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Melatonin Mitigates the Progression of Chemically Induced Hepatocellular Carcinoma in Rats via Targeting Wnt/B-Catenin Pathway, and Small Noncoding miR-let-7b

Nesma Mohammed Bahaa Eldeen[1], Moataz Maher Kamel[1], Abbas Mohamed[1], Samaa Samir Kamar[3,4], Laila Rashed[1], Asmaa Mohammed ShamsEldeen*[2]

Abstract

Background: Melatonin, the controlling hormone of the sleep–wake cycle, has acquired attention due to its role in immunomodulation, anti-inflammation, as well as its proapoptotic effects. Wnt/β-catenin signaling can modulate cancer progression by promoting cell division and migration, while miR-let-7b may inhibit cell growth, migration, and invasion by affecting the function of adaptive immune cells. This work was designed to detect the effect of using melatonin as an immunomodulating therapeutic approach to control the progression of chemically induced hepatocellular carcinoma (HCC).

Methods: Thirty male rats were equally divided into control, HCC, and melatonin-HCC groups. Animals in the HCC and melatonin-HCC groups were injected with diethylnitrosamine (intraperitoneal single dose) followed by repeated carbon-tetrachloride subcutaneous injection once weekly for six weeks. Melatonin was given from the first week of the study and continued during the process of HCC induction.

Results: In the HCC group, the levels of tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), and Wnt/β-catenin expression significantly increased, while there was a downregulation of microRNA Let7b. Melatonin administration reversed these changes, along with an increase in hepatic content of interleukin-2 (IL-2) and caspase-3.

Conclusions: Melatonin exerted hepatic immunomodulating changes, in addition to proapoptotic and antiangiogenic effects, illustrated by increased IL-2, caspase-3, and decreased VEGF levels, respectively. Moreover, the use of melatonin during hepatocarcinogenesis positively modulated the disrupted expression of microRNA let7b and Wnt/β-catenin significantly.

Keywords: Hepatocellular carcinoma, Melatonin, IL-2, miRNA Let7b, Wnt pathway, β-catenin.

Introduction

Hepatocellular carcinoma (HCC) stands as a highly prevalent primary liver malignancy and a leading cause of cancer-related mortality worldwide. Despite multiple available treatment modalities, only liver transplantation or surgical resection is curative, necessitating exploration into additional therapeutic approaches, including molecular and immune-targeted therapies (1). Wnt protein signaling pathways encompass both canonical "β-catenin-dependent" and non-canonical "β-catenin-independent" pathways (2). These pathways play a crucial role in cellular functions such as proliferation, differentiation, migration, genetic stability, and apoptosis (3). The actions of Wnt hinge on its Frizzled (Frz)

1: Department of Biochemistry, Faculty of Medicine, Cairo University, Cairo, Egypt.
2: Department of Physiology, Faculty of Medicine, Cairo University, Cairo, Egypt.
3: Histology Department, Faculty of Medicine, Cairo University.
4: Histology Department, Armed Forces College of Medicine, Cairo, Egypt.
***Corresponding author: Asmaa Mohammed ShamsEldeen; Tel: +2 001112694267; E-mail: Asmaa.abdulwahab@kasralainy.edu.eg.**
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receptors' binding ability. The canonical Wnt pathway induces cytoplasmic accumulation of β-catenin, subsequently translocating into the nucleus to regulate the transcription of Wnt target genes (4), including c-Myc and Cdkn1a (5).

Alterations in Wnt/β-catenin signaling have been implicated in the development of liver diseases, particularly HCC (6). Additionally, changes in β-catenin signaling are associated with hepatic stellate cell activation, contributing to liver fibrosis pathophysiology (7). Enhanced Wnt activation is correlated with "immune evasion" and impaired immune cell recruitment to the tumor area (3).

C-Myc, a downstream regulator of Wnt/β-catenin, acts as an oncogene stimulating cell proliferation. C-Myc collaborates with nuclear factor kappa B (NF-kB) to promote carcinogenesis (8). Therefore, further exploration of the modulatory effects of Wnt/β-catenin on HCC is increasingly attractive.

Small noncoding RNAs, particularly let7-b, have been proposed as valuable for malignancy diagnosis and prognosis prediction (9). In HCC tissues, let-7b has been observed to be down-regulated, associated with the over-expression of key oncogenic factors like cyclin D, RAS, and c-Myc in human cancer colon cell lines (11).

Melatonin, primarily produced by the pineal gland and human lymphocytes, can regulate human monocyte activity, promoting cytotoxicity and the secretion of interleukin-1 and 6 (IL-1 and IL-6). It also induces reactive oxygen species (ROS) production in cancer cells, leading to apoptosis. Melatonin is linked to the direct activity of natural killer (NK) cells, augmenting NK immunosurveillance and enhancing cytokine production, including IL-2 and interferon γ (IFN γ) (12-15).

Hence, there is a particular interest in determining the precise target of melatonin monotherapy in regulating chemically induced HCC, investigating the immunomodulatory effect of melatonin, and studying the molecular changes in let-7b associated with Wnt/β-catenin signaling in this model.

Materials and Methods

Experimental work & study groups

The experimental work was conducted in the Animal House of the Faculty of Medicine, Cairo University, and the molecular studies were carried out in the Physiology and Biochemistry Departments. This study is a case-control experimental study, and all experimental work and procedures received approval from the Cairo University Institutional Animal Care and Use Committee (CU-IACUC) Review Board (approval number: CU/III/F/37/21).

Thirty male adult Wistar albino rats, aged 10-12 weeks and weighing 200 to 250 grams, were included in the current study. After acclimatization to ordinary environmental living conditions, the rats had free access to food and water throughout the experimental procedure and were randomly classified into three groups.

Group I; Control (n=10): This group represents the negative control group (normal healthy rats). Animals in this group were given a single intraperitoneal injection of 0.1 ml of isotonic saline, followed by a weekly subcutaneous injection of 3ml/kg of isotonic saline for 6 weeks.

Group II; HCC group (n=10): Rats in which experimental HCC was induced (pathological control) (16).

Group III; Melatonin-HCC group (n=10): These are HCC rats that were subjected to melatonin administration. Melatonin was prepared three times per week by dissolving 16 mg of the powder in 250μL of ethanol. Then, this solution was diluted with drinking water to get a final concentration of 20 mg/L for 6 weeks. Melatonin was given from the first week of the study and throughout the process of HCC induction.

Induction of Hepatocarcinogenesis

The 20 rats in groups II and III underwent hepatocellular carcinoma (HCC) induction by receiving Diethylnitrosamine (DENA) and

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carbon-tetrachloride (CCl4). DENA, an initiator of DNA damage, was administered as a single intraperitoneal injection at a dose of 200 mg/kg body weight, followed by weekly subcutaneous injections of CCl4 at a dose of 3 mL/kg body weight for 6 weeks (16).

Sample Collection

After 6 weeks, just before scarification, blood samples were collected from the retro-bulbar plexus, centrifuged to separate plasma, and stored at a temperature ≤ -20°C for subsequent assessment of alanine transaminase (ALT), aspartate aminotransferase (AST), and alpha-fetoprotein (AFP) levels. At the planned time, animals were sacrificed by cervical dislocation. Liver tissues were dissected and divided into two samples for biochemical assessment of TNF-α, IL-2, caspase-3, and vascular endothelial growth factor (VEGF) levels using rat ELISA kits. Additionally, Wnt, β-catenin, NF-kB, and miRNA Let7b gene expression levels were assessed using real-time PCR. Liver specimens were processed as paraffin blocks, and deparaffinized sections were used for histopathological evaluation with Hematoxylin and Eosin (H&E) stain.

Chemicals

DENA (carcinogenesis initiator) was obtained from Sigma-Aldrich Egypt (Cat No. C1900) in solution form and diluted with castor oil at a 1:1 ratio. CCl4 (propagator of DNA damage) was purchased from Sigma-Aldrich Egypt (Cat No. C1900) in solution form. Melatonin, purchased as a powder (Product Number: M5250), was obtained from Sigma, USA.

Estimation of ALT, AST, and AFP using Rat ELISA Kits

Serum samples were utilized for estimating ALT using Rat ALT ELISA Kit (Cat No. ab234579, Abcam; Cambridge, United Kingdom), AST using Rat AST ELISA Kit (Cat No. ab263883, Abcam; Cambridge, United Kingdom), and AFP using Rat AFP ELISA Kit (Cat No. MBS267612, MyBioSource; San Diego, United States). All steps were performed following the manufacturer's instructions.

Determination of Serum and Hepatic Levels of TNF-α, IL-2, Caspase 3, and VEGF Using Rat ELISA Kits

After liver tissue dissection, 10 mg of tissue was added to 100 µL of phosphate buffer saline (PBS). Homogenization was performed, and the supernatant was collected for estimating TNF-α, IL-2, caspase 3, and VEGF levels using respective ELISA kits.

Estimation of the Expression Level of Wnt, β-catenin, NF-kB, and miRNA Let7b in Liver Tissue by Quantitative Real-Time PCR

Total RNA extraction, including small non-coding RNA, was performed using miRNeasy Mini kit (50) (Qiagen, Germany). RNA samples were quantified, and purity was assessed using the NanoDrop® (ND)-1000 spectrophotometer. Reverse transcription and qPCR were carried out using TransScript® Green One-Step qRT-PCR SuperMix (Beijing, China). The assay was conducted with primers normalized to the internal β-actin gene, serving as the housekeeping gene. The synthesized cDNA was detected using SYBR Green fluorescence. Table 1 shows the sequences were used.

For detection of miRNA, cDNA was reverse transcribed from micro total RNA samples using the (TransScript® miRNA First-Strand cDNA Synthesis SuperMix, Beijing, China) Cat no. AT351. Then, quantitative Real-time PCR (qPCR) was done using a SYBR Green PCR kit (PerfectStartTM Green qPCR SuperMiX, Beijing, China) Cat No., AQ601.The assay was done with the primer that was normalized to the snU6RNA that represented the housekeeping gene as shown in Table 1.

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Table 1. Primers sequence for Wnt, β-catenin, NFKβ, and miRNA-Let7b.

Gene symbol		Primer sequence from 5′- 3′	Gene bank accession number
Wnt 10a	Forward	TCCTGTTCTTCCTACTGCTGCT	XM_032901368.1
	Reverse	ACGCACACACACCTCCATC	
β-catenin	Forward	ACAGCACCTTCAGCACTCT	XM_054595383.1
	Reverse	AAGTTCTTGGCTATTACGACA	
NF-kB	Forward	GTGCAGAAAGAAGACATTGAGGTG	XM_032891140.1
	Reverse	AGGCTAGGGTCAGCGTATGG	
β-actin	Forward	CATGTGCAAGGCCGGCTTCG	XM_055114891.1
	Reverse	GTAGCAGGAGAAGTTGTTGG	
miRNA-Let 7b	Forward	UGAGGUAGUAGGUUGUGUGGUU	XR_009280412.1
	Reverse	CCACACAACCUACUACCUCATT	
snU6RNA	Forward	ATACAGAGAAGATTAGCATGGC	XR_006594310.1
	Reverse	GTCCAGTTTTTTTTTTTTTTTCGAC	

For detection of miRNA, cDNA was reverse transcribed from micro total RNA samples using the (TransScript® miRNA First-Strand cDNA Synthesis SuperMix, Beijing, China) Cat no. AT351. Then, quantitative Real-time PCR (qPCR) was done using a SYBR Green PCR kit (PerfectStartTM Green qPCR SuperMiX, Beijing, China) Cat No., AQ601.The assay was done with the primer that was normalized to the snU6RNA that represented the housekeeping gene as shown in Table 1.

Correlation Study

A correlation study was conducted to explore the relationships between the expression levels of miRNA Let7b, Wnt, and β-catenin, as well as between miRNA Let7b and caspase-3 expression levels. The study involved the comprehensive dataset collected from all experimental groups. Additionally, another correlation study was performed to assess the associations between IL-2 levels and liver enzymes (ALT and AST).

Histological Examination

At the conclusion of the experiment, hepatic sections were dissected, fixed in 10% formalin for 48 hours, and subsequently processed as paraffin blocks. Serial sections, with a thickness of 5-7 μm, were cut for hematoxylin and eosin staining.

Statistical Methods

All results were coded and analyzed using the statistical package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). Data were presented as mean and standard deviation (SD). Group comparisons were performed using analysis of variance (ANOVA) with Bonferroni post hoc test (17). Correlations between quantitative parameters were assessed using the Pearson correlation coefficient (18). A significance level of $P< 0.05$ was considered statistically significant.

Results

Melatonin Treatment Improves Liver Functions and AFP Levels in Chemically Induced HCC in Rats

Figure 1 illustrates that liver enzymes (ALT and AST) and AFP significantly increased in the HCC group compared to the control group. However, melatonin treatment decreased the damaging effects of chemically induced HCC, evidenced by a significant decrease in ALT, AST, and AFP levels in the melatonin-treated group compared to the HCC group.

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The Protective Effect of Melatonin: Immunomodulation, Proapoptosis, and Antiangiogenesis

Figure 2 depicts significant decreases in IL-2 and caspase 3 levels in the HCC group compared to the control group, while TNF-α and VEGF levels increased significantly in the HCC group. Melatonin treatment led to a significant increase in IL-2 and caspase 3 levels and a significant decrease in TNF-α and VEGF levels compared to the HCC group.

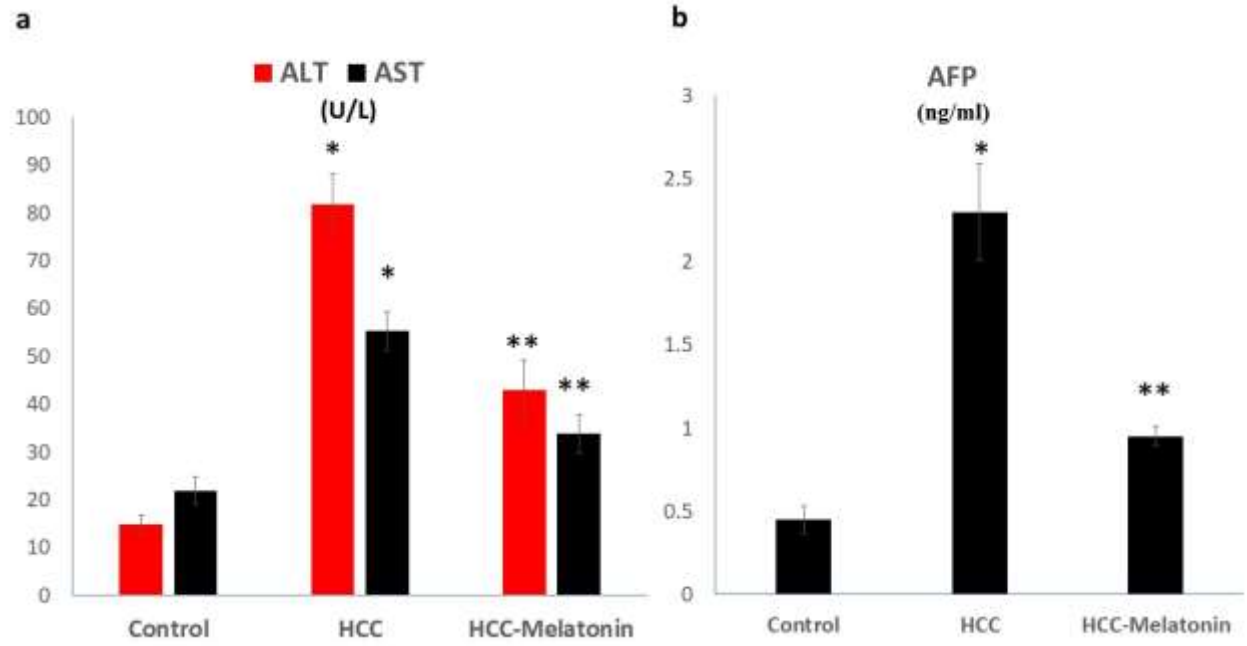

Fig. 1. The levels of serum ALT, AST (a), and the levels of AFP (b) in all studied groups. Data presented as mean and SD, *: Statistically significant compared to the control group (P< 0.05), **: Statistically significant compared to the HCC group (P< 0.05).

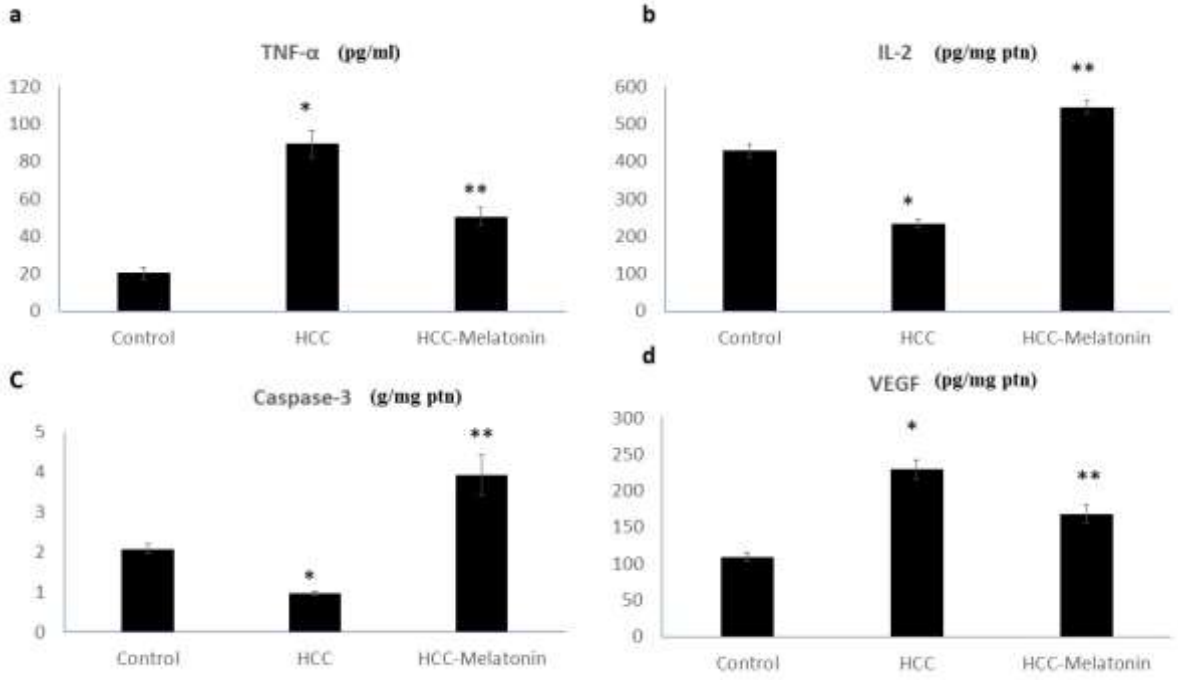

Fig. 2. Changes in TNF-α (a), IL-2 (b), Caspase-3 (c), and VEGF (d) in all studied groups. Data presented as mean and SD, *: Statistically significant compared to the control group (P< 0.05), **: Statistically significant compared to the HCC group (P< 0.05).

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Enhanced Expression of miRNA-Let7b Associated with Decreased Wnt/β-catenin and NF-kB

To explore melatonin's role in chemically induced HCC, expression levels of Wnt/β-catenin and NF-kB were investigated. Results revealed a significant reduction in their expression levels in the HCC group compared to the control group. Melatonin administration further decreased their expression levels significantly compared to the HCC group.

miRNA-Let7b Expression and its Prognostic Relevance

The data demonstrated a significant decrease in miRNA-Let7b expression in the HCC group compared to the control group. Melatonin intake significantly increased its expression level compared to the HCC group (Fig. 3), suggesting a potential role in HCC prognosis.

Correlation Results

Our findings unveiled a strong negative correlation between miRNA-Let7b and the expression levels of Wnt (R= -0.977) and β-Catenin (R= -0.966) (P< 0.001).

Additionally, a positive correlation was observed between miRNA-Let7b and the expression level of Caspase-3 (R= 0.520, P< 0.001) (Fig. 4). Another significant negative correlation was identified between IL-2 levels and liver enzymes (ALT: r= -0.674, P< 0.001; AST: r= -0.717, P< 0.001) (Fig. 5).

Histological Results

In rats with induced HCC, liver tissues exhibited solid sheets of atypical cells with pleomorphism, increased nuclear/cytoplasmic ratio, and necrosis. Some portal tracts displayed inflammatory cell infiltration and congested portal vessels. Conversely, the melatonin-treated group showed smaller focal lesions with fewer cellular alterations (Fig. 6).

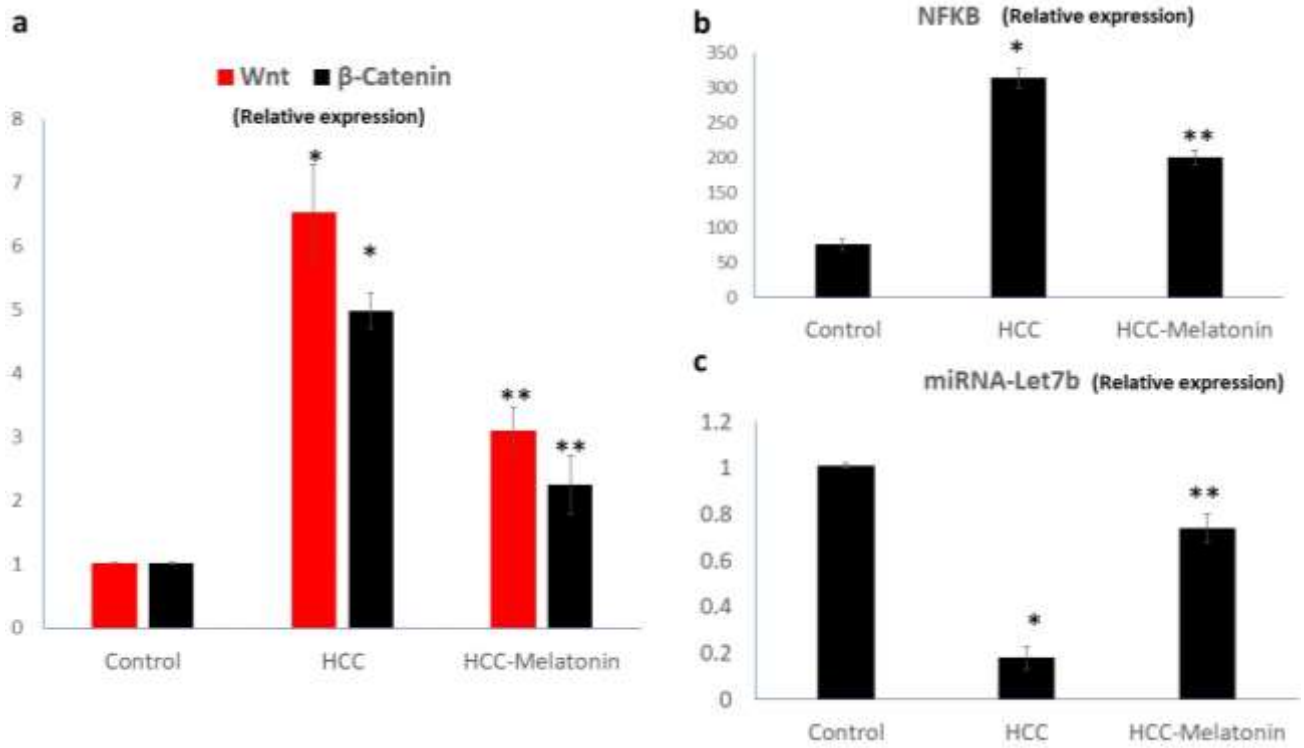

Fig. 3. The relative expression levels of Wnt/β-catenin (a), NFKB-β (b), and miRNA-Let7b (c) in all studied groups. Data presented as mean and SD, *: Statistically significant compared to the control group (P< 0.05), **: Statistically significant compared to the HCC group (P< 0.05).

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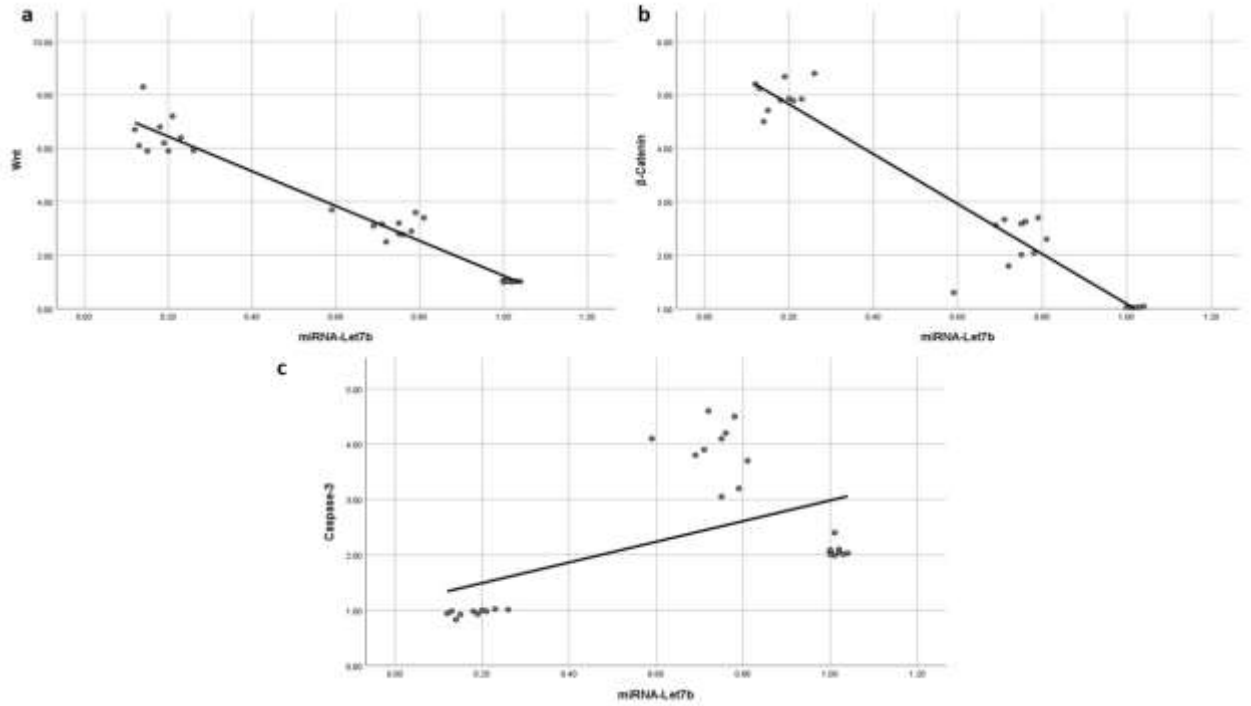

Fig. 4. A negative correlation study between miRNA-Let7b from one side and the expression levels of Wnt /β-Catenin on the other side (P< 0.001) (a, b). a positive correlation between miRNA-Let7b and the expression levels of Caspase-3 (P< 0.001) (c).

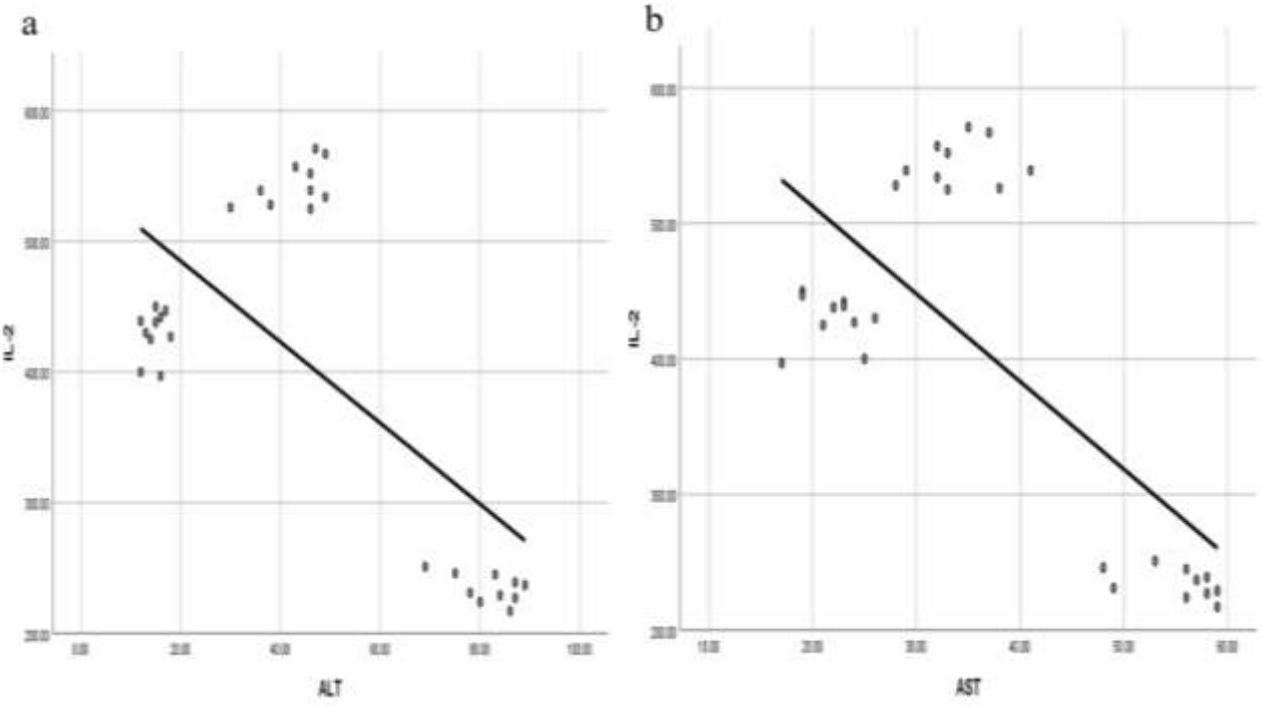

Fig. 5. A negative correlation was observed between the levels of IL-2 and the liver enzymes, ALT (R= -0.674, P < 0.001), and AST (R=-0.717, P< 0.001.

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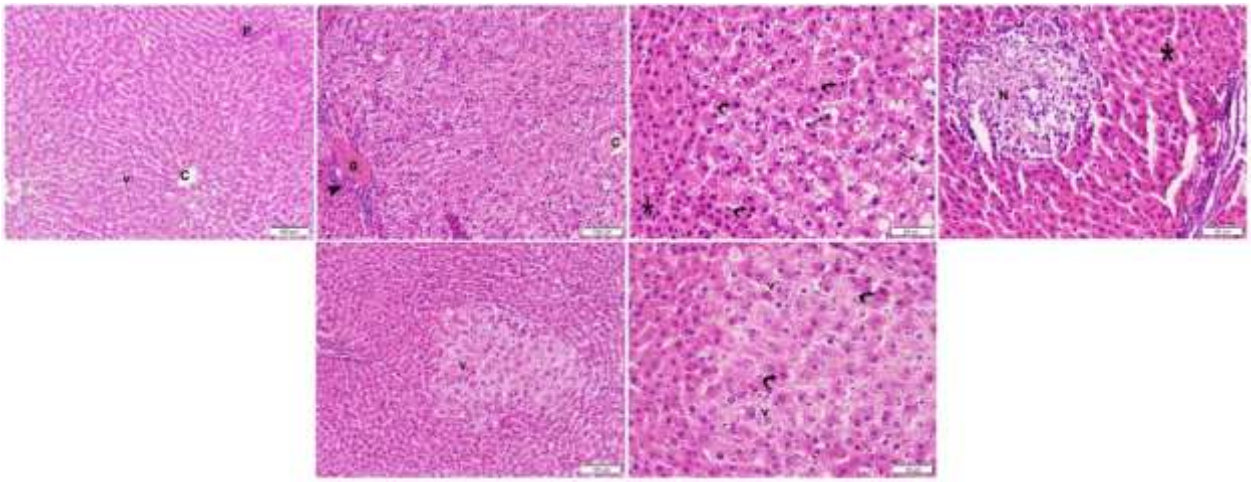

Fig. 6. Photomicrograph of H&E-stained liver sections showing melatonin alleviating induced hepatotoxicity. a) Control group: the normal architecture of liver parenchyma displaying, portal tract (P) and central vein (C) with radiating hepatic cords separated by blood sinusoids. Hepatocytes show vesicular nuclei (v) and acidophilic cytoplasm. b-d) HCC-induced group: large area of dysplastic cells with mild compression of the surrounding parenchyma (asterisk) displaying loss of normal architecture and mixed growth pattern of atypical cells. Some cells are enlarged with vacuolated cytoplasm and dense eccentric nuclei (arrow), others with dark minimal cytoplasm and pyknotic nuclei (curved arrow) showing high nuclear/cytoplasmic ratio. Besides, small area of necrosis (N), congested vessel (g), dilated central vein (C), and portal tract infiltrated by a mononuclear cell arrowhead) are noted. e-f) Induced Melatonin-HCC: small focal area of cellular alteration with disturbed architecture. Most cells show vesicular nuclei (v) while few with increased acidophilia and pyknotic nuclei (curved arrow).

Discussion

The main objective of this study was to examine the Wnt/β-catenin axis and miR-let-7b in a model of chemically induced HCC. We successfully induced a rat model of hepatocarcinogenesis, confirmed by the level of AFP and histopathological examination.

Exposure to DENA and CCL4 resulted in liver damage, proliferation of hepatic cells, and the development of both fibrosis and cirrhosis. All these changes, along with disorganized local immunity, oxidative stress (19), and disrupted vasculature, mimic the usual events observed during human HCC development (20). The current results reported the development of an HCC rat model confirmed by significantly elevated AFP levels in the HCC group compared to the control group, along with histopathological changes such as fibrosis with multiple foci of liver necrosis, inflammation, and dysplastic changes. Hepatic sections in the HCC group also exhibited irregularly arranged hepatocytes. Most of the hepatocytes showed clear vacuolated cytoplasm, while other cells were shrunken with dark pyknotic nuclei and occasional cell ballooning, indicating heterogeneity and different cell patterns.

Most HCC cases are associated with either liver fibrosis or cirrhosis together with an impaired anti-cancer immune response. Despite variable pathogenesis mechanisms, they all are involved in carcinogenic pathways (21). In agreement with previous work (22), AFP is the most used tumor marker for the diagnosis and follow-up of HCC. Also, TNF-α can be utilized as a biomarker that may help in the early diagnosis of HCC (23). The state of chronic inflammation is known to participate in the development of HCC and usually plays a crucial role in the initiation as well as the progression of liver tumors (24,15). In accordance with our results, Li and colleagues (25) reported higher TNF-α expression in tumor tissues compared with tumor-adjacent free tissues.

The current results showed a significantly elevated VEGF level in the HCC group compared with the control group, while it showed a significant decrease in the melatonin-treated group compared to the HCC group. Therefore, targeting VEGF became the most currently approved line of treatment for advanced HCC. The VEGF/VEGF receptor

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(VEGFR) signaling pathway has been validated as a therapeutic intervention for targeting HCC (26). Yang et al. (27) reported that melatonin was able to inhibit VEGF receptor-2 expression and delay VEGF signaling transduction.

Taniguchi and colleagues (28) found that the constitutive activation of NF-kB signaling and its related transcription factors are the hallmarks of cancer pathophysiology. NF-kB modulates many aspects of tumor biology and promotes the survival of tumor cells and their resistance to therapy by enhancing the expression of other cytokines, antiapoptotic factors, and adhesion molecules. Consequently, a therapeutic approach that may suppress NF-kB activity can disturb its anti-apoptotic functions and may be useful for preventing the progression of hepatic carcinogenesis (29). Many clinical studies have demonstrated that abnormal stimulation of Wnt/β-catenin signaling contributes to hepatocarcinogenesis (30,31).

Cellular inhibitors of apoptosis are produced by the expression of NF-κB target genes; they result in cell division and resistance to cancer therapy via suppressing caspase activity (29). In this context, the current data showed that caspase-3 protein levels significantly decreased in the HCC group compared to the control group.

In the present study, Let7b, NF-κB, and Wnt/β-catenin genes' expression was measured by real-time PCR. Let7b gene expression decreased significantly in the HCC group compared to the control group, whereas the gene expression levels of NF-κB and Wnt/β-catenin significantly increased.

Let-7, the important small noncoding RNA, is frequently downregulated in several types of cancer (32). Previous studies revealed that miR-let-7b is the key player in immune responses during carcinogenesis. It may inhibit cell growth, migration, invasion, and metastasis, subsequently inducing apoptosis and cell cycle arrest (33).

Additionally, Hui et al. (34) reported that let-7b expression levels showed a significant downregulation in HCC tissues compared with the surrounding normal tissues. Moreover, the authors concluded that a lower expression of Let-7b was associated with poor prognosis of HCC. On the contrary, over-expression of Let-7b suppressed the growth and progression of implanted HCC tumors in mice and enhanced innate immune activity. In this context, Cai and colleagues (32) reported that activation of the Wnt–β-catenin pathway suppresses mature let-7 miRNAs at the post-transcriptional level.

For hepatic tissues, in particular, IL-2 acts as a fundamental upstream regulator of liver homeostasis (35). Under physiological conditions, the expression of IL-2 in liver tissue is closely related to the regeneration and defensive functions against hepatic infection. However, during pathological conditions, IL-2 boosts antitumor mechanisms and acts to suppress tumor progression by promoting cancer cell death (36). In the last decades, the relevance of melatonin to human physiology and pathology has become evident. It is already well-established that melatonin is not only a hormone but also functions as a cell protector and an immune activator (37). Melatonin was also able to inhibit epithelial to mesenchymal transition, which is the fundamental process underlying cancer metastasis in MCF-7 cells because it induced the degradation of β-catenin (38).

In the current study, the results showed a remarkable decrease in TNF-α and VEGF in the melatonin-treated HCC group compared to the HCC group. In addition, a significant elevation in hepatic IL-2, caspase-3, and miRNA Let7 expression levels in the melatonin-treated HCC group compared to the HCC group. The results obtained from the current work indicate the contribution of melatonin in cytokine induction and cancer-mediated immune-therapy. In line with our findings, the administration of IL-2 was able to increase the number of intra-hepatic Treg-cells and ameliorate CCl_4-induced hepatic damage (35).

Currently, melatonin treatment was able to enhance miRNA let7i-3b expression, which potentially dampens the activity of the oncogenic downstream pathways and suppresses tumorigenesis in liver cells.

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Furthermore, melatonin was proved not to be cytotoxic, so it can help improve the patients' quality of life if utilized in the management of hepatic malignancies (9). According to Wang's results (9), overexpression of miRNA let-7b resulted in decreased β-catenin expression in liver tumor cells. Moreover, the inhibitor of Wnt/β-catenin signaling succeeded in blocking hepatic cancer cell proliferation, giving the same results that may be obtained secondary to let-7b overexpression. They also recommended that efficient modulation of let-7b may serve as a novel therapy for human liver cancer in the future.

In conclusion, melatonin, the old circadian rhythm-regulating hormone, could play a central role in hepatic cell protection against chemically induced HCC and exert immunomodulation by enhancing local hepatic production of IL-2, caspase-3, and miRNA Let7b. Melatonin successfully downregulated Wnt/β-catenin signaling and consequently modified the progression of hepatocarcinogenesis.

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Ethics section
The study was conducted by the Declaration of Helsinki and approved by the Institutional Animal Care and Use Committee (IACUC) Review Board (Etheical code: CU/III/F/37/21).

Conflicts of Interest
Nothing to be declared.

Acknowledgment
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