Effect of diosmin on the expression of matrix metalloproteinase-2 and-9 and p53 in the liver of N-Ndea-induced experimental rats.

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Abstract

Hepatocellular carcinoma most commonly occurs in people with liver disease, particularly in people with chronic hepatitis B and C. Diosmin is a type of plant derived chemical compound found mainly in citrus fruit. Understanding the advances of the molecular pathogenesis of HCC have led to identification of critical driver mutations, however the most prevalent of these are not yet druggable targets. Chemotherapeutic agent, an antineoplastic agent inhibits the uncontrolled proliferation of cancer cells in our body. In a previous study diosmin is used to treat hemorrhoids and thus has anti-cancer properties. To analyse the effect of diosmin on the expression of matrix metalloproteinase 2 and 9 and p53 in the liver of N-NDEA-induced experimental rats. Healthy male albino rats of wistar strain were divided into three groups with six animals each. mRNA expression of matrix metalloproteinase and p53 expression was studied in hepatocellular carcinoma induced animals treated with diosmin. The data were analyzed statistically by a one-way Analysis of Variance (ANOVA) followed by Duncan's multiple range tests was used to see the statistical significance among the groups. The results with the $p<0.05$ level were considered to be statistically significant. Hepatocellular induced rats exhibited a significant increase in MMP2 and MMP9 and decrease in p53 marker. Treatment of N-NDEA induced rats with diosmin shows a reversal in the levels of markers which was equivalent to the control groups.

Keywords: Diosmin, Hepatocellular cancer, Anticancer, Chemotherapeutic agent, Innovative technology, Novel method.

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Introduction

Hepatocellular Carcinoma (HCC) is one of the leading causes of cancer-related death worldwide. It is the sixth most common cancer and third most common cause of cancer deaths as of 2020, with an estimated 905,677 new cases and 830,180 deaths. The incidence was highest in East Asia, at 17.9 per 100,000 population [1]. Cancer is not a reportable disease in India and the cancer registries in India are mostly urban. The HCC incident rate in india for men ranges from 0.7 to 7.5 and for women 0.2 to 2.2 per 10,000 population per year, mostly people between the age of 40 to 70 years were affected [2]. Liver cirrhosis is tightly linked with HCC: A chronic liver insult, which is able to produce inflammation of the liver parenchyma and can in turn trigger the activation of hepatic stellate cells and portal myofibroblasts, resulting in progressive liver fibrosis and, finally, in liver cirrhosis. The complex nature of HCC, therapeutic decisions in HCC clinics are dependent on disease staging, location and size of tumor, presence and extent of extrahepatic spread and underlying hepatic function. Understanding the molecular pathogenesis of HCC and its advancement have led to the identification of critical driver mutations, the most prevalent of these are not yet druggable targets. The molecular classification of HCC is not established [3].

Cirrhosis develops as a result of Chronic Liver Disease (CLD), which is marked by an excess of Extracellular Matrix (ECM) protein aggregation. Matrix Metalloproteinases (MMPs) such as MMP-1, 2, 8, 9 and Transforming Growth Factor beta 1 (TGF-1) play critical roles in CLD and HCC progression [4]. MMP-2 levels were slightly higher in chronic liver disease patients relative to controls, and MMP-2 levels increased with Child-Pugh class. MMP-2 and liver activity (bilirubin, albumin, and prothrombin time) had a strong positive correlation, whereas MMP-9 and these parameters had a strong negative correlation. MMP-2 levels were slightly higher in patients with HCC than in controls, but were similar to patients with chronic liver disease who did not have this malignancy hence in the present study, the expression of MMP2 and 9 were studied to analyze their role in HCC [5]. Diosmin which is a flavonoid glycoside decreases cancer cell number and proliferative activity in different ways which is also proven in our study that, when NDEA induced hepatocellular carcinogenic rats are treated with diosmin the number of cancer cells is reduced **[6]**.

Flavonoids are plant-derived natural compounds that are thought to have a long-term positive impact on human health. They are considered important ingredients in the human diet, despite the fact that their daily intake varies depending on nutritional habits [7-9]. Citrus flavonoids have been shown to have a wide range of biological effects, including anti-cancer properties. Based on this activity, citrus plants and its flavonoids can be considered as chemopreventive molecules. As a flavonoid, diosmin is thought to play a significant role in the extravagance of a variety of illnesses [10,11]. Diosmin is a flavonoid that is naturally derived from flavone glycoside and is a major flavonoid in citrus. Diosmin can be used as a

vascular-protecting and phlebotonic drug, and it is now being researched for other uses such as anticancer, treating premenstrual syndrome, diabetes, and colitis [12,13]. The model of NDEA- induced HCC rats elicited significant increase in alfafetoproteina, lipid peroxidation and increase in anti-apoptotic proteins like Bcl-2, Bcl-xl and Mcl-1 with an concomitant significance decline in the liver antioxidant enzymes. Results of the study suggest that diosmin may be one of a pharmacological and therapeutic representative against hepatocellular carcinoma [14]. Our team has extensive knowledge and research experience that has translate into high quality publications [15-28]. Therefore, the present study was designed to study the effect of diosmin against the NDEA induced rats.

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 Wister 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°C ± 2°C) with constant 12 h light and 12 h dark schedule at Biomedical Research Unit and Lab Animal Research (BRULAC), Saveetha Dental College and Hospitals, Saveetha University, Chennai-600 077, India. They were fed with a standard rat pellet diet (Lipton India, Mumbai, India), and clean drinking water was made available ad libitum.

Experimental design

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 b.wt/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 4°C for 5 min. Later, the contents were centrifuged at 12,000 xg for 15 min at 4°C. 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.

Centrifugation of the content at 12000 xg for 10 min at 4°C, 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 [29]. 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 below) in total volume of 45 µl expect the cDNA was made, mixed intensively and spun down. In individual PCR vials, about 5 of control DNA for positive control, 5 µl of water for negative control and 5 µ l of template cDNA for samples were taken and reaction mixture (45 µ l) 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.

Statistical Analysis

The triplicate analysis results of the experiments performed on control and treated rats were expressed as mean ± standard deviation. Results were analyzed statistically by a one-way Analysis of Variance (ANOVA) and significant differences between the mean value were measured using Duncan's multiple range test 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 levels of MMP-2 in HCC-induced animals

Effect of diosmin on the expression of MMP-2 mRNA levels is shown in Figure 1. These mRNA expressions were significantly increased in HCC-induced animals. Treatment with diosmin significantly reduced the same to that of the control level ($p<0.05$).

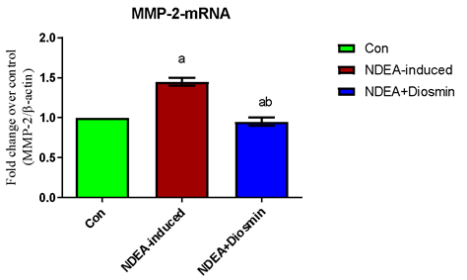

Figure 1. *Represents the effect of diosmin on the mRNA expressions of MMP-2 mRNA expression in liver tissue of NDEA-induced experimental rats. The y-axis represents the mRNA expression of MMP-2 expressed in fold change over control. Light green represents Group 1 the controlled rats. Brown represents Group 2 NDEA-induced hepatocellular carcinogenic rats (0.01% NDEA orally for 16 weeks) and blue represents Group 3 Cancer-bearing rats treated with diosmin (200 mg/kg b.wt/day) orally for 28 days. The expression of MMP-2 mRNA was assessed by Real Time-PCR. Each bar represents the mean ± SEM of 6 animals. Significance at P<0.05, a: Compared with control, b: Compared with NDEA-induced.*

Effect of diosmin on MMP-9 protein expression in HCC-induced animals

Effect of diosmin on the expression of MMP-9 mRNA levels is shown in Figure 2. These mRNA expressions were significantly increased in HCC-induced animals. Treatment with diosmin significantly reduced the same to that of the control level (p<0.05).

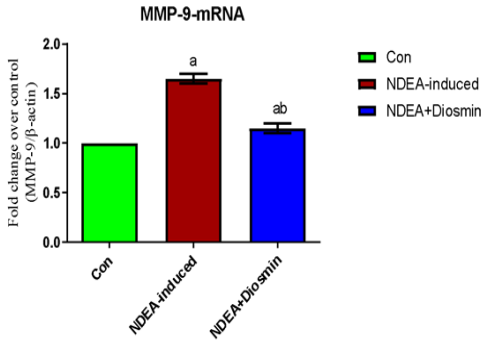

Figure 2. *Represents the effect of diosmin on the mRNA expressions of MMP-9 mRNA expression in liver tissue of NDEA-induced experimental rats. The y-axis represents the mRNA expression of MMP-9 expressed in fold change over control. Light green represents Group 1 the controlled rats, Brown represents Group 2 NDEA-induced hepatocellular carcinogenic rats (0.01% NDEA orally for 16 weeks) and blue represents Group 3 Cancer-bearing rats treated with diosmin (200 mg/kg b.wt/day) orally for 28 days The expression of MMP-9 mRNA was assessed by Real Time-PCR. Each bar represents the Mean ± SEM of 6 animals. Significance at P<0.05, a: Compared with control, b: Compared with NDEA-induced.*

Effect of diosmin on mRNA levels of p53 in HCC-induced animals

Effect of diosmin on the expression of p53 mRNA levels is shown in Figure 3. These mRNA expressions were significantly increased in HCC-induced animals. Treatment with diosmin significantly reduced the same to that of the control level (p<0.05).

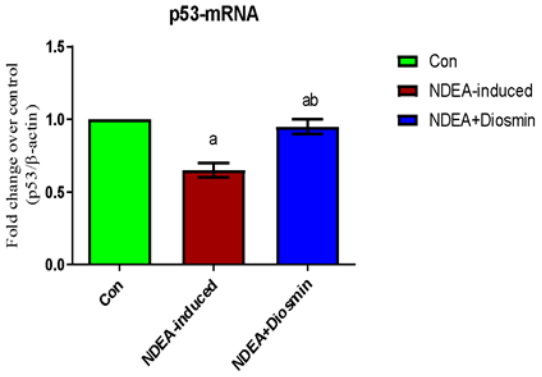

Figure 3. *Represents the effect of diosmin on the mRNA expressions of P53 mRNA expression in liver tissue of NDEA-induced experimental rats. The y-axis represents the mRNA expression of p53 expressed in fold change over control. Light green represents Group 1 the controlled rats, Brown represents Group 2 NDEA-induced hepatocellular carcinogenic rats (0.01% NDEA orally for 16 weeks) and Blue represents Group 3 Cancer-bearing rats treated with diosmin (200 mg/kg b.wt/day) orally for 28 days. The expression of P53 mRNA was assessed by Real Time-PCR. Each bar represents Mean SEM of 6 animals. Significance at P<0.05, a: Compared with control, b: Compared with NDEA-induced.*

Discussion

A number of epidemiological studies have indicated that a diet rich in fruits and vegetables is associated with the reduction of cancer risk in humans, suggesting that certain dietary constituents may thus be effective in preventing cancer **[30]**. These natural agents generally down-regulate signaling pathways which have been activated in malignant cells, and block the proliferation of initiated cells with minimal damage to normal cells. Most cancer preventive agents are natural phytochemicals which act by preventing enzymes involved in carcinogen activation and proliferation [31]. Oxidative stress plays an important role in pathophysiology of many human disorders, while antioxidant diosmin prevents various adverse symptoms, especially in Chronic Venous Insufficiency (CVI), it also have ability to scavenge the oxygen free radicals and hence reduce the level of oxidative stress biomarkers [32].

Diosmin is a well-known flavonoid having a broad spectrum of biological activities, including antioxidant [33] anti-inflammatory and anti-apoptotic activities. Several studies reported that diosmin has a beneficial effect in many pathological conditions as hyperlipidaemia, diabetes mellitus

and peptic ulcer [34]. Hence this study is carried out to investigate the molecular mechanism of Diosmin administration in N-nitrosodiethylamine-induced liver cancer.

Matrix Metalloproteinases (MMPs) such as MMP-1, 2, 8, 9 and Transforming Growth Factor beta 1 (TGF-1) play critical roles in CLD and HCC progression. MMP-2/9 expression is more likely to be upregulated in highly invasive HCC cells than in lowly invasive and non-invasive cells, according to numerous reports. Furthermore, MMP-2/9 overexpression has been linked to HCC invasiveness and metastasis, and MMP-9 outperforms MMP-2 in terms of predicting tumour recurrence and survival in HCC [35]. In line with these findings, our current research found that MMP-9 and MMP-2 mRNA and protein levels were significantly higher in NDEA-induced HCC rats than in control rats. Furthermore, when diosmin was administered, the levels of MMP9 and MMP-2 were greatly reduced to normal levels when compared to control rats. Since diosmin has previously been shown to suppress tumour development, this new finding adds to the evidence that diosmin has anti-cancer properties [36]. Diosmin shows an anti-inflammatory effect by inhibiting the expression of proinflammatory cytokines through blocking the activation of NF-κB pathways and reduction of T cell receptors [37].

In at least half of HCC patients, the p53 cell cycle pathway is disrupted. In HCC, the p53 pathway may be disrupted by mutations in the p53 gene itself [38]. Apoptosis signalling mediated by the p53 family is also disrupted at various levels in HCC. In cancer-bearing animals, the level of p53 mRNA was found to be significantly increased. In diosmin-treated animals, however, the amount of p53 was restored to normal. In the control animals, there was no significant variance [39].

Conclusion

In conclusion, our study suggests that diosmin may play a protective effect on N-nitrosodiethylamine-induced liver cancer, which could be due to its antioxidant activities and anti-cancer activity. Diosmin with its anticancer property provides biological evidence supporting the usefulness to protect Liver cancer induced by toxins like NDEA.

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Conflict of Interest

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

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