endothelial cell adhesion molecule-1 (PECAM-1)[10] and consequently represses the actions of T and B cells as is evident from the inhibition of their proliferation, antibody production, and secretion of lymphokines[10].

It has been reported that resveratrol also hinder the production of thromboxane A2 by mimicking the action of aspirin which inhibits the platelet-specific inflammatory enzyme cyclooxygenase-1 (COX-1)[10]. However, various other pro-inflammatory agents like Interleukin-1β induces its isozyme cyclooxygenase-2

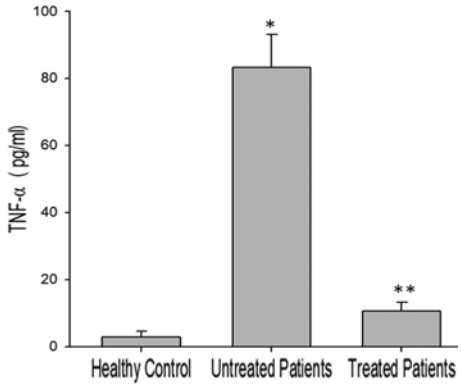

Fig. 3—TNF-α levels in 24 h monocyte culture supernatants: Monocytes from PBMC's of healthy controls and patients with MI were isolated and cultured for 24 h with or without resveratrol as described in methods. The data represents mean ± S.E.M, n =20; * P <0.001 compared with healthy control group and ** P <0.001 compared with untreated patients group.

(COX-2) and causes inflammation. Resveratrol induced a decrease in the activity of COX-2 has also been well documented[10]. Additionally, the production of leukotriene B4 (LTB4) and matrix metalloproteinases (MMPs)[10], where both are involved in the development of atherosclerosis, has been reported to be suppressed by resveratrol.

The results of the present study clearly demonstrate the protective effect of resveratrol on monocyte cultures obtained from MI patients. Treatment with resveratrol showed considerable improvement in inflammation as is evident by the decreased levels of IL-1, IL-6, and TNF-α.

In the ischemic-reperfused myocardium, ROS generated by neutrophils[29] immediately stimulate the release of pro-inflammatory factors, along with an enhanced transcription of factors through NF-κB. These factors again activate neutrophils to generate ROS and various cytokines like TNF-α, IL-1, and IL-6.

The present study showed high levels of TNF-α, IL-1, and IL-6 in 24 h cultures of monocytes isolated from PBMCs of MI patients as compared to the basal levels observed to those in healthy controls. Treatment of these cells with resveratrol ameliorated the TNF-α, IL-1 and IL-6 levels, and GSH to normal or near-normal range. This indicates that resveratrol possess anti-inflammatory and antioxidant properties and can efficiently scavenge the ROS production.

Furthermore, resveratrol hinder the deleterious effects of lipid peroxidation mainly by scavenging the peroxyl radicals[30]. MDA the main lipid peroxidation product is found to be controlled/regulated by

resveratrol treatment in our study. There is an inhibitory effect of resveratrol on NF-κB signalling pathway after cellular exposure to metal-induced radicals[31]. This antioxidant action of phenolics like resveratrol requires the presence of the meta-hydroxyl configuration in ring A and the 4'hydroxyl group in ring B. As the number of hydroxyl groups on the chemical structure of the phenolic increases, its ROS suppressing activity increases[32].

Next, glutathione is known to be one of the major non-enzymatic antioxidant, ubiquitously present in every cell type and mainly distributed in the nucleus, endoplasmic reticulum, and mitochondria[33]. It is a tripeptide containing three amino acids, namely, glutamate, cysteine and lysine and exist in two forms *i.e*. reduced (GSH) and oxidized (GSSG). Glutathione by the reversible oxidation of its active thiol group actively participates in redox reactions[33].

In addition to the above, GSH act as a coenzyme for various enzymes that participates in cellular defense in a process termed as glutathionylation by covalently binding to proteins[33]. Glutathione is also a substrate for the enzyme glutathione peroxidase (GPx) and helps in the conversion of a variety of organic and inorganic hydroperoxides to the corresponding hydroxy compounds. *In vivo*, they have a defensive role against toxic reactive compounds produced during oxidative stress[33]. In addition, the GSH plays a crucial role in preventing cytotoxicity mediated by ROS[33].

In our study, GSH levels were significantly decreased in the cultures of patients monocyte in comparison to healthy controls due to compromised antioxidant status. However, co-culturing of monocytes isolated from PBMC's of patients with MI with resveratrol ameliorated the intramonocyte glutathione levels.

In summary, resveratrol –a natural antioxidant, ameliorated glutathione levels with a simultaneous decrease in MDA levels. In addition, the levels of TNF-α, IL-1 and IL-6 expressions in cell culture studies were also decreased in cell culture supernatants from cells treated with resveratrol. Further studies are required to elucidate the role of resveratrol at the molecular level.

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