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Anti-proliferative and anti-invasive effects of garcinol from *Garcinia indica* on gallbladder carcinoma cells

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Received February 10, 2018, accepted March 16, 2018

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Pharmazie 73: 413-417 (2018) *doi: 10.1691/ph.2018.8366*

Garcinol, a natural histone acetyltransferase inhibitor, has been reported to exhibit significant anti-proliferative activity in various cancer cell types. However, no information is available about the anti-cancer effects of garcinol on gallbladder carcinoma cells (GBC). In this study, GBC cells (GBC-SD and NOZ) were treated by garcinol and subjected to Cell Counting Kit-8 (CCK-8), and GBC-SD cells were selected for further transwell chamber assay, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis. Our results indicated that garcinol could significantly inhibit the growth of GBC cells in a dose- and time-dependent manner. It also inhibited the invasion of GBC-SD cells in a dose-dependent manner. Garcinol treatment decreased the activity of matrix metalloproteinase 2 (MMP2) and MMP9 by the downregulation of mRNA levels, and these two enzymes are critical to tumor invasion. Treatment with garcinol also decreased Stat3 and Akt activation in GBC-SD cells. Taken together, the effects of garcinol on GBC-SD cells may be associated with the suppression of Stat3 and Akt signaling pathways, which may contribute to inhibiting their downstream targets such as mRNA levels of MMP2 and MMP9.

1. Introduction

Gallbladder carcinoma (GBC) is the most common malignant tumor of the biliary tract and one of the common malignant tumor of the gastrointestinal tract (Zhu et al. 2010). Early diagnosis of GBC is difficult due to a lack of specific and reliable tumor markers (Sharma et al. 2017). Most GBC patients are diagnosed at an advanced, inoperable stage with detectable distant metastases. Up to now, surgery is considered as the unique curative therapy for GBC. However, postoperative recurrence is an intractable problem. It is therefore important to identify reliable drugs for prevention and treatment of GBC.

Cancer therapy with phytochemicals has attracted great attention because of its safety and effectiveness (Wang et al. 2014; Hosseini and Ghorbani 2015; Zhang et al. 2018). Garcinol ($C_{38}H_{50}O_6$, Fig. 1) is a polyisoprenylated benzophenone extracted from the fruit of *Garcinia indica* (Balasubramanyam et al. 2004). It has been reported to exhibit various pharmaceutical effects, including anti-bacterial, anti-oxidative, gastroprotective, anti-inflammatory and anti-cancer properties (Liu et al. 2015; Behera et al. 2016). Garcinol has exerted significant anti-tumor effects on lung cancer cell lines (H441 and A549) (Wang et al. 2017; Huang et al. 2018), hepatic cancer cell lines (C3A, HUH-7, PLC/PRF5, and HepG2) (Sethi et al. 2014), prostate cancer cell lines (DU-145, PC-3, and LNCaP) (Wang et al. 2015), oral cancer cell lines (SCC-4, SCC-9 and SCC-25) (Aggarwal and Das 2016), breast cancer cell line (MCF-7) (Ye et al. 2014), colon cancer cell line (HT-29) (Ranjbarnejad et al. 2017), pancreatic cancer cell lines (BxPC-3 and Panc-1) (Parasramka and Gupta 2011), leukemic cell line (HL-60) (Pan et al. 2001). However, no information is available about the anti-cancer effects of garcinol on GBC. Therefore, this study investigated the anti-cancer activity of garcinol towards the human GBC cell lines (GBC-SD and NOZ) *in vitro*. Our results in this study will help expand the understanding of the anti-proliferative and anti-metastatic effects of garcinol and the related mechanism(s) on GBC. Meanwhile, this study will also contribute to the development of garcinol as a potent anti-cancer agent for GBC.

2. Investigations and results

2.1. Garcinol inhibited the proliferation of GBC cells

The proliferation inhibition effect of garcinol on GBC cells (GBC-SD and NOZ) was determined using Cell Counting Kit-8 (CCK-8). Within definite dose and time, garcinol could indeed inhibit cell proliferation in a dose- and time-dependence manner

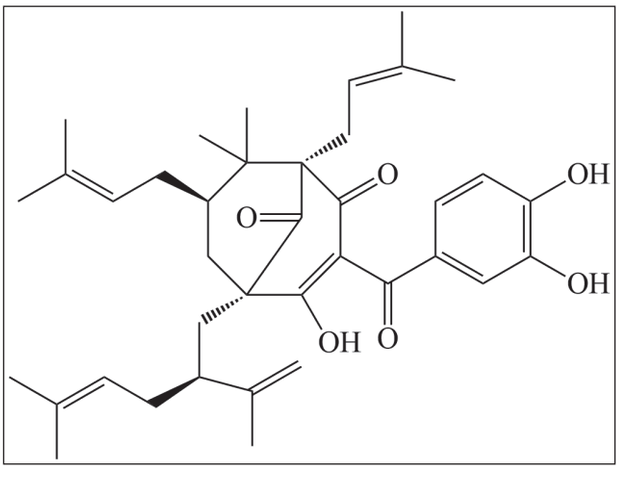


Fig. 1: Structure of garcinol.

(Fig. 2). Meanwhile, the IC_{50} values of garcinol at the same exposure time for GBC-SD cells were slightly less than that for NOZ cells (e.g., IC_{50} values for GBC-SD cells at 24 and 48 h were 13.52±0.47 μM and 10.14±0.25 μM, respectively, and the values for NOZ cells were 15.76±0.66 μM and 10.77±0.68 μM, respectively). So GBC-SD cells were selected to further investigate the effect of garcinol on the invasion of GBC cells.

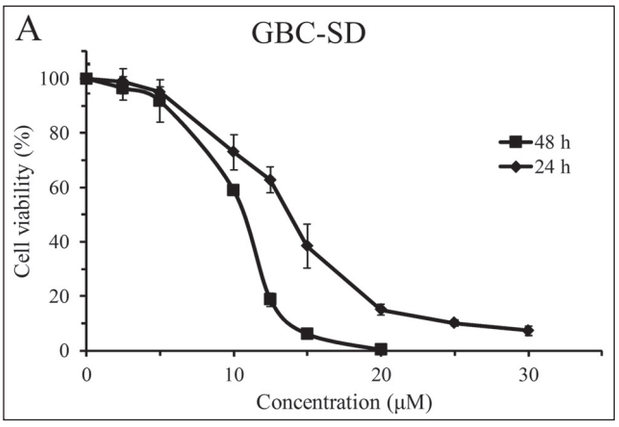


Fig. 2: Garcinol inhibited cell proliferation in gallbladder cancer (GBC) cells. GBC-SD (A) and NOZ (B) cells were treated with various concentrations of garcinol for 24 and 48 h. Cell viability was determined by the CCK-8 assay and calculated as a ratio, comparing it to the vehicle-treated cells. Data are presented as mean ± standard deviation (SD) from three independent experiments.

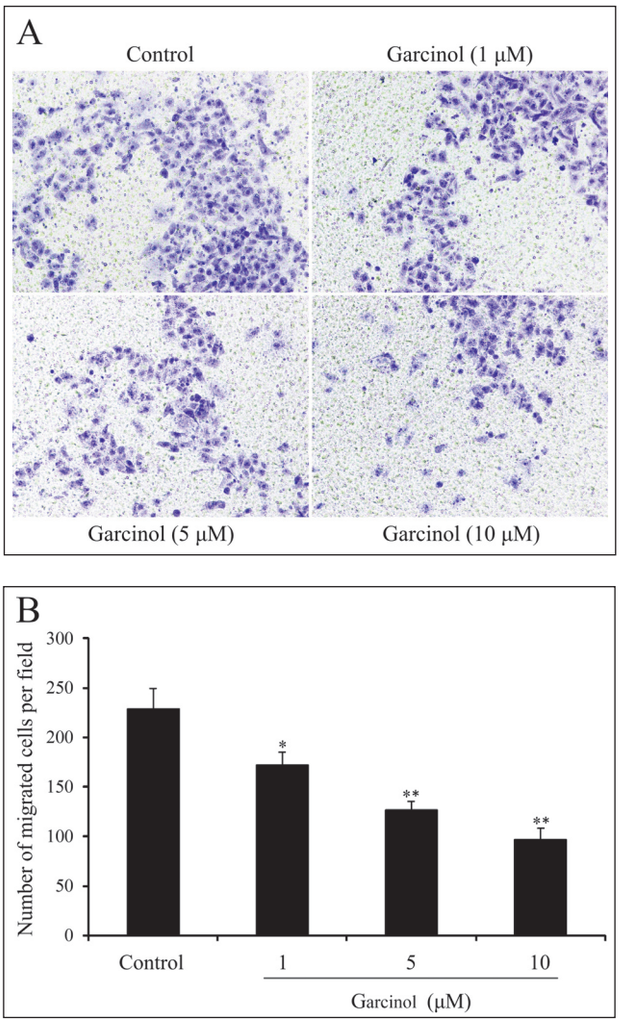


Fig. 3: Effects of garcinol on GBC-SD cell invasion using a transwell assay. Cells suspended in serum-free DMEM were overlaid in the upper chamber of each transwell. Following incubation with different concentrations of garcinol for 24 h, penetrating cells were stained with crystal violet and recorded under a microscope mounted with a CCD camera. A. Images depicted invasion of GBC-SD cells. B. Quantified data are expressed as mean ± SD from three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. control group (0 μM).

2.2. *Garcinol inhibited the invasion of GBC-SD cells*

Given that most clinically diagnosed patients with GBC have invasion and/or metastasis, this study focuses on the effect of garcinol on the invasion of GBC cells. As shown in Fig. 3, the collagen invasion assay showed that garcinol at the concentrations of 1-10 μM significantly reduced the rate of GBC-SD cell invasion when compared with the control group after the cells were treated for 24 h ($P < 0.01$ or 0.001). Furthermore, garcinol at concentrations of 1 and 5 μM did not significantly reduce the viability of GBC-SD cells after the cells were treated for 24 h (Fig. 2A). These results suggested that the inhibition of GBC-SD cell invasion by garcinol was not the result from a reduction of cell viability. These observations suggested that garcinol could inhibit the invasive capacity of GBC-SD cells in a dose-dependent manner.

2.3. *Inhibition of MMP2 and MMP9 expression by garcinol*

Matrix metalloproteinases (MMPs) are responsible for the degradation of extracellular matrix. Among these MMPs, MMP2 and MMP9 are known to induce aggressive invasion and metastasis of different cancer types (Gao et al. 2015; Tang et al. 2016a; Zeng et al. 2016b). Quantitative real-time polymerase chain reaction (qRT-PCR) detection demonstrated that garcinol at 5 and 10 μM significantly reduced the mRNA levels of MMP2 and MMP9 in GBC-SD cells after the cells were treated for 24 h (Fig. 4).

2.4. *The effects of garcinol on GBC-SD cells partly through Stat3 and Akt signaling pathways*

Previous studies showed that garcinol could suppress the growth of cancer cells *via* modulation of various signaling pathways (e.g., NF-κB, Stat3 etc.) (Behera et al. 2016). Three key regulatory pathways (NF-κB, Stat3, and Akt) were selected to further investigate the effect of garcinol on GBC cells. The endogenous levels of Stat3 and phosphorylated Stat3 [p-Stat3 (Tyr705 or Ser727)] were inhibited dose dependently when GBC-SD cells were treated with garcinol and analyzed by Western blotting (Fig. 5A). The activation of Stat3 involves phosphorylation at Tyr705 and Ser727 (Sakaguchi et al. 2012), and these results suggest a potent inhibitory action of garcinol on constitutive Stat3 signaling in GBC-SD cells, which is consistent with previous reports in other cancer cell-line models (Ahmad et al. 2012). As shown in Fig. 5B, GBC-SD cells with garcinol treatment also induced a significant and dose-dependent

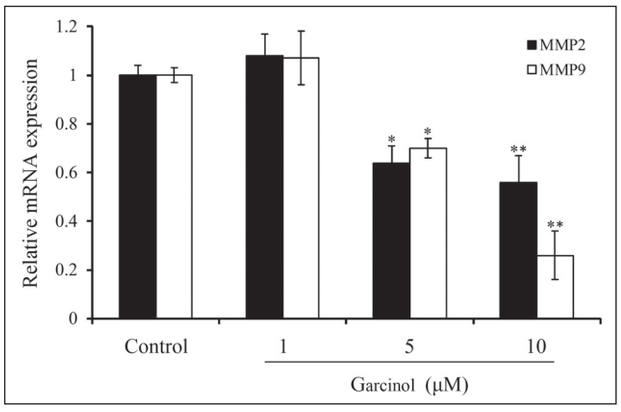
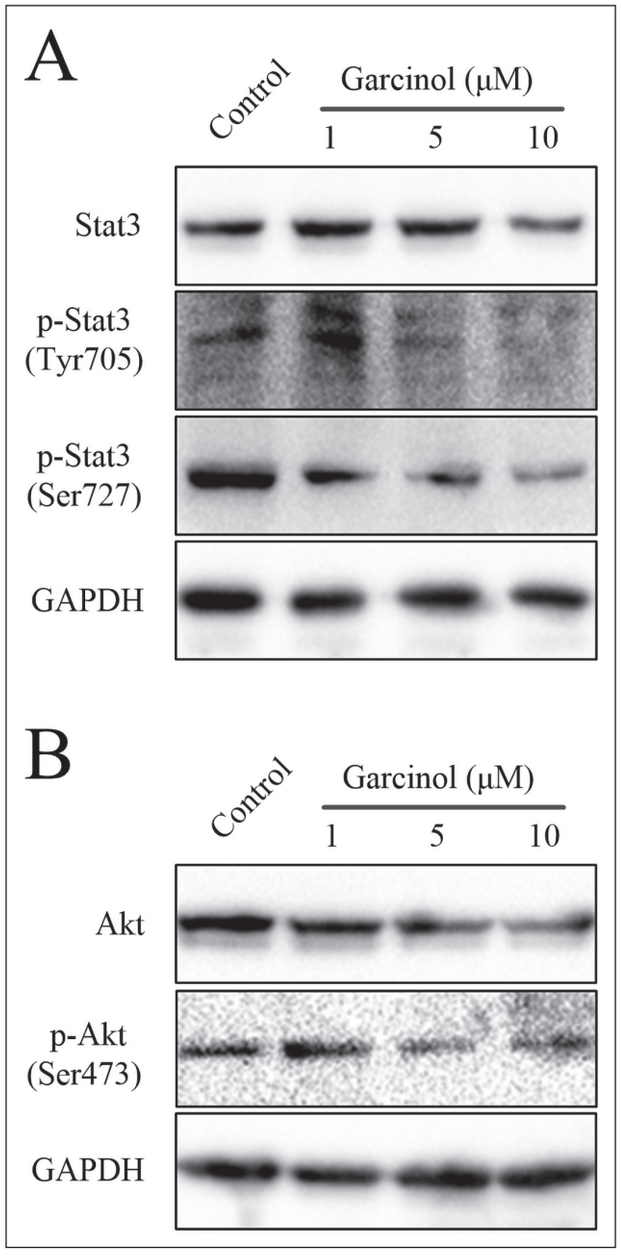


Fig. 4: Garcinol suppressed mRNA expression of MMP2 and MMP9. GBC-SD cells were treated with different concentrations of garcinol for 24 h. Expression of MMP2 and MMP9 were determined by qRT-PCR and normalized against GAPDH control. Quantified data are expressed as mean ± SD from three independent experiments. $*P < 0.01$, $**P < 0.001$ vs. control group (0 μM).



reduction in total Akt and p-Akt (Ser473). However, garcinol did not cause significant changes in the expression of total NF-κB p65 and p-NF-κB p65 (Ser536) (Fig. 5C). Taken together, these results indicated that garcinol inhibited Stat3 and Akt signaling pathways, suggesting that these changes mediated the effects of garcinol on GBC-SD cells.

3. Discussion

Garcinol is a natural compound extracted from *Garcinia indica* fruit rind. Dietary phytochemical agents are very favorable because of their safety, low toxicity and general acceptance (Dai and Mumper 2010). Garcinol exerted significant anti-proliferative activity in multiple cancer cell lines but it had little impact on the growth of normal cells [human bronchial epithelial cell line BEAS-2B (Huang et al. 2018) and peripheral blood mononuclear cells (Aggarwal and Das 2016)]. These observations suggest the probable tumor cell-selective and normal cell safety of garcinol therapeutic activity. In addition, most clinically diagnosed patients with GBC have invasion and/or metastasis and so anti-invasion is important for the treatment of GBC. In this study, we have shown that garcinol as a potential anti-invasive agent markedly inhibited the invasive capacity of GBC-SD cells in a dose-dependent manner. The anti-invasive capacity of garcinol may be important in decreasing mortality and improving the survival time in gallbladder cancer patients.

Garcinol could positively or negatively regulate some different cellular pathways (e.g., NF-κB, Stat3 etc.) (Behera et al. 2016). Garcinol had no significant effect on NF-κB pathway in this study. However, many reports showed that garcinol could lead to downregulation of the NF-κB pathway, which appeared to be the underlying mechanism in the induction of apoptosis (Ahmad et al. 2010, 2011). Low concentrations of garcinol (1-10 μM) might be not enough to induce apoptosis and influence NF-κB pathway. Furthermore, we also demonstrated that garcinol inhibited Akt and Stat3 signaling pathways, which was consistent with results obtained in previous studies (Liao et al. 2005; Ahmad et al. 2012; Li et al. 2013). Akt was involved in the regulation of the Stat3 signaling pathway (Subramaniam et al. 2013). Thus, it is possible that the inhibition of Akt pathways may contribute to Stat3 inhibitory effect of garcinol as observed in GBC-SD cells. In addition, we then determined mRNA expression levels of MMP2 and MMP9 were significantly downregulated by garcinol in GBC-SD cells. It

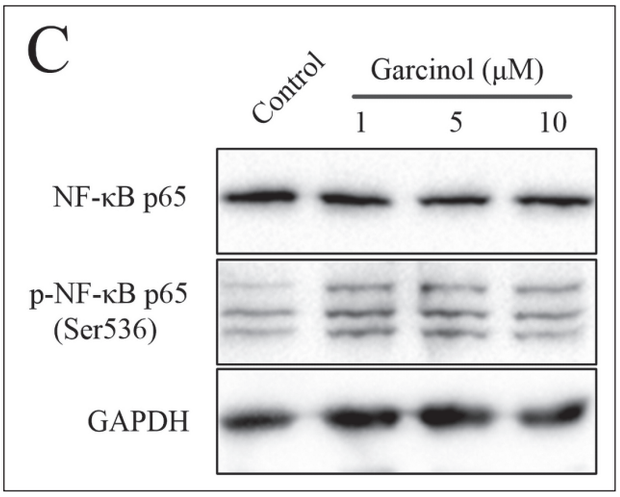


Fig. 5: Effect of garcinol treatment on the expression of total Stat-3, p-Stat-3 (Tyr705), p-Stat-3 (Ser727), Akt, p-Akt (Ser473), NF-κB p65 and p-NF-κB p65 (Ser536) in GBC-SD cells. Cells were treated with 0, 1, 5 or 10 μM garcinol for 24 h (A) or 6 h (B and C), and these proteins were detected by Western blot assay. GAPDH was used as the internal loading control.

is suggested that MMP2 and MMP9 can be induced in Akt and Stat3 signaling pathways (Gao et al. 2015a; Zhang et al. 2015b). So the downregulation of Akt and Stat3 pathways might reduce the mRNA levels of MMP2 and MMP9. Future studies are required to analyze the precise mechanism(s) of garcinol and to exploit its full potential for gallbladder carcinoma chemotherapy.

4. Experimental

4.1. Cell culture and garcinol treatment

The human gallbladder carcinoma cell lines GBC-SD and NOZ which showed correct short tandem repeat (STR) profiles, were purchased from Guangzhou Cellcook Biotech Co., Ltd (china). GBC-SD and NOZ cells were cultured in high-glucose DMEM medium (Hyclone, GE Healthcare Life Sciences, Piscataway, NJ, USA) supplemented with 10% fetal bovine serum (FBS) (Gemini, Calabasas, CA, USA) at 37 ºC in 5% CO_2 humidified atmosphere, and routinely passaged at 2-3 day intervals. Garcinol was isolated from *Garcinia indica* fruit rind according to a method described previously (Sang et al. 2001). Garcinol was dissolved in DMSO (Solarbio Science and Technology, Beijing, China) to a 50 mM stock concentration. The final DMSO concentration was accounted for less than 0.1% (v/v). 0.05% DMSO treated cells were taken as a vehicle control.

4.2. Cell viability assay

The effect of garcinol on cell viability was assessed by using CCK-8 (Dojindo, Shanghai, China). A total of 1.2×10^4 cells/well were seeded in 96-well plates overnight and then treated with varying concentrations of garcinol (0, 2.5, 5, 10, 12.5, 15, 20, 25, 30 μM). Cells were either incubated for 24 or 48 h at 37 °C in a humidified incubator, and then 10 μL CCK-8 solution was added into each well and incubated for another 1 h. Absorbances were measured at 450 nm in each well by a microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA). The results were the average of three independent experiments done over multiple days. The percentage of cell viability was calculated using the following formula: Percentage cell viability = (OD of the experiment samples/OD of the control) × 100. The IC_{50} value of garcinol against GBC-SD and NOZ cells was calculated using GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA, USA).

4.3. Cell invasion assay

Cell invasion assay was performed using 8-μm pore size transwell chambers (Corning, NY, USA). The upper side of the transwell filter inserts was coated with 80 μL of diluted (1:8 in serum-free medium) Matrigel (BD Biosciences, Bedford, MA, USA) in 24-well plates. GBC-SD (1×10^5) cells in 200 μL of serum-free high-glucose DMEM medium and added to the upper chamber containing various concentrations of garcinol (1, 5, or 10 μM). The bottom chambers were filled with 500 μL high-glucose DMEM medium containing 20% FBS. After 24 h, non-invasion cells were removed, and the invasive cells were fixed with 95% ethanol, stained with 0.1% crystal violet, photographed (×100). Tests were replicated via three independent experiments.

4.4. Western blot analysis

After 6 or 24 h of garcinol (0, 1, 5 and 10 μM) treatment, GBC-SD cells were washed with PBS and lysed in cell lysis buffer. Total cell lysates were separated on 8% SDS-PAGE gels, and then transferred onto polyvinylidene difluoride (PVDF) membranes in a standard transfer buffer. After blocking with 1% BSA (blocking solution) for 1.5 h at room temperature, the membranes were incubated with primary antibodies diluted in blocking solution overnight at 4 °C. After the membranes were washed three times with TBST, they were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. After extensive washing in TBST, the protein signals were visualized using enhanced chemiluminescence (ECL) Western Blotting Substrate (Proteintech, Wuhan, China) and ECL system (Bio-Rad, Hercules, CA, USA). Equal protein loading was assessed by the expression of GAPDH.

4.5. RNA isolation and qRT-PCR

In brief, cells from garcinol-treated (1, 5 and 10 μM) and -untreated groups were seeded in 6-well plates (1×10^6 cells/well) and cultured for 24 h. Total RNA was extracted from each experimental group using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified by spectrophotometry. Next, 1 μg of total RNA was reverse transcribed with the PrimeScript™ RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. QRT-PCR analyses for MMP2 and MMP9 were performed using an EvaGreen 2 × qPCR MasterMix-No Dye Kit (Abm, Vancouver, Canada). Gene expression levels were analyzed following normalization to the gene GAPDH using the $2^{-\Delta\Delta CT}$ method. The specific primer pairs used were: MMP2, 5′-CCCCAAAACGGACAAAGAG-3′ (forward) and 5′-CTTCAGCACAAACAGGT TGC-3′ (reverse); MMP9, 5′-TACCACCTCGAACTTTGAC-3′ (forward) and 5′-CT GAGGAATGATCTAAGCCC-3′ (reverse); GAPDH, 5′-CAGGAGGCATTGCTGAT GAT-3′ (forward) and 5′-GAAGGCTGGGGCTCATTT-3′ (reverse).

4.6. Statistical analysis

All data are expressed as mean±SD. Statistical analysis was performed using Statistical Product and Service Solutions (SPSS, Chicago, Illinois, USA) Vision 22.0. Results were considered statistically significant only if the *P* value was less than 0.05 using one-way ANOVA.

Acknowledgement: This research was supported by the National Natural Science Foundation of China (31670859), CAMS Innovation Fund for Medical Science (2017-I2M-1-016), and Natural Science Foundation of Tianjin (15KPXM01SF056).

Conflicts of Interest: None declared.

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