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ORIGINAL ARTICLE

Melatonin Ameliorates 5-Fluorouracil-Induced Cytotoxicity and Apoptosis in H9c2 Cell Line: Insights into Cytoprotection and Anti-Apoptotic Mechanisms

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ABSTRACT

Cardiotoxicity represents a significant adverse effect associated with 5-fluorouracil (5-FU), a widely used chemotherapeutic agent. Melatonin (MLT), a powerful antioxidant and agent that prevents apoptosis, has shown promise in mitigating various toxicities. This study evaluated the cardioprotective effect of MLT on 5-FU-induced cardiotoxicity (5-FU-IC) in the H9c2 cardiomyoblast cell line.

The cells were grown in DMEM + FBS and divided into four groups: control (untreated), 5-FU-treated (varying concentrations for 48 hours), MLT-treated (varying concentrations), and 5-FU plus MLT-treated (combined treatment for 48 hours). The cell viability was evaluated using the MTT assay, while apoptosis was analyzed through flow cytometry following Annexin V staining and caspase-3/7 (Cas-3/7) activity assays.

Treatment with 5-FU led to a significant decrease in the viability of H9c2 cells in a dose-dependent fashion, with an estimated IC50 value of 400 µM. Co-treatment with MLT at 100 and 200 µM significantly enhanced cell viability and reduced apoptosis induced by 5-FU, as demonstrated by flow Cytometry and reduced Cas-3/7 activity. These results emphasize the protective effects of MLT against 5-FU-IC, primarily through its anti-apoptotic mechanisms.

These findings underscore the importance of MLT to protect against 5-FU-IC through its anti-apoptotic properties. MLT shows promise as a cardioprotective agent in mitigating 5-FU-IC, providing perspectives on its potential therapeutic application in mitigating cardiac risks linked to chemotherapy.

Keywords: Melatonin, 5-Fluorouracil, Apoptosis, Cardiotoxicity, Chemotherapy, H9c2 cells.

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Introduction

Melatonin (MLT), a key modulator of circadian rhythms, is produced by the pineal gland and other tissues throughout the body (1). MLT (N-acetyl-5-methoxytryptamine) is synthesized from serotonin following N-acetylation and then O-methylation (2). A growing body of evidence underscores the therapeutic potential of MLT, emphasizing its robust anti-inflammatory and antioxidant actions (3), along with its role in reducing pyroptosis in adipose and endothelial tissues (4). Because of its highly lipophilic properties, MLT has the potential to easily penetrate cardiomyocyte cells and their nuclear membranes, exerting a cardioprotective effect in various pathophysiological conditions (5, 6).

Numerous studies have documented that MLT provides protective effects against heart dysfunction caused by ischemia and/or reperfusion (I/R) insults (7). It has also been demonstrated that MLT offers protection against cardiac I/R injury in both *in vitro* and *in vivo* models through its modulation of oxidative stress and apoptotic pathways (8). Moreover, it has been observed that MLT provides protective benefits against cardiac damage and apoptosis caused by long-term intermittent hypoxia in rats by modulating calcium homeostasis in the sarcoplasmic reticulum, as well as apoptosis and autophagy (7). Experimental evidence has shown that MLT can inhibit apoptosis in myocardial cells at the molecular level by increasing the pro-survival Bcl-2 protein levels and decreasing the activity of the apoptotic enzyme caspase-3 (9). Recently, a study on a mouse model of myocardial injury induced by 5-FU has investigated the effects of resveratrol. The results indicated that simultaneous treatment of H9c2 cells with 5-FU and resveratrol led to increased cell viability and modulation of apoptosis caused by ferroptosis under *in vitro* conditions (10).

Despite the availability of various chemotherapy drugs that have been shown to enhance the survival rates and quality of life for colon cancer patients, 5-FU remains the most commonly used medication in the clinical treatment of colorectal and metastatic cancers even after 30 years of research (11, 12). However, despite its effectiveness in cancer treatment, its clinical use is restricted due to significant side effects, including myelosuppression (13), cardiotoxicity (14), neurotoxicity (15), and gastrointestinal disturbances (16). 5-FU mainly induces its cytotoxic effects by activating the cellular mechanisms of autophagy and apoptosis, which in turn stimulates the activity of caspase enzymes (17). A study's findings indicate that 5-FU triggers mitochondrial-mediated apoptosis by upregulating caspase-8 and caspase-9 in cultured corneal epithelial cells (18), critical effectors in the intrinsic apoptotic pathway. This mechanism contributes to the cytotoxicity observed in both malignant and normal cells exposed to 5-FU.

In this study, H9c2 cardiomyocytes were utilized as a model for treatment with 5-FU to replicate the effects of cardiotoxicity observed *in vivo*. Apoptosis is a cellular process that removes damaged cells in multi-cellular organisms. The programmed death of myocardial cells, commonly known as "myocardial apoptosis," frequently occurs in cases of myocardial infarction and various heart diseases (19). Caspase-3 and caspase-7 are critical effector proteases in the apoptotic pathway, executing the terminal stages of cell death (20). Caspase-3 is regarded as the primary executioner caspase, cleaving various substrates, including poly-ADP ribose polymerase, leading to DNA fragmentation and cytoskeletal breakdown (21).

Caspase-7, while functionally similar to caspase-3, complements its activity and is particularly efficient in cleaving substrates involved in cellular repair and maintenance (22). Both enzymes are activated downstream of initiator caspases and serve as pivotal regulators of the morphological and biochemical alterations associated with apoptosis. However, the mechanism of 5-FU induced cardiotoxicity requires further investigation. Therefore, given the importance of this topic, this study was designed and conducted to investigate the effects of 5-FU on the cytotoxicity of H9c2 cardiomyoblast cells and to examine the protective effects of various concentrations of melatonin on these cells under *in vitro* conditions. Consequently, *in vitro* studies are essential for dissecting the complex mechanisms underlying the actions of cytotoxic or protective agents such as 5-FU and MLT, contributing to a deeper understanding of their therapeutic potential and limitations in cancer therapy.

Methods

Chemicals and Reagents

MLT was obtained from Merck Co. (Germany). Fetal bovine serum (FBS) and DMEM were purchased

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from BioIdea (Tehran, Iran). MTT reagent was obtained from WI, USA. 5-FU, Annexin V and propidium iodide were purchased from Sigma Aldrich Co., USA.

Cell Culture Protocol and Viability Evaluation Method

The rat myocardial cell lines, H9c2, were purchased from the Pasteur Institute of Iran (Tehran, Iran). In brief, the cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Beyotime Institute of Biotechnology, China). The cells were kept in a 5% CO2 incubator at 37°C and split every 2–3 days using a 0.15% trypsin solution. The cells were divided into four groups: the control group was treated with DMEM FBS, 5-FU group was treated with varying concentrations of 5-FU for 48 hours. MLT groups were pretreated with varying concentrations of MLT for 48 h, and 5-FU plus MLT-treated groups (combined treatment for 48 hours).

Cells in the 5-FU cardiac damage group were screened at eight concentrations of 5-FU (0, 50, 75, 100, 150, 200, 300, and 400 µM) for 48 hours. The control group cells were cultured under similar conditions but without 5-FU.

To investigate the role of melatonin, H9c2 cardiomyocytes in MLT groups were treated with MLT at concentrations of 0, 12.5, 25, 50, 100, 200, and 400 µM, utilizing a two-fold dilution series. Melatonin was dissolved in DMSO, and the final concentration of DMSO in all treatment and control wells was adjusted to 0.1% (v/v) to avoid cytotoxic effects while ensuring equal vehicle exposure across groups.

To evaluate the impact of MLT on the cytotoxic effects caused by 5-FU, the cells were categorized into ten distinct groups: a control group pre-treated with DMEM and FBS, groups treated separately with 300 and 400 µM concentrations of 5-FU, three cell groups that were simultaneously treated with 300 µM 5-FU and concentrations of 50, 100, and 200 µM MLT, and three cell groups that were simultaneously treated with 400 µM 5-FU and concentrations of 50, 100, and 200 µM MLT. 5-FU and MLT were dissolved in DMEM supplemented with 10% (v/v) FBS to obtain final concentrations. Cells in the 5-FU group received 300 and 400 µM for 48 hours. Cells in the MLT + 5-FU groups were treated with various concentrations of MLT and 5-FU for 48 h. The pharmaceutical compounds were added to the cells at room temperature, ranging from 18 to 25 °C. To estimate the half-maximal inhibitory concentration (IC50) of 5-FU, cells were seeded in flat-bottomed 96-well plates at a density of 7×10^3 cells per well and cultured overnight in complete growth medium. The medium was subsequently removed and substituted with fresh DMEM containing 5-FU and/or MLT, as previously described.

Forty-eight hours after cell treatments, the cells were collected and examined using the MTT assay to assess cell viability and cytotoxicity. This colorimetric method measures cell viability by reducing the yellow MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) into purple formazan crystals. Following treatment, 50 µL of MTT reagent (Roche Diagnostics (Mannheim, Germany) was added to each well, and the plates were incubated in the dark at 37°C for an additional 2 hours.

After incubation, 150 µL of DMSO was added to each well to dissolve the formazan crystals formed during the experiment. The spectrophotometric absorbance of the samples was measured at a wavelength of 570 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (BioTek, USA). The absorbance value at this wavelength is directly proportional to the number of viable cells in each well. The proportion of cell viability was calculated using the formula below: percentage of cell viability = [100 × (sample absorbance) / (control absorbance)] (23).

Quantifying Apoptotic Changes Using Flow Cytometry and Annexin V Labeling

This study utilized a flow cytometer (Becton Dickinson, USA) equipped with spatially separated lasers, fluorescence detectors, and light scatter detectors. Apoptosis in H9c2 cell suspensions was analyzed using fluorescent markers, including fluorescein isothiocyanate (FITC)-conjugated Annexin V (Anx V) and propidium iodide (PI; Sigma Aldrich Co., USA). Cells were plated in 6-well dishes at a density of 1×10^6 cells per well using suitable growth media and left to incubate overnight to establish a monolayer. Then, since the IC50 of 5-FU in this study was determined to be 400 µM, a concentration that inhibits cell growth by 50%, in the next phase of the study, cells were incubated either with 400 µM 5-FU alone or with 5-FU in combination with MLT for 48

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hours. The cells were ultimately harvested using a 0.5% trypsin solution, then washed twice with cold FBS. The samples were centrifuged in a cold centrifuge, and the trypsin was carefully aspirated. Subsequently, 100 µL of sample, 5 µL of PI, and 5 µL of Anx V-FITC were added to the tubes.

The samples were kept in the dark at room temperature for 15 minutes. The samples were examined on a flow cytometer with 488 nm excitation for PI and 633 nm excitation for Anx V-apoptotic cells conjugate. Data were carefully calibrated by adjusting the logarithmic amplification scale and compensating between the green and orange channels using Flowjo V10 software. The cell fractions were categorized as follows: 1. Quadrant 1 (Q1): necrotic cells were negative for Anx V but positive for PI; 1. Quadrant 2 (Q2): late apoptotic cells were positive for both Anx V and PI; Quadrant 3 (Q3): early apoptotic cells were positive for Anx V but negative for PI and Quadrant 4 (Q4): viable cells were negative for both Anx V and PI; (24, 25).

Measurement of Caspase-3/-7 Activity via Fluorescent Assay

Caspase-3/7 (Cas-3/7) activity was assessed using fluorescent assay kits designed to detect enzymatic activity in cell lysates. The assay employed a fluorogenic substrate specifically cleaved by activated Cas-3/7. A reaction solution was prepared on ice, consisting of 50 mM HEPES (pH 7.5), 10% sucrose, 0.5% Triton X-100, and 10 mM dithiothreitol. To initiate the reaction, 25 µL of each sample lysate was mixed with 75 µL of the substrate solution. The fluorescence intensity, corresponding to Cas-3/7 activity, was measured at 405 nm using a spectrophotometer over 30 minutes. Two closely related caspases, Cas-3 and Cas-7, share similar substrate specificity (26). Since it was not possible to measure the activity of these enzymes separately, further analysis was conducted on their combined activity. To provide a cohesive visual overview of the research process and its findings, the graphical abstract of this study is presented in Figure 1.

Graphical Abstract

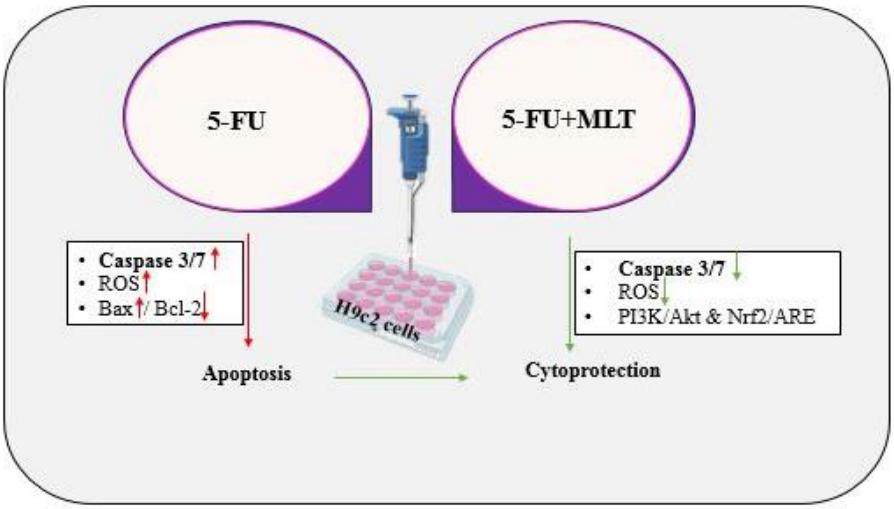

Figure 1. 5-Fluorouracil (5-FU) induces apoptosis in H9c2 cardiomyoblasts primarily by activating the executioner caspases 3/7 and associated pro-apoptotic signaling pathways. In contrast, co-treatment with melatonin (MLT) counteracts this effect by modulating key survival signaling pathways. MLT activates the PI3K/Akt and Nrf2/ARE axes, which leads to the suppression of caspase 3/7 activity and a reduction in oxidative stress, ultimately resulting in decreased apoptosis and enhanced cell protection.

Statistical analysis

The data were assessed using GraphPad Prism software, version 8. The data were analyzed statistically using the t-test when the distribution was normal and the Mann–Whitney Test when the distribution was not normal. In every instance, the significance level was set at $\alpha = 0.05$ or lower. All experiments were performed in three independent biological replicates, each with three technical replicates, to ensure statistical validity and reliability of the data.

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Results

Determination of 5-FU Cytotoxicity in H9c2 Cells

Our model cell line was treated with two drugs with distinct mechanisms of action: 5-FU, which induces cellular damage, and MLT, to evaluate its protective effects against 5-FU-induced cytotoxicity. H9c2 cells were exposed to a range of 5-FU concentrations (0, 50, 75, 100, 150, 200, 300, and 400 μM) for 48 hours. The conventional MTT cytotoxicity test demonstrated that the viability of H9c2 cells was affected by 5-FU treatment. After 48 hours of incubation, no significant changes in cell viability were observed at concentrations of 50 and 75 μM. However, a substantial decrease in viability was noted at a concentration of 200 μM ($P < 0.001$), which resulted in cell viability reaching only 75% of the control group treated with 0 μM 5-FU (Figure 2A). The results indicate that the 50% cytotoxic concentration, or half-maximal inhibitory concentration (IC50), is defined as the concentration of 5-FU that reduces cell viability by 50% compared to the untreated control. The IC50 value for 5-FU in H9c2 cells after 48 hours of incubation was determined to be 400 ± 1.15 μM. The findings showed a dose-dependent decline in cell viability, with notable reductions detected at higher concentrations of 300 μM ($P < 0.01$) and 400 μM ($P < 0.001$) compared to the untreated control group. Non-linear regression analysis determined an IC50 value of 400 μM for 5-FU.

Additionally, to evaluate the effect of MLT on cell viability in H9c2 cardiomyoblast cells, we conducted an MTT assay following a standard experimental protocol and determined the IC50 after 48 hours of incubation. Cells were seeded at a density of 7×10^3 cells per well in a 96-well plate and exposed to varying concentrations of MLT (0, 12.5, 25, 50, 75, 100, 200, and 400 μM) in serum-free medium. However, as illustrated in the dose-response curve in (Figure 2B), Viability estimates were initially recorded at levels exceeding 100% at the beginning of the experimentation. Cell viability values decreased in a concentration-dependent manner. In contrast, doses of 100, 200, and 400 μM of MLT significantly decreased cell viability percentages compared to the control group ($P < 0.001$).

In the next phase of the study, we investigated the effects of combined treatment with MLT and 5-FU on cell viability percentages. By MTT assay, we examined the impact of MLT concentrations of 50, 100, and 200 μM alongside 5-FU concentrations of 300 and 400 μM on viability after 48 hours in culture. The viability of H9c2 cells exposed to 300 μM 5-FU was 60.5 ± 1.69%. In contrast, for H9c2 cells co-treated with 300 μM 5-FU and MLT at concentrations of 50, 100, and 200 μM, the viability percentages were 65.1 ± 1.6%, 72.3 ± 1.25%, and 88.1 ± 2.5%, respectively. As displayed in (Figure 3), a substantial increase in viability was detected for the groups treated with 300 μM 5-FU combined with MLT at concentrations of 100 μM and 200 μM ($p < 0.001$ and $p < 0.0001$, respectively) compared to the group treated with 5-FU alone. Our results indicated no significant differences in viability percentages for cells treated with 300 μM 5-FU and 50 μM MLT when compared to the group treated with only 5-FU.

On the other hand, when the effects of various doses of melatonin on the cytotoxicity induced by 400 μM 5-FU were examined, as shown in (Figure 3), it was observed that, unlike the combination treatment of 50 μM MLT with 5-FU, which did not considerably affect cell viability, the doses of 100 and 200 μM MLT significantly reduced the cytotoxicity caused by 400 μM 5-FU when compared to the cells treated with only 5-FU ($p < 0.001$ and $p < 0.0001$, respectively).

Flow Cytometry Findings on the Effects of MLT and 5-FU on Cell Viability

We aimed to demonstrate that the flow cytometry (FCM) study of apoptotic and necrotic cells supports the results obtained from the MTT assay conducted on H9c2 cell cultures (Figure 4). In the untreated control group, the percentage of live cells was 96.7% (±1.5), with early apoptotic cells at 0.88% (±0.1), late apoptotic cells at 1.13% (±0.2), and necrotic cells at 1.29% (±0.3). At the same time, as illustrated in (Figure 4), treatment with 400 μM of 5-FU resulted in a marked decrease in viable cells to 48.96% (±3.2, $p<0.001$ vs. control) and markedly increased late apoptotic cells to 48.1% (±2.8, $p<0.001$) and necrotic cells to 2.85% (±0.5, $p<0.01$).

The analysis of FCM findings from samples stained with Anx V/PI indicates that in the groups of cells incubated with 5-FU + MLT for 48 hours resulted in a dose-dependent protective effect. At 50 μM melatonin, live cells increased to 65% (±2.5, $p<0.05$ vs. 5-FU alone), while early apoptotic cells rose to 8% (±1.2), late apoptotic cells decreased to 17% (±1.6, $p<0.01$), and necrotic cells increased to 10% (±1.8).

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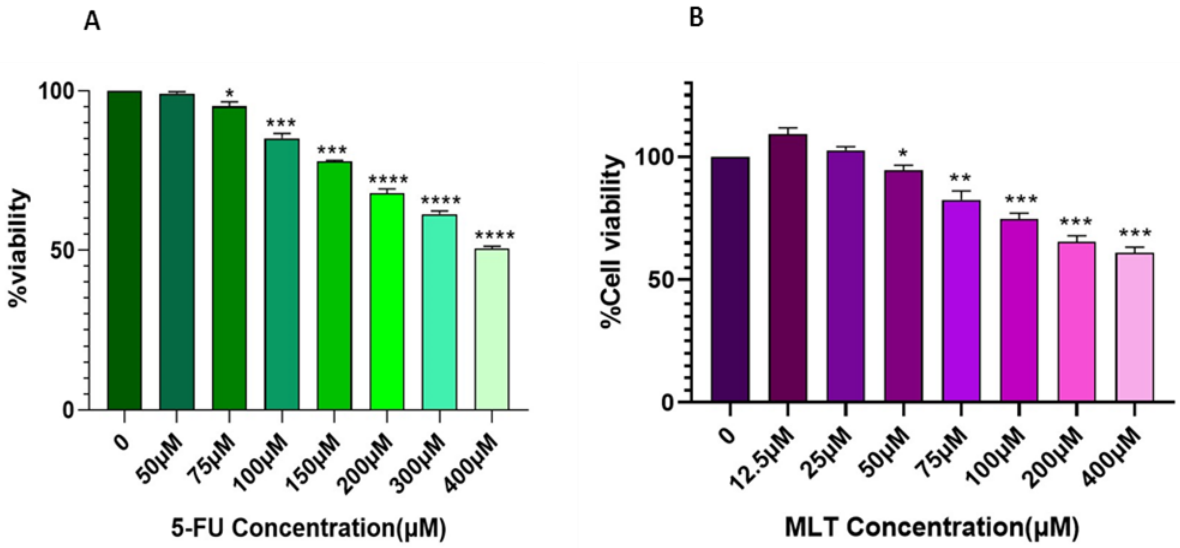

Figure 2. Cytotoxicity of 5-FU in H9c2 cells. Cells were exposed to different concentrations of 5-Fluorouracil (5-FU) ranging from 0 µM to 400 µM for an incubation period of 48 h. At the end of the exposure, MTT cell viability was determined as explained in the materials and methods (A). The MTT assay results obtained from H9c2 treated cells with different concentrations of Melatonin (MLT) ranging from 0 µM to 400 µM for an incubation period of 48 h. The data represented are the mean ± SD of three identical experiments made in three replicates. The dashed lines represent a 50% decrease in the MTT assay results (B).

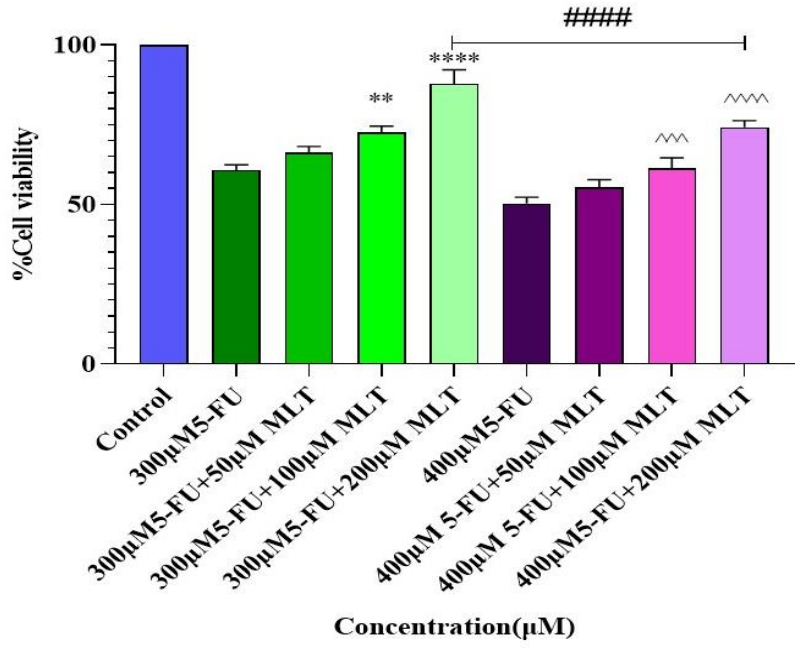

Figure 3. The cell viability of H9c2 cell line treated with different concentrations of MLT and 5-FU for 48 h. The data are presented as mean ± SD of three independent each performed in three replicates. **p<0.03, ****p<0.0001 compared to cells treated with 5-FU 300 µM. ^^^p<0.001, ^^^^p<0.0001 compared to cells treated with 5-FU 400 µM. ####p<0.0001 compared among the treated groups with the highest viability percentages.

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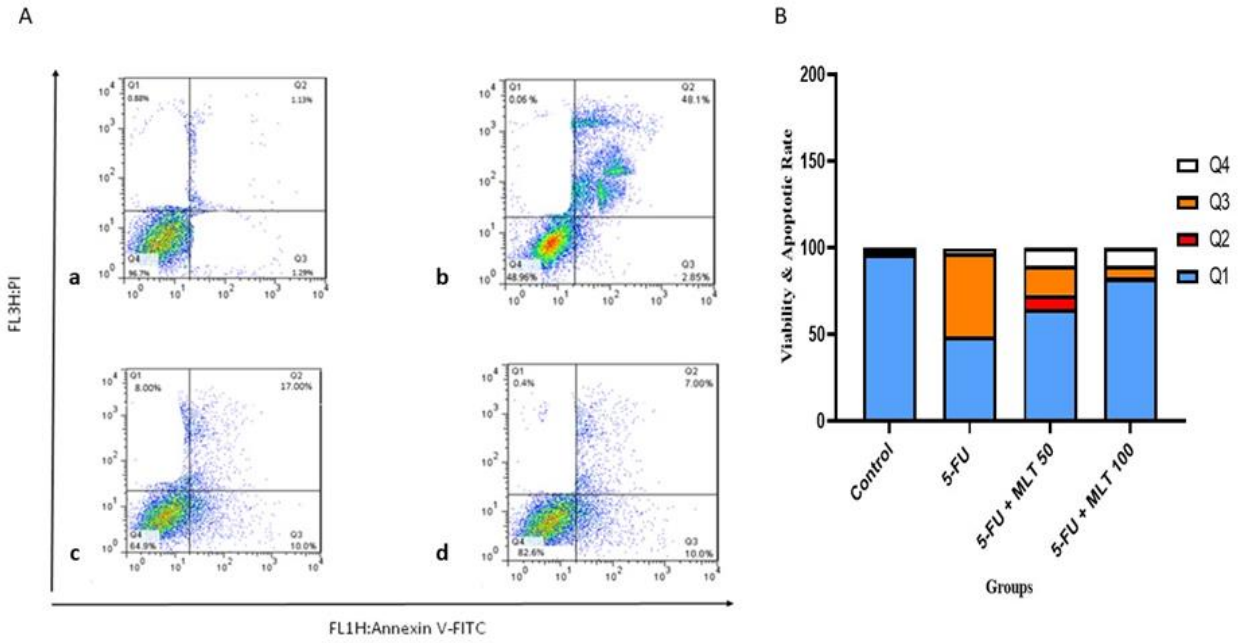

Figure 4. Flow cytometric analysis of 5-FU and/or MLT exposure using AnnexinV/PI staining. H9c2 cells were treated with different concentrations of test compounds. (a) control group cells, (b) Cells treated with 5-FU 400 µM, (c) cells co-treated with 5-FU 400 µM and MLT 50 µM, (d) cells co-treated with 5-FU 400 µM and MLT 100 µM (A). The apoptotic cells were measured by flow cytometry, and the percentage of apoptosis of cells labeled as Anx V (+) PI (−) was determined. (Q1) early apoptotic cells, (Q2) late apoptotic cells, (Q3) necrotic cells, and (Q4) viable cells (B).

At 100 µM melatonin, live cells further increased to 82.6% (±2.1, $p<0.01$), early apoptotic cells were 0.4% (±0.08), late apoptotic cells decreased to 7% (±1.1, $p<0.001$), and necrotic cells were maintained at 10% (±1.5). The statistical comparison of the findings from the two groups treated with 5-FU+ 50 µM MLT and 5-FU + 100 µM MLT showed that the percentage of viable cells in the 5-FU + 100 µM MLT group was significantly greater than in the 5-FU + 50 µM MLT group ($p<0.01$). Conversely, the percentages of early apoptotic cells and late apoptotic cells were significantly lower in the 5-FU + 100 µM MLT group compared to the cells treated with 5-FU + 50 µM MLT ($P < 0.05$; $P < 0.01$, respectively). There was no substantial difference in the percentage of necrotic cells between these two groups.

Melatonin Attenuates 5-FU-Induced Apoptosis via Decreasing Caspase Activity

To further characterize the apoptotic effects of 5-FU and MLT on the H9c2 cell line, we analyzed Cas-3/7 activity using fluorescent assays. The observed alterations in cell viability and the changes in FCM analysis of apoptotic and necrotic cells after 48 hours of treatment with 5-FU and MLT were associated with the induction of apoptosis in H9c2 cells. Data collected from Cas-3/7 activity confirmed significant toxicity following incubation with 5-FU. The quantification of caspase activities in apoptotic cardiomyocyte cells treated with various concentrations of 5-FU and/or MLT *in vitro* is illustrated in (Figure 5). As shown in this figure, incubation of H9c2 cells with 300 and 400 µM of 5-FU for 48 hours significantly increased Cas-3/7 activity in a dose-dependent manner in comparison to the control group ($P < 0.001$ and $P < 0.0001$, respectively).

Additionally, the comparison of spectroscopic findings indicated that the fluorescence intensity in cells treated with 400 µM 5-FU was significantly higher than that in the group treated with 300 µM ($P < 0.05$). In contrast, treatment with MLT in conjunction with 5-FU significantly decreased the fraction of apoptotic cells while simultaneously increasing the fraction of viable cells. Specifically, incubation with 300 µM 5-FU alongside 100 µM MLT significantly reduced caspase activity ($P < 0.05$) compared to cells treated with only 300 µM 5-FU. Furthermore, there was a statistically significant difference in Cas-3/7

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activity between the treatment group receiving 400 µM 5-FU plus 100 or 200 µM MLT and the cells treated with only 5-FU ($P < 0.05$ and $P < 0.001$, respectively). The statistical comparison of caspase activity in cells treated simultaneously with 5-FU and MLT at concentrations of 100 and 200 µM also revealed significant differences ($P < 0.05$). These results align well with the beneficial effects of MLT against the cytotoxic actions of 5-FU.

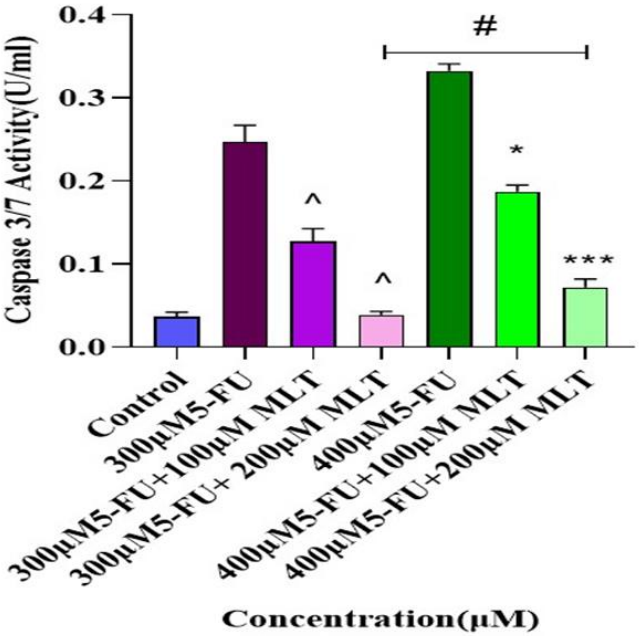

Figure 5. Effect of 5-FU and MLT on the caspase-3/7 activity in H9c2 cardiomyocyte cell line *in vitro*. Caspase-3/7 activity was examined by using fluorescent assay kits. The values are in mean ± SD. *p<0.05 compared to cells treated with 5-FU 300 µM, ^p<0.05 and ^^^p<0.001, compared to cells treated with 5-FU 400 µM, #p<0.05 compared among the treated groups with the lowest caspase 3/7 activities.

Discussion

This study aimed to evaluate the protective effects of MLT against 5-FU-IC in the H9c2 cardiomyocyte cell line, with a particular focus on apoptotic pathways and caspase activity. Our findings from the MTT assay, flow cytometry, and caspase activity analysis revealed that 5-FU exerts significant cytotoxic effects on H9c2 cells. The MTT assay demonstrated a concentration-dependent reduction in cell viability following exposure to 5-FU, with an IC50 of 400 ± 1.15 µM after 48 hours, indicating its potent cytotoxicity. This IC50 value reflects the concentration at which 5-FU reduces cell viability by 50% compared to the untreated control group. Flow cytometric analysis corroborated these findings by showing a substantial increase in the percentage of viable, late apoptotic cells, and also the percentage of necrotic cells in the 5-FU-treated group. These results indicate that high concentrations of 5-FU induce substantial cytotoxic effects, leading to increased late apoptosis and necrosis. Additionally, caspase activity assays revealed a significant elevation in Cas-3/7 activity, further confirming the apoptotic effects of 5-FU. Collectively, these results underscore the detrimental impact of 5-FU on cardiomyocyte viability and highlight the necessity of exploring protective interventions for mitigating its cardiotoxic effects.

Conversely, MLT demonstrated a concentration-dependent protective effect on both cell viability and apoptotic pathways. Co-treatment of cells with 5-FU and MLT exhibited a dose-dependent protective effect. The results indicated that when MLT was used alongside 5-FU, it significantly increased the percentage of viable cells at both concentrations while reducing the percentage of apoptotic cells compared to the group treated with only 5-FU. This protective effect was more pronounced at the 100 µM concentration of MLT. Additionally, measurements of Cas-3/7 activity using fluorescent assays showed that MLT treatment

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significantly mitigated the cytotoxic effects of 5-FU by reducing Cas-3/7 activity and decreasing the fraction of apoptotic cells in a concentration-dependent manner. Since Cas-3/7 are key executioner caspases in the apoptotic pathways, this reduction strongly suggests that MLT effectively interrupts apoptotic signaling triggered by 5-FU. Interestingly, also reported similar protective effects in H9c2 cells, where resveratrol alleviated the cytotoxicity induced by 5-FU (10). In addition to resveratrol, there are several other compounds that have demonstrated cardioprotective potential against chemotherapy-induced cardiotoxicity.

N-Acetylcysteine (NAC) has been shown to exert protective effects through modulation of TLR4/NF-κB and Nrf2/HO-1 pathways (27). Taxifolin has been demonstrated to mitigate oxidative stress, apoptosis, and inflammation via Sirt1/Nrf2/HO-1 signaling, and curcumin has been shown to reduce apoptosis while promoting autophagy in cardiac cells (28, 29). These findings emphasize the therapeutic value of targeting key signaling pathways and highlight the novelty of melatonin as a cardioprotective agent. In other studies, the IC50 of 5-FU varied across cell types, such as 11.3 μM for HCT-116 cells after three days and 11.25 μM for HT-29 cells after five days (16), illustrating the influence of cellular context on drug sensitivity (30). The elevated IC50 values observed in H9c2 cells, in comparison to cancer cell lines, are indicative of their non-cancerous nature and relative resistance to cytotoxic agents. This observation underscores the cell-line-specific response to 5-FU (31).

5-FU is a generally used chemotherapeutic agent primarily employed in the treatment of various cancers, including colorectal, breast, and head and neck cancers (32). Its mechanism of action involves the inhibition of thymidylate synthase, leading to a disruption in DNA synthesis and ultimately inducing cytotoxic effects in rapidly dividing cancer cells (33). However, 5-FU is also associated with substantial side effects, including cardiotoxicity, which can limit its clinical efficacy. The incidence of cardiotoxicity associated with 5-FU is reported to range from 1% to 18% among patients exposed to fluoropyrimidine therapies (14).

In terms of comparison, one study has indicated that 10–15% of cells treated with Doxorubicin exhibited spectroscopic changes in fluorescence and morphological characteristics indicative of necrosis, including increased cell size, vacuolization, and alterations in plasma membrane structure (34). It has been proposed that less than 5% of the entire MLT in mammalian cells is synthesized by the pineal gland. In other words, the majority of MLT in the body is generated outside the pineal gland and does not enter the bloodstream. Instead, it is released from mitochondria into the cytosol, where it interacts with receptors located on the outer mitochondrial membrane (1). The mechanisms underlying the protective effects of MLT may involve several pathways. Melatonin is recognized for its antioxidant properties, which could play crucial roles in decreasing oxidative stress and subsequent cellular damage caused by chemotherapeutic agents (35).

In normal cells, MLT exerts its mitochondrial antioxidant effects via two main pathways: by directly neutralizing free radicals and reactive oxygen species (ROS) and by indirectly boosting the expression and activity of the body's natural antioxidant enzymes (36). Additionally, MLT may regulate various signaling pathways associated with apoptosis, further enhancing its protective role through caspase-dependent mechanisms, thereby exerting its anti-apoptotic effects (37). Despite this information, to the best of our knowledge, the protective effects of MLT against the cytotoxicity induced by 5-FU have not been demonstrated in *in vitro* studies using H9c2 cell lines.

We investigated whether the percentage of cell viability corresponded with apoptosis in H9c2 cardiomyocytes by comparing different constructs using a cell viability assay combined with Annexin V/PI staining, followed by flow cytometry analysis. For all treated cells with 5-FU 400 μM, a significant reduction in cell viability was observed due to an increase in the percentage of apoptotic cells by 48.1% compared to the control group. Therefore, it appears that there is a correlation between cell viability and the rate of apoptosis, with a decrease in cell viability resulting in an increase in the rate of cellular apoptosis. However, simultaneous treatment of the cells with 5-FU and melatonin at concentrations of 50 or 100 μM significantly reduced the percentage of cells undergoing early apoptosis, specifically those that were AnxV positive but PI negative. This treatment resulted in a notable increase in the percentage of viable cells that were AnxV negative and PI negative.

Therefore, it appears that the beneficial effect of MLT against the cytotoxicity induced by 5-FU is at least partially mediated by its ability to protect against

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the pro-apoptotic effects of 5-FU. This protective effect of melatonin against 5-FU-IC in culture may arise from its antioxidant properties, which warrant further investigation into the impact of melatonin on oxidative stress markers under *in vitro* conditions. Interestingly, these findings are further supported by the results of the apoptosis study based on Cas-3/7 activity, as analyzed using fluorescent assays. The results of the present study revealed that Cas-3/7 activity in H9c2 cells significantly increased following exposure to 5-FU compared to the control group. However, simultaneous treatment with MLT led to a significant and substantial reduction in Cas-3/7 activity. Taken together, these data suggest that the protective role of melatonin against 5-FU-IC may occur, at least in part, through the modulation of the Cas-3/7 signaling pathway.

These results contradict the findings of a prior study by Song J. et al., which indicated that melatonin treatment induces a pro-apoptotic effect on SGC-7901 GC cells by increasing Cas-3 activity through a mitochondrial apoptotic pathway (38). This difference is likely related to methodological variations and the different types of cell lines used in the two studies. Additionally, an earlier study on the effects of MLT at concentrations of 1 and 2 mM on Cas-3/7 activity in SRC cell lines showed that melatonin significantly increased the rate of apoptosis through enhanced caspase-3/7 activity at both concentrations (39).

However, this finding seems to contradict our study's results; in fact, it supports our findings, as our study also indicated that cell viability decreased at higher concentrations of melatonin. As illustrated in Figure 2, the percentage of cell viability significantly reduced with a 400 μM concentration of melatonin, suggesting a dual effect of melatonin that is both protective and cytotoxic, depending on its concentration. It has been proposed that Cas-3 and Cas-7 play critical roles in driving the final stages of apoptosis by cleaving specific substrates and regulating the terminal events of the apoptotic signaling pathway in a coordinated manner (40).

Recent studies have demonstrated that MLT exerts cardioprotective effects by modulating key signaling pathways, including PI3K/Akt and SIRT1/Nrf2. In cardiac hypoxia models, melatonin enhances phosphorylation of PI3K and Akt, thereby activating the PI3K/Akt pathway and reducing apoptosis in H9c2 cardiomyocytes. The use of the PI3K/Akt inhibitor LY294002 significantly attenuates these protective effects, confirming their dependence on this pathway (41). Furthermore, a recent study reported that melatonin has activated the SIRT1/Nrf2 pathway, mitigating oxidative stress, apoptosis, and pyroptosis induced by doxorubicin in both H9c2 cells and mouse models. Treatment with doxorubicin led to a reduction in SIRT1 and Nrf2 expression, while melatonin administration resulted in an increase in these proteins and demonstrated cardioprotective effects.

Inhibition of SIRT1 or Nrf2 significantly diminished the protective effects of melatonin, highlighting the critical role of this pathway in mediating its anti-apoptotic and antioxidant actions (42). These findings collectively suggest that the activation of the PI3K/Akt and SIRT1/Nrf2 pathways may serve as a potential therapeutic strategy to protect against cardiotoxicity induced by chemotherapeutic agents such as doxorubicin. Thus, our findings align with the hypothesis that MLT mitigates 5-FU-IC, at least partially, through the modulation of Cas-3/7 signaling pathways. These results offer a foundation for future research into the therapeutic potential of MLT as a cardioprotective agent in clinical oncology. Further studies are warranted to explore the molecular pathways underlying melatonin's protective effects, including its impact on oxidative stress markers, mitochondrial dynamics, and cell signaling pathways.

The dual role of melatonin, as observed at varying concentrations, underscores the necessity for further investigations to ascertain its therapeutic window, particularly in long-term and multi-dose scenarios. However, the precise mechanisms underlying these dual actions remain to be fully elucidated. Given that our findings are derived from an in vitro system, they should be interpreted with caution, given the inherent limitations in directly translating them to in vivo or clinical settings.

In conclusion, this *in vitro* study provides novel insights into the protective effects of MLT against 5-FU-IC in H9c2 cardiomyocytes. Our findings demonstrate that MLT, at optimal concentrations, significantly mitigates 5-FU-induced apoptosis by reducing apoptotic rate, Cas-3/7 activity and protecting against cell death. These effects are likely mediated through melatonin's antioxidant properties and its ability to modulate apoptotic pathways. However, we also observed a concentration-dependent dual role of melatonin, where higher doses exhibited cytotoxic

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effects. This underscores the necessity for careful dose optimization in potential therapeutic applications. Melatonin's protective role against 5-FU-induced cardiotoxicity has significant clinical implications, particularly for patients undergoing chemotherapy. Its ability to reduce apoptosis in cardiomyocytes suggests potential as a co-adjunct therapy to improve cardiovascular safety profiles during cancer treatment. However, it is important to investigate potential interactions between MLT and other chemotherapeutic agents to understand its broader implications as an adjuvant therapy.

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