Studies on the N-nitrosodiethylamine induced changes in the cell cycle regulatory protein in the liver of adult male express: therapeutic role of diosmin.

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Abstract

Diosmin, a flavonoids rich in citrus fruits, are plant derived dietary polyphenols with natural antioxidant properties. Diosmin inhibits pro apoptotic activator as a chemo prevent agent thus acts as a promising anticancer agent. The effect of diosmin in the expression of cell cycle regulatory protein, such as cyclin D1 and cyclin D4 in NDEA induced experimental animals were studied. The study concludes that diosmin has an anticancer effect against NDEA induced experimental HCC by modulating the expression of cell cycle regulatory proteins and thereby reveals the anti-carcinogenic potential of diosmin can be used as future therapeutic agents against liver cancer. The effect of diosmin in the expression of cell cycle regulatory protein, such as cyclin D1 and cyclin D4 in NDEA induced experimental animals were studied. Male Wistar albino rats were divided into 3 groups. Group 1: Normal control, Group 2: NDEA induced rats (for 16 weeks), Group 3: NDEA induced rats treated with diosmin (for 28 days). The data were analysed statistically by a one way Analysis of Variance (ANOVA) followed by Duncan's multiple range test was used to see the statistical significance among the groups. The results with the $p<0.05$ level were considered to be statistically significant. The hepatocellular induced rats show significant increase ($p<0.05$) in mRNA expressions of cyclin D1 and D4 in the liver compared to control rats. The study concludes that diosmin has an anticancer effect against NDEA induced experimental HCC by modulating the expression of cell cycle regulatory proteins and thereby reveals the anti-carcinogenic potential of diosmin can be used as future therapeutic agents against liver cancer.

Keywords: Diosmin, NDEA, Cyclin D1, Cyclin D4, Liver cancer, Apoptosis, Innovative technology, Novel method.

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Introduction

Liver Cancer is the 5th most commonly occurring cancer in men and 9th most commonly in women. The American cancer society estimated out of 42,230 new cases diagnosed with liver cancer, about 30,230 people die of it. Liver cancer is the sixth most common cancer worldwide **[1]**. Hepatocellular Carcinoma (HCC) is associated with people who have chronic liver diseases, such as hep B or hep C infection **[2,3]**. Cyclin D1 protein expression level is directly related to hepatocellular carcinoma histological grades. Cyclin D is synthesised during the G1 Phase which is involved in cell cycle progression **[4]**. And the compounds involved in the diagnosis of changes associated with signalling pathways are much more expensive **[5]**.

Diosmin is a plant derived dietary polyphenolic compound. It is a flavonoid richly present in citrus plants. It has pro-apoptotic activity, antioxidant, anti-inflammatory and anti-apoptotic activities **[6]**. Owing to its antioxidant property the compound can be used to scavenge free radicals and its associated oxidative stress. Non-pathological disorders such as diabetes, cancer, ageing is associated with an imbalance between oxidants and antioxidants ratio. Diosmin being a flavonoid can be used as a potential free radical scavenger and thus can be tested against various cancers **[7]**.

Several studies also reported that diosmin has a beneficial effect in many pathological conditions **[8]**. The protective role of diosmin against oxidative stress in NDEA induced liver cancer was investigated in this study. Cyclin D is a member of the cyclin protein family which is involved in regulating cell cycle progression **[9]**. In the regulation of cell cycle cyclin D1 plays a central role in the regulation of proliferation and extracellular signalling environment to cell cycle progression **[10]**. Cyclin D synthesis is initiated during G1 **[11]**. A programmed cell death which occurs in the multicellular organisms is called apoptosis **[12]**. It can eliminate the unwanted cells in the early stage [13,14]. Some foods cause apoptosis like beta carotene, a carotenoid in orange. Apoptosis plays a major role in preventing cancer [15-17]. Our team has extensive knowledge and research experience that has translate into high quality publications [18-34]. The aim of this study is to analyse anticancer role of diosmin against NDEA induced experimental HCC by modulating the expression of cell cycle regulatory proteins.

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°C ± 2°C) with constant 12 h light and 12 h dark schedule at 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

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 1: Normal control; Group 2: Hepatocellular carcinogen induced rats (0.01% NDEA orally for 16 weeks); Group 3: 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.

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. 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×g 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 second and placed on ice for 10 min. After centrifugation of the content at 12000 ×g 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 [35]. 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 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 (95ɵC for 5 min, 95°C for 5 second, 60°C for 20 second and 72°C for 40 second) 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

Results were analysed statistically by a one way Analysis of Variance (ANOVA) and significant differences between the mean values 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.

Results

Effects of diosmin on the expression of cyclin D1 and D4 mRNA were shown in the Figure 1 and Figure 2. These mRNA expressions were significantly increased in HCC-induced animals. Treatment with the diosmin significantly reduced the same to that control level.

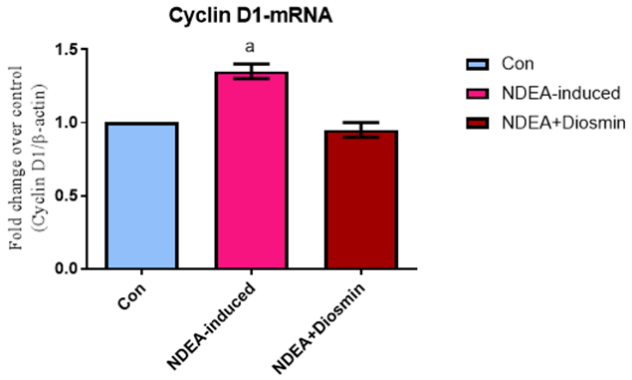

Figure 1. *The effect of diosmin on the mRNA expression of cyclin D1 in the liver tissue of NDEA induced experimental rats. The x-axis represents mRNA expressions of Cyclin D1-mRNA expression in liver tissue of NDEA induced and NDEA +diosmin experimental rats. The y-axis represents the mRNA expression of cyclin D1 expressed in fold change over control. The blue colour represents the controlled rats, pink colour represents the NDEA induced hepatocellular carcinogenic rats and red colour represents cancer-bearing rats that are treated with NDEA + diosmin. The expressions of cyclin d D1 mRNA were assessed by real time PCR. Each bar represents Mean S.E.M. of 6 animals. Significance at p<0.05, a: Compared with control, b: Compared with NDEA induced.*

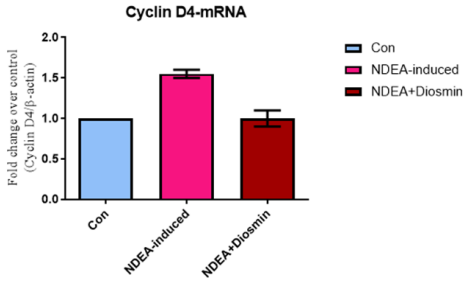

Figure 2. *The effect of diosmin on the mRNA expression of cyclin D4 in the liver tissue of NDEA induced experimental rats. The x-axis represents mRNA expressions of Cyclin D4-mRNA expression in liver tissue of NDEA induced and NDEA +diosmin experimental rats. The y-axis represents the mRNA expression of cyclin D4 expressed in fold change over control. The blue colour represents the controlled rats, pink colour represents the NDEA induced hepatocellular carcinogenic rats and red colour represents cancer-bearing rats that are treated with NDEA+diosmin. The expressions of cyclin d D4 mRNA were assessed by real time PCR. Each bar represents the Mean S.E.M. of 6 animals. Significance at p<0.05,a: Compared with control,b: Compared with NDEA induced.*

Discussion

Most cancer preventive agents are natural phytochemicals which act by preventing enzymes involved in carcinogen activation and proliferation [35**]**. Flavonoids like diosmin are significant ingredients in the human diet, as it differs with their dietary practices. Citrus plants have large number of flavonoid glycosides. The reduced cell viability and proliferation due to anti-cancer and antioxidant potential of diosmin [36].

Cyclin D1 is present in the members of cell cycle regulatory proteins. It can function as a transcriptional co regulator. Cyclin D1 is more important for the development of several cancers. Functions are cellular migration and chromosome stability **[37]**. In line with these findings, our current research found that cyclin D1 and cyclin D4 were significantly increased in NDEA induced HCC rats than in control rats **[38]**.

Furthermore, when diosmin was administered the levels of cyclin D1 and D4 mRNA were greatly reduced to normal levels when compared to the control rats. Since diosmin has previously shown about the cell cycle regulatory protein, the new findings adds to the evidence that diosmin has anti-cancer properties. In accordance with the present study out previous a study has shown that has reduced the expression of anti-apoptotic drugs such as Bcl2 and Bcl-Xl and Mcl-1 in NDEA induced hepatocellular carcinoma in adult male rats **[39]**. Conversely the pro apoptotic molecules such as bax, band and the expression of caspase-3 and caspase9 protein expression effectively increased at the dose of 200 mg per kg but moreover the authors have found that their expressions were normalised by quiching the free radical formation such as lipid peroxidation as a results of increased levels of endogenous enzyme antioxidants such as SOD, cat and GPX levels in NDEA-induced rats **[40]**.Therefore in the present study also the diosmine treated reduced levels of the mRNA expressions of cell cycle regulatory protein might be due to the increased levels of endogenous antioxidant system and thereby it reduced the production lipid peroxidation and this study suggest that diosmin could be a therapeutic option inhibiting the cancer cell progression in liver.

Conclusion

It is suggested that diosmin has shown promising anticancer effect against NDEA induced experimental HCC. Our finding strongly reveals the anti-carcinogenic potential of diosmin may be directly or indirectly antioxidant properties.

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Conflict of Interest

The authors declare no conflict of interest.

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