European Review for Medical and Pharmacological Sciences 2017; 21: 5837-5842

Anti-proliferative effect of digoxin on breast cancer cells via inducing apoptosis

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Abstract. – OBJECTIVE: Digoxin is a kind of plant-derived cardiac glycoside that is mainly used to treat heart diseases, especially in congestive heart failure or arrhythmia. However, its potentiality presented in anti-tumor remains unexplored. The purpose of this study was designed to investigate the beneficial pharmacological activity of digoxin on breast cancer cell line (MDA-MB-231, MM231).

MATERIALS AND METHODS: The methyl thiazolyl tetrazolium (MTT) assay was utilized to detect the proliferation of the breast cancer MM231. The apoptotic cell numbers were determined by the flow cytometry analysis. The expressions of Bcl-2 (B-cell lymphoma-2) and Bax (Bcl2-associated X protein) were detected by Western blot analysis.

RESULTS: Digoxin dose-dependently blocked the cell growth of the breast cancer MM231 through MTT assay, whereas the apoptotic numbers were significantly elevated as reflected in acridine orange staining and flow cytometry analysis. In addition, findings from Western blotting method indicated that digoxin intervention showed reduced Bcl-2 expression and elevated Bax level in MM231 cells, characterized by increased Bax/Bcl-2 ratio.

CONCLUSIONS: Digoxin plays a potential anti-tumor role in breast cancer *in vitro*, possibly by inducing mitochondria-dependent apoptosis.

Key Words:
Digoxin, Breast cancer, Apoptosis.

Introduction

Triple negative breast cancer (TNBC) represents a disease that contains three different phenotypes of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER-2)[1]. As one special subtype, TNBC accounts for 10-18% of breast cancer[2]. Unfortunately, TNBC has a particularly poor prognosis due to insensitivity to therapies that target the receptors they lack. There is a vital need for effective targeted therapeutics for TNBC patients.[3]. Thus, it is necessary to seek an alternative drug for managing TNBC.

Physiologically, MDA-MB-231 breast cancer cell line derives from human epithelial tissue, which is characterized by its aggressive biologic biobehavior and similarity with TNBC[4-6]. Recently, accumulating evidence highlights that digoxin exerts potential anticancer properties with selective cytotoxic effect *in vitro* and *in vivo*, including small cell lung cancer, leukemia, neuroblastoma, and other tumor cells[7]. In addition, previous reports indicated that digoxin mediated the inhibitory effect on the proliferation and induced the apoptosis of certain cancer cells. However, more data should be provided in this attractive application in breast cancer, especially underlying mechanism of digoxin affecting MM231. In the present study, we aimed to investigate the potential therapeutic effect of digoxin on MM-231 breast cancer cell line.

Materials and Methods

Materials

Digoxin was provided by Jianglai Biological Technology Corporation (Shanghai, China). digoxin was dissolved in dimethyl sulfoxide (DMSO) Sigma-Aldrich (St. Louis, MO, USA) to prepare as 100 mM stock solution and was stored at 4°C for further use.

Culture of MM-231 Cells

The MM-231 breast cancer cell line was obtained from Jeanniou Biological Technology Corporation (Guangzhou, China). When the cells underwent logarithmic growth phase, the different concentration of the digoxin was diluted into 50 nmol/L (50 nM), 125 nmol/L (125 nM), 200 nmol/L (200 nM) from final solution. Simultaneously, equal volume of 75% ethanol was set as vehicle control (0 nM).

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MTT assay

Cell proliferation mediated by digoxin on MM-231cell was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were cultured as 1×10^7cells/mL and seeded in 96-well plates. After 24 h incubation, they were exposed to stepwise concentrations of digoxin and the ethanol vehicle. At the end of 24 h, 48 h and 72 h co-incubations, each well was added with 20 μL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, St. Louis, MO, USA) following 4 h of reaction at 37°C. ELISA test was used to determine the optical density (OD) of each well at 490 nm using a microculture plate reader (Bio-Tek Instruments, Winooski, VT, USA). The inhibition rate of cell growth was measured by the formula as follows: % inhibition=$[(1-OD_{\text{administered average}})/OD_{\text{control average}}\times100\%]$.

Apoptotic Morphological Screening

Acridine orange staining was applied to evaluate morphological changes in MM 231 cells. After the incubation for 48 h, the cells were digested by 0.25% trypsogen before they were washed twice with phosphate-buffered saline (PBS) (pH 7.4). When the cell density was confirmed in 5×10^6/mL, 95 uL cell suspension was collected adding 0.1% acridine orange solution. Mixture dyes (10 ul) were added in slides, and the cell morphology was captured via a fluorescence microscopy (Olympus, Tokyo, Japan).

Flow Cytometry Detection

After the intervention with digoxin for 48 h, cell density was proportioned as 1×10^6/mL for test. Annexin V-FITC combined propidium iodide (PI) staining was introduced in apoptosis-related assessment of MM231 breast cancer cells. Apoptosis-like tumor cells showed the outcomes of positive Annexin V- FITC for apoptotic count and negative PI for non-apoptotic count. Finally, the results were harvested after flow cytometry instrument (Epics-XL, Beckman Coulter, Brea, CA, USA) determination.

Western Blotting Analysis

Cell total protein was sampled by radioimmunoprecipitation assay (RIPA) lysis buffer (50 mmol/L Tris, pH 7.12; 1 mmol/L EDTA; 1 mmol/L PMSF, 1:1000). Protein content was identified through Bradford method with a spectrophotometer. An equal amount of protein samples from each group was electrophorezed by using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for 20 min, followed by proteins (50 μg) transferring to the polyvinylidene difluoride (PVDF) membrane. Rabbit-monoclonal antibodies of Bcl-2 and Bax (1:1200; Sigma-Aldrich, St. Louis, MO, USA) were added in the protein-contained membrane at 4°C overnight, followed by the incubation with anti-rabbit secondary antibody (1:400; Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 1 h. The OD value of positive band was determined by enhanced chemiluminescence (ECL) system (Beyotime, Jiangsu, China), and the final data were presented as a ratio to GAPDH control.

Statistical Analysis

Results were expressed as the mean ± SD. Comparisons between different age control groups were tested using one way analysis of variance (ANOVA) and Fisher's *t*-test. Furthermore, LSD was used to validate ANOVA. The *p*-value less than 0.05 was considered as significant difference.

Results

Digoxin Inhibited MM231 Cell Proliferation

MTT assay was conducted to evaluate the digoxin-mediated anti-proliferative effect on MM231 cell. Compared to vehicle control, digoxin treated MM231 showed attenuated cell proliferation in a dose-dependent manner ($p<0.05$), marked by reductions of cells count in different concentrations of digoxin (Figure 1A). As shown in OD value, that reflects the inhibitory effect, digoxin groups resulted in time-dependently blocking the cell growth of MM231 with notably reduced OD value, when compared to that in vehicle groups. Particularly, high-dose of digoxin (200 nM) had the most robust inhibitory effect on MM231 proliferation (Figure 1B).

Digoxin Induced MM231 Cell Apoptosis

Acridine orange staining and flow cytometry assay were carried out in MM231 cells for morphological examination and apoptotic assessment. As shown in Figure 2, untreated cells in vehicle control had few intracellular lesions and plenty of elliptical cells, whereas digoxin-administered MM231 cells presented significant morphological alterations, such as vacuolar degeneration, cell collapse or fragmentation, and apoptotic patterns. The flow cytometry analysis showed that with digoxin increased positive proportion of apoptosis cells in dose-dependent manner (Figure 3).

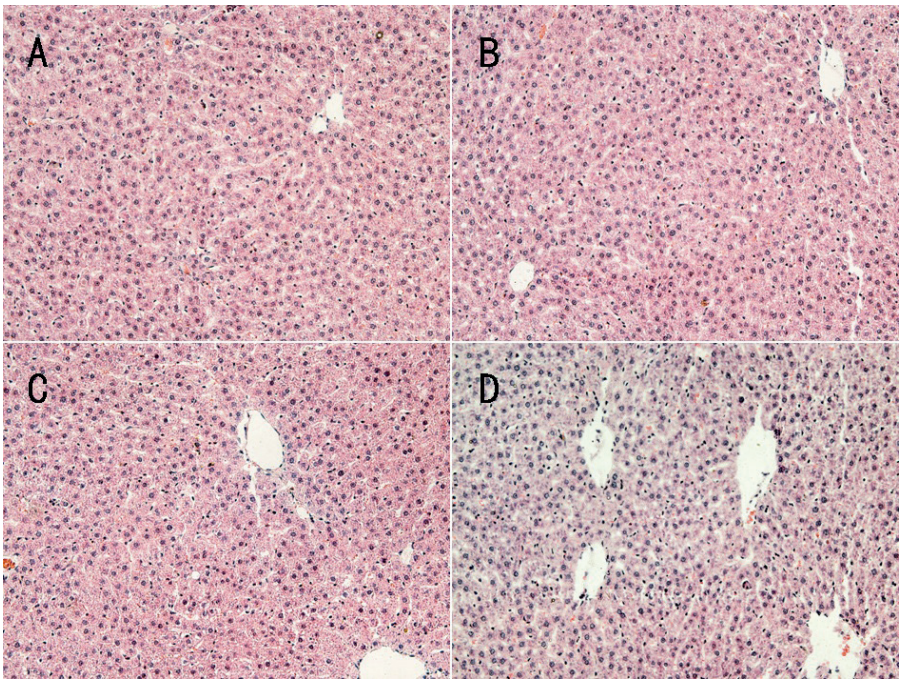

Figure 1. ***(A)*** Digoxin resulted in the inhibition of MM231 cell proliferation through MTT assay at the endpoint of 24, 48, 72 h. ***(B)*** Digoxin blocked the cell growth of MM231 characterized by decreased OD value. Five independent experiments were repeatedly conducted in this study. Data were determined with one-way ANOVA followed by LSD post-hoc test, and final results were expressed as the mean ± SD. Notes: **p*<0.05 *vs*. vehicle control (0 nM).

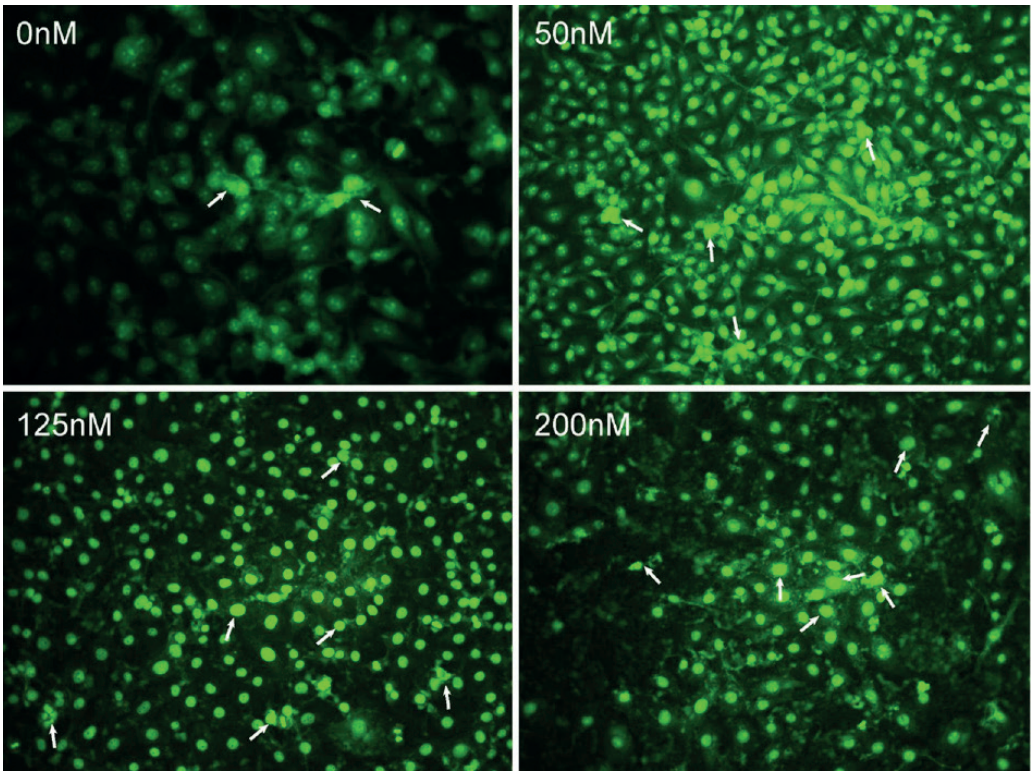

Figure 2. Digoxin induced morphological lesions in MM231 cell as shown in acridine orange staining (200×). *Arrows* pointed to the lesioned cells. Data were determined with one-way ANOVA followed by LSD post-hoc test, and final results were expressed as the mean± SD. Notes: **p*<0.05 *vs*. vehicle control (0 nM).

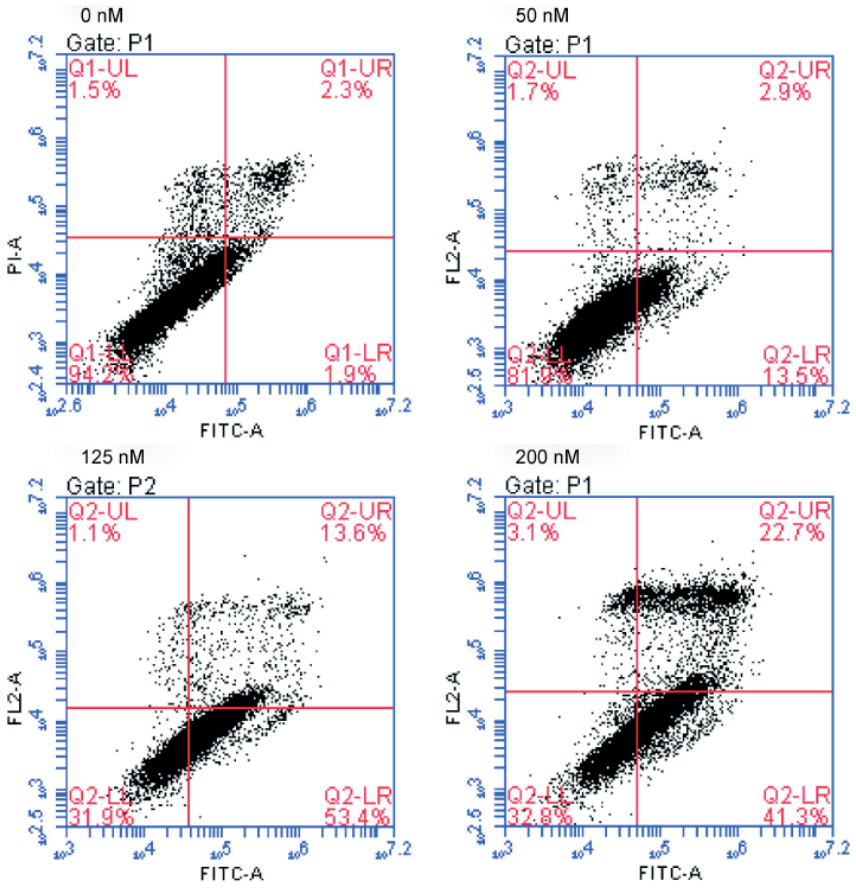

Figure 3. Digoxin elevated the apoptotic proportions through flow cytometry analysis in MM231 cells. Five independent experiments were repeatedly conducted in this study. Data were determined with one-way ANOVA followed by LSD post-hoc test, and final results were expressed as the mean±SD. Notes: *$p<0.05$ *vs.* vehicle control (0 nM).

Digoxin Lowered Bcl-2 Expression and up-Regulated Bax Production

Further, some key regulatory proteins associated apoptosis (such as Bcl-2 and Bax) were tested in digoxin-administered cells. Western blotting data showed that protein expression of Bcl-2 in MM231 cells was effectively down-regulated after digoxin (50, 125, 200 nM) exposure for 48 h, whereas Bax protein level was notably increased ($p<0.05$). Accordingly, the Bax/Bcl-2 ratios in digoxin-administered groups were distinctly elevated ($p<0.05$) (Figure 3).

Discussion

Universally, tumorigenesis and its development may be due to the failure of controlling tumor's proliferation. Currently, the effectiveness of intervention on cancer is evaluated by, at least in part, the ability of inhibiting tumor cells growth or inducing apoptosis[8]. In comparison with existing chemotherapy, digoxin exhibited anti-tumor effects through suppressing cell proliferation or inducing its apoptosis, whereas digoxin therapy showed beneficial effect in relieving breast cancer symptoms[9]. Here, we conducted the experiments using as a cell model *in vitro* to explore the underlying mechanism of digoxin-mediated anti-tumor role on MM231 breast cancer.

MTT serves as a tool for developing anti-tumor medication[10]. In this study, the data showed that tumor cells in vehicle treated group resulted in uncontrolled growth and reflected the high proliferative characteristic of breast cancer cells. Instead, digoxin-treated MM231 cell counts were significantly decreased, implying that initial pharmacological activity of digoxin was due to the anti-proliferative action.

Apoptosis presents a phenomenon of cell death in specialized machinery to destruct itself[11]. If apoptotic process is dysregulated, tumor tissue will develop rapidly, leading to a malignant

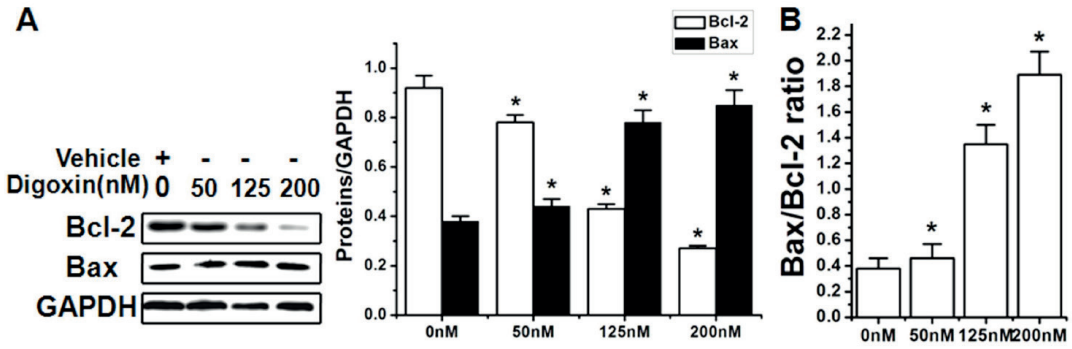

Figure 4. ***(A)*** Digoxin down-regulated Bcl-2 protein level and increased Bax expression, whereas the Bax/Bcl-2 ratio in MM231 cells was elevated ***(B)***. Data were determined with one-way ANOVA followed by LSD post-hoc test, and final results were expressed as the mean±SD. Notes: **p*<0.05 *vs*. vehicle control (0 nM).

transformation. Bcl-2, a regulator classified as an oncogene that mediates cell death, functioned as a vital anti-apoptotic protein[12]. As shown in pathogenetic process, the over-production of the anti-apoptotic Bcl-2 protein in cell can accelerate the cancer development. Bcl-2 may form heterodimers and play as an anti-apoptotic regulator. In contrast, apoptosis regulator Bax triggers cell death via binding to and offsetting the Bcl-2 protein[13]. To induce apoptosis in cancer tissue, Bax first undergoes the conformational change, followed by functional shift via interacting with organelle membrane, especially in mitochondrial membrane[14]. In this work, vehicle treated MM231 cells showed elevated Bcl-2 protein and decreased Bax expression, suggested that MM231 cells had the physiological feature of reducing apoptosis through counter regulation of Bcl-2/Bax ratio. After administration of digoxin, the cell endogenous proteins were altered as a result of increased Bax/Bcl-2 ratio. digoxin-mediated anti-tumor benefits were accordant with the presented outcome of elevated apoptotic numbers. Therefore, we extrapolated that digoxin-administered MM231 cell elicits apoptosis via changing intracellular Bax/Bcl-2 proportion, resulting in perforation of mitochondrial membrane, and inducing downstream cascaded events associated with apoptosis.

Conclusions

We observed that digoxin effectively inhibits breast cancer cell proliferation via induction of apoptosis. Thus, this study provides the evidence that digoxin is expected to become a promising candidate for breast cancer therapy.

Acknowledgments

This work was funded by grants from the two National Natural Science Foundation of China (81302395 and 81072254), the Natural Science Foundation of Hebei Province (C2011401024), and the Science and Technology Research Projects in the Colleges and Universities of Hebei Province (ZD20131035).

Conflict of Interest

The Authors declare that they have no conflict of interest.

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