Diosmin attenuates NDEA-induced changes in the mRNA expression of stress signalling in the liver of adult male rats.

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Abstract

This study is designed based on the beneficial role of diosmin, a biologically active flavonoid. It is a citrus flavone with antioxidant and anti-inflammatory properties which underlays its protection against cardiac, hepatic and renal injuries. Hepatocellular carcinoma is the most common form of liver cancer that occurs in people with liver disease, particularly in people with chronic hepatitis B and C. To study the diosmin effects in N-Nitrosodiethylamine (NDEA)-induced rats and to evaluate the mRNA expression of stress signalling in the liver of adult male rats. Healthy male albino rats were divided into three groups. First one being the control group, the second one being the NDEA induced group and the third one being the diosmin treated group. The animals were then sacrificed and the tissues were excised. Then the RNA was isolated and it was converted to cDNA using a reverse transcriptase enzyme. The obtained results were plotted using quantitative Real Time Polymerase Chain Reaction (RT-PCR) on a graph. The data were analysed statistically by one way Analysis of Variance (ANOVA) followed by Duncan's multiple range test that was used to see the statistical significance among the groups. Results with $p<0.05$ level were considered to be statistically significant. diosmin plays significant role on stress signaling molecules in liver cells and hence, it could be considered as an anti-cancer therapeutic natural drug for liver cancer.

Keywords: Diosmin, Anti-cancer, mRNA expression, Hepatocellular carcinoma, Innovative technology, Novel method.

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Introduction

Hepatocellular Carcinoma (HCC), an inflammation related cancer. More than 90% of HCCs are due to hepatic injury and inflammation. It is the 5th most common malignancy and 3rd leading cause of cancer related to death, worldwide. Chronic inflammation is associated with hepatic injury leading to sequential development of fibrosis, cirrhosis and HCC [1]. Hepatocellular Carcinoma (HCC) is derived from hepatocytes. In India, the incidence rate of HCC seems to be in the 4th to 7th decade of life. Alcohol consumption, hepatitis B, C virus is the risk factors leading to HCC **[2-4]**. Hepatitis B and C are one of the risk factors for the development of cirrhosis. Cirrhosis remains the most important risk factor for the development of HCC **[5,6]**.

Nuclear factor erythroid 2-related factor 3 (Nrf2) is an essential component of cellular defense and it is a master regulator of cellular protection *via* induction of anti-inflammatory, antioxidant and cyto-protection gene expression [7]. Heme Oxygenase-1 is highly expressed in spleen and liver and the evidence supports its role in cyto-protection against oxidative stress and stimuli. The activation of HO-1 gene expression is considered to be an adaptive cellular response and it is an inducible isoform of the rate-limiting enzyme of heme degradation **[8-10]**. Liver cancer is divided into primary and metastatic liver cancer. It is an aggressive tumor that occurs in the setting of chronic liver disease and cirrhosis. Diosmin is a flavone glycoside that is found in citrus fruit. It is a safe, non-toxic and well tolerated drug. It is demonstrated to activate the rate limiting enzymes of carbohydrate metabolism.

It is found to reverse the abnormalities in glycoprotein components. The biosynthesis of flavonoids in citrus plants has a special significance that determines the chemical structure and bioaccumulation of the bioactive compounds. Diosmin possesses diverse pharmacological activities, including anti-inflammation, anti-cancer, anti-oxidation, etc; diosmin is limited in clinical applications because of its low water solubility. This research aims to provide better drugs for the treatment of hepatocellular carcinoma. Our team has extensive knowledge and research experience that has translate into high quality publications [11-31].

Materials and Methods

Animals

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethics Committee (BRULAC/SDCH/SIMATS/IAEC/02-2019/016). Healthy male albino rats of wistar strain (Rattus norvegicus) weighing 180–210 g (150–180 days old) were used in this study. Animals were obtained and maintained in clean polypropylene cages under specific humidity (65 ± 5%) and temperature (27 ± 2°C) with a constant 12 h light and 12 h dark schedule at the Central animal house facility, University of Madras (Taramani campus). They were fed with a standard rat

pellet diet (Lipton India, Mumbai, India), and clean drinking water was made available ad libitum.

Experimental design

Healthy adult male albino rats were divided into four groups consisting of six animals each. In the present study, diosmin dose (200 mg/kg body weight) was selected based on the study from our laboratory.

Group I—Normal control.

Group II—Hepatocellular carcinogen induced rats (0.01% NDEA orally for 16 weeks).

Group III—Cancer-bearing rats were treated with diosmin (200 mg/kg/ body weight/day) orally for 28 days.

At the end of the experimental period, animals were subjected to ether anesthesia; blood was collected from retro orbital plexus and serum was separated by centrifugation. Animals were sacrificed by cervical decapitation and liver tissues from control and treated animals were excised, washed in ice-cold saline and blotted to dryness. A 10% homogenate of the tissue was prepared in 0.1 M Tris–HCl buffer (pH 7.4), centrifuged and the clear supernatant was used for further analysis.

mRNA expression analysis

Total RNA isolation, cDNA conversion and real-time PCR Using a Total RNA Isolation Reagent Invitrogen kit (TRIR), total RNA was isolated from control and experimental samples. In brief, to 100 mg fresh tissue, 1 ml of TRIR was added and homogenized. The content was transferred to a microcentrifuge tube instantly and 0.2 ml of chloroform was added, vortexed for 1 min then kept at 40C for 5 min. Later, the contents were centrifuged at 12,000 ×g for 15 min at 40C. The aqueous phase (upper layer) was carefully transferred to a fresh microfuge tube and an equal volume of isopropanol was added, vortexed for 15 S and placed on ice for 10 min. After centrifugation of the content at 12000×g for 10 min at 40C, the supernatant was discarded and RNA pellet was washed with 1 ml of 75% ethanol by the vortex. The isolated RNA was estimated spectrometrically by the method of Fourney et al. The RNA concentration was expressed in micrograms (µg). By using the reverse transcriptase kit from Eurogentec (Seraing, Belgium), complementary DNA (cDNA) was synthesized from 2 µg of total RNA as stated in the manufacturer's protocol. To perform real-time PCR, the reaction mixture containing 2x reaction buffer (Takara SyBr green master mix), Forward and reverse primers of the target gene and house-keeping gene, water and β-actin (the primer sequences were listed) in total volume of 45 µl expect the cDNA was made, mixed intensively and spun down. In individual PCR vials, about 5 µl of control DNA for positive control, 5 µl of water for negative control and 5 µl of template cDNA for samples were taken and reaction mixture (45 µl) were added. 40 cycles (95°C for 5 min, 95°C for 5 s, 60°C for 20 s and 72°C for 40 s) was set up for the reaction and obtained results were plotted by the PCR machine (CFX96 Touch Real-Time PCR Detection System) on a graph. Relative quantification was calculated from the melt and amplification curves analysis.

Rat Nrf2

FW: 5’-TTGTAGATGACCATGA GTCGC-3’

RW: 5'-TTC CTG CTG TAT GCT GCT -3’

Rat HO-1

FW: 5'-GTGTGTGTGT-3”

RW: 5-GCCC ATTGCC AGGCAT CTC TTC-3'

Rat β-actin

FW: 5’- TACACCTTGGCGACGACT - 3’

RW: 5’- TCTC GAGAGAGAGAGAGAGA - 3’

Statistical analysis

The triplicate analysis results of the experiments performed on control and treated rat were expressed as mean standard deviation. Results were analyzed statistically by a one-way Analysis of Variance (ANOVA) and significant differences between the mean values were measured using Duncan’s multiple range tests using Graph Pad Prism version 5. The results with the $p< 0.05$ level were considered to be statistically significant.

Result

Effect of diosmin on mRNA expression of Nrf2 (Figure 1) and heme oxygenase(Figure 2) in NDEA-induced liver cancer. The mRNA expressions were assessed by real-time PCR. Each bar represents the mean SEM (n=6). Significance at $p< 0.05$, significantly different from the control group.

Real time -PCR expression of Nrf2

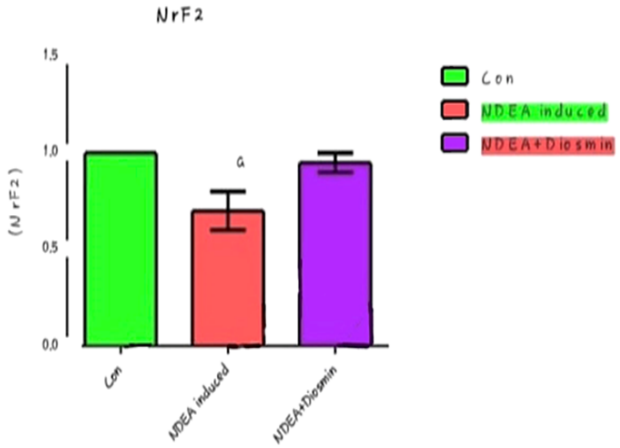

Figure 1. *Diosmin's effect on Nrf2 mRNA expression in NDEA-induced liver cancer rats. Real-time PCR was used to determine the mRNA expression levels. Each bar represents the mean SEM (n=6). Significance at p<0.05, significantly different from the control group.*

Real time -PCR expression of heme oxygenase

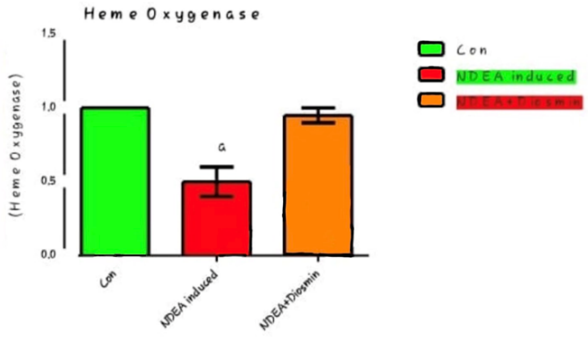

Figure 2. *Effects of diosmin on heme oxygenase mRNA expression in NDEA-induced liver cancer rats. Real-time PCR was used to determine the mRNA expression levels. Each bar represents the mean SEM (n=6). Significance at p<0.05, significantly different from the control group.*

Discussion

The aim of the study is to study the diosmin effects in N-nitrosodimethylamine (NDEA)-induced rats and to evaluate the mRNA expression of stress signalling in the liver of adult male rats. In the present study diosmin restored NDEA-induced detrimental changes in the expression of Nrf2/HO signalling molecule in liver tissue might be due to the potential antioxidant properties of the flavonoid compound, the diosmin suggesting that it has a significant role in the activator of antioxidant signalling mechanism. In accordance with the present study, studies have shown that diosmin possesses potential anticancer activity due to its antioxidant properties in other cancers.

The study by evaluated the antioxidant and chemopreventive efficacy of diosmin against N-nitrosodiethylamine (NDEA) induced hepatocarcinogenesis in adult male rats. The model of NDEA-induced HCC rats showed significant increase in Alpha-Fetoprotein (AFP), Lipid Peroxidation (LPO) and increase in anti-apoptotic, proapoptotic and caspase 3 and 9 proteins. The study by evaluated the radio protective efficiency of diosmin, a natural citrus flavone of hesperidin derivative on radiation induced activity such as biochemical estimations, histopathological alteration. Diosmin is a famous natural flavonoid for treating chronic venous insufficiency and due to its low water solubility, diosmin is limited in clinical applications. Mechanisms of diosmin in mediating cellular processes with high specificity are still needed.

Hepatocellular Carcinoma (HCC) is the most serious and dreaded complication of chronic liver disease. It is regarded as largely a complication of alcoholic cirrhosis, hemochromatosis, or cirrhosis of unknown etiology. This phenomenon emerged with the discovery of the Hepatitis B Virus (HBV) and the evidence that the viral infection was endemic to these same parts of the world. In recent years, the incidence of HCC in the United States has begun to show a great increase. This rise has occurred with the emergence of Hepatitis C Virus (HCV) infection as a major form of liver disease. Diosmin is naturally found in many citrus fruits known to have anti-inflammatory and antimutagenic properties. Diosmin prevents the development and progression of HBP carcinomas through the inhibition of signaling and its downstream events. Therefore, diosmin functions as a potent inhibitor of tumor development and progression by targeting the signaling that may be an ideal candidate for cancer chemoprevention.

Conclusion

According to the findings of this study, diosmin, a natural flavonoid, might be employed as an anticancer therapeutic natural medication for the treatment of liver cancer. Its phlebotonic effect is known to improve the venous tone, stabilize capillary permeability and increase lymphatic drainage, and also its well established safety profile. In order to determine diosmin's potential, more research into its molecular mechanism of action is required.

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Statement of Conflict of Interest

The author declares that there is no conflict of interest in the present study.

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