Bratisl Lek Listy 2009; 110 (5)
276–279

EXPERIMENTAL STUDY

The effects of melatonin on human hepatoma (Hep G2) cell line

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Abstract: *Background:* Melatonin has been suggested to have antiproliferative effects on cancer cells. These effects can be attributed to immunomodulation, growth factor inhibition, induction of apoptosis and prooxidant properties. Melatonin is considered as a safe drug with minimal adverse effects.
Objectives: We planned to investigate the effects of melatonin in hepatoma (Hep G2) cell line. In this study, different concentrations of melatonin were studied to assess its effects on human hepatoma (Hep G2) cell line in vitro.
Methods: In this study, different doses ($5x10^{-5}$ M, $5x10^{-4}$ M, 10^{-3} M) of melatonin were administered into hepatocellular carcinoma cell line in vitro. After an incubation period of 72 hours, the studied and control groups were evaluated for cell cycle, morphology, proliferating index and apoptosis percentage.
Results: A significant decrease in percentage of phase G0/G1 cells was found in high-dose melatonin group (10^{-3} M) compared to control group. Melatonin increased the cell counts in S phase of cell cycle at high doses as well. However, phase G2/M cell percentage did not change with the administration of melatonin. Cell proliferation was increased in all melatonin groups, but the only statistically significant difference was found between the high-dose and control groups. There was a significant increase in proliferative index between the control group and high-dose melatonin group.
Conclusion: High dose of melatonin increases the cell count in S phase and shows an antiproliferative effect on hepatoma cells. This indicates that melatonin can be considered a promising drug when used along with other antineoplastic agents for the treatment of hepatoma.However, it has no effect on apoptosis and colony counts *(Tab. 1, Fig. 2, Ref. 19)*. Full Text (Free, PDF) *www.bmj.sk.*
Key words: melatonin, hepatoma, cell line, Hep G2.

Melatonin (N-acetyl-5-metoxytryptamin) is secreted mainly from the pineal gland. It was shown to be secreted from retina as well as bowels. This hormone plays a major role in the regulation of many physiological and psychological processes (1, 2). Melatonin is a strong antioxidant (1, 2). It has an anti-oxidant effect at pharmacological doses. Hovewer, in some studies, it has been shown to have pro-oxidant activity beside its antioxidant activities (3).

In clinical practice, melatonin has been used in the treatment of jet-lag and insomnia. Hovewer, it has been used in some cancer diseases in experimental studies (1, 2). The treatment dose range is usually between 1–3 mg. Melatonin has been shown to be effective in some cancer types (4–8). In breast cancer with low estrogen receptor, and prostate cancer, serum levels of melatonin were found to be lower. There are some studies showing that melatonin is effective in breast and prostate cancer (4, 6). Melatonin has been found to decrease proliferation in epithelial breast cancer cell line and malign melanoma cell line (6). In addition there are some studies showing that it is effective in colon, pancreas, gastric and lung cancer metastatic to liver (9). But the mechanism of this action is unclear.

In this study we planned to investigate the effects of different doses of melatonin molecules on human hepatoma (HepG2) cell line.

Material and method

Hepatoma (Hep G2) cell culture

This study was done by addition of melatonin in different doses into hepatocellular carcinoma cell line in vitro. Hepatocellular carcinoma cell line HepG2 was obtained from American Type Culture Collection (ATCC). Cells were proliferated by incubation in RPMI medium containing 100 µg/ml streptomycine, 100 u/ml penicillin (Llorente Laboratories, Madrid, Spain), 10 % fetal calf serum (Sigma), at 37 °C with 5 % CO_2. Cells were seperated from the flask by trypsination process with 0.25 % trypsine, when the cellular flask of 25 ml became 75 % confluent. Later the medium were prepared by using RPMI 1640 (Sigma), 10 % fetal calf serum, 100 unit/ml penicilline and 100 µg/ml streptomicine at pH 7.4.

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Tab. 1. The effects of different melatonin doses on apoptosis of hepatoma cells.

	G0/G1	S	G2/M	Apoptosis	Colony counts	Proliferation index
Control	51.9±2.9	28.0±2.2	20.1±3.8	38.3±7.8	37.8±6.3	48.1±2.9
Grup 1 (Melatonin $5x10^{-5}$ M)	48.7±2.1	30.7±1.6	20.7±2.0	47.1±26	43.5±7.9	51.3±2.2
Grup 2 (Melatonin $5x10^{-4}$ M)	44.3±9.6	36.4±15	19.4±5.3	51.6±26	42.7±5.2	55.7±9.6
Grup 3 (Melatonin 10^{-3} M)	45.8±1.6	34.4±2.5	19.9±3.2	46.8±23	48.3±8.1	54.3±1.4
p	p=0.0041	p=0.0058	NS	NS	NS	p=0.0024

NS — non-significant

Melatonin (Sigma) was taken, and dissolved in ethanol (95 %) to form 1 ml of stock solution (10^{-1} M). Thereafter, final concentrations were obtained by dilution of stock solution with RPMI 1640 ($1x10^{-3}$, $5x10^{-4}$, $5x10^{-5}$ M). The level of final Ethanol concentrations was less than 1 %.

Studied groups

Cells were distributed into a cell plate with 24 wells, the last volume was 1 ml in each well. This culture plate was divided into four groups with 6 wells each, while one of them was chosen as a control group; the others were added with melatonin at different doses as follows below:

Control group
Group 1: Melatonin $5x10^{-5}$ M
Group 2: Melatonin $5x10^{-4}$ M
Group 3: Melatonin 10^{-3} M

These plates were incubated for 72 hours at 37 degrees with 5 % CO_2. After the incubation period of 72 hours, groups were evaluated for cell cycle, morphology, proliferating index and apoptosis percentage.

Evaluation of apoptosis, cell cycle analysis and proliferative index

The Coulter DNA-prep reagent system(Miami FL,USA) was used to stain the DNA of the cultured cells with propidium iodide (PI) for the quantitative measurement of cellular deoxyribonucleic acid (DNA) content by flow cytometry. The reagents were used in conjunction with the Coulter DNA Prep workstation (Florida USA). Flowcytometry was performed on Coulter Epics Elite Flowcytometry (Florida USA). Data were analysed for apoptosis and cell cycle using the Multicycle Software (Phoenix Flow Systems, San Diego, CA) (10). Apoptosis was evaluated with measurement of expression of annexin V (Immunotech, Marseille, France) by flow cytometry (11). Apoptosis ratio of cultured mononuclear cells were measured as percentage of hipodiploidic pic. The proliferation ratio of cultured cells was assessed, using the formula as follows below (12)

$$\text{Proliferative index (\%)} = 100 \times \frac{\text{Cell number in mitosis+Cell number in S-phase}}{\text{Total cell number}}$$

Evaluation of colony counts

Cells were observed through the inverted microscope on first, second and third days. After 72 hours, photographs of formed cell colonies (>20 cells = 1 colony) were taken with inverted microscope.

Statistical analysis

Data were evaluated with Kruskal-Wallis method of multiple group analysis for proliferative index, apoptosis, colony count and cell cycle. Data were expressed as mean + standart deviation. P Value of less then 0.05 was accepted as being significant.

As a result of variation analysis, in the post-hoc matching, Mann-Whitney-U test was used by lowering the error level (13). As this is a subgroup analysis, here the p value of less then 0.008 (0.05/6) was accepted as statistically significant.

Results

The results of this study are summarized in Table 1.

The effects of melatonin on cell cycle of hepatoma cell line (Hep G2)

Cellular percentages in the phase of G0/G1 between the groups were different (p<0.0041). In the subgroup analysis, this difference was found between the control (51.9±2.9) and group 3 (45.8±1.6) (p=0.0039). In the comparison of the control group and groups 1 and 2, the cellular percentage in G0/G1 phase was not found to be significant. There was no significant difference between the group 1 and groups 2 and 3, and between the groups 1 and 2. It has been seen that the variation at G0/G1 phase has come from group 3.

There was a statistical difference between the groups in terms of cell percentage at S phase of cell cycle (p=0.0058). In subgroup analysis, cell percentage at S phase of cell cycle was found to be significant between the control (28.0±2.2) and group 3 (34.4±2.5) (p<0.0039). When the control group was matched with groups 1 and 2 seperately, the difference in cell percentage at S phase was not statistically significant. There was no significant difference between group 1 and groups 2 and 3, and between group 1 and group 2. The variation at S phase was seen to be due to group 3.

There was no significant difference between the groups in terms of cell percentage at phase G2/M (p>0.05).

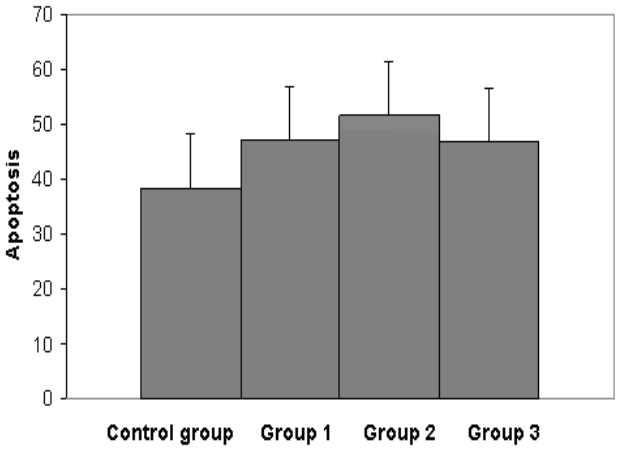

Fig. 1. The effects of different melatonin doses on apoptosis of hepatoma cells.

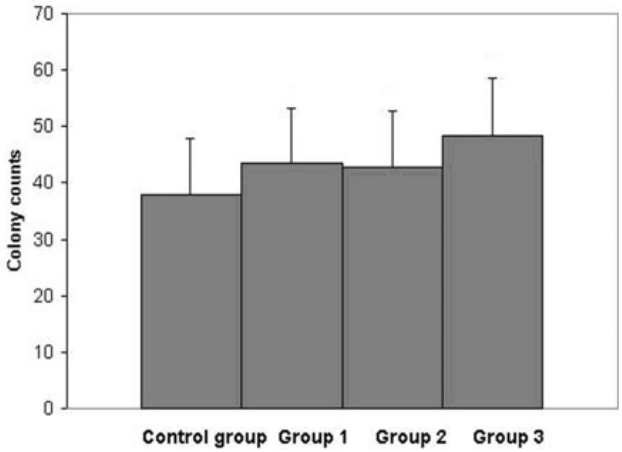

Fig. 2. The effects of melatonin in different doses on colony counts of hepatoma cells.

The effects of melatonin on apoptosis of hepatoma cell line (Hep G2)

Apoptotic cell percentage of the groups was not statistically significant (p>0.005) (Fig. 1).

The effects of melatonin on proliferation index of hepatoma cell line (Hep G2)

The proliferation index was significant among the groups (p=0.0024). In subgroup analysis, the difference between the control (48.1±2.9) and the group 3 (54.3±1.4) was found to be significant in proliferation index (p=0.0039). When the control group was compared with groups 1 and 2 seperately, the proliferation index was not found to be statistically significant. There was no difference between group1 and groups 2 and 3, and between groups 1 and 2.

The effects of melatonin on colony counts of hepatoma cell line (Hep G2)

The groups were not found to be statistically different (Fig. 2).

Discussion

In some recent studies, melatonin has been shown to be effective in different cancer types (3–9, 13, 14). There are few experimental studies about the usage of melatonin in hepatocellular carcinoma (13, 14). In these studies, melatonin is generally added to other chemotherapeutics. In literature, there is no study dealing with efficiency of melatonin in human hepatoma cell line in respect of proliferation parameters and cell cycle.

In this study, melatonin decreased the cell percentages at the phase of G0/G1 in all groups compared to control group. In subgroup analysis, the decrement in the cell count passing through G0/G1 phase was between the control group and group 1 when highest dose was used. Melatonin was found to inhibit the proliferation of tumor cells by decreasing the cell count passing through G0/G1 phase at high doses. Cini showed that melatonin increased cells at G0/G1 phase in rat hepatoma 130 AH cell line (7). Urata and colleaques reported that melatonin increased the cell population at the phase of G0/G1 in human vascular endothelial cell line through increasing the production of glutation (15). The results of these two studies were similar to our results. However, the cell lines used were different. Chemotherapeutic agents are generally known to be more effective at the proliferation stage of cancer cells. In tumor growth, it is known that with some stimulants, resting cells pass into the cell cycle and proliferate. If the cell population at this stage decrease as seen in our study, the cell count entering the cell cycle will decrease too. As a result it is possible that melatonin may slow the tumor growth by decreasing the cell count passing through G0/G1 phase.

Melatonin was found to increase the cell count at S phase of cell cycle in all of the groups. But only the difference in cell counts at S phase between group 3 and control group was significant when the groups were compared separately. According to these findings, melatonin leads to proliferation of tumor cells by increasing the cell counts passing through S phase at high doses. Shiu and colleagues reported that melatonin slowed down the passing through the S phase in human choriocarcinoma cell line by inhibiting the factors promoting the tumor growth and antiproliferative effects (16). Cos and colleagues showed that melatonin, at concentration of 10^{-9} M with an incubation time of 96 hours, increased the cell counts entering the S phase in estrogen positive MCF-7 breast cancer cell line (17). The results of these two studies were different. However they were done in different cell lines. In our study, the results were similar to those of Cos et al. The increasing effect of entering the S phase may be related to dose or incubation time. Cancer cells are known to be sensitive to chemotherapeutics at the proliferating stage. Melatonin may increase the effects of antineoplastic agents specific to S phase (18, 19).

We obseved that melatonin increased the proliferative index in all of the groups. When the groups were compared separately,

this effect was observed to be due to the difference between the control group and group 3. Other groups did not demonstrate this effect. Hermann et al reported that melatonin had antiproliferative effects on Mouse hepatoma cell line (HEPA 1–6) (14). The effective dose of melatonin was found to be 640 microgram in this study. Karasek et al did not observe antiproliferative effects on hamster hepatoma cell lines but found antiproliferative effects in vivo (13). They put forward that these antiproliferative effects of melatonin might be through the immune system, neuroendocrine system or direct effects. Similar to Karasek's study, in this in-vitro study, we did not observe antiproliferative effects of melatonin on hepatoma cell lines.

The usage of high doses of melatonin in hepatoma cells increases the cell count in S phase and shows an antiproliferative effect. The latter facts demonstrate that melatonin may be a promising drug together with antineoplastic agents in hepatoma therapy.

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Received September 17, 2008.
Accepted January 22, 2209.