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Efficacy of hesperidin (HDN), on apoptotic markers in tissue of normal and myocardial ischemic rats

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ABSTRACT

Myocardial Infarction (MI) results from the interruption of blood supply to a part of the heart causes heart cells to die. In this process, cardiomyocytereach the state of ischemia. Hesperidin (HDN) is naturally occuring flavonoids in citrus and vegetables, have potent antioxidant property. In this study, myocardial ischemia was induced in the experimental rats by subcutaneous injection of Isoproterenol hydrochloride 85 mg/kg b.w., dissolved in saline for two consecutive days. The optimum dose of HDN 200 mg/ kg b.w., was administered to rats post orally for seven days after ISO-induction. In this study, expression of apoptotic markers TNF-α, Fas, Caspase-3, -9, Bax, Bcl-2, Bcl-xL proteins were analyzed by western blot. ISO induced ischemic rats showed up regulation of TNF-α, Fas, Caspase-3, -9 and Bax and all are down regulated after adminstration of HDN. The heart of ischemic rats showed the down regulated expression of Bcl-2 and Bcl-xL. Administration of HDN of ischemic rats, upregulated the expression of Bcl-2 and Bcl-xL expression. Based on this findings HDN conforms the anti apoptotic property.

Keywords: Hesperidin, Myocardial Infarction, Apoptosis, Isoproterenol

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INTRODUCTION

Apoptosis or programmed cell death, is a genetically regulated, cellular suicide response that plays a pivotal role in a variety of homeostatic and pathological processes. Apoptosis is distinguished from necrosis, another form of cell death, by a number of morphological and biochemical criteria, such as fragmentation of chromosomal DNA that can be recognized by electrophoresis on an agarose gel as a characteristicpattern of DNA ladder formation. Growing evidence implicates that apoptosis significantly contributes to acute myocardial infarction (AMI), one of the leading causes of mortality worldwide [1-3].

Myocardial infarction has been induced by the administrationof isoproterenol. It is non-selective b adrenoceptor agonist andsynthetic. It produces physiological disturbances between antioxi-dant defense mechanism and free radical formation [4,5]. Altered antioxidant enzymes and increased lipid peroxidation have beenobserved in acute myocardial necrosis [6]. ISO-induced DNA damage in cardiomyoblasts causes cardiacwall hypertrophy and also induces apoptosis through free radicalsformation and mitochondrial dysfunction in cardiac cells [7].

Flavonoids are a large class of natural polyphenolic compounds, occurring in fruits and vegetables regularly consumed by humans.Hesperidin (HDN), one of the bioflavonoid in citrus fruits and grapefruit, possesses significant anti-inflammatory, analgesic [8], antioxidant [9] and antihyperlipidemic [10] activities. We wereinterested in testing this citrus bioflavonoid as a potential agent protect the apoptosis in the ISO-induced myocardialinjury in experimental rats.The present study was designed to examine the efficacy of HDNon apoptotic markers in tissue of normal and myocardial ischemic rats

MATERIALS AND METHODS

Chemicals

Isoproterenol hydrochloride and hesperidin were purchasedfrom Sigma–Aldrich (St. Louis, MO, USA). All other chemicals usedin this study were of analytical grade obtained from E. Merck andHIMEDIA, India.

Animals

Healthy male albino Wistar rats (160–180 g), were obtainedfrom the Central Animal House, Department of ExperimentalMedicine, Rajah Muthiah Medical College and Hospital, AnnamalaiUniversity and maintained in an air-conditioned room (25± 3ºC)with a 12 h light/12 h dark cycle. Feed and water were provided adlibitum to all the animals. The study protocols were approved by theInstitutional Animal Ethics Committee of Rajah Muthiah

MedicalCollege and Hospital (Reg No. 160/1999/CPCSEA, Proposal number:541), Annamalai University, Annamalainagar.

Induction of myocardial ischemia

Myocardial ischemia was induced by subcutaneous injection(s.c.) of isoproterenol hydrochloride (85 mg/kg BW, twice at aninterval of 24 h) for two consecutive days [11].

Experimental design

Twenty-four rats were randomly divided in to four groups withsix rats each. HDN dissolved in carboxyl methyl-cellulose (CMC)(0.5%) was administered post-orally (p.o.) to group II and IV rats.CMC treatment alone was given to control rats.

Group I: control (0.5% CMC p.o. for seven days)

Group II: control + HDN (200 mg/kg BW, p.o. for seven days)

Group III: ISO-control (85 mg/kg BW, s.c. for first two consecutive days)

Group IV: ISO (85 mg/kg BW, s.c. for first two consecutive days) + HDN

(200 mg/kg BW, p.o. for seven days)

The total duration of the study was nine days. At day ten, ratswere anaesthetized with an intramuscular injection of ketaminehydrochloride (24 mg/kg BW), and sacrificed by cervical dislocation.

SDS-PAGE and Western blot analysis

Western blotting was performed to analyze the expression pattern of Bax, Bcl-2, Bcl-xL, caspase-3, -9, TNF-a and Fas [12]. The heart tissue samples were homogenised in an ice-cold RIPA buffer (1% Triton, 0.1% SDS, 0.5% deoxycholate, 1 mmol/L EDTA, 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 10 mmol/L NaF, and 0.1 mmol/L phenylmethyl sulfonyl fluoride (PMSF)). The homogenate was centrifuged at 12,000 rpm/min for 15 min at 4 ºC to remove debris. Protein concentration was measured by the method of Lowry et al. [13]. Samples containing 50 mg of total cellular proteins were loaded and separated using 10 % SDS polyacrylamide gel electrophoresis. The resolved proteins wereblot transferred on to a PVDF membrane (Millipore). The membranes were incubated with the blocking buffer containing 5 % BSA for 2 h to reduce non-specific binding sites and then incubated with Bax and b-actin (rabbit polyclonal; 1:500 dilution in 5 % BSA in Tris-buffered saline and 0.05 % Tween-20 (TBST)), Bcl-2 and Bcl-xL (rabbit polyclonal; 1:750), caspase-3, -9, Fas (rabbit polyclonal; 1:1000), TNF-a (goat polyclonal); 1:700) with gentle shaking overnight at 4 ºC. After this, membranes were incubated with their corresponding secondary antibodies (anti-rabbit or anti-goat IgG conjugated to horseradish peroxidase) for 2 h at room temperature. Membranes were washed thrice with TBST for 30 min. Protein bands were visualized by an enhanced chemiluminescence method using ECL kit (GenScript ECL kit, USA). Bands were scanned using a scanner and quantitated by Image J, a public domain Java image processing software, Wayne Rasband, NIH, Bethesda, MD, USA, which of control was set to 1.

Statistical analysis

Values are expressed as means ±S.D. The data were statisticallyanalysed by one-way analysis of variance (ANOVA) followed byDuncan's Multiple Range Test (DMRT) using a statistical packageprogram (SPSS 10.0 for Windows). p-values of less than 0.05 wereconsidered as statistically significant.

RESULTS AND DISCUSSION

Apoptosis is a type of cell death, regulated in an orderly way by a series of signal cascades under certain situations. It has a central role in the pathogenesis of human disease when the genes controlling the apoptotic process are suppressed, overexpressed or altered by mutation [14]. The myocyte death with cardiac disease occurs by both apoptosis and necrosis in response to hypoxia and ischaemia [15]. Cardiomyocyte apoptosis is increased in heart failure [16].

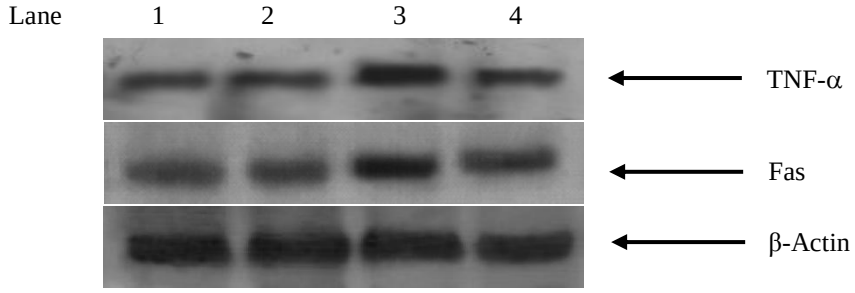
Figures 1 A and B show the expression of TNF-α and Fas proteins in the heart tissue of control and ISO-induced rats. ISO-induced rats showed upregulation of TNF-α and Fas protein expressions and treatment with HDN showed the downregulation.

Simeonova and Luster [17] have shown that intracellularly generated ROS can stimulate TNF-αexpression in alveolar macrophages. Also, earlier report state that GSH depletion observed during ISO-induction can result in enhancement of TNF-α expression [18]. It has been reported that TNF-α/Fas pathway can be activated in the ischemic myocardium [19]. Fas is a member of the TNF-α superfamily and is implicated in the initiation of apoptotic signaling in many tissues. The mRNA for the prototypical death receptor Fas was shown to be detectable in several different organs, including the heart, and in atherosclerotic lesions [20]. Zhu et al. [21] reported that, the conformity between the degree of Fas expression and apoptosis increase which indicates that the change in the expression of Fas was closely related to cardiomyocyte apoptosis. The Present study showed over-expression of TNF-α and Fas proteins during myocardial infarction suggesting that these expressions might play an important role in myocardial injury. HDN administration prevented ISO-induced over-expression of TNF-α and Fas. This finding reveal that the cardioprotective effect of HDN is associated with decreased TNF-α and Fas expressions in the heart, which could be due to the free radical scavenging potential of HDN thereby preventing the depletion of GSH.

Figures 2 A and B represents the westernblot analysis of caspase-3 and -9 in the heart tissue of control and experimental groups. The expressions of caspase-3 and -9 were upregulated in ISO-rats and downregulated after administration of HDN.

During ischemia cytochrome c is released from the mitochondria; it then in concert with Apaf-1 (Apoptosis protease activating factor-1) and ATP causes the processing of procaspase-9 to its active form. Caspase-9 can then follow the apoptotic cascade by activating executioner caspase-3 [22, 23]. Caspase-3 has now been implicated as a key protease that promotes the cleavage of cytoskeletal and nuclear proteins, resulting in the biochemical and morphological hallmarks of apoptosis after ischemia. Pollack et al. [24] reported that ISO-induced mitochondrial oxidative damage which in turn triggers mitochondrial cytochrome c release, along with significant upregulation of caspase-9 and caspase-3 as observed in this study. HDN administration downregulated caspase-3 and caspase-9 expression.

Figure 1A:



Lane 1: Control; 2: Control + HDN; 3: ISO; 4: ISO + HDN.

Figure 1B:

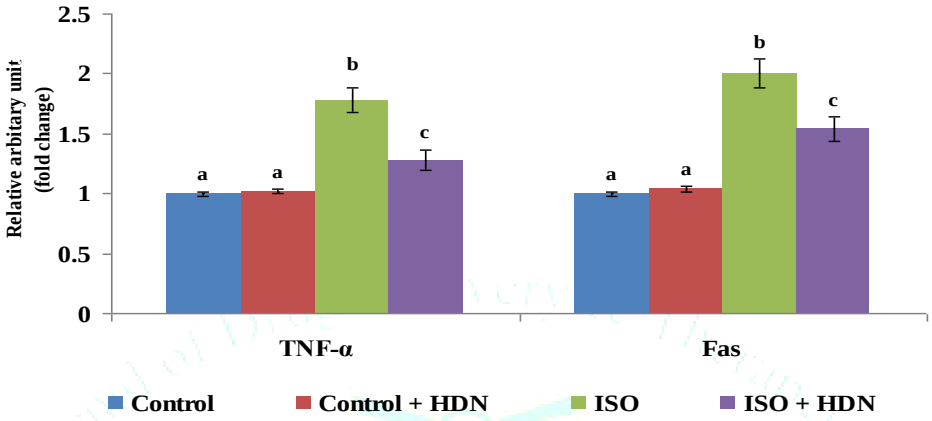
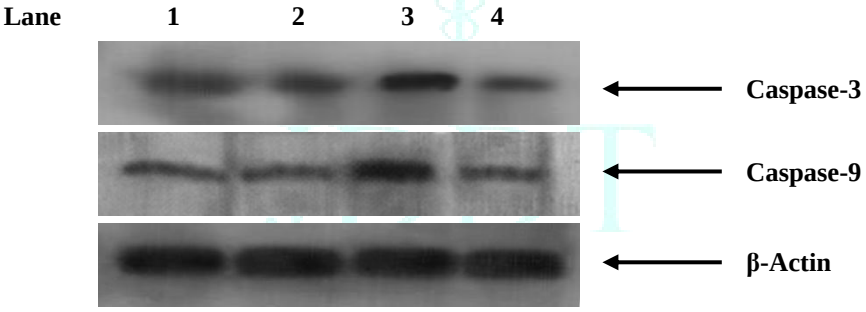


Figure 1.Effect of HDN on TNF-α and Fas protein expressions in the heart tissue of control and ISO-rats. A. Westernblot Analysis. B. Intensities scanned by densitometer.Histogram depicts quantization of three independent experiments (means ± S.D), with data normalized by defining the control group, with TNF-α and Fas protein, as 1 unit. Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

Figure 2A:



Lane 1: Control; 2: Control + HDN; 3: ISO; 4: ISO + HDN.

Figure 2B: Intensities scanned by densitometer.

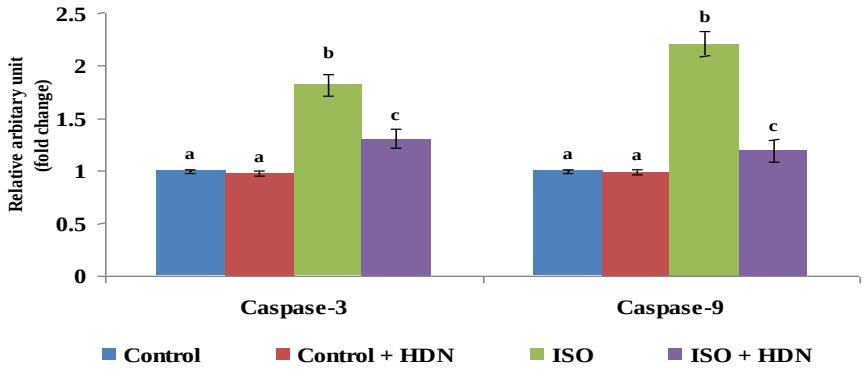
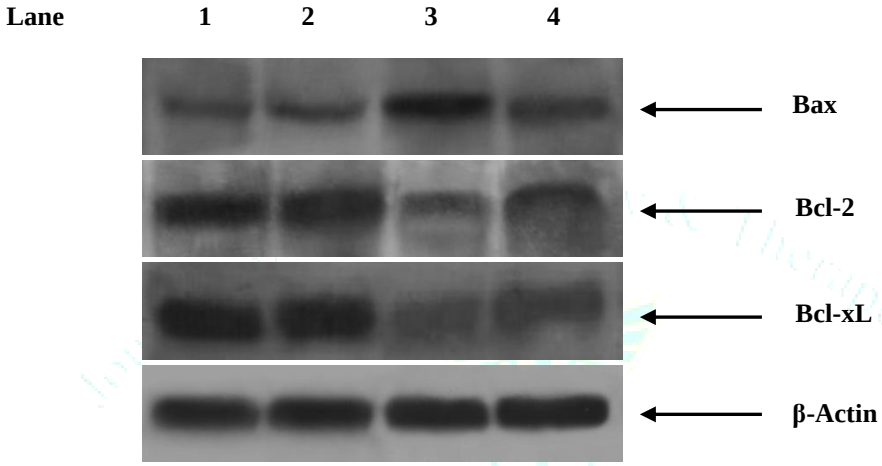


Figure 2.Effect of HDN on caspase-3 and -9 protein expressions in the heart tissue of control and ISO-rats. A. Westernblot analysis. B. Intensities scanned by densitometer.Histogram depicts quantization of three independent experiments (means ± S.D), with data normalized by defining the control group, with caspase-3 and -9 protein, as 1 unit. Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

Figures 3 A and B depicts the expression of Bax, Bcl-2 and Bcl-xL proteins. The heart of ISO-induced rats showed the downregulated expression of Bcl-2 and Bcl-xL and increased Bax expression. Administration of HDN to ISO-induced rats upregulated the expression of Bcl-2 and Bcl-xL and attenuated Bax expression.

Bcl-2, an apoptosis inhibitor, is not expressed in non-infarcted myocardial tissue but it is expressed in cardiomyocytes surrounding infarcted areas soon after the onset of infarction. However, no Bcl-2 was found in the infarcted area itself. Bax is a member of the Bcl-2 family and, when overexpressed, accelerates apoptosis. The pro-apoptotic proteins (Bad, Bax or Bid) are often found in the cytosol where they act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic Bcl-2 proteins and can lead to the formation of pores in the mitochondria and the release of cytochrome c and other pro-apoptotic molecules from the intermembrane space. This in turn leads to the formation of the apoptosome and the activation of the caspase cascade [25]. In this study, the expression of Bcl-2 and Bcl-xL proteins were downregulated and Bax protein was upregulated in ISO-induced rats. Administration of HDN increased the expression of the anti-apoptotic proteins and decreased the expression of the pro-apoptotic protein.

Figrue 3A:



Lane 1: Control; 2: Control + HDN; 3: ISO; 4: ISO + HDN.

Figrue 3B: Intensities scanned by densitometer

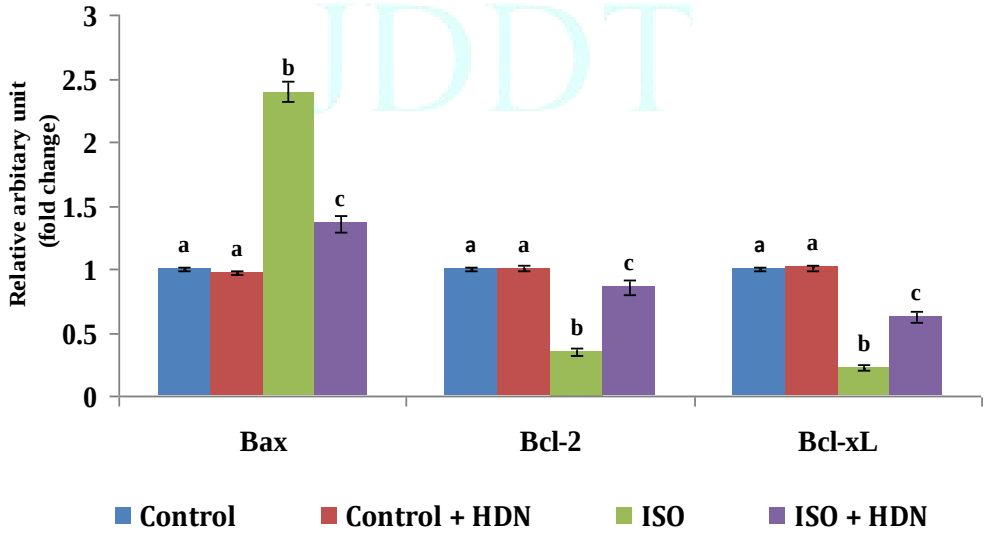


Figure 3.Effect of HDN on Bax, Bcl-2, Bcl-xLprote--in expressions in the heart tissue of control and ISO-rats.A. Westernblot analysis. B. Intensities scanned by densitometer.Histogram depicts quantization of three independent experiments (means ± S.D), with data normalized by defining the control group, with Bax, Bcl-2 and Bcl-xL, as 1 unit. Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

CONCLUSION

The present study shows that the antiapoptotic effect of hesperidin might be by downregulated expressions of TNF-α, Fas, caspase-3, caspase-9, Bax and upregulated expressions of Bcl-2, Bcl-xL in heart tissue. Studies on cardiovascular signaling pathways are needed to fully evaluate HDN role in human health.

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