Diosmin modulates the expression of wnt/β-Catenin mRNA expression in N-Nitrosodiethylamine (NDEA)-induced hepatocellular carcinoma in experimental rats.

Faaizah Fathima, Gayathri R*, V Vishnupriya, J Selvaraj, S Kavitha

Department of Biochemistry, Saveetha Institute of Medical and Technical Sciences, Chennai, India

Abstract

The current pharmacotherapies associated with cancer are very expensive. Many people cannot afford these drugs which might lead to numerous deaths in the future. The purpose of this study is to find a new potential anticancer herbal drug, which might help in treating cancer. To determine whether diosmin modulates the expression of Wnt/beta catenin mRNA expression in N-nitrosodiethylamine (NDEA)-induced hepato cellular carcinoma in experimental rats. The present result showed that NDEA - induction significantly raised the ($p<0.05$) mRNA expression of both Wnt and beta catenin in the liver compared to control rats, suggesting that NDEA induces cancer *via* Wnt/beta catenin signaling. However, diosmin treatment reversed the gene expression of the same ($p<0.05$) to that of the control.

Keywords: PCB, Diabetes, Vitamin C and E, Adult male Wistar albino rats, Innovative technology, Novel method

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Introduction

Liver cancer is associated with the uncontrolled division of abnormal cells in the liver. It's one of the most prevalent cancers in the world. The most common liver cancer is called hepato cellular carcinoma and begins in the cells called hepatocytes [1]. The incident rate of this cancer in India is approximately 0.2 to 2% among women and 0.7 to 7.5% among men [2]. This cancer is also one of the leading causes of death in the world's most populated country, China [3]. The symptoms of liver cancer are weight loss, stomach pain, vomiting and yellowed skin. There are many treatments such as combination therapy that can be used for liver cancer [4]

In the development of HCC many genetic mutations and abnormal activation of signal transduction pathways is involved [5-9]. The Wnt signalling pathways are a group of pathways which begin with proteins that pass signals into a cell through cell surface receptors. Wnt/beta catenin can possibly manage the embryonic development and adult homeostasis [10]. Wnt can activate pathways with beta catenin but can also activate it without beta catenin [11]. When the level of Wnt/beta catenin is controlled, proliferation, differentiation and other activities of the cells are under control but when this pathway is subjected to mutations it can lead to defects and diseases [12]. Wnt/beta catenin pathway controls not only proliferation and differentiation, but also many other activities of the cells. NDEA, an organic compound, is carcinogenic and mutagenic in nature and can induce hepato cellular cancer. Its continued use in many countries is still up for debate. NDEA can alter the Wnt/beta catenin pathway and promote liver cancer. In hepato cellular cancer Wnt/beta catenin is generally up regulated and activated which might lead to tumour formation due to excessive proliferation [13].

The cost of anti-cancer drugs has led to many problems making it more unaffordable for people below the poverty line [14]. Diosmin is a Plant-derived dietary polyphenolic compound, such as natural flavonoid usually extracted from citrus plants with cancer cell-specific pro-apoptotic activity [15] antioxidant, anti-inflammatory, anti-apoptotic activities and chemopreventive potential [16,17]. Several studies also reported that Diosmin has a beneficial effect in many pathological conditions such as hyperlipidaemia, diabetes mellitus and peptic ulcer [18,19]. Due to its anti-inflammatory and analgesic properties it helps in the treatment of vascular pain [20]. Flavonoids have been known far and wide for their anti-cancer property [21]. One such flavonoid, diosmin, is a safe compound with a good protective effect on oxidative stress and cellular damage. Diosmin being a flavonoid has a potential antiproliferative effect that might be helpful in treating hepato cellular carcinoma [22]. Our team has extensive knowledge and research experience that has translate into high quality publications [23-44]. The aim of the study is to investigate the anticancer activity of diosmin in NDEA liver cancer by analysing the Wnt/beta catenin pathway.

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*) which weighed around 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. They were maintained at the central animal house facility at

Saveetha Dental College and Hospitals, Chennai-600 077, India. They were fed with a standard rat pelleted 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 carcinoma 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.

After 28 days, the animals were subjected to ether anaesthesia at the end of the experimental period; blood was collected from retro orbital plexus and serum was separated by centrifugation. Cervical decapitation was used to sacrifice the animals and the 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 TRIR kit (Total RNA Isolation Reagent Invitrogen), total RNA was isolated from control and experimental samples.1 ml of TRIR was added to 100 mg fresh tissue 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. An equal volume of isopropanol was added to the aqueous phase(upper layer) which was carefully transferred into a fresh microfuge tube earlier , vortexed for 15 S and placed on ice for 10 min. After 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 method of Fourney et al. was used to spectrometrically estimate the isolated RNA. The RNA concentration was expressed in microgram (µg). The reverse transcriptase kit from Eurogentec (Seraing,Belgium) was used and complementary DNA (cDNA) was synthesized from 2 µg of total RNA as stated in the manufacturer's protocol. A reaction mixture of 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 Table 1) in a total volume of 45 µl except the cDNA was made, mixed intensively and spun down to perform real-time PCR. 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 in individual PCR vials 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. The melt and amplification curves were analysed to calculate the relative quantification.

Statistical analysis

The triplicate analysis results of the experiments performed on control and treated rats were expressed as mean ± standard deviation. One-way Analysis Of Variance (ANOVA) was used to analyze the results 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.

Results

Effect of diosmin on Wnt mRNA expressions in HCC induced rats

Effect of diosmin on Wnt mRNA expressions in HCC induced rats is shown in Figure 1. This expression was remarkably increased in HCC induced rats and the same expressions were reduced in diosmin treated rats which is equal to the control ($p<0.05$).

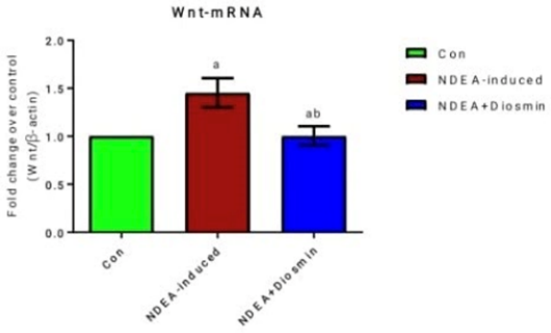

***Figure 1**. The effect of diosmin on the mRNA expression of Wnt mRNA expression in the liver tissue of NDEA-induced experimental rats. The y-axis represents the mRNA expression of Wnt expressed in fold change over control. Light green represents Group 1- the controlled rats, Brown represents Group 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/body weight/day) orally for 28 days. The expression of Wnt 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 beta-catenin mRNA expressions in HCC induced rats

Effect of diosmin on beta-catenin mRNA expressions in HCC induced rats is shown in Figure 2. This expression was remarkably increased in HCC induced rats and the same was reduced in diosmin treated rats which is equivalent to the control ($p<0.05$).

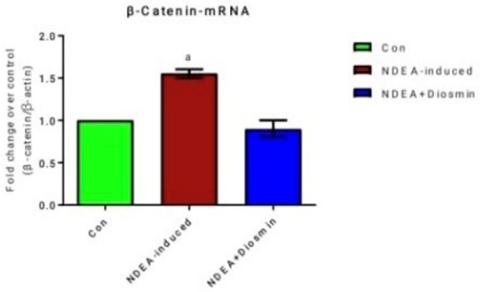

Figure 2. *Effect of diosmin on the mRNA expression of β-Catenin mRNA expression in the liver tissue of NDEA-induced experimental rats. The y-axis represents the mRNA expression of Catenin expressed in fold change over control. Light green represents Group 1- the controlled rats, Brown represents Group 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/body weight/day) orally for 28 days. The expression of Catenin 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.*

Discussion

Flavonoids are compounds that are present in many fruits and vegetables. They have been used as herbal medicine because of its medicinal properties. Diosmin, a flavonoid, is predominantly found in citrus fruits. Diosmin being a dietary compound might help prevent liver cancer. Wnt/beta catenin pathway regulates multiple cellular processes that are involved in the differentiation and apoptosis of hepato cellular carcinoma. Wnt Beta catenin pathway is also involved in the proliferation, cell migration, apoptosis, genetic stability and stem cell renewal.

The same was seen over expressed in Group 2 animals induced with NDEA. Diosmin treated animals (group 3) has exhibited a decrease in the expression of Wnt/beta catenin mRNA,which was equivalent to the control. These results suggest that increased expression of Wnt/beta catenin in the tumour should be investigated and validated. The process of Wnt signalling is increased during liver cancer. Wnt can act as a target in treating liver diseases. The signalling process of Wnt pathway is increased during liver cancer according to various articles and the same is found through the results obtained. In accordance with the present study our study has shown that diosmin reduces the expression of auntie of antiapoptotic proteins such as BCL, BCLXL and Mcl 1 in NDEA-induced hepatocellular carcinoma in adult male rats conversely the proapoptotic molecules such as bax, band and the expression of caspase-3 and caspase 9 protein expression effectively increased at the dose of 200 mg/kg. More over the authors have found that the three expressions were normalized by quiching the free radical formation such as lipid peroxidation as a result of increased levels of endogenous enzymatic antioxidants such as sop,cat and gpx levels in NDEA-induced rats. Therefore, in the present study also the diosmin treated had reduced levels of the mRNA expression of Wnt/beta catenin and it might be due to the increased levels of endogenous antioxidant system and thereby it reduced the production of lipid peroxidation and this study suggests that diosmin could be a therapeutic option inhibiting the cancer cell progression in liver.

Conclusion

From the study it was evident that diosmin, a flavonoid, is a potential antioxidant and has an anticancer effect which can fight against liver cancer. Wnt/beta catenin pathway has shown significant promise as a potential target for novel molecular therapies. There by diosmin can regulate the Wnt/beta catenin mRNA expression in NDEA induced hepato cellular carcinogenic rats. This study thereby concludes that diosmin modulates the Wnt/beta catenin mRNA expression in N-nitrosodimethylamine (NDEA) induced hepatocellular carcinoma in experimental rats. And hence diosmin can be considered a potential flavonoid rich natural drug for the treatment of hepato cellular carcinoma.

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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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*Corresponding to:

Gayathri R

Department of Biochemistry

Saveetha Institute of Medical and Technical Sciences

Chennai

India

E-mail: gayathri.sdc@saveetha.com