

ORIGINAL ARTICLE

CYCLOPHOSPHAMIDE ALTERED THE MYOCARDIAL MARKER ENZYMES: PROTECTION PROVOKED BY HESPERIDIN IN RATS**Meenu Shrivastava*^a, Vasundra Kar ^b, Shobhit Shrivastava^c**^{a,c} Department of Pharmaceutical Biotechnology, Bansal College of Pharmacy, Kokta Anand nagar, Raisen road, Bhopal, (M.P.) 462021.^b Department of Pharmaceutical Biotechnology, IEC Institute of engineering and technology, Greater Noida (U.P.)**ABSTRACT**

Our present experimentation was carried out to evaluate the efficacy of hesperidin (HDN) (100mg/kg body weight), administered orally for 7 days on cyclophosphamide (CP) elicited oxidative damage on rat heart. Cardiotoxicity inflicted by single intraperitoneal injection of CP (200mg/kg body weight) was manifested by exalted levels of CPK (creatinine phosphokinase), ALT (alanine transaminase), AST (aspartate transaminase) and LDH (lactate dehydrogenase). CP induced group depicted significant amelioration in level of MDA (malondialdehyde), HDN treated group demonstrated inflated levels of above enzymes and decreased level of MDA. These levels were reversed to normal levels in HDN treated group. Thus result of our study is in concordance with the notion that HDN is adept in combating myocardial free radical damage provoked by CP thus proving its protective potential.

Keywords: Antioxidants; Cardiotoxicity; Cyclophosphamide; Hesperidin; Oxidative stress.

Corresponding Author: Meenu Shrivastava, Department of Pharmaceutical Biotechnology, Bansal College of Pharmacy, Kokta Anand nagar, Raisen road, Bhopal, (M.P.) 462021

INTRODUCTION

It is quite prevalent that anticancer agents caused mild to severe side effects; therefore without besmirching the name of these curative agents, we emphasized our study on toxicity occurring due to their overdose regimens. CP, a nitrogen mustard alkylating agent, is widely used in the treatment of variety of human malignancies, including breast cancer (Hirano et al., 2008), carcinoma of the lung (Zhang et al., 2006), acute leukemia (Pagel et al., 2005). Like most of the anticancer drugs, CP also causes various toxic effects, the commonest of which is the dose-dependent cardio toxicity that ultimately leads to acute and chronic heart

failure (Tiersten et al., 2004; Burnett et al., 2002). Biotransformation of CP mediates through involvement of cytochrome p450 mixed function oxidases with the formation of metabolites phosphoramidate mustard and acrolein which are highly toxic (Lindley et al., 2002; Murgo et al., 1993; Kern et al., 2002). Through this pathway CP has potential to generate superfluous reactive oxygen species (Ahmadi et al., 2008; Sudharsan et al., 2006). Due to the presence of less developed antioxidant defense mechanism, heart is particularly vulnerable to injury by reactive oxygen species. This facilitated the notion that CP also caused a significant decrease in heart weight / body weight ratio, which indicate loss of myofibrils and cytoplasmic vacuolization in myocytes (Lushnikova et al., 2007; Vleet et al., 1986) of cardiac tissue. Antioxidant system in our body inactivates the free radicals to retard the destruction performed by them. Some antioxidants such as, superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione (GSH), sulfhydryl group, CoQ 10 and thioredoxin (Patel et al., 1985). The rationale for choosing nature's treasure such as flavonoid was to obtain maximum benefit with their antioxidant property. One of them is "flavonoids" like hesperidin, naringin and diosmin (Simplicio et al., 2008; Lee et al., 2004). In the present investigation the potential of HDN was deeply evaluated in condition of oxidative stress and it was significantly observed that comparable amounts of free radicals were quenched by it (Chopra et al., 2006). HDN (30, 5, 9-dihydroxy-40-methoxy-7-O-rutinosyl Flavanone), a naturally occurring flavonoid, is a usual constituent of fruits and vegetables (Park et al., 2008). By going with the previous findings which established its bolstering role in oxidative stress, it was considered as safe model for protection against free radicals. There is substantial evidence to suggest that HDN exerts protective action in cardiac tissue by its antihypertensive and antioxidant properties (Wilmsen et al., 2005; Menon et al., 2007). Present study was postulated in the fact that HDN delayed the progression of CP induced myocardial injury which was in concordance with previous studies which revealed antioxidant role of HDN in conditions of oxidative stress.

MATERIALS AND METHODS

Drugs and chemicals

CP (Ledoxan) was purchased from Dabur Pharma Limited, New Delhi, India. HDN was purchased from Sigma Aldrich Chemical Company, Bangalore, India. All other chemicals and solvents were of the highest purity and analytical grade.

Animals

The study was conducted on male Wistar rats (150±10 g). Animals were obtained from the Animal House, Vel's college of pharmacy, the Tamil Nadu Dr. M.G.R. Medical University, Chennai, India. Animals were provided with commercially available standard rat pellet feed (M/s Pranav Agro Industries Ltd., India) under the trade name Amrut rat/mice feed and water ad libitum. The animals were deprived of food for 24 hr before experimentation but allowed free access to sterile water. The rats were housed under conditions of controlled temperature (25±2°C) and were accclamatized to 12 hr light: 12 hr dark cycles. Experiment was conducted after obtaining prior permission according to the university and institutional legislation as regulated by the committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Thus experiment was performed under defined guidelines laid down by the committee.

Experimental Protocol

The experimental animals were randomized into four groups of six rats each as follows:

Group I: Rats were given saline (1ml/kg b.wt.) as vehicle for 7 days which served as control.

Group II: Rats were given intraperitoneal injection of CP (200mg/kg b.wt.) dissolved in saline, on the first day of the experimental period.

Group III: Rats received only HDN (100mg/kg b.wt.) orally for 7 days.

Group IV: Rats were administered with CP as in group II, and immediately supplemented with HDN intraperitoneally for 7 days.

Seven days after the experimental period (i.e., on the 8th day), all the animals were anaesthetized and killed by decapitation. Cardiac tissues were excised and washed in ice-cold physiological saline. The cardiac tissues were homogenized in 0.01 M Tris – HCL buffer (pH 7.4) in homogenizer and aliquots of this homogenate were used for the assays. After collecting the blood, serum was centrifuged for analysis of biochemical parameters.

Biochemical Estimations

Estimation of Diagnostic Heart Markers:

Activities of AST and ALT were determined by the method of Reitman and Frankel et al

(1957). LDH was assayed according to the method of King et al (1965a). CPK was assayed by the method of Broad et al (1948).

Estimation of Lipid Peroxides:

Serum lipid peroxide content was estimated by the method of Yagi et al (1976). The level of lipid peroxides in tissue was determined by 'Thio barbituric acid' reaction as described by Ohkawa et al (1979). Absorbance was measured at 532 nm and 1, 1', 3; 3'-tetramethoxypropane was used as standard. Tissue lipid peroxide levels were expressed as nmoles of MDA/mg protein.

Statistical Analysis

All the grouped data were statistically evaluated with Statistical Package for Social Sciences (SPSS), Version 7.5. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. A 'P' value of less than 0.05 was considered to indicate statistical significance. All the results were expressed as mean + S.D. for six animals in each group.

RESULTS

Effect of HDN on the levels of diagnostic marker enzymes

Oxidative damage in the cardiac tissue resulted with the single dose of CP (200mg/kg b.wt.) as was evidenced by following biochemical changes. A significant ($p < 0.05$) increase in the level of diagnostic marker enzymes (CPK, ALT, AST and LDH) was observed in (group II) CP administered rats as compared to group I control rats (Table 1). Subsequent administration of hesperidin in (group IV) CP+HDN restored the levels of enzymes to ($p < 0.05$) normalcy when compared to (group II) CP induced rats. No significant differences were observed in HDN alone administered rats (group III) compared to (group I) control rats.

Effect of HDN on the levels of cardiac enzymes

Decreased activity of cardiac enzymes was observed in CP administered rats (group II), that demonstrate cardiac tissue degradation (Table 2) when compared to hesperidin treated rats (Group IV) CP+HDN, the level of enzymes were restored to near normal status ($p < 0.05$).

Effect of HDN on the levels of MDA (malondialdehyde) in cardiac tissue

Lipid peroxidation resulted due to CP administration (group II) was assessed by measuring MDA (Figure 1), CP+HDN treated group significantly ($p < 0.05$) lowered the CP induced degradation of membrane lipids (group IV).

Table 1: Effect of Cyclophosphamide and Hesperidin on the activities of cardiac marker enzymes in serum

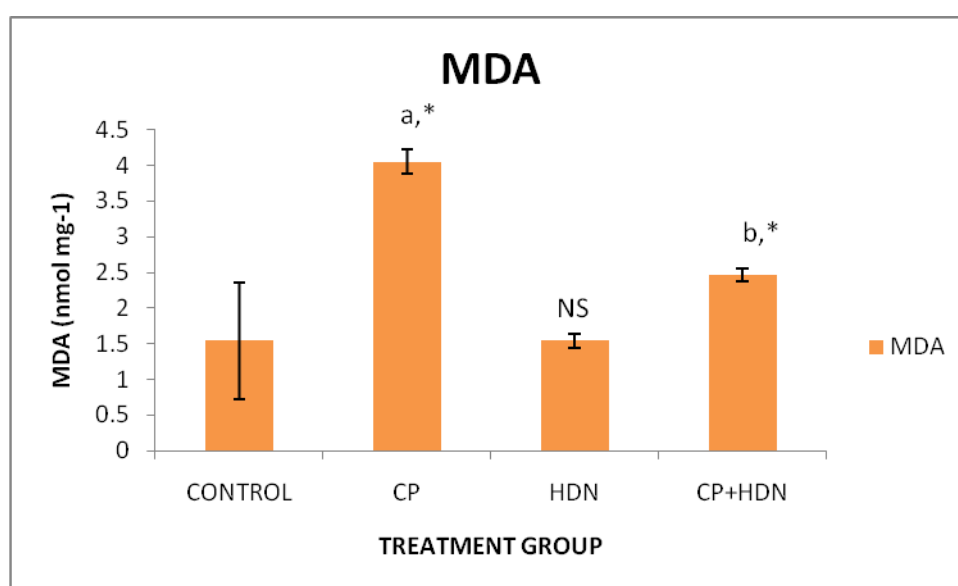
Groups	CPK (IU L ⁻¹)	ALT (IU L ⁻¹)	LDH (IU L ⁻¹)	AST (IU L ⁻¹)
Group I (Control)	125.46 ±2.54	50.95 ±0.74	274.73 ±1.44	83.04 ±2.16
Group II (CP)	290.89 ±7.57* ^a	185.74 ±11.44* ^a	453.26 ±48.31* ^a	275.35 ±18.75* ^a
Group III (HDN)	125.71 ±2.66 ^{NS}	53.59 ±1.72 ^{NS}	280.40 ±7.50 ^{NS}	85.05 ±2.98 ^{NS}
Group IV (HDN+CP)	136.76 ±10.84* ^b	59.15 ±2.66* ^b	299.75 ±14.14* ^b	101.78 ±6.83* ^b

Results are expressed as mean ±S.D. for 6 rats. Comparisons are made between: ^a-group I and group II; ^b-group II and group IV. *Statistically significant ($p < 0.05$); NS – non significant.

Table 2: Effect of Cyclophosphamide and Hesperidin on the activities of cardiac marker enzymes in tissue

Groups	CPK (IU mg ⁻¹ protein)	ALT (IU mg ⁻¹ protein)	LDH (IU mg ⁻¹ protein)	AST (IU mg ⁻¹ protein)
Group I (Control)	22.59 ±1.48	6.16 ±0.22	33.88 ±1.50	7.00 ±0.44
Group II (CP)	7.43 ±0.45 ^{*a}	2.68 ±0.10 ^{*a}	14.52 ±1.08 ^{*a}	3.32 ±0.15 ^{*a}
Group III (HDN)	23.30 ±1.53 ^{NS}	6.16 ±0.35 ^{NS}	34.71 ±0.95 ^{NS}	7.19 ±0.87 ^{NS}
Group IV (HDN+CP)	17.92 ±0.276 ^{*b}	4.61 ±0.10 ^{*b}	28.94 ±1.83 ^{*b}	7.13 ±0.56 ^{*b}

Results are expressed as mean ±S.D. for 6 rats. Comparisons are made between: ^a-group I and group II; ^b-group II and group IV. *Statistically significant ($p < 0.05$); NS – non significant.

Figure 1: Levels of MDA in the heart of experimental animals.

Results are given as mean $\pm$ S.D. For 6 rats. Comparisons are made between: ^a-group I and group II; ^b-group II and group IV. *Statistically significant ($p < 0.05$); NS – non significant.

DISCUSSION

Preceding explorations revealed CP after ingestion converts into active metabolites, 4-OH-cyclophosphamide and aldophosphamide. Aldophosphamide, as assured primally dissociates into phosphoramidate mustard and acrolein, which is a toxic by product (Schimmel et al., 2004; Murgo et al., 1993). Acrolein can also react directly with glutathione (non enzymic antioxidant); forming irreversible conjugates (Grafton et al., 1988). Molecules such as proteins, membrane lipids and RNA may interact with either of these metabolites resulting in production of unstable reactive oxygen species (ROS). Mounting studies substantiated that CP cardiotoxicity proceeded via production of free radicals (Varalakshmi et al., 2006). These free radicals cause profound deterioration to the membrane integrity of a cell as conglomerated previously. This is accompanied by endothelial and vascular damage to the myocardium. Mounting reports assured that there was a poignant amelioration noticed in the levels of diagnostic marker enzymes such as ALT, AST, LDH and CPK (Devaki et al., 2005). This indicated that these enzymes, leaking from disrupted cardiac tissue because of their tissue specificity and catalytic activity are the best markers of myocardium injury (Varalakshmi et al., 2004). In the present study, administration of HDN maintained the level of these enzymes to near normalcy thereby restoring the membrane function (Park et al., 2008; Chopra et al., 2005). Previous documentations reported that HDN due to its surefootedness in providing protective effect is efficient in combating free radicals causing oxidative stress (Chopra et al., 2006). CP induction provokes lipid bilayer repercussion by breaking down membrane phospholipids (Varalakshmi et al., 2006) therefore MDA, product of lipid peroxidation acts as a marker for lipid bilayer damage (Abraham et al., 2008). As the membrane damage progress, it results in the buildup of free radicals in normal animals which leads to greater membrane damage and inactivation or alteration of membrane bound enzymes (Wills et al., 1971). On treatment with rats, HDN decreased MDA level signifying attenuation in lipid peroxidation thereby proving its stabilizing power on membranes (Menon et al., 2007). Enough evidence has been garnered for HDN proving to be effective antioxidant in CP mediated oxidative stress (Ahmadi et al., 2008). Thus concluding, the cardioprotective effect of HDN is probably related to its membrane stabilizing action and scavenging of free radicals generated by CP.

REFERENCES

1. Abraham P, Sugumar E. Increased glutathione levels and activity of PON1 (phenyl acetate esterase) in the liver of rats after a single dose of cyclophosphamide a defense mechanism. *Exp Toxicol Pathol*. 2008, 59(5): 301-6.
2. Ahmadi A, Hosseinimehr S.J, Naghshvar F, Hajir E, Ghahremani M. Chemoprotective effects of hesperidin against genotoxicity induced by cyclophosphamide in mice bone marrow cells. *Arch Pharm Res*. 2008, 31(6): 794-7.
3. Amy T, Jennifer Wo, Caron J, Aaron Weitzman, Tamara Horwich, Charles Hesdorffer, David Savage, Andrea Troxel. Cardiac toxicity observed in association with high dose Cyclophosphamide – based chemotherapy for metastatic breast cancer. *The Breast*. 2004 , 13: 341-346.
4. Annida Balakrishnan, Venugopal P, Menon. Effect of hesperidin on matrix metalloproteinases and antioxidant status nicotine – induced toxicity. *Toxicology*. 2007, 238: 90-98.
5. Broad J, Sirota, J H. Renal clearance of endogenous creatinine in man. *J. Clin Invest*. 1948, 27: 646.
6. Lushnikova E L, Tolstikova T G, Nepomnyashchikh L M, Klinnikova M G, Molodykh O P, Sviridov E A, Sorokina I V, and Zhukova N A. Cardiomyocyte count in rat myocardium under the effect of antitumor agents cyclophosphamide and triterpenoids. *Bulletin of Experimental Biology and Medicine*. 2007, 144: 3.
7. Willis E D. Effects of lipid peroxidation on membrane – bound enzymes of the Endoplasmic reticulum. *Biochem J*. 1971, 123: 983- 991.
8. Elangovan Selvakumar, Chidambaram Prahalathan, Periyasamy Thandavan Sudharshan, Palaninathan Varalakshmi. Protective effect of lipoic acid on Cyclophosphamide induced testicular toxicity. *Clinica Chimica Acta*. 2006, 367: 114-119.
9. Gaganjit Kaur, Naveen Tirkey, Kanwaljit Chopra. Beneficial effect of hesperidin on lipopolysaccharide-induced hepatotoxicity. *Toxicology*. 2006, 226: 152-160.
10. Grafstrom R C, Dypbukt J M, Willey J C, Sundqvist K, Edman C, Atzori L, and Harris C C. Pathobiological effects of acrolein in cultured human bronchial epithelial cells. *Cancer Res*. 1988, 48: 1717–1721.
11. Hirano A, Shimizu T, Watanabe O, Kinoshita J, Kimura K, Kamimura M, Domoto K, Aiba M, Ogawa K. Epirubicin and cyclophosphamide followed by docetaxel as primary systemic chemotherapy in locally advanced breast cancer. *Anticancer Res*. 2008, 28(6B): 4137-42.
12. Van Vleet, J F, and Ferrans, V J. Myocardial diseases of animals. *American Journal of Pathology*. 1986, 124: 98-178.
13. John M P, Frederick R A, Janet F E, Joseph R, Darrell R F, Ted G, Katherine R, Eneida N, Eileen S, Larry D, Jeanette C, Mary M H, Oliver W P, Ajay K G, Paul J M, Irwin D B and Dana C Matthews. 131I-anti-CD45 antibody plus busulfan and cyclophosphamide before allogeneic hematopoietic cell transplantation for treatment of acute myeloid leukemia in first remission. *Blood*. 2005, 107(5): 2184–2191.
14. Kannampalli Pradeep, Sang Hyun Park, Kyong Cheol Ko. Hesperidin a flavanoglycone protects against gamma-irradiation induced hepatocellular damage and oxidative stress in Sprague-Dawley rats. *European journal of pharmacology*. 2008, 587: 273-280.
15. Kern Julie C, Kehrer James P. Acrolein-induced cell death: a caspase-influenced decision between apoptosis and oncosis/necrosis. *Chemico-biological interactions*.

- 2002, 139: 79-95.
16. King J. The dehydrogenase or oxidoreductase-lactate dehydrogenase. In: Practical clinical enzymology Van D (ED). Nostrand Company Limited, London, 1965a, 83-93.
 17. Kirsten J M, Schimmel Dick J, Richel, Renee B A, Van den Brink, Henk-Jan Guchelaar. Cardiotoxicity of cytotoxic drugs. Cancer treatment reviews. 2004, 30: 181-191.
 18. Lindley C M G, Hamilton J S, McCune S, Faucette S S, Shord R L, Hawke H, Wang Gilbert D S, Jolley B Yan and Lecluyse E L. The effect of Cyclophosphamide with and without dexamethasone on cytochrome p450 3A4 and 2B6 in human hepatocytes. Drug Metabolism and Disposition. 2002, 30: 814-822.
 19. Gharib M I, Burnett A K. Chemotherapy-induced cardiotoxicity: current practice and prospects of prophylaxis. European Journal of Heart Failure. 2002, 4: 235-242.
 20. Murgo A J, Weinberger B B. Pharmacological bone marrow purging in autologous transplantation: focus on the cyclophosphamide derivatives. Crit Rev Oncol Hematol. 1993, 14(1): 41-60.
 21. Mythili, Y., Sudharsan, P.T., Selvakumar, E., Varalakshmi, P., Protective effect of DL-alpha-lipoic acid on cyclophosphamide induced oxidative cardiac injury. Chem Biol Interact. 2004, 151(1): 13-9.
 22. Naveen Tirkey, Sangeeta Pilkhwal, Anurag Kuhad and Kanwaljit Chopra. Hesperidin, a citrus bioflavonoid, decreases the oxidative stress produced by carbon tetrachloride in rat liver and kidney. BMC Pharmacology. 2005, 5: 2.
 23. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979, 95: 351-358.
 24. Patel J M, Block E R. Cyclophosphamide-induced depression of the antioxidant defense mechanisms of the lung. Exp Lung Res. 1985, 8(2-3): 153-65.
 25. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol. 1957, 28: 56-63.
 26. Serra H, Mendes T, Bronze M R, Simplício A L. Prediction of intestinal absorption and metabolism of pharmacologically active flavones and flavanones. Bioorg Med Chem. 2008, 16(7): 4009-18.
 27. Subramanian Senthlikumar, Thiruvengadam Devaki, Bhakthavatchalam Murali Manohar, Munismay Suresh Babu. Effect of squalene on Cyclophosphamide induced toxicity. Clinica Chimica Acta.. 2005, 364: 335-342.
 28. Sudharsan, Periyasamy Thandavan, Mythili, Yenjerla, Selvakumar, Elangovan, Varalakshmi, Palaninathan. Lupeol and Its Ester Exhibit Protective Role against Cyclophosphamide-Induced Cardiac Mitochondrial Toxicity. Journal of Cardiovascular Pharmacology. 2006, 47(2): 205-210.
 29. Un Ju Jung, Mi-Kyung Lee, Kyu-Shik Jeong and Myung-Sook Choi. The Hypoglycemic Effects of Hesperidin and Naringin Are Partly Mediated by Hepatic Glucose-Regulating Enzymes in C57BL/KsJ-db/db Mice. The American Society for Nutritional Sciences J. Nutr. 2004, 134: 2499-2503.
 30. Wilmsen P K, Spada D S, Salvador M. Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. J Agric Food Chem. 2005, 53(12): 4757-61.
 31. Yagi K. A simple fluorometric assay for lipid peroxide in blood plasma. Biochem Med. 1976, 15: 212-216.
 32. Zhang Jing, Tian Quan, Zhou Shu-Feng. Clinical Pharmacology of Cyclophosphamide and Ifosfamide. Current Drug Therapy. 2006, 1: 55- 84(30).