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Cardioprotection by the phytoestrogen genistein in experimental myocardial ischaemia-reperfusion injury

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1 Soybean phytoestrogens have no oestrogen agonist effects on the reproductive system and therefore it is reasonable to explore the potential of these naturally occurring plant oestrogens in the cardiovascular pathology. We therefore investigated the effects of genistein in a rat model of myocardial ischaemia-reperfusion injury.

2 Anaesthetized rats were subjected to total occlusion (45 min) of the left main coronary artery followed by 5 h reperfusion (MI/R). Sham operated rats were used as controls. Myocardial necrosis, myocardial myeloperoxidase activity (MPO), serum creatinine phosphokinase activity (CPK), serum and macrophage Tumour Necrosis Factor-α (TNF-α), cardiac intercellular adhesion molecule-1 (ICAM-1) immunostaining, cardiac mRNA for ICAM-1 evaluated by the means of reverse transcriptase polymerase chain reaction (RT–PCR), ventricular arrhythmias and myocardial contractility (left ventricle dP/dt_{max}) were evaluated.

3 Myocardial ischaemia and reperfusion in untreated rats produced marked myocardial necrosis, increased serum CPK activity and MPO activity both in the area-at-risk and in the necrotic area, reduced myocardial contractility, caused ventricular arrhythmias and induced a marked increase in serum and macrophage TNF-α. Furthermore myocardial ischaemia-reperfusion injury increased ICAM-1 expression in the myocardium.

4 Administration of genistein (1 mg kg^{-1}, i.v., 5 min after coronary artery occlusion) lowered myocardial necrosis and MPO activity in the area-at-risk and in the necrotic area, decreased serum CPK activity, increased myocardial contractility, decreased the occurrence of ventricular arrhythmias, reduced serum and macrophages levels of TNF-α and blunted ICAM-1 expression in the injured myocardium. Finally genistein added *in vitro* to peritoneal macrophages collected from untreated rats subjected to myocardial ischaemia-reperfusion injury significantly reduced TNF-α production.

5 Our data suggest that genistein limits the inflammatory response and protects against myocardial ischaemia-reperfusion injury.

Keywords: Genistein; myocardial ischaemia-reperfusion injury; ICAM-1; leukocytes

Abbreviations: ICAM-1, intercellular adhesion molecule-1; MI/R, myocardial ischaemia-reperfusion; TNF-α, tumour necrosis factor-α

Introduction

A large body of evidence suggests that oestrogens play a protective role on cardiovascular system (Barret-Connor & Bush, 1991), and several mechanisms have been proposed to explain their positive effects (Wahl *et al*., 1983; Stampfer & Colditz, 1991; McAde & Berra, 1992; White *et al*., 1997; Guetta *et al*., 1997).

However, oestrogens also have adverse effects on the reproductive system that limit their therapeutical potential. Because of this, it seems reasonable to study the therapeutic effects of naturally occurring plant oestrogens such as the isoflavonoid genistein, a soy derived phytoestrogen. This compound is in fact oestrogen agonist for bone, liver, and the cardiovascular system but does not appear to have agonist effects on the female reproductive system (Washburn *et al*., 1993; Black *et al*., 1994; Anthony *et al*., 1996).

It was already demonstrated that genistein has a hypocholesterolemic effect in animals and humans and is able to inhibit LDL oxidation, endothelial cell proliferation and angiogenesis (Fotsis *et al*., 1993), and to enhance the dilator response to acetylcholine of atherosclerotic arteries (Honore *et al*., 1997), all effects that may predict a favourable impact on the cardiovascular system. Furthermore genistein is able to shut down the earliest steps of atherogenesis and those associated with plaque rupture by inhibiting the tyrosine kinase which is involved in the cascade of events that lead to the formation of thrombi and subsequent inflammation (Raines & Ross, 1995; Wilcox & Blumenthal, 1995).

An inflammatory response has already been shown to have an important role in the pathogenesis of myocardial ischaemia-reperfusion injury (Lucchesi, 1990). Leukocyte accumulation in the myocardium may amplify tissue damage by producing cell activation of the myocytes and by releasing deleterious

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substances such as leukotrienes, thromboxane A_2 (Coker & Parratt, 1985), oxygen free radicals (McCord, 1985) and platelet activating factor (Braquet *et al*., 1987).

Adhesion molecules such as ICAM-1, (a member of the immunoglobulin supergene family) also play a pivotal role in the localization and development of the inflammatory reaction. This molecule is normally expressed at a low basal level on endothelial cells, but its expression can be enhanced by several inflammatory mediators (TNF-α, IL-1), (Wertheimer *et al*., 1992). Previous findings have suggested that cardiac myocytes express ICAM-1 in response to cytokine stimulation and that it serves as an adhesive molecule for neutrophils on this cell type (Smith *et al*., 1991). Furthermore, passive immunization with specific antibodies raised against ICAM-1 reduces infarct size and myocardial leukocyte accumulation in an experimental model of myocardial ischaemia in the rat (Ioculano *et al*., 1994).

Since oestrogens have already been shown to exert protective effects on myocardial injury by limiting the ICAM-1 mediated leukocyte accumulation (Squadrito *et al*., 1997), we investigated whether their natural occurring analogue genistein causes the same degree of cardiovascular protection and inhibition of the inflammatory response in a rat model of myocardial ischaemia-reperfusion injury.

Methods

Animal preparation

Female ovariectomized Sprague-Dawley rats weighing 225–250 g were permitted access to food and water *ad libitum*. Experiments were approved by the Ethical Committee of the University of Messina and were consistent with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No 8523, revised 1987). Rats were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.) and placed on a heated operating table. Polyethylene catheters (PE 50) were inserted into the common carotid artery for the measurement of blood pressure and heart rate, as reported previously (Caputi *et al*., 1980). After tracheotomy, the animals were ventilated with room air with a respirator for small rodents (model 7025 Ugo Basile, Varese, Italy) with a stroke volume of 15 ml kg^{-1} and a rate of 54 strokes min^{-1} to maintain normal pO_2, pCO_2 and pH parameters. An incision was made on the left side of the chest and the fourth intercostal space was exposed. Sutures were placed through the overlapping skin and muscles to permit rapid closure of the chest wall after the surgical procedures. The chest was then opened and the ribs were gently spread. The heart was quickly expressed out of the thoracic cavity, inverted and a 4.0 silk ligature was placed under the visualized left main coronary artery. The ligature was then tied. The heart was returned quickly to the thoracic cavity, the tips of the suture used to produce the coronary ligation were exteriorized through the chest wall and, after the removal of air in the chest with a syringe, the incision was closed by tying the previously placed sutures (Smith *et al*., 1989). The tips of the sutures were removed after 45 min and the heart was taken out after 5 h of reperfusion (MI/R rats). Sham operated animals underwent all the previously described procedures apart from the fact that the suture passing around the left coronary artery was not tied (sham operated rats). Rats subjected to myocardial ischaemia-reperfusion injury and sham operation were treated with either genistein (0.25, 0.5, 1.0, 1.5, 3 and 5 mg kg^{-1}) or vehicle (1 ml kg^{-1}) 5 min after the coronary occlusion.

Quantification of myocardial damage

Infarcted and perfused areas were evaluated with the triphenyl tetrazolium chloride-Evan's blue technique (Klein *et al*., 1981). At the end of the reperfusion period, the ligature around the left main coronary artery was retightened; 2 ml of Evan's blue dye (2 mg ml^{-1} solution) was injected into the jugular vein to stain the area of the myocardium perfused by the patent coronary arteries. The area at risk was, therefore, determined by negative staining. The atria, right ventricle and the major blood vessels were subsequently removed from the heart. The left ventricle was then sliced into sections 3 mm thick parallel to the atrioventricular groove. The unstained portion of the myocardium (i.e., the area at risk) was separated from the stained portion (i.e., the area not at risk). The unstained portion was again sliced into 1 mm-thick sections and incubated in a 1% solution of the triphenyl tetrazolium chloride stain in 20 mM phosphate buffer, pH 7.4 at 37°C for 20 min to detect the presence of coenzyme and dehydrogenase. The necrotic portion of the myocardium, which did not stain, was separated from the stained portion (i.e., the non-necrotic area at risk). Samples from all three portions of left ventricular cardiac tissue (i.e., non-ischaemic, ischaemic non-necrotic and ischaemic necrotic) were weighed and stored at −70°C for subsequent assay of myeloperoxidase activity.

Biological assay for tumour necrosis factor-α activity

Killing of L 929 mouse tumour cells was used to measure TNF-α levels in serum and in peritoneal macrophage supernatants on the basis of a standard microelisa assay (Ruff & Gifford, 1980). L 929 cells in RPMI 1640 medium containing 5% foetal calf serum were seeded at 3×10^4 cells $well^{-1}$ in 96-well microdilution plates and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. Serial 1 : 2 dilutions of serum (drawn at the end of reperfusion) and the supernatants of peritoneal macrophages (we measured only the spontaneous release of the cytokine by the macrophages), harvested at the same time as the serum using a previously described method (Altavilla *et al*., 1989), were made in the above-described method containing 1.0 μg ml^{-1} of actinomycin D, and 100-μl volumes of each dilution were added to the wells. To test whether the cytotoxicity was due to the presence of TNF-α or to other factor(s), we preincubated our samples for 2 h at 37°C with an excess of rabbit anti-recombinant murine TNF-α polyclonal antibodies (Nuclear Laser Medicine, Milan, Italy), or with control rabbit serum. Our results showed that cytotoxicity against L929 cells was completely neutralized by rabbit anti-recombinant TNF-α polyclonal antibodies, but not by control rabbit serum. For the *in vitro* studies macrophages collected from untreated MI/R rats and sham operated rats were incubated for 3 h either with RPMI 1640 medium or several doses of genistein (25, 50 and 100 μM). One TNF-α unit was defined as the amount causing 50% cytotoxicity. The TNF-α content in the sample was calculated by comparison with a calibration curve obtained with recombinant murine TNF-α (Nuclear Laser Medicine, Italy).

Serum creatinine phosphokinase activity

Samples of arterial blood were drawn from the carotid catheter at different time points and collected in polyethylene tubes. The blood was kept at 4°C until it was centrifuged at $2400 \times g$ at 4°C for 15 min. The serum was decanted off and aliquots were used for the determination of creatinine phosphokinase

activity (CPK) using a commercial kit (CK-NAC activated, Boehringer-Mannheim).

Myeloperoxidase activity

Polymorphonuclear neutrophil accumulation was investigated using myeloperoxidase activity as a measure (Mullane *et al*., 1985). MPO activity was determined from cardiac tissue samples (obtained as described above after the end of the reperfusion period), thereby permitting the simultaneous assessment of PMN infiltration and myocardial injury. The samples were first homogenized in a solution containing 20 mM of potassium phosphate buffer (pH 7.4), 0.01 M EDTA, 50 U ml^{-1} of a protease inhibitor (aprotinin) in proportions of 1 : 10 (w v^{-1}) and then centrifuged for 30 min at 20 000 × *g* at 4°C. The supernatant of each sample was then discarded and the pellet was immediately frozen on dry ice. The samples were kept at a temperature of 0°C for 14 h before sonication. After thawing, the resulting pellet was added to a buffer solution consisting of 0.5% hexacyltrimethylammonium bromide (Sigma, St. Louis, MO, U.S.A.) dissolved in 50 mM potassium phosphate buffer (pH 6) containing 30 U ml^{-1} of protease inhibitor. Each sample was then sonicated for 1 min at intensity 2 and at a temperature of 4°C. After sonication, the samples were chilled on ice for approximately 30 min, and were then centrifuged for 30 min, at 40 000 × *g* at 4°C. An aliquot of the supernatant was then allowed to react with 0.167 mg ml^{-1} o-dianisidine dihydrocloride (Sigma) and 0.001% H_2O_2 and the rate in absorbance was measured at 405 nm in a microtitre plate reader. MPO activity was defined as the quantity of enzyme degrading 1 μmol min^{-1} of peroxide at 25°C and was expressed in milliunits per g weight (mU g^{-1} tissue).

Immunohistochemistry

ICAM-1 staining was studied in the area-at-risk at the end of reperfusion period. For immunohistochemical evaluation 5-μm-thick cryostat sections were stained according to the avidin-biotin-peroxidase complex procedure (Hsu *et al*., 1981). An average of seven sections per immunohistochemical stain was cut from each sample, air-dried for 30 min and then fixed in cold acetone for 10 min. Endogenous peroxidases were blocked with horse serum for 15 min at room temperature prior to incubation with primary antibodies. Monoclonal antibodies consisted of mouse monoclonal antibodies raised against rat ICAM-1 (clone: IA 29, subclass IgG_1) and were obtained from British Biotechnology Products Ltd (Abingdon, U.K.). A monoclonal mouse IgG_1 antibody was used for the controls. Biotinylated, specific-specific second layer reagents were then applied, followed by avidin-biotin-horse radish peroxidase complex as a chromogenic substrate, as previously reported (Hsu *et al*., 1981). The microscopy image was sent to a computer-assisted image analyser that analysed the changes in staining. Densitometric analysis of the captured image was performed on a PC using image analysis software.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT–PCR)

Total cellular RNA was extracted from heart sections (area-at-risk) at 5 h of reperfusion. The methods used in the current study have been described elsewhere (Yamada *et al*., 1994). In brief, approximately 100 mg^{-1} of cardiac tissue was homogenized with 800 μl^{-1} RNAZOL STAT (Teltest, Firendswood, TX, U.S.A.) in a microfuge tube, after which 80 μl chloroform was added. After vortexing and centrifugation, the aqueous phase was transferred to a new microfuge tube containing an equal volume of cold isopropanol and the RNA recovered by precipitation by chilling at −80°C for 15 min. The pellet was washed with cold ethanol 70%, centrifuged, dried in speed vacuum, centrifuged a second time and then dissolved in 20 μl of buffer. A 2 μg portion of total RNA was subjected to first strand cDNA synthesis in a 20 μl reaction mixture containing the AMV reverse transcriptase (Superscript II; BRL, U.S.A.), each dNTP, the specific primers, Tris-HCl, and $MgCl_2$.

After dilution of the product with distilled water, 5 μl were used for each polymerase chain reaction (PCR) which contained the Taq polymerase (Perkin Elmer), the buffer as supplied with the enzyme, each dNTP and the specific primers, designed to cross introns and to avoid confusion between mRNA expression and genomic contamination.

The following oligonucleotide pairs were used (5′ oligo/3′ oligo), each sequence as 5′ to 3′: ICAM-1: AGGTGGATACCGGTAGA/CCTTCTAAGTCCTCCAACA; GAPDH: ACCACCATGGAGAAGGCTGG/CTCAGTGTAGCCCAGGATGGC.

The optimal cycle number for ICAM-1 was 25 and we used a PCR negative and a PCR positive control without cDNA or with a known cDNA, respectively. A portion of the PCR product was electrophoresed and transferred to a nylon membrane which was prehybridized with oligonucleotide probes, radiolabelled with [^{32}P]-ATP by a T4 oligonucleotide kinase. After an overnight hybridization at 55°C, filters underwent to the autoradiography.

Haemodynamic measurements

For the haemodynamic measurements a cannula was inserted into the left common carotid artery, as described elsewhere (Squadrito *et al*., 1999) and connected to a pressure transducer (Mac Lab/4E transducer module, AD Instruments, Hastings, U.K.). Changes in electrical activity of the myocardium were detected by electrocardiogram (ECG) (Mac LAB/4E ECG module, AD Instruments, Hastings, U.K.) in lead II during reperfusion. All the data obtained from each module of the system were elaborated by a computer software (Charter windows 3.5, AD Instruments, Hastings, U.K.) and the derivatives parameters LV dP/dt_{max} were displayed on a computer monitor (Squadrito *et al*., 1999). Ventricular arrhythmias during reperfusion were analysed by evaluating electrocardiogram (ECG) in lead II.

Statistical analysis

Data are expressed as means ± s.e.mean and were analysed by analysis of variance for multiple comparison of results; Duncan's multiple range test was used to compare group means. In all cases, a probability error of less than 0.05 was selected as criterion for statistical significance.

Results

Myocardial infarct size

The area-at-risk, determined by negative staining following reperfusion with Evan's blue stain, showed no significant difference between experimental groups (Table 1) indicating that a similar amount of tissue was jeopardized by occlusion of

Table 1 Effects of vehicle of genistein on infarct size (%) after ischaemia-reperfusion injury (MI/R)

Experimental group	*Area-at-risk total* (%)	*Necrotic area-at-risk* (%)	*Necrotic total* (%)
MI/R + vehicle (1 ml kg^{-1})	51 ± 3.1	48 ± 2.2	29 ± 2.3
MI/R + genistein (0.25 mg kg^{-1})	50 ± 3.2	29 ± 2.9*	19 ± 4.1*
MI/R + genistein (0.5 mg kg^{-1})	49 ± 3.7	26 ± 2.5*	16 ± 3.3*
MI/R + genistein (1 mg kg^{-1})	49 ± 4.1	11 ± 1.9†	7 ± 1.2†
MI/R + genistein (1.5 mg kg^{-1})	50 ± 4.5	49 ± 3.1	28 ± 2.8
MI/R + genistein (3 mg kg^{-1})	52 ± 3.6	51 ± 3.7	26 ± 3.8
MI/R + genistein (5 mg kg^{-1})	48 ± 2.9	47 ± 4.5	27 ± 2.7

Each point represents the mean ± s.e.mean of six experiments. *P < 0.05 vs MI/R + vehicle. †P < 0.01 vs MI/R + vehicle.

the main left coronary artery in each group. In contrast, the necrotic area, which was measured by negative staining with triphenyl tetrazolium chloride, indicated that a relatively large amount of the myocardium at risk became necrotic in the MI/R vehicle treated rats (Table 1).

In a dose-response study we have selected the dose to be used in the further studies. Genistein, at a dosage ranging from 0.25 to 1 mg kg^{-1}, showed a marked cardioprotection (Table 1). At higher doses (up to 1 mg kg^{-1}) genistein loses this cardioprotective effect and does not modify infarct size (Table 1). Therefore we have selected the 1 mg kg^{-1} dose as the most effective (Table 1) and we used it in the further experiments.

Serum and macrophage TNF-α

Serum and macrophage levels of TNF-α were not detectable in sham-operated rats treated with either vehicle or genistein. TNF-α was significantly increased in both serum and macrophages collected from infarcted rats at the end of reperfusion period (Figure 1A,B). The administration of genistein significantly blunted the macrophages and serum levels of this cytokine (Figure 1A,B). Furthermore, genistein, added *in vitro* to macrophages collected from untreated rats subjected to myocardial ischaemia-reperfusion injury significantly reduced TNF-α (Table 2).

Serum creatinine phosphokinase

Sham-operated rats given either vehicle or genistein exhibited no significant differences in creatinine phosphokinase (CPK) levels (results not shown). A significant increase of this enzyme was found in the serum of rats subjected to myocardial ischaemia-reperfusion injury and given vehicle (Figure 2).

Administration of genistein (Figure 2) resulted in blunting of CPK activity depletion. These data further support a cardioprotective effect of genistein in acute myocardial infarction in rats.

Myeloperoxidase activity

Very low myeloperoxidase (MPO) activity was measured in sham-operated rats (Figure 3). In contrast, elevated myeloperoxidase activities were found in the area-at-risk and in the necrotic area of the untreated myocardial ischaemia-reperfusion injured rats (Figure 3). Administration of genistein (Figure 3) blunted the rise in myocardial myeloperoxidase activity both in the area-at-risk and in the necrotic area. Thus, genistein limits neutrophil infiltration into the ischaemic/reperfused myocardium.

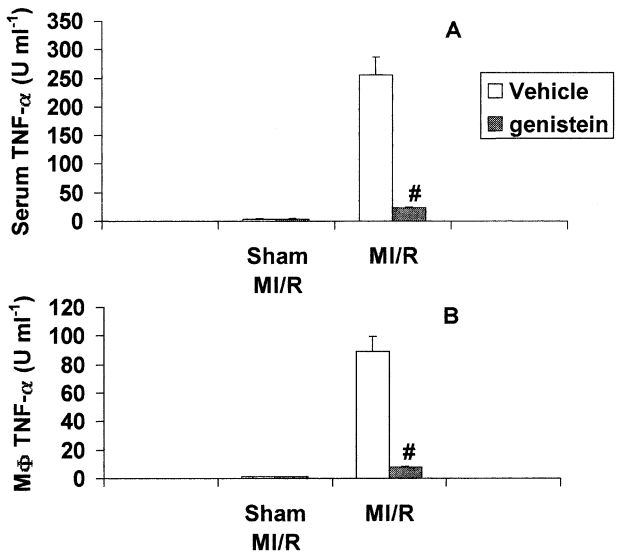

Figure 1 Effects of vehicle (1 ml kg^{-1}) or genistein (1 mg kg^{-1}) given 5 min after the occlusion of the coronary artery on serum (A) and macrophage (B) (MΦ) tumour necrosis factor (TNF-α) in myocardial ischaemia-reperfusion injury (MI/R). Bar heights represent the mean ± s.e.mean of five experiments. #P < 0.001 vs MI/R + vehicle.

Table 2 *In vitro* effects on genistein on Tumour Necrosis Factor (TNF-α) release

Treatments	*TNF-α* (U ml^{-1})
RPMI 290	290 ± 10
Genistein 25 μM	189 ± 12
Genistein 50 μM	70 ± 13*
Genistein 100 μM	23 ± 7†

Peritoneal macrophages were collected from untreated rats subjected to myocardial ischaemia-reperfusion injury. Each point represents the mean ± s.e.mean of five experiments. *P < 0.05 vs RPMI; †P < 0.001 vs RPMI.

ICAM-1 expression in myocardium at risk

ICAM-1 staining was studied in myocardium at risk. Immunohistochemical evaluation indicated that a very low constitutive staining of ICAM-1 was present in the myocardium of sham-operated animals (Figure 4) and in non-

ischaemic myocardium of infarcted rats (results not shown). In contrast, samples of the area at risk obtained from MI/R rats showed an increase in ICAM-1 staining. Genistein reduced the increased staining of ICAM-1 (Figure 4). In agreement with these findings, enhanced cardiac mRNA levels for ICAM-1 were found in the myocardium at risk at 5 h of reperfusion (Figure 5) and genistein treatment markedly suppressed ICAM-1 gene activation (Figure 5).

Haemodynamic parameters

Left ventricular derivative developed force (LV dP/dt_{max}) was monitored throughout the experiment at several time intervals (0, 10 and 45 min after coronary occlusion and 0.5, 2.5 and 5 h following reperfusion; Figure 6). Genistein did not modify this parameter in sham-operated rats (results not shown).

The maximum value of the derivative LV dP/dt_{max} was severely lowered (Figure 6) at 45 min of occlusion. LV dP/dt_{max}, increased promptly upon the release of occlusion but it was always significantly decreased, when compared to the basal values, during the 5 h of reperfusion (Figure 6). Genistein (Figure 6) did not modify LV dP/dt_{max} during coronary occlusion. In contrast, the drug significantly ameliorated myocardial contractility and performance during reperfusion (Figure 6).

Animals subjected to myocardial ischaemia-reperfusion injury showed, during the reperfusion, a high incidence of ventricular arrhythmias (i.e. premature ventricular complexes and ventricular fibrillation). Phytoestrogen treatment reduced the occurrence of these ventricular arrhythmias (Figure 7).

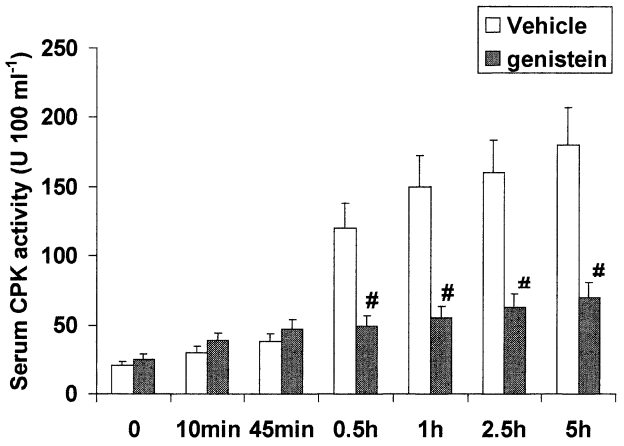

Figure 2 Effects of vehicle (1 ml kg^{-1}) or genistein (1 mg kg^{-1}) given 5 min after the occlusion of the coronary artery on serum creatinine phosphokinase (CPK) activity in myocardial ischaemia-reperfusion injury (MI/R). Bar heights represents the mean ± s.e.mean of five experiments. #$P<0.001$ vs MI/R + vehicle.

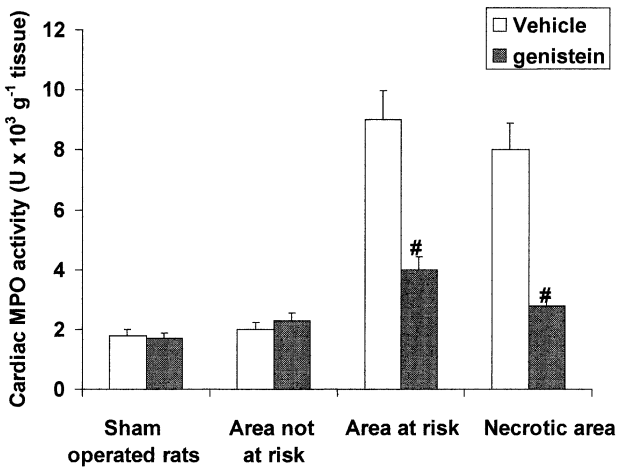

Figure 3 Effects of vehicle (1 ml kg^{-1}) or genistein (1 mg kg^{-1}) given 5 min after the occlusion of the coronary artery on myeloperoxidase (MPO) activity in the area not at risk, area-at-risk and area of necrosis from rats subjected to myocardial ischaemia-reperfusion injury and in cardiac samples from sham-operated rats (MI/R). Bar heights represent the mean ± s.e.mean of five experiments. #$P<0.001$ vs MI/R + vehicle.

Discussion

Genistein is a naturally occurring plant-derived oestrogen-like compound. The precursors of this biologically active compound originate in soybean products, whole grain cereal food, seeds, berries and nuts and they are converted by intestinal bacteria into hormone-like compounds with weak antioxidative and oestrogenic activity (Adlercreutz *et al.*, 1993).

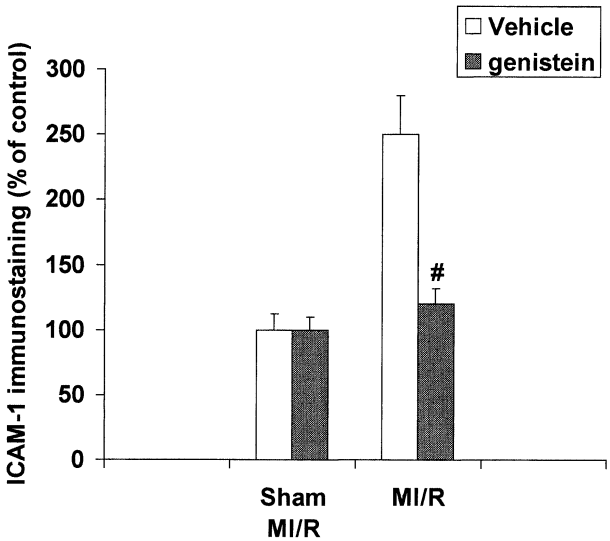

Figure 4 Effects of vehicle (1 ml kg^{-1}) or genistein (1 mg kg^{-1}) given 5 min after the occlusion of the coronary artery on ICAM-1 immunostaining in the myocardium at risk from sham-operated rats and from rats subjected to myocardial ischaemia-reperfusion injury (MI/R). Bar heights represents the mean ± s.e.mean of five experiments. #$P<0.005$ vs MI/R + vehicle.

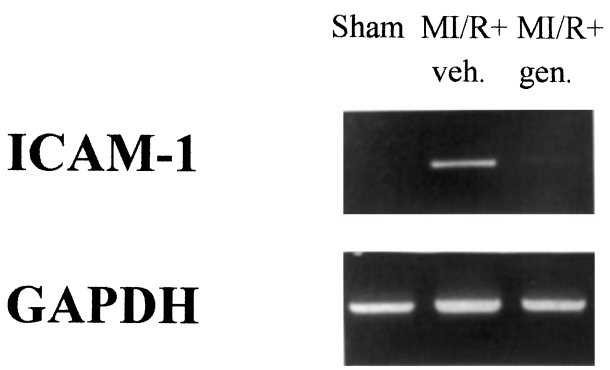

Figure 5 Cardiac ICAM-1 mRNA expression in samples of myocardium obtained at the end of reperfusion and treated with vehicle (veh; 1 ml kg^{-1}) or genistein (gen; 1 mg kg^{-1}) given 5 min after the occlusion of the coronary artery.

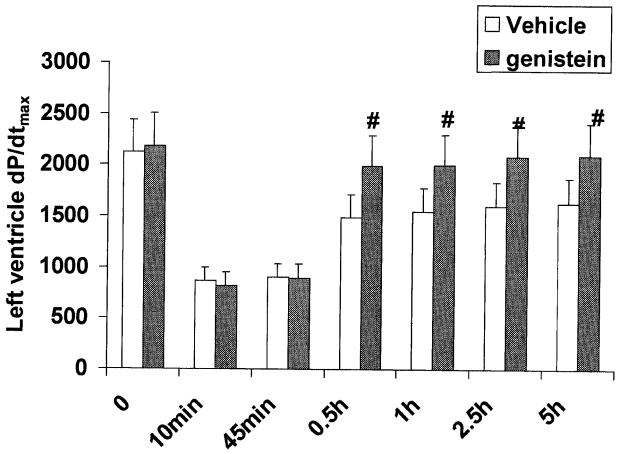

Figure 6 Effects of vehicle (1 ml kg^{-1}) or genistein (1 mg kg^{-1}) given 5 min after the occlusion of the coronary artery on left ventricular dP/dt_{max} in rats subjected to myocardial ischaemia-reperfusion injury (MI/R). Bar heights represents the mean ± s.e.mean of six experiments. #$P<0.001$ vs MI/R + vehicle.

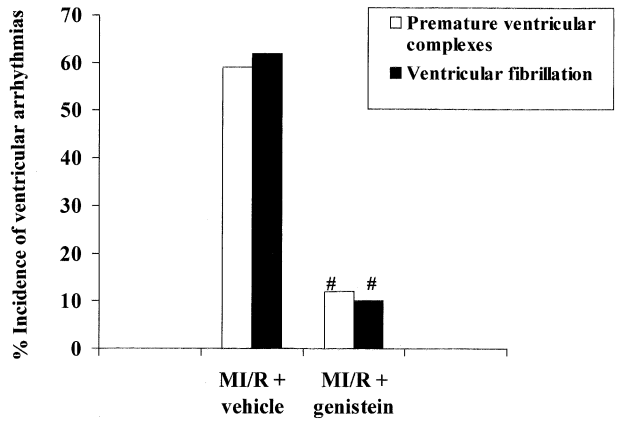

Figure 7 Effects of vehicle (1 ml kg^{-1}) or genistein (1 mg kg^{-1}) given 5 min after the occlusion of the coronary artery on the incidence (%) of reperfusion-induced ventricular arrhythmias in rats subjected to myocardial ischaemia-reperfusion injury (MI/R). Ventricular arrhythmias were evaluated during reperfusion. #$P<0.001$ vs MI/R + vehicle.

Genistein, the principal isoflavone found in soy (Eldridge, 1982) is structurally similar to 17β estradiol and diethylbestrol, binds to oestrogen receptors and exhibits oestrogenic properties in some tissue.

Although the precise mechanisms of myocardial injury following ischaemia with reperfusion are not fully understood, experimental studies have led to the idea that post-ischaemic reperfusion activates interconnected inflammatory networks (Seekamp *et al*., 1994). The presence of induced adhesion molecule gene expression and adhesive receptors within the ischaemic myocardium, therefore represents a myocardial response to injury and the modulation of this response may have therapeutic implications for myocardial ischaemia. In agreement with this hypothesis 17β estradiol has been shown to reduce the inflammatory response in turn causing a marked cardioprotection in myocardial ischaemia with reperfusion (Squadrito *et al*., 1997). Since genistein possesses an oestrogen-like activity, we investigated whether genistein might have an overlapping anti-inflammatory and cardioprotective effect. This experiment could contribute to indirectly ascertain if genistein may substitute for 17β-estradiol in the prevention of cardiovascular diseases.

To test this hypothesis we studied the effects of genistein in a model of myocardial ischaemia-reperfusion injury in rats. Previous findings suggested that the increase in serum TNF-α levels is likely to be involved in this type of experimental myocardial injury (Ioculano *et al*., 1994). Furthermore, leukocyte accumulation in the myocardium has been shown to represent an important aspect of myocardial ischaemia with reperfusion (Ioculano *et al*., 1994). A major factor involved in leukocyte recruitment into inflammatory tissues is thought to be the expression on activated endothelial cells of cytokine inducible adhesion molecules for leukocytes (Harlan, 1987; Osborn, 1990).

Our findings suggest that the deleterious leukocyte accumulation in ischaemic cardiac tissue *in vivo* is mediated by the adhesion molecule ICAM-1, as previously shown (Weyrich *et al*., 1995). This result is also in agreement with previous *in vitro* experiments showing that isolated cardiac myocytes express ICAM-1 in response to TNF-α stimulation and that ICAM-1 serves as an adhesive molecule for neutrophils on this cell type (Smith *et al*., 1991).

Genistein reduced the enhanced macrophage and serum levels of TNF-α. This effect was a consequence of a direct inhibition of this inflammatory cytokine: in fact genistein added *in vitro* to macrophages collected from untreated rats subjected to myocardial ischaemia-reperfusion injury caused a marked inhibition of the inflammatory cytokine.

The phytoestrogen also reduced ICAM-1 expression and decreased myeloperoxidase activity, an index of leukocytes accumulation. Since leukocyte endothelial-interaction (more specifically the ICAM-1-dependent leukocytes adhesion) is primed by TNF-α, it can be proposed that genistein, by blunting this inflammatory cytokine, inhibits ICAM-1 expression, limits leukocytes accumulation and reduces infarct size.

Our data also showed that cardioprotection mediated by this treatment was also accompanied by a reduction in both oxygen consumption and occurrence of ventricular arrhythmias. These effects are most probably due to the consequence of inhibition of leukocyte accumulation into the ischaemic myocardium and can be explained by the significant decrease in cardiac ICAM-1 expression induced by genistein. Alternatively, the reduced leukocyte accumulation could also result from a blunted cell migration from the bloodstream into the ischaemic tissues as a consequence of the decreased serum levels of TNF-α.

Indeed, the present findings are in disagreement with previous data showing that genistein, by inhibiting also tyrosine kinase (Akiyama *et al*., 1987) attenuates or abolishes the cardioprotective effects of ischaemic preconditioning (Fatehi-Hassanabad & Parratt, 1997; Imagawa *et al*., 1997; Fryer *et al*., 1998). However, ischaemic preconditioning is a totally different model. Myocardial infarction is induced after brief period(s) of ischaemia that turn(s) on several mechanisms that are not operative during the typical ischaemia with reperfusion model. In addition the dose used in the preconditioning experiments is higher (5 mg kg^{-1}) (Fatehi-Hassanabad & Parratt, 1997; Imagawa *et al*., 1997; Fryer *et al*., 1998) than the dose range of phytoestrogen (from 0.25 to 1 mg kg^{-1}) that, under our experimental conditions, causes a marked reduction in infarct size. In addition higher doses of genistein (up to 1 mg kg^{-1}) or treatment with more selective inhibitors of tyrosine kinase, such as lavendustin have no effect on myocardial infarction without preconditioning (Imagawa *et al*., 1997) and we were able in our dose-response study to reproduce this finding. Finally, genistein behaves as a tyrosine

kinase inhibitor only at higher doses (up to 1 mg kg^{-1}) while at lower doses it exerts oestrogen-like activity (Akiyama *et al*., 1987). We tried to reproduce these oestrogenic properties to investigate whether the phytoestrogen shows cardioprotective effects as 17β estradiol does. More specifically the hypothesis is that genistein exerts cardioprotection by its ability to serve as an oestrogen agonist. However, besides the oestrogen-like activity, a weak inhibition of tyrosine kinase, which appears to be involved in the mediation of TNF-α-stimulated nuclear factor-κB mobilization and induction of cell adhesion molecules, might also concur to determine the anti-inflammatory effects of genistein and might enhance its cardioprotective effects. By this mechanism in fact, genistein has been shown to cause a dose-dependent inhibition of TNF-induced intracellular adhesion molecule 1 and vascular cell adhesion molecule 1 up regulation *in vitro* (Weber *et al*., 1995).

In conclusion the present findings suggest that the phytoestrogen genistein causes cardioprotection in experimental myocardial ischaemia-reperfusion injury.

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