

Quercetin Inhibits Left Ventricular Dysfunction Induced by Chronic Stress in Rats

La Quercetina Inhibe la Disfunción Ventricular Izquierda Inducida por el Estrés Crónico en Ratas

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SUMMARY: Complications of chronic stress including cardiovascular disease are among the common public health problems that affect the lives of millions of people around the globe. We sought to determine whether the anti-oxidant and anti-apoptotic agent, quercetin can inhibit chronic stress-induced left ventricular dysfunction (LVD). Chronic unpredictable stress (CUS) was induced in rats using a variety of stressors in the presence and absence of quercetin (50 mg/kg body weight/day). Harvested tissues from the left ventricles (LV) of these animals were examined using basic histological staining. In addition, LV tissue homogenates were assayed for markers of oxidative and anti-oxidative stress that are known to be modulated in cardiac dysfunction. Furthermore, LV pressure was monitored by a pressure catheter inserted directly into the LV. Histopathological examinations of the LV in the model group (CUS) showed a profound damage to LV compared to the control group as demonstrated by a severe damage of cardiomyocytes and an increase of inflammatory cell infiltration, which was prevented by quercetin. CUS increased LV end-diastolic pressure that was significantly blocked by quercetin. In addition, quercetin significantly ($p<0.05$) blocked CUS-induced inhibition of the anti-oxidant superoxide dismutase (SOD) and the survival Bcl-2 proteins. Quercetin also significantly ($p<0.05$) inhibited CUS-induced augmentation of the oxidative stress TBARS and the apoptotic protein caspase-3. We conclude that LVD induced by CUS possibly via activation of oxidative and apoptosis pathways can be inhibited by quercetin; thus may offer therapeutic potential in humans.

KEY WORDS: Quercetin; Chronic stress; Heart failure; Left ventricular dysfunction; Antioxidant; Apoptosis.

INTRODUCTION

Stress is any uncomfortable emotional experience that could lead to biochemical, physiological and behavioral changes, and can affect people of all ages and genders (Baum, 1990). A prolonged exposure to stressors lead to chronic stress that could affect the immune, cardiovascular, endocrine and central nervous systems (Anderson, 1998; Rozanski *et al.*; 1999; Brydon *et al.*, 2006). Indeed, the risk of heart disease is doubled in people suffering from stress related anxiety and depression (Kivimäki *et al.*, 2006), and stress significantly increased the risk of myocardial infarction in parents who lost a child (Li *et al.*, 2002) and ischemic and coronary heart disease in people who suffered stressful childhood experiences (Krantz & McCeney, 2002; Dong *et al.*, 2004). Furthermore, chronic stress caused a 27 % death from cardiac arrest in serotonin receptor knockout mice (Carnevali *et al.*, 2012). On the other hand, treating psychological distress reduced coronary artery disease (Rozanski *et al.*, 2005).

Tissue oxidative stress is believed to be the main cause behind damage occurring in animals and humans exposed to traumatic events including chronic stress. Indeed, stress-induced overproduction of reactive oxygen species (ROS) caused apoptosis and enhanced levels of lipid peroxidation (TBARS) and peroxynitrite that damages DNA in brain and heart tissues (Matsumoto *et al.*, 1999; Wang *et al.*, 2013).

Quercetin is a flavonoid antioxidant found in fruits, vegetables and grains, and is the most abundant flavonol in the human diet (Burda & Oleszek, 2001). Quercetin has been widely known to have a potent cardiovascular protective and therapeutic effect via scavenging ROS (Burda & Oleszek; Park *et al.*, 2003), inhibition of lipid peroxidation (Laughton *et al.*, 1991), antihypertensive effects (Duarte *et al.*, 2001), inhibition of platelet aggregation and thrombus formation (Hubbard *et al.*, 2004), and preventing apoptosis and promoting cell survival (Choi *et al.*, 2005).

Damage to the left ventricle, the major heart pumping chamber, caused by severe emotional and/or physical stress in humans is called Takotsubo cardiomyopathy, also named “broken-heart syndrome” (Roshanzamir & Showkathali, 2013). It is characterised by weakening of the left ventricle, and accounts for about 2 % of heart attack cases and mostly in women (Eshtehardi *et al.*, 2009; Roshanzamir & Showkathali). However, very little is known about the effect of chronic stress on left ventricle in animal models, and the effect of chronic unpredictable stress (CUS) on the left ventricle (LV) in rats in the presence and absence of quercetin has not been addressed before. Therefore, this study was designed to investigate the biochemical, molecular, physiological and histological changes occur in LV upon CUS induction that can be reversed by quercetin.

MATERIALS AND METHOD

Animals. Experiments were performed with the approval of the Research Ethics Committee at the College of Medicine, King Khalid University, Abha, Saudi Arabia, and all procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Male Wistar rats of 8 weeks of age and weighing 230 ± 10 g were used for the experiments and obtained and maintained at the animal house of the College of Medicine, where they fed standard rat pellets and allowed free access to water. The housing conditions were a controlled ambient temperature of 25 ± 2 °C and 50 ± 10 % relative humidity, with 12-h light/12-h dark cycles.

Experimental design. After one week adaptation period, rats were randomly assigned to 4 groups (n = 6 each) as follows: 1. Control group: received normal saline. 2. Quercetin treated group (Control+Qur): received Qur (50 mg/kg). 3. Chronic unpredictable stress (CUS) group: a model group and were exposed to CUS protocol, as detailed below and received normal saline. 4. CUS + Qur treated group: were exposed to CUS with a concomitant daily dose of Qur (50 mg/kg). All treatments were administered as 1 ml, i.p. for three consecutive weeks on daily basis. QUR was always prepared fresh, daily, by dissolving in normal saline to the final concentration used in the experimental procedure. Quercetin dose and mode of administration selected is in according to previously published research that showed poor absorption and protective effects of QUR when administered orally, where as its i.p. Administration (10-50 mg/kg) showed pharmacological and therapeutic effect against many disease conditions (Yoshida *et al.*, 1990; Anjaneyulu *et al.*, 2003; Murota & Terao, 2003).

Chronic unpredictable stress (CUS) protocol. According to the method established by Harro *et al.* (1999), a set of chronic unpredictable mild stressors were used to induced depression in rats that lasted for 3 weeks.

Cardiac hemodynamic measurements. Eight hours after the last dose on day 21, cardiac hemodynamic measurements were assessed in each group. In brief, rats were anaesthetized with 1 % solution of sodium pentobarbital (50 mg/kg; i.p.) and placed on a heating pad to maintain body temperature. After performing tracheal intubation and ventilation, the right carotid artery was cannulated using a polyethylene pressure catheter (SPR-320 pressure catheter, AD Instruments, Sydney, Australia) prefilled with heparin (50 U/mL) and connected to a pre-calibrated bridge amplifier. Subsequently, after stabilization of the cardiovascular parameters, the systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MAP). After that, the carotid artery was closed and an open chest surgery was performed. The pressure catheter was inserted directly into the LV to measure LV systolic pressure (LVSP), LV end diastolic pressure (LVEDP), maximal rate of rise in LV pressure (+dP/dt), and maximal rate of decline in LV pressure (-dP/dt). All data were recorded and analyzed with a PowerLab data acquisition system (ML780 PowerLab/8channels, AD Instruments Ltd., Australia).

Collection of LV homogenates. Anesthetized rats were sacrificed by cervical dislocation. Parts of the LV obtained from rats were homogenized in cold phosphate buffer, containing ethylenediaminetetraacetic acid (EDTA). The supernatant obtained was stored at -70 °C for biochemical assays. Other parts of these LVs were frozen at -80 °C and used for RNA extraction. Also, some LV samples were fixed in 10 % formalin for histopathological evaluation.

Biochemical analysis in LV homogenates. Malondialdehyde (MDA) levels as Lipid peroxidation marker were measured as levels of Thiobarbituric acid reactive substances (TBARS) using a commercial assay kit (Cat No. NWK-MDA01, NWLSS, USA). Superoxide dismutase (SOD) activities were measured using a commercial assay kits (Cat. No. 706002, Cayman Chemical, Ann Arbor, MI, USA). Levels of caspase- 3 and Bcl-2 were measured using ELISA commercial kits purchased from STZ ELISA Company, USA. (Cat. No. R5814 and Cat. No. R6813, respectively).

Histopathological studies. Parts of the LV were rapidly fixed in 10 % neutral buffered formalin, dehydrated in ascending concentrations of ethyl alcohol (70-100 %) and then prepared using standard procedures for Hematoxylin and Eosin (H&E) staining.

Statistical analysis. Statistical analyses were performed by using Graphpad prism statistical software package (version 6). Data were presented as means with their standard Deviation (mean ±SD). Normality and homogeneity of the data were confirmed before ANOVA, differences among the experimental groups were assessed by one-way ANOVA followed by Tukey's t test.

RESULTS

Quercetin inhibits chronic stress-induced changes in left ventricular hemodynamics. To test the hypothesis that chronic stress can induce changes in the left ventricular hemodynamics in an animal model of CUS and whether quercetin is able to block these changes, we inserted a pressure catheter directly into the LV to measure LV systolic pressure (LVSP), LV end diastolic pressure (LVEDP), maximal rate of rise in LV pressure (+dP/dt), and maximal rate of decline in LV pressure (-dP/dt). Table I shows data on left ventricular hemodynamics. Compared to control unstressed rat groups,

CUS group showed 8-fold increase in LVEDP and a significant decrease in LVSP, LV +dP/dt and LV -dp/dt. Simultaneous quercetin treatment (CUS+QUR group) inhibited LVEDP augmentation and restored LVSP and LV -dp/dt to their normal levels. Quercetin also significantly increased LV +dp/dt as compared to CUS group.

Quercetin inhibits chronic stress-induced modulation of biomarkers of oxidative and anti-oxidative stress in the left ventricle tissue. Tissue oxidative stress is known to be involved in the pathology of heart disease (Dubois-Randé *et al.*, 1994; Hu *et al.*, 2016). To test the hypothesis that quercetin can modulate oxidative and anti-oxidative biomarkers in LV tissue, we measured the free radicals as TBARS and the superoxide dismutase (SOD) by ELISA in LV homogenates. As shown in Figure 1, CUS significantly (p<0.5) increased TBARS (Fig. 1A) and suppressed the anti-oxidant enzyme, SOD (Fig. 1B). Quercetin treatment significantly (p<0.5) inhibited TBARS (Fig. 1A) and augmented SOD (Fig. 1B). However, the degree of TBARS inhibition (about 75 %) by quercetin was higher than SOD increase (about 50 %) by the same agent.

Table I. Left ventricle hemodynamic measurements for all the animal groups.

Animal groups	LVSP (mmHg)	LVEDP (mmHg)	LV pressure (+dP/dt)	LV pressure (-dP/dt)
Control	110.3±4.7	2.07±0.9	1656.7±137.7	1168±79.3
Control + Quercetin	107.7±3.8	2.2±0.6	1541.2±150.2	1186±123.1
Chronic stress	48.5±4.8*	17.2±3.3*	778±67*	555.8±21.2*
Chronic stress + Quercetin	102.3±9.7-	2.9±0.6-	1178±140.6*-	971.7±35.7-

Values are expressed as Mean ± SD for 6 rats in each group. Analysis by one way ANOVA and Tukeys t-test. Values were considered significantly different at P < 0.05. *: Significant in comparison to Control group, l:Significant in comparison to Chronic stress group. Abbreviations: LVSP, left ventricle systolic pressure; LVEDP, left ventricle end diastolic pressure; (+dP/dt), maximal rate of rise in LV pressure (mmHg/sec); (-dP/dt), maximal rate of decline in LV pressure (mmHg/sec).

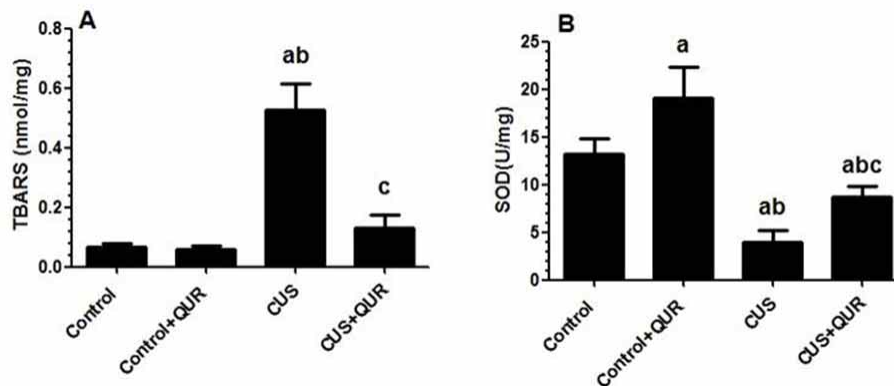


Fig. 1. Effects of treatment by quercetin on TBARS and SOD levels in CUS-induced LV dysfunction. LV tissue levels of TBARS (A) and SOD (B) were measured after 3 weeks by TBARS assay and ELISA in 4 groups of rats; control, control + quercetin, CUS, and CUS + quercetin groups. Results represent the mean (±SD); n=6 for each group. Experiments were performed in triplicate. Values were considered significantly different at P < 0.05. a: Significantly different to control group. b:Significantly different to Control+QUR. c:Significantly different to CUS.

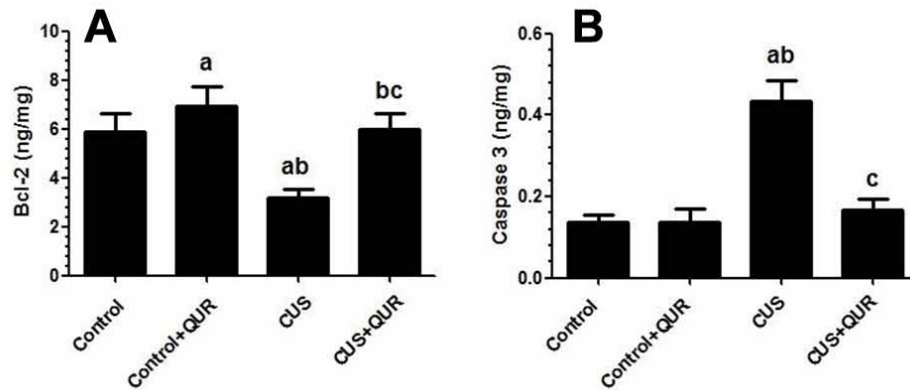


Fig. 2. Effects of treatment by quercetin on Bcl-2 and Caspase-3 levels in CUS-induced LV dysfunction. LV tissue levels of Bcl-2 (A) and caspase-3 (B) were measured after 3 weeks in 4 groups of rats; control, control + quercetin, CUS, and CUS + quercetin groups. Results represent the mean (\pm SD); n=6 for each group. Experiments were performed in triplicate. Values were considered significantly different at $P < 0.05$. a:Significantly different to control group. b:Significantly different to Control+QUR. c:Significantly different to CUS.

Quercetin inhibits chronic stress-induced modulation of biomarkers of survival and apoptosis in the left ventricle tissue. Both the survival and apoptosis biomarkers have been previously shown to be modulated in cardiovascular disease (Dubois-Randé *et al.*; Salimi *et al.*, 2017). To assess whether CUS can ameliorate the survival protein Bcl-2 and augments the apoptotic protein caspase-3 in LV tissue and whether

quercetin is able to inhibit CUS effects, the levels of Bcl-2 and caspase-3 were quantified in LV tissue homogenates of the four rat groups. CUS caused one-fold reduction in Bcl-2, which was significantly ($p < 0.5$) augmented by quercetin to a level comparable to control (Fig. 2A). Whereas, CUS caused a three-fold increase in caspase-3 and quercetin inhibited the enzyme to a level comparable to controls (Fig. 2B).

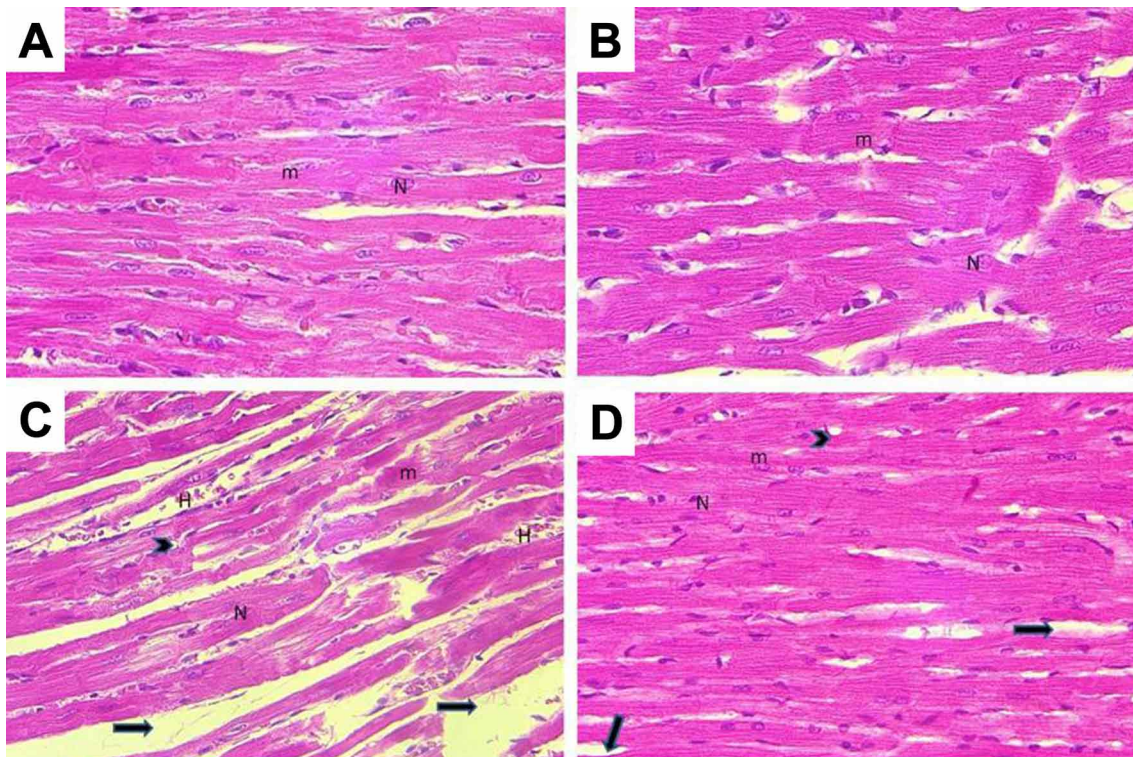


Fig. 3. Quercetin protected the LV against CUS in rats. H&E stained sections (x400) of LV after 3 weeks from control (A), control + quercetin (B), CUS (C), and CUS + quercetin (D) groups. Abbreviations: N, nucleus; m, myofibrils; H, haemorrhage. Note that arrows point to the intramyofiber edema, and arrow heads point to vacuoles.

Quercetin protects against chronic stress-induced left ventricle tissue damage. To investigate whether chronic stress can induce pathological changes in the left ventricle in an animal model of CUS and to assess the potential protective effect of quercetin, sections from LV were examined by light microscopy after staining with H&E. Compared to control groups (Fig. 3A and 3B) that demonstrated normal myocardial architectures with intact myofibrils, rats exposed to CUS (Fig. 3C) showed intensive myocytes damage that revealed areas of cardiac muscle degeneration with loss of myofibrils, wide areas of intramyofiber edema and vacuoles. Histological changes also show tissue haemorrhage and severely pyknotic nuclei. Treatment with quercetin (Fig. 3D) substantially prevented LV tissue damages and protected the architecture of cardiac muscle cells especially the myofibrils, and less vacuoles and edema.

DISCUSSION

This report investigated the effects of chronic stress on the left ventricle pathophysiology using animal model of CUS, and tested the polyphenolic compound quercetin as a potential protective agent to the left ventricle upon chronic stress induction. The principal finding of our study was that CUS caused marked abnormalities in the left ventricular hemodynamics and in the histology and pathology of the left ventricle. Quercetin strongly inhibited CUS-induced damages to left ventricle's structure and function. These studies are the first to report a thorough investigation on the impact of CUS in an animal model on left ventricular dysfunction in the presence and absence of quercetin. This conclusion is supported by the data (Table I) indicating that CUS markedly increased LVEDP and significantly decreased LVSP and both maximum LV contractility (+dp/dt max) and maximum LV relaxation (-dp/dt max), which are indications of heart failure (Danzmann *et al.*, 2008) that were corrected by quercetin. Furthermore, CUS caused a substantial destruction to the LV tissue (Fig. 3) and increased apoptotic and oxidative stress biomarkers as well as decreased survival and anti-oxidative proteins in LV homogenates (Figs. 1 and 2), which were substantially prevented by quercetin. However, CUS did not alter blood lipid profile (data not shown) that is a known factor in cardiovascular disease. Nonetheless, no significant association between stress and serum lipids was also reported before (Hershock & Vogel, 1989; Huang *et al.*, 1990; Andersson *et al.*, 2009).

Our data which points to an effective rat model of CUS that caused substantial LV damage are in agreement with previous studies on CUS animal models; where mild

type of CUS in rats induced the proapoptotic Bax protein in the myocardium (Dang *et al.*, 2016), caused myocardial injury and increased cardiomyocyte apoptosis in cultured cells (Xinxing *et al.*, 2014). In addition, the beneficial effects of quercetin on Wistar rats that was recently reported (Barteková *et al.*, 2015) to (i) protect the left ventricle ultrastructure from damages cause by the chemotherapy drug doxorubicin; (ii) inhibited doxorubicin-induced caspase-3 in left ventricle tissues; and (iii) reversed the down-regulation of SOD by doxorubicin in left ventricle tissues, are in agreement with our data shown in Figures 1 and 3 on the protection of the same species of rats' left ventricles by quercetin upon CUS induction. However, their *ex vivo* work on measuring left ventricle hemodynamics done by removal of the heart and placed onto the Langendorff setup for perfusion (Barteková *et al.*) are in partial agreement with our *in vivo* approaches (Table I and method section in this study). Like ours, quercetin improved the indexes of contraction (+dp/dt max) and relaxation (-dp/dt max) after ischemia/reperfusion injury, but there were no significant differences in the baseline values of all measured parameters between their different animal groups.

Elevated levels of caspase-3, TBARS, and LVEDP, and a reduction in SOD, Bcl-2, and the indexes of contraction (+dp/dt max) and relaxation (-dp/dt max) values are suggestive of heart injury (Chidambaram & Carani Venkatraman, 2010) and a sensitive index of the ventricular damage (Gradman & Alfayoumi, 2006). These reports are in agreement with our findings (Table I & Figs. 1 and 3), and the return levels of these biomarkers close to the baseline upon treatment with quercetin might account for the substantial improvement of the left ventricle histology that was observed.

Taken together, our data supports the conclusion that quercetin ameliorates CUS-induced left ventricular dysfunction (LVD). Further studies are required to investigate CUS-induced myocardial infarction as a type of cardiac dysfunction that could lead to LVD with and without quercetin as a potential protective agent.

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RESUMEN: Las complicaciones del estrés crónico, incluyendo las enfermedades cardiovasculares, se encuentran entre los problemas comunes de salud pública que afectan la vida de millones de personas en todo el mundo. En este trabajo se buscó determinar si la quercetina, agente antioxidante y antiapoptótico, puede inhibir la disfunción ventricular izquierda (DVI) inducida por estrés crónico. Se indujo estrés crónico impredecible (ECI) en ratas utilizando una variedad de factores de estrés, en presencia y ausencia de quercetina (50 mg / kg de peso corporal / día). Las muestras recolectadas de los ventrículos izquierdos (VI) de estos animales se examinaron usando tinción histológica básica. Además, los homogenados de tejido de VI se utilizaron para detectar marcadores de estrés oxidativo y anti-oxidativo que se conocen están modulados en la disfunción cardíaca. Además, la presión del VI se controló mediante un catéter de presión insertado directamente en el VI. Los exámenes histopatológicos del VI en el grupo modelo (ECI) mostraron un daño profundo al VI en comparación con el grupo control, como lo demuestra un grave daño de los cardiomiocitos y un aumento de la infiltración de células inflamatorias, que fue evitado por la quercetina. El ECI aumentó la presión diastólica final del VI, que fue bloqueada significativamente por la quercetina. Además, la quercetina bloqueó en forma significativa ($p < 0,05$) el ECI inducida por la inhibición de la antioxidante superóxido dismutasa (SOD) y la supervivencia de proteínas Bcl-2. Quercetina también inhibió en forma significativa ($p < 0,05$) el ECI inducido por el aumento de la tensión oxidativa y la proteína apoptótica caspasa-3. Llegamos a la conclusión de que la DVI inducida por ECI, posiblemente a través de la activación de las vía oxidativa y la apoptosis, pueden ser inhibidos por la quercetina, pudiéndose ofrecer entonces como un potencial terapéutico en los seres humanos.

PALABRAS CLAVE: Quercetina; Estrés crónico; Insuficiencia cardíaca; Disfunción ventricular izquierda; Antioxidante; Apoptosis.

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