Original Article

The effect of melatonin on apoptosis in hepatocellular carcinoma cell line: An experimental study

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Summary

Introduction: *Apoptosis resistance in Hepatocellular Carcinoma (HCC) is considered as one of the most important factors involved in hepatocarcinogenesis and its progression. Recent experimental research of melatonin highlights its anticancer function through its antioxidant effect, induction of apoptosis and cell cycle arrest, inhibition of metastasis, and suppression of angiogenesis. The aim of our study is to explore the effect of melatonin on casp 8 mRNA expression and Alpha-fetoprotein (AFP) mRNA expression in HepG2 HCC cell line by using real time PCR technique to proof its apoptotic effect.* ***Methods***: *HepG2 cells were treated with 75mg melatonin then AFP mRNA expression and casp8 expression is investigated by RT PCR.* ***Results***: *Melatonin significantly increased caspase 8 expression. The relative expression was 2.51, 2.48 and1.89 respectively as compared to control (P <0.001). While the expression of AFP was down regulated by melatonin administration. The relative expression of AFP was 0.051, 0.052 and 0.046 respectively as compared to control (P <0.001).* ***Conclusion***: *melatonin decreases AFP gene expression and stimulates apoptosis via up regulation of caspase 8 expression in HCC. Melatonin can be considered as promising adjuvant for chemotherapy in HCC.*

Keywords: *Alpha fetoprotein (AFP), Hepatocellular Carcinoma (HCC), Apoptosis, melatonin*

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Introduction

HCC represents a serious public health issue in Egypt, where liver cancer counts for 11.75% of the malignancies of the digestive organs and 1.68% of the total malignancies. HCC forms 70.48% of all liver tumors among Egyptians and it is considered the main complication of cirrhosis[1]. A number of strategies such as surgery, chemotherapy, molecular targeted therapy, radiation, and liver transplantation have been utilized for the treatment of HCC; however, most of the therapeutic treatments have a low response rate and are accompanied with significant side effects. Moreover, the high fatality rate of HCC has not yet improved, primarily because of uncontrolled metastasis, liver failure, and its resistance to therapeutic agents. To overcome these clinical shortcomings, genetic therapy has been considered as a new therapeutic approach for HCC[2]. AFP has become a target for liver cancer immunotherapy [3]. It is a diagnostic marker for HCC. A direct relationship between poor prognosis and the concentration of serum AFP has been observed[4]. Apoptosis is one of the main mechanisms implicated in cell death, and its inactivation contributes to tumour progression and chemotherapy resistance[5]. The intrinsic pathway involves the mitochondrial release of cytochrome c and consequently procaspases activation. And this process is regulated by the Bcl-2 family of proteins[6]. The cascade of apo-ptosis signal transduction begins by the action of initiator caspases that are recruited and activated by autocatalytic processing. Among these caspases, caspase 8 and 9 are the main initiators of programmed cell death; caspase 8 is stimulated in response to extrinsic death ligands, while caspase 9 is necessary for the activation of executor caspase3[7]. Targeting both the intrinsic and

extrinsic pathways reduces the growth of different tumour types, and a large number of studies demonstrate that different drugs alone or in combination enhance apop-tosis in cancerous cells, including HCC[8]. A large number of studies have demonstrated that Melatonin has important oncostatic propert-ies. It inhibits cell proliferation in several cancer cell lines including human B-lymphoma cells, human myeloid leukemia cells and human neuroblastoma cancer cells[9]. Melatonin decr-eased the growth rates of tumors in vivo both in transplantable animal model and the animal model induced by the administration of carci-nogens[10]. This study aimed to investigate the effects of melatonin on apoptosis and AFP mRNA expression in HepG2 cell line by revers transcription polymerase chain reaction (RT-PCR) in order to develop a new adjuvant therapy for HCC.

Materials and methods

Melatonin (M.W 232.28 g/mol) was purcha-sed from Sigma chemicals (Sigma Chemical Co., St. Louis, MO, USA) Melatonin was provided as 5 mg powder. The powder was dissolved in ethanol 95% forming 1 ml stock solution then it was diluted as 50 mol/L solution.

Cell culture

Hepatic cancer cell line (HepG-2) was kindly supplied from tissue culture department VACSERA - Egypt. The cells was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. When cells became >80% confluent, cells were seeded into 96-well plates for 24 h in DMEM culture medium before they were treated with 75mg melatonin for 48 h before RT- PCR assay.

Assessment of casp 8 and AFP gene exp-ression by real-time quantitative reverse transcription polymerase chain reaction

Total RNA was isolated using the RNeasy extraction kit (Qiagen- Germany) according to the manufacturer's protocol. The RNA con-centration was measured using nanodrop (Biowave II Germany).The RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, USA). Quantitative PCR was performed using the Ready Mix PCR Reaction Mix kit (iScriptTM One-Step RT-PCR Kit with SYBR® Green (Bio-Rad, USA). Thermal cycling conditions were: 10 min at 50°C, 5 min at 95°C then 40 cycles 10 sec at 95°C 30 sec at 55°C, 1 min at 55°C using Rotorgene real time PCR system and the related software for analysis and interpretation (Qiagen- S.Korea). Data were analyzed using the comparative Ct ($2^{-\Delta\Delta ct}$) method[11,12]. The PCR primers sequen-ces were as follows: * Casp8 F, 5'-CATCCA GTCACTTTGCCAGA -3', * Casp8 R, 5'-GC ATCTGTTTCCCCATGTTT-3', * AFP F 5′ GTCCTTTCTTCCTCCTGGAGAT-3′,* AFP R 5′-CTGTCACTGCT- GATTTCTCTGG-3′, * β -actin was used as a reference gene for internal control, * β-actin F 5'-GTGACATCC ACACCCAGAGG-3, * β-actin'R 5'-ACAG GATGTCAAAACTGCCC-3'.

Statistical analysis

Data are presented as the mean ± SD of three independent experiments. The data were tabu-lated and analyzed by SPSS (statistical package for the social science software) version 20.0. Statistical significance was analyzed using Student t test and differences were considered statistically significant when P value < 0.05.

Results

To determine the effect of melatonin on apop-tosis, we performed RT-PCR to demonstrate gene expression level of caspases 8, the intia-ting enzyme for extrinsic pathway of apoptosis with demonstration of the effect of melatonin on AFP gene expression by RT-PCR assay. All experiements are repeated 3 times for more accuracy of the results. Melatonin significantly increased caspase 8 expression. The relative expression was 2.51, 2.48 and 1.89 respectively as compared to control. While the expression of AFP was down regulated by melatonin admi-nistration. The relative expression of AFP was 0.051, 0.052 and 0.046 respectively as compared to control. The fold change for caspase 8, AFP is presented in table (1) and figure (1) as mean±SD.

Table (1) fold change of Casp 8 and AFP after melatonin and treatment.

Sample data	Results Fold Change	
Sample code	**Casp8**	**AFP**
Melatonin	**2.29±0.071**	**0.496±0.032**
Cont.HepG2	**1**	**1**

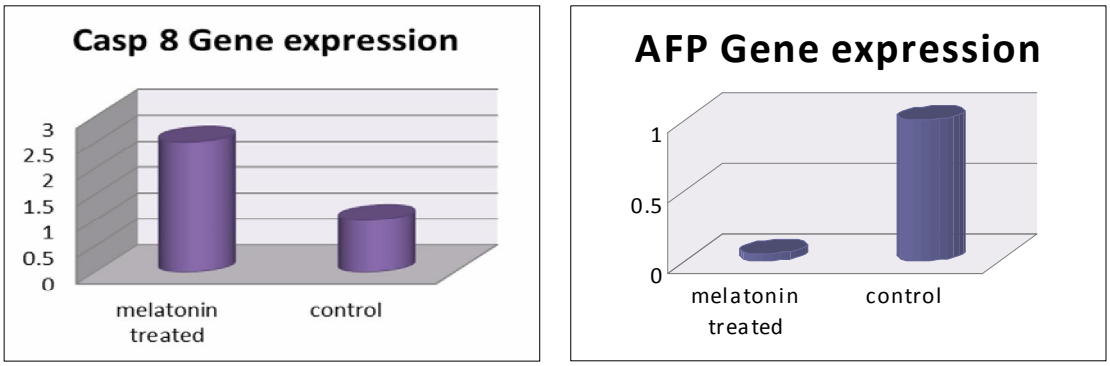

Fig (1) Expression of caspase 8, AFP in melatonin treated groups as compared to non-treated control group showed that it significantly increased caspase 8 expression ($P < 0.001$). Indicating that melatonin promoted apoptosis through initiation of extrinsic pathway, and significantly decreased AFP gene expression ($P < 0.001$).

Discussion

Surgical resection, local ablation or liver transplantation are possible treatments for a proportion of patients with early stages of HCC. However, these options will be restricted in advanced stages[13]. HCC has limited therapeutic options due to its frequent recurrence, metastasis[14], and easy relapsing post-operative[15]. Resisting cell apoptosis and sustaining cell growth are two major hallmarks of cancer. During the process of hepatocarcinogenesis, the balance between cell growth and apoptosis will be disrupted[16]. Therefore, selective apoptosis indu-ction in liver cancer cells has emerged as a promising possibility for developing selective cancer therapies[17]. Despite extensive research efforts in this area, there is still controversy con-cerning the most suitable therapeutic approach to target HCC[18]. Drugs used for treatment of HCC are cytotoxic, and impose a high risk of side effects to the patients. Moreover, it has been recognized that HCC cells are chemo resistant to conventional chemotherapeutic drugs[17]. When sorafenib was firstly tried as a therapy for advanced HCC, it gave a significant survival benefit but unresolved issues are still present Sorafenib is the only presently approved systemic treatment which showed statistically respectable advance in survival and slowed down tumor progression[19]. Recently, there is an increased interest in the potential use of melatonin in clinic[18]. Melatonin is a secretory product of the pineal gland and gastrointestinal tract during night and daytime, respectively[20]. Melatonin is responsible for a variety of physiological functions including antioxidant. Antiapoptotic[21]. and pro-apoptotic activities[16]. So, in this study we aimed to evaluate the effect of melatonin on casp8 mRNA expression and AFP mRNA expression in Hep G2 HCC cell line. According to our study, melatonin decreased AFP expression and stimulates apoptosis via stimulation of casp 8 expression in HCC. Melatonin can be considered a promising adjuvant for chemotherapy in HCC. Previously Shibo et al., 2017. showed that melatonin synergistically enhanced sorafenib-induced apoptosis through induction of caspase-3[22] expression. Based on these data, it is highly suggestive that the combination treatment with both melatonin and sorafenib together may offer better therapeutic potential for patients with HCC. Also in 2008 and in agreement to our results, Rendedo et al.[23] showed that melatonin treatment in HepG2 cell line significantly increased casp 8 activity control versus control cell lines. Up till the date no studies known demonstrate the effect of melatonin on AFP gene expression especially at HCC to compare our results with them. It has been reported in clinical trials that supplemental melatonin could abate chemotherapy-related side effects and improve patients' survival by combating several mali-gnant tumors. Melatonin induces apoptosis, promotes cell

cycle arrest, and suppresses angiogenesis and metastasis[16]. Without being toxic to normal cells[24]. Our study introduces melatonin as inhibitor for AFP expression and apoptosis inducer via stimulation of casp 8 expression in HCC. So, Melatonin could be considered a promising adjuvant for chemot-herapy in HCC.

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