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Experimental Research

Protective and sensitive effects of melatonin combined with adriamycin on ER+ (estrogen receptor) breast cancer

C. Ma[1], L.X. Li[2], Y. Zhang[1], C. Xiang[1], T. Ma[1], Z.Q. Ma[1], Z.P. Zhang[1]

[1] Department of Oncology, the First Hospital of Shijiazhuang, Shijiazhuang
[2] Department of Clinical Laboratory, the First Hospital of Shijiazhuang, Shijiazhuang (P.R. China)

Summary

Objective: This study aims to investigate the protective and sensitive effects of melatonin (MLT) in the treatment of breast cancer. *Materials and Methods:* ER+ breast cancer rat model was established and then rats were randomly divided into five different groups as follows: control group, Diss group, adriamycin (ADM) group, MLT group, and MLT combined with adriamycin (M+A) group. Tumor weights and one month survival rate were compared among these groups. In addition, changes of tumor tissues and expression of E-cadherin were observed under optical microscopy or electro-microscopy. *Results:* Tumor weights were significantly lighter in M+A group than those in ADM group ($p < 0.05$). Under optical and electro-microscopy, tumor cell apoptosis was obviously increased in MLT group, and tumor cell injury was more severe in M+A group than that in ADM group; additionally, expression of E-cadherin was higher in MLT group and M+A group than that in other groups. Moreover, MLT group had the highest one month survival rate (100%), there was the poorest life quality in ADM group, but the best life quality in MLT. *Conclusion:* MLT could enhance the sensitivity of tumor to ADM in vivo and improve patient's life quality.

Key words: Melatonin; Breast cancer; Adriamycin; Metastasis.

Introduction

Breast cancer is a malignant tumor with a high incidence in females from more developed Western countries. Although historically the incidence of breast cancer in women in China was low, it has been on the increase in the past 20 years. In large cities, such as Beijing and Shanghai, breast cancer is the most common type of malignant tumor diagnosed in women. As the country with the highest population in the world, there are a large total number of cases annually. Anthracycline chemotherapeutic agents, including adriamycin (ADM), are among the main agents used for breast cancer chemotherapy. ADM has been essential in breast cancer therapy, particularly in prolonging the survival of patients with advanced and metastatic breast cancer. However, due to the dose-limiting cardiotoxicity of ADM, it is not suitable for use in patients with cardiac disorders. The administration of ADM may cause varying degrees of myocardial toxicity, seriously affecting the patients' quality of life, and in certain cases has caused toxicity-related mortality [1-3].

In recent years, studies of myocardial toxicity have been reported, but no drug has been satisfactorily applied in a clinical environment [4-6]. With developments in the treatment of malignant tumors, tumor reduction and disease relief in the short term are no longer sufficient and steps to improve quality of life and prolong the survival of patients are drawing an increasing amount of attention. There has been a strong clinical demand for cardioprotective drugs for use in tumor patients [7]. There is a need for a drug that is not only helpful in the treatment of breast cancer, but also alleviates ADM-induced cardiotoxicity.

Melatonin, produced in the pineal gland which is outside of the blood-brain barrier, acts as an endocrine hormone and is widely distributed in different organs. In animals, melatonin (MLT) is associated with several biological functions. Some studies have indicated that MLT inhibited the growth of many malignant tumors, such as ER+ breast cancer [8-11]. The authors' previous study also demonstrated that exogenous MLT suppressed the proliferation of breast carcinoma cell lines and enhanced the sensitivity of ER+ breast carcinoma cell line MCF-7 to ADM [12, 13], but we did not perform further study in animal model. In this study, the authors established rat model with ER+ breast cancer and further confirmed the effect and mechanism of MLT on proliferation and resistant to ADM in breast cancer rat model in order to provide basic evidences for developing safer and more effective drugs.

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Materials and Methods

Animals

The study included 140 Sprague-Dawley (SD) female rats, about 200-250 gram weight, were purchased from animal center of Hebei medical university. All rats were fed under light-dark conditions that was 12 hours light time (6:00-18:00) and 12 hours dark time (18:00-6:00) by turns. Room temperature was 25 ± 2°C and the humility in the air was 45% - 50%. All rats underwent experiments after being fed for three weeks. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Hospital of Shijiazhuang.

Establishing breast cancer rat model

According to Russo methods [14], 130 of SD female rats were injected with N-nitroso-N-methylurea (NMU) to induce breast cancer. Ten SD female rats were injected with Diss as control.

All rats were weighed and numbered through Dagar method. Rats were treated with 50 mg/kg NMU through intraperitoneal injection. Then rats were weighted again after two weeks, and then treated once again. Rat breast were checked once a week to observe tumor growth.

Groups

Rats were divided into five groups: blank group; Diss group; MLT group that rats were treated with 10 mg/kg/day MLT for 15 days; ADM group with rats that were treated with 2.5 mg/ml ADM, qod, for seven times; M+A group that rats were treated with 10 mg/kg/d MLT for 15 days and 2.5 mg/ml ADM were administered from the third day, qod, for seven times. MLT was injected before ADM treatment. Some rats were sacrificed at 18 days after tumors were taken out and weighed, and others were observed while still alive.

Electro-microscopy observation

The tumor tissue was placed in a 10 ml flasket, then 4% glutaraldehyde was added for immersion. After fixation at -4°C for one hour, the sample was washed with phosphate buffer solution (PBS) for three times within ten minutes, followed by fixation using 1% osmic acid at -4°C for two hours. Then PBS was used for washing for three times within ten minutes. The dehydration was conducted using ethanol with gradient concentration (50%, 70%, 80%, 90%, 100%, and 100%; ten minutes for each step), followed by dehydration using acetone for two times (ten minutes for each time). The sample was immersed with mixture liquid of acetone and resin (3:1, 1:1, and 1:3, respectively) for 15 minutes, followed by immersion with pure resin for 30 minutes. Then the sample was embedded in epoxy resin (type 812, 815), followed by polymerization (successive 37 °C, 45 °C, and 60°C; 24 hours for each temperature). The ultrathin sections were prepared in a ultramicrotome, followed by double electron staining using lead citrate for 30 minutes. Finally, the sections were observed and photographed using H-7500 transmission electron microscope.

Immunohistochemistry assay

Four-μm tumor tissues were sliced and then dewaxed with dimethylbenzene, absolute alcohol, 95% alcohol, 80% alcohol, 70% alcohol, respectively, and then added ethylenediamine tetracetic acid (EDTA) solution, after treatment of primary antibodies, tissues were incubated overnight at 4°C, then washed with PBS, followed by treated with EnVision reagent to incubate for 30 seconds at room temperature, then stained with 3,3-diminobenzidine (DAB) which was stopped through observation under light microscopy, and then dehydrated with 70% alcohol, 80% alcohol, 95% alcohol, absolute alcohol, and dimethylbenzene, respectively. Finally, sections were observed under light microscopy.

Results of immunohistochemistry were judged as follows: most of positive primrose or brown-yellow particles were located in cytoplasm; few positive particles were located in nucleus. According to counting positive cells and color depth to score under five different fields: 0 score: proportions of positive cells were less than 5%, 1 score: proportions were 5%-24%, 2 scores: proportions were 25%-49%, 3 scores: proportions were 50%-74%; 4 scores: proportions were more than 75%. In addition, according the color depth, no-staining color was 0 score, light yellow presented 1 score, yellow presented 2 scores, and brown and yellow presented 3 scores. If positive cells scores × color scores < 1 score, this case were a negative expression of ER or E-cadherin, others were judged as positive expression of ER or E-cadherin

Recording of survival condition

Rats from every group were recorded and observed feeding condition and mental status. One month survival rate was also recorded.

Statistical analysis

Statistical analysis was performed by SPSS 13.0 software and all results were presented as mean±SD. One-way analysis of variance was used to compare with different groups and χ^2 test was used for comparisons of one month survival rate. A p value < 0.05 denoted a significant statistical difference.

Table 1. — *Comparisons of tumor weight among groups.*

Group	n	Tumor weight (grams)
Blank	8	23.45 ± 5.08
Diss	8	27.52 ± 4.93
MLT	8	22.51 ± 4.26
ADM	8	14.84 ± 2.99
M+A	8	10.06 ± 3.13

Blank and Diss: $p = 0.052$. Blank and MLT: $p = 0.645$. Blank and ADM: $p = 0.000$. Blank and M+A: $p = 0.000$. MLT and M+A: $p = 0.000$. ADM and M+A: $p = 0.007$.

Results

Tumor formation

Only one rat died without any reason in vehicle group; seven rats died during injection or before dividing into groups in treatment groups. Seven rats failed to establish breast tumor model. Therefore, 116 rats were randomly divided into treatment groups. Tumor formation rate was 91.5%. Eight rats from every group were sacrificed and then developed tumors; others were observed at one month survival rate.

Tumor weight

The tumor weights of blank group, Diss group, MLT group, ADM group, and M+A group were 23.45 ± 5.08, 27.52 ± 4.93, 22.51 ± 4.26, 14.84 ± 2.99, and 10.06 ± 3.13 grams, respectively, and there was no significant difference between blank group and Diss group ($p > 0.05$). Moreover, there was also no significant difference between MLT group and blank group ($p > 0.05$), but tumor weights from ADM

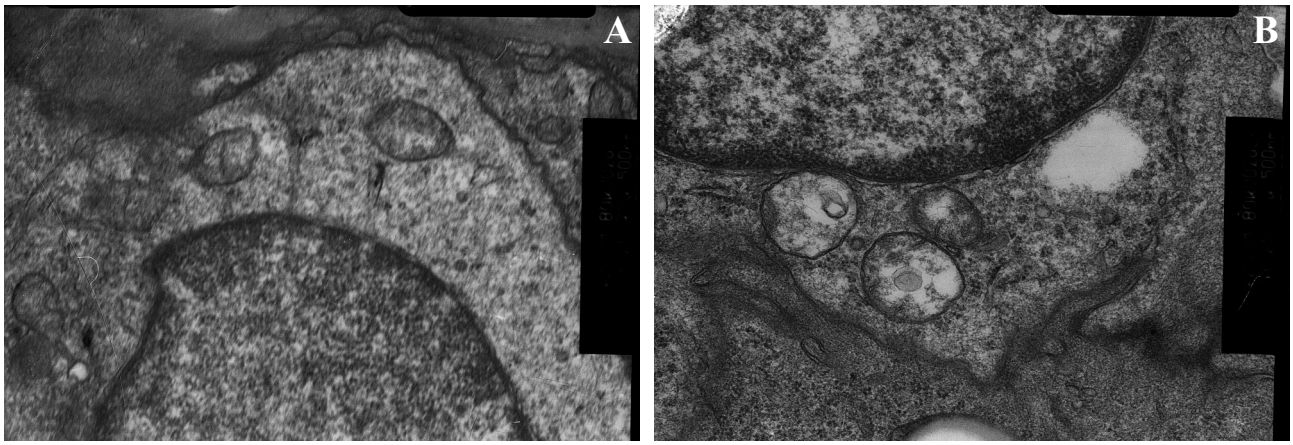

Figure 1. — A: The damage of tumor cell in ADM group by high power under electro-microscopy (×20.0KX). B: The damage of tumor cell in M+A group by high power under electro-microscopy (×20.0KX).

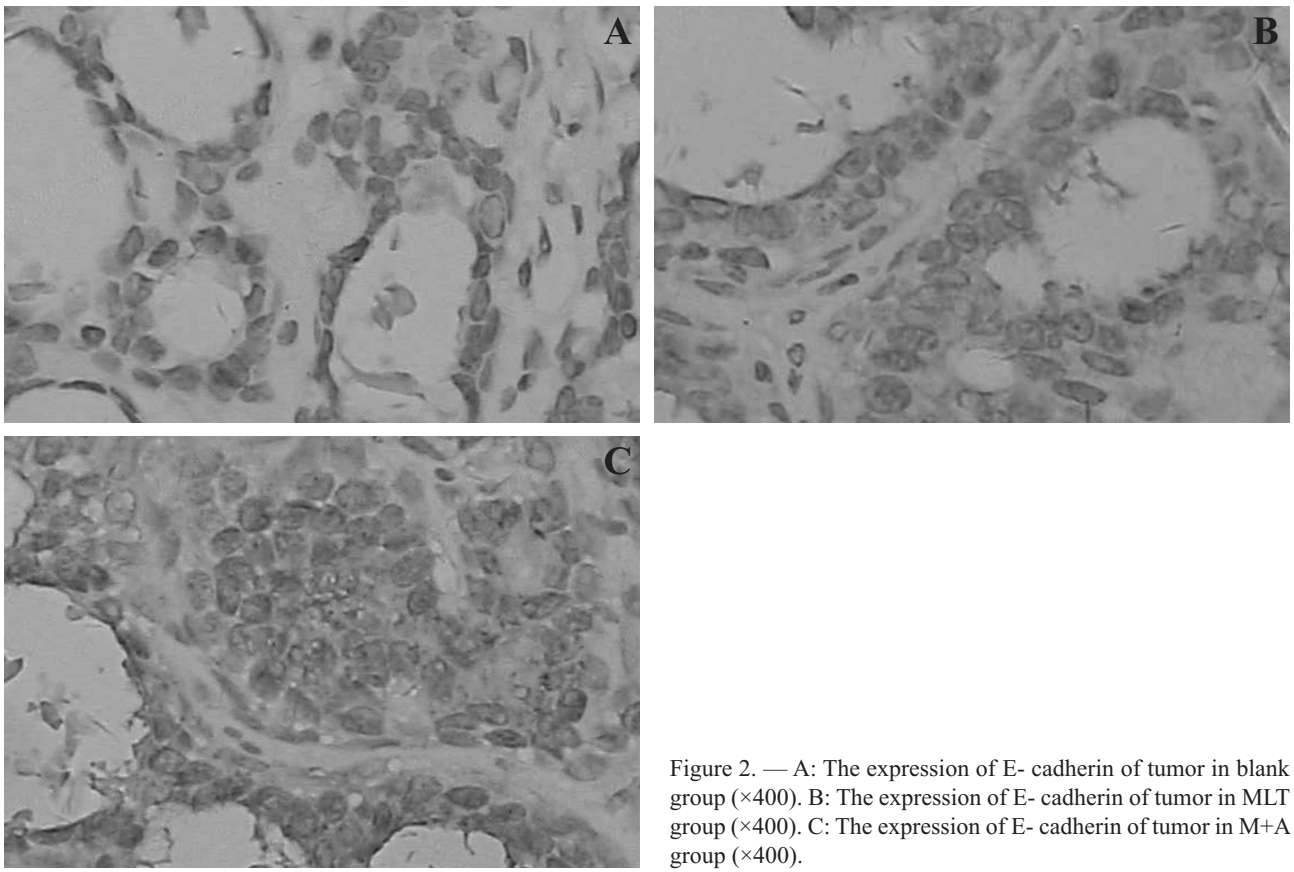

Figure 2. — A: The expression of E- cadherin of tumor in blank group (×400). B: The expression of E- cadherin of tumor in MLT group (×400). C: The expression of E- cadherin of tumor in M+A group (×400).

group and M+A group had an obvious difference from those from blank group ($p < 0.01$), while, tumor weights of M+A group was lighter than those of ADM group ($p < 0.01$) (Table 1).

General changes

In MLT group and M+A group, tumors had relative integrity and smooth membrane envelopes and were easily stripped off, but there was reverse phenomenon in blank group, Diss group, and ADM group, in which the tumors adhered with adjacent tissues and grew aggressively, and some tumors boundary were not clear.

Changes of tumor tissues under electro-microscopy

Tumor tissues in blank group and Diss group were not observed obvious abnormal changes; tumor cell apoptosis

Table 2. — *Comparisons of one month survival rate among groups*.

Group	n	One month survival number
Blank	16	4
Diss	16	6
MLT	14	14
ADM	16	5
M+A	14	11

Blank and Diss: $x^2 = 1.45$, $p = 0.35$. Blank and MLT: $x^2 = 17.5$, $p = 0.000$. Blank and ADM: $x^2 = 0.00$, $p = 1.00$. Blank and M+A: $x^2 = 8.57$, $p = 0.005$. MLT and M+A: $x^2 = 3.36$, $p = 0.11$. ADM and M+A: $x^2 = 6.71$, $p = 0.012$.

was dramatically increased in MLT group; loss of most iliac crests in mitochondria and reduction of rough endoplasmic reticulums and free ribosomes were observed (Figure 1A).

Tumor tissues were severely injured in M+A group, and many iliac crests of mitochondria disappeared and many cellular organs were decreased (Figure 1B).

Expression of E-cadherin in tumor tissues among groups

Immunohistochemistry assay confirmed that expression of E-cadherin was not obvious in blank group, Diss group, and ADM group. Light yellow area was seen by survivin staining, and overall scores were ranged from 0-2 (Figure 2A). In MLT group and M+A group, there were many brown cells around tubules and proportion of positive expression of E-cadherin area was more than 50%, in which overall scores were 3-6, thus indicating that there was a strong positive expression of E-cadherin (Figure 2B, 2C).

Comparisons of one month survival condition among groups

One month survival conditions of blank group and Diss group were 4/16 and 6/16, respectively; there was no significant difference between two groups ($p = 0.35$). All rats were alive in MLT group (14/14); moreover, one month survival rate was significantly higher in M+A group (11/14) than that in ADM group (5/14) ($p = 0.012$). There was no obvious difference between MLT group and M+A group ($p = 0.11$) (Table 2).

Comparisons of general conditions

In blank group and Diss group, weight, fair diet, and response of rat was worsened with the enlargement of tumor size. Although tumor was obviously small in ADM group, general condition was still weak and ADM group had the highest mortality rate, tumor size was not significantly decreased in MLT group, but had good general condition. The M+T group had the best conditions.

Discussion

Previous studies have confirmed that MLT inhibited the growth of breast cancer cell lines and had a reverse effect on MCF-7/ADM resistant to ADM in vitro [12, 13]. However, there are complicated factors in vivo, as it is necessary to systemically evaluate anti-tumor effect of MLT and sensitivity of MLT to ADM in vivo.

According to references protocol [14] and considering immune function and endocrine factors, breast cancer SD rat model with positive estrogen receptor induced by NMU peritoneal injection method was applied instead of the xenograft in nude rat model. On the one hand, one of anti-tumor mechanisms of MLT is to regulate the immune and endocrine function [15, 16], and the other hand, breast cancer SD rat model, established by injecting twice 50 mg/kg, had a high tumor formation rate (91.5%), was valuable, and had a clinical significance. This method had some advantages, such as high tumor formation rate and similar tumor formation time, but also had disadvantages, such as long modeling period. Results from pathology and immunohistochemical analyses showed a positive expression of estrogen receptor, which confirmed that the model was established successfully.

Previous study found that MLT had anti-tumor effect through direct or indirect complex mechanisms, which made a great part of tumor cells in non-proliferation state, with even apoptosis [17, 18]. The present study also observed similar results. Tumor size became smaller in SD rat model after treatment of exogenous MLT, but there was no significant difference between control group and treatment group, which was relevant to short observing time. Additionally, some researches also reported that MLT enhanced the sensitivity to chemotherapy [19]. The present results confirmed that tumor weight was lighter in ADM group than that in MLT group, control and vehicle group ($p < 0.01$), but heavier in ADM than that in ADM combined with MLT group ($p < 0.01$). This result further demonstrated that MLT enhanced the sensitivity of tumor cells to ADM. Attentively, one month survival rate was highest and no death in MLT group. Many studies indicated that MLT seemed to have a fundamental role as a system regulator in haemopoiesis and immune-enhancement [20, 21], moreover, MLT protected against stress damage [22-25]. The present authors also reported that MLT had a protective role in the myocardium by reducing ADM-induced myocardial oxidative damage [26]. These protective effects made tumor animal maintain good survival condition, which complied with therapeutic aim for late cancer patients. The rats presented poor conditions in the ADM group; however the same group did not show a significant difference with regards to the survival rate when compared to the control group due to a strong toxic reaction. Although ADM had some advantage in anti-tumor effects, its cyto-toxicity was also considered in clinic. A balance point should be found that not only suppresses the growth of tumor, but also improves life quality of patients with cancer. Interestingly, the authors observed that tumor weight and one month survival rate were better in MLT combined with ADM group than those in ADM group. Obviously, MLT enhances the toler-

ance of rate to chemotherapy. This provides a new thought that MLT combined with high-dose ADM increases complete remission rate for good condition patients, but MLT combined with low-dose ADM for poor condition patients also reduces tumor load and improved life quality. Thus, MLT combined with ADM had many obvious advantages, for example, increasing the sensitivity of tumor to ADM and complete remission rate, moreover, toxicity is evidently reduced in MLT combined with ADM group, compared with ADM group. In other words, tumor is suppressed at the utmost; meanwhile, patients never pay dearly for antitumoral chemotherapy. This regimen has with no doubt a significant meaning in clinical treatment.

The metastasis of carcinoma is a major life-threatening factor. Recent studies found that the reduction of adhesive capability of tumor cells led to metastasis, and E-cadherin is the well-studied member of the cadherin family and in epithelial cells. E-cadherin-containing cell-to-cell junctions are often adjacent to actin-containing filaments of the cytoskeleton. Furthermore, loss of E-cadherin function or expression has been implicated in cancer progression and metastasis. Additionally, E-cadherin, a suppressor gene, down-regulation decreases the strength of cellular adhesion with a tissue, resulting in promoting the development of tumor. Previous studies confirmed that MLT induced the expression of E-cadherin and increased cell-to-cell junctions [27, 28]. In this study, the authors obviously observed that both in MLT group and MLT combined ADM group, membrane of tumor cell was clear and tumor tissue had obvious boundary with normal tissues. These results indicated that tumor cells had weak metastasis capacity. Conversely, in control group and ADM group, tumor tissues adhered with normal tissues and tumor cell membranes were unclear, moreover, there were few expression of E-cadherin. These imply that MLT might reduce the incidence of tumor metastasis.

In conclusion, the authors demonstrated that MLT could inhibit the proliferation and metastasis of tumor, enhance the sensitivity of tumor to ADM, and have a protective effect against ADM-induced cardiotoxicity in ER+ breast cancer rat model. Although many studies have reported that MLT selectively inhibited tumor formation and protected normal tissues, the exact mechanisms are still to be elucidated [29]. Nonetheless, the present results indicate that MLT probably becomes one of important ancillary drugs to treat breast cancer. The authors also believe that with the deeper basic study and large-scale randomized controlled studies, MLT will be widely used in clinic.

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Address reprint requests to:
Y. ZHANG, M.D.
Department of Oncology,
The First Hospital of Shijiazhuang,
No 36 Fanxi Road,
Shijiazhuang 050010 (P.R. China)
e-mail: yanzhangdoc@163.com