Sanjie Yiliu Formula Inhibits Colorectal Cancer Growth by Suppression of Proliferation and Induction of Apoptosis

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ABSTRACT: Colorectal cancer (CRC) is one of the most common malignancies worldwide. As current therapies toward CRC, including chemotherapy and radiotherapy, pose limitations, such as multidrug resistance (MDR) as well as the intrinsic and potential cytotoxic effects, necessitating to find more effective treatment options with fewer side effects, traditional Chinese medicine (TCM) has an advantage in complementary therapies. In the present study, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assays), trypan blue staining, colony formation, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, cell cycle determination, and Annexin V-FITC/PI staining were used to examine the efficacy of Sanjie Yiliu Formula (SJYLF) against CRC proliferation and to investigate its underlying molecular mechanisms through protein expression of various proapoptotic factors by quantitative polymerase chain reaction (q-PCR) and Western blotting. This four-herb-TCM SJYLF can be suggested as one of the decoctions clinically effective in late-stage cancer treatment. Our results suggest that SJYLF robustly decreased the viability of only CRC cell lines (HCT-8, SW-480, HT-29, and DLD-1) and not the normal human kidney cells (HK-2). Moreover, SJYLF significantly suppressed proliferation and induced apoptosis in HCT-8 and downregulated cyclin D1, CDK4, and BCL-2, while Bax expression was upregulated at both mRNA and protein expression levels.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common and fatal malignancies worldwide. An increasing number of CRC cases, late diagnosis, high risk of tumor recurrence, and multidrug resistance (MDR) make it the fourth cause of cancer-related deaths globally.1−3 It is estimated that the number of new CRC cases may increase to 2.5 million annually by 2035.4 Despite multiple available treatments like chemotherapy, surgical resection, targeted therapy, and radiotherapy, CRC diagnosis remains poor in patients. CRC is mostly diagnosed in the last stages of the disease, making eradicating cancer impossible.5 Conventional chemotherapy, making use of 5-fluorouracil (5-FU), FOLFOX, and platinum-based drugs, e.g., cisplatin and oxaliplatin,4,5 has its limitations, such as low efficacy, multidrug resistance (MDR), intrinsic and potential cytotoxic effects, and relapse in more than 50% cases.5,6 Therefore, finding more selective and effective chemotherapeutic options with fewer side effects remains an urgent need and a challenge to treat CRC patients.

Traditional Chinese medicines (TCMs) have been appreciated for more than 5000 years and have evolved their diagnosis methods, treatment procedures, and therapies.7 Since the past decade, TCM has gained considerable attention as a complementary and alternative treatment for various types of cancers.8,9 Recent studies appreciate the contribution of TCM for the cure of CRC with certain benefits, including toxicity attenuation, inhibition of tumorigenesis, effective remedy, and decreased risks of metastasis.10,11 TCM herbals in a discrete dose and form of concoction/formula have proved to be a better long-term remedy in stage II and III CRC patients.12,13 Furthermore, TCM-based remedies in combination with chemotherapy, radiotherapy, and conventional medicine were found more effective in cancer patients.14−16 For these reasons, TCM can be considered as an advantageous and alternative remedy for CRC.

Several TCM is being used as a concoction or formula composed of various herbs, minerals, and animal extracts or their components. Their synergistic action seems to be more effective against cancer and other related diseases.17 Xiaotan Sanjie and Ruanjian Sanjie are multitherb decoctions found effective in gastric and breast cancers, respectively. These
formulae were found to induce apoptosis in cancer cells.\textsuperscript{18,19} Another multitherb decoction, BushenShugan Formula (BSF), was found effective in treating middle- and late-stage lung cancers,\textsuperscript{20} while Ginseng Radix and Astragali Radix showed promising therapeutic results against CRC.\textsuperscript{21,22} TCM Sanjie Yiliu Formula (SJYLF) is a four-component anticancer decoction composed of Rhizoma Pinelliae Preparatum (Fabanxia), Glabrous Sarcandra herb, Thunberg Fritillary bulb, and ground beetle species (Figure 1). Both meta-analysis and systematic review show that individual components of SJYLF may play an important role in enhancing life quality in cancer patients by relieving symptoms and decreasing the harmful effects of undergoing chemotherapy\textsuperscript{23,24} (China patents CN101744849A, CN102688404A). Nevertheless, no detailed studies are available about the precise anticancer activities and mechanisms of the SJYLF component decoction. In the present study, we have investigated the antiproliferative activity of SJYLF. An SJYLF concentration of 0–1.5 mg/mL was selected for subsequent analysis.

**RESULTS**

**SJYLF Suppresses Colorectal Tumor Growth.** For 24 and 48 h, the anticancer effect of SJYLF was evaluated with the help of MTT assays using CRC cell lines (HCT-8, SW-480 HT-29, and DLD-1). An increasing SJYLF extract concentration (0–2 mg/mL) was added to the cells. Cell viability was also monitored for human kidney cell lines (HK-2). Figure 2a–d shows a marked reduction of the CRC cell line viability by SJYLF, in terms of both time- and dose-dependent manner. Figure 2e shows that SJYLF is selectively toxic to the CRC cell line and not toward normal human cells (HK-2).

Table 1 shows that IC50 values for SJYLF were 1.25 ± 0.20 and 0.91 ± 0.11 mg/mL for HCT-8 cells; 1.94 ± 0.09 and 1.09 ± 0.10 mg/mL for SW-480 cells; 2.64 ± 0.04 and 1.54 ± 0.08 mg/mL for HT-29 cells; and 1.90 ± 0.17 and 1.19 ± 0.08 mg/mL for DLD-1 cells at 24 and 48 h, respectively. HCT-8 cells show growth inhibition by 0.91 ± 0.11 mg/mL with the lowest effective dose of SJYLF. SJYLF antiproliferative activity was found to be concentration-dependent. As SJYLF extract showed better IC50 against HCT-8 cell line, these cells were further used to determine the mechanism of action for SJYLF. An SJYLF concentration of 0–1.5 mg/mL was selected for subsequent analysis.

**SJYLF Suppresses the Proliferation of HCT-8 Cells.** To investigate the inhibition mechanism and confirm the proliferation suppressant effect of SJYLF toward HCT-8 cells, trypan blue staining, colony formation, and DAPI immunofluorescence staining methods were utilized. Cells were treated with 0.5, 1.0, and 1.5 mg/mL of SJYLF and stained with trypan blue to analyze proliferation factors.
SJYLF Stimulates Apoptosis in HCT-8 Cells. Flow cytometric analysis was used to confirm the apoptotic effects of SJYLF on HCT-8 cells. For 48 h, the cells were treated with an increasing concentration of SJYLF (0.5, 1.0, and 1.5 mg/mL). Annexin V-FITC and PI stains were used for labeling. Figure 6a–d indicates that after the treatment of SJYLF, there was a considerable increase in the percentage of apoptotic cells in a dose-dependent manner. At a concentration of 1.5 mg/mL, SJYLF induced 23.42 ± 2.58% apoptotic events in comparison to that of 0.87 ± 1.71% apoptotic events in the negative control (Figure 6e). Consequently, it described that SJYLF induced apoptosis in HCT-8 cells.

Effects of SJYLF on Cell Cycle Progression in HCT-8 Cells. Cell cycle involves a sequential process starting from the cell’s last to current mitosis. It comprises four phases including the Gap 1 phase (G1 phase), deoxyribonucleic acid (DNA) synthesis phase (S phase), Gap 2 phase (G2 phase), and M phase (mitosis phase). Interphase includes G0, S, and G2 phases. After the interphase and mitotic phase, a parental cell divides into two progeny cells with similar genetic material. Figure 7a–d shows that cell apoptosis was induced by SJYLF through cell cycle arrest. Treatment with SJYLF enhanced the cells’ concentration in the G0 and G1 phases, while the decreased concentration of cells in the G2 phases was also observed. SJYLF treatment (at a concentration of 1.5 mg/mL) induced the cell cycle in the G0/G1 phase considerably; the HCT-8 cell population was increased from 51.08 to 66.71% (Figure 7a–e). These results propose that SJYLF inhibits the progression of the cell cycle to induce apoptosis.

Expression of Upstream and Downstream Apoptosis and Cycle-Related Proteins of the Cyclin D-CDK4 Signaling Pathway. The key controlling point of the cell reproductive cycle where proliferation starts is between the G1 and S phases. The classical approach to relate the signals and the cell restriction point is the signal pathway whose core is cyclin-CDK4/6.

Different proteins control the signaling of apoptosis. Significant proteins of apoptosis include proapoptotic Bax and antiapoptotic Bcl-2. As compared to the control group (0 mg/mL), SJYLF (0.5, 1.0, and 1.5 mg/mL) downregulated the expression of Bcl-2 protein and upregulated the expression of Bax protein. A considerable increase in the Bax/Bcl-2 ratio suggests that SJYLF has a profound induction effect on apoptosis proteins (Figure 7a–c). Apoptosis is caused by the activation of caspase waterfall and elevated Bax/Bcl-2 that result in the permeability changes in the mitochondrial membrane. Proteins related to the cell cycle declined the expression of CDK4 and cyclin D1 proteins, and the inhibition of the G0/G1 phase progression was also noticed.

As seen in Figure 8a, SJYLF-treated HCT-8 cells show a considerable decrease (>50%) in the relative mRNA expression for cyclin D1, CDK4, and Bcl-2, while Bax mRNA level was increased about 25% (Figure 8a). When protein levels of cyclin D1, CDK4, Bcl-2, and Bax were measured with Western blotting, similar protein expression patterns were observed (Figure 8b,c). Therefore, the induction of apoptosis seems to be associated with the increased expression ratio of proapoptotic Bax/Bcl-2 proteins, suggesting that the SJYLF treatment makes the HCT-8 cells sensitive to apoptotic stimuli.
DISCUSSION

Traditional Chinese medicines (TCMs) are suitable, effective, and usually pose less harmful effects. These medicines are being used for thousands of years while improving clinical symptoms and life quality in many fatal diseases, including cancer. In this aspect, TCM as a single herb formulation or in the form of decoction/formula has gathered worldwide attention for the treatment of various types of cancers, including CRC. Ruanjian Sanjie (RJSJ), Huanglian Jiedu Tang, Yanshu Injection, Feiji Recipe, Jianpi Yangzheng Xiaozheng Recipe, and Jiedu Xiaozheng Yin are few reported formulas showing antitumor activities in various cancers. TCM Sanjie Yiliu Formula (SJYLF) is a four-component anti-CRC decoction involved in relieving the...
symptoms and can work in synergism with chemotherapy. This study has reported the antitumor, antiproliferative, and apoptosis-inducing activity of SJYLF (Figure 1).

Apoptosis, no doubt, is a significant self-regulating mechanism to maintain stability in the internal tissue environment. One common method in cancer therapy is the induction of apoptosis, and it rendered an important index of evaluating the anticancer efficacy of drugs. SJYLF shows the antitumor and antiproliferative activities against CRC cell lines, i.e., HCT-8 cells with minimal effects on normal cells, i.e., HK-2 cells, in a time- and dose-dependent manner (Figures 2 and 3). MTT, colony-forming, trypan blue, and DAPI immunofluorescence assays are in good agreement with the anticancer activity of SJYLF. Selective cytotoxicity, effectiveness, and efficacy against HCT-8 cells make it important to elucidate the SJYLF mechanism of action. A herbal formulation named C168, in the form of methanol extract (CME), was also found to show the antiproliferative activity against CRC by halting the cell cycle in G2/M and inducing apoptosis.

SJYLF components, including Rhizoma Pinelliae Preparatum (Fabanxia), Glabrous Sarcandra herb, Thunberg Fritillary bulb, and ground beetle species, have already been reported to exert antitumor activity against colorectal cancer cells. Rhizoma Pinelliae Preparatum (Fabanxia) and Glabrous Sarcandra herb have been reported to show antitumor and antiproliferative properties by arresting the cell cycle and inducing apoptosis in human cell lines. Thunberg Fritillary bulb has also been reported to inhibit uncontrolled cell growth. Many TCMs prepared from insects have been reported to possess immune-modulating antitumor, antiproliferative, and apoptosis-inducing properties. Extracts from Paederus beetle (Paederus fuscipes) have been found to inhibit cancer cell growth by MTT and colony-forming assay. Pertinent to that, SJYLF components, Glabrous Sarcandra herb and Thunberg Fritillary bulb, were previously reported as nontoxic.

Though Rhizoma Pinelliae Preparatum (Fabanxia) is considered a toxic herb, its amount and ratio in SJYLF are nontoxic. Increasing drug concentration levels caused a considerable increase in the apoptotic cell proportion while downregulating the antiapoptosis protein, Bcl-2 (Figures 6 and 8).

SJYLF was found to induce apoptosis via cyclin D1, CDK4, Bcl-2, and Bax proteins that have been reported to involve in many cancers, including CRC. Regulation of cyclin D1 and CDK4 in tumor cells may make SJYLF an effective complementary therapy to combat CRC. Many anticancer agents have been reported to arrest the cell cycle at the G0/G1 phase by regulating Bcl-2 and Bax proteins.

Bcl-2 belongs to a special protein family in which certain members promote apoptosis, including Bax, Bid, and Bad, whereas certain members like Bcl-2 have an inhibition effect on the release of cytochrome c in the cytoplasm from mitochondria, resulting in apoptosis inhibition. Bcl-2 protein is chiefly localized in mitochondria, nuclear membrane, endoplasmic reticulum, and various tumor cells. It has the potential to increase the potential of mitochondrial membrane permeability, inhibiting the calcium ion release, preventing endonuclease activation, resulting in an antiapoptotic effect. It is reported that p53 can inhibit Bcl-2, while it also activates Bax and Bak proteins. Bax protein tends to activate the death effect factor caspase or change the cell membrane permeability, resulting in the release of small molecules and ions, by cytochrome C, through the cell membrane, therefore promoting the apoptosis of the cell. When Bcl-2 is dominant, then the change in Bcl-2/Bax can regulate apoptosis, and cell possesses antiapoptotic effects. On the other hand, the overexpression of Bax makes the cells a victim of apoptosis.

An important role is played by CDK4 and cyclin D1 in regulating the progression of the cell cycle. Western blotting, qPCR, and cell cycle test showed that SJYLF could block A549 cells in the G0/G1 phase and significantly reduce CDK4 and cyclin expression level D1 (Figures 3 and 7). Cell cycle is regulated by CDKs and cyclins to pass from the G1-to-S phase. CDK4 and CDK6 act as switches for cell cycle entry from G1 to S phase. Although some studies report that CDK4 is not involved in the mammalian cell cycle, it still plays an important role in the control of cancer proliferation. In SJYLF-treated
cells, CDK4 levels were found to significantly decrease, while cyclin D1 levels were also marginally decreased (Figure 8b,c). These results propose that SJYLF initiates apoptosis by arresting the cell cycle, where Bcl-2, cyclin D1, and CDK4 protein are downregulated, and Bax protein expression was induced. This preclinical study shows that some additional investigation for SJYLF is required regarding p53 and other proteins, and characterization of active components will help a better understanding of the SJYLF mechanism.

**CONCLUSIONS**

Our results demonstrate that SJYLF possesses remarkable anti-CRC activity with fewer toxic effects on normal cells. Moreover, SJYLF is involved in the suppression of proliferation and induction of apoptosis in cancer cells.

**MATERIALS AND METHODS**

**Ethical Approval and Consent to Participate.** All procedures performed in studies involving human participants were in accordance with the institutional and/or national research committee’s ethical standards and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

**Consent for Publication.** Informed consent was obtained from all individual participants included in the study.

**Reagents and Cell Lines.** CRC cell lines (HCT-8, SW-480, HT-29, and DLD-1) and normal human kidney cell line HK-2 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and subjected to short tandem repeat profiling to determine their authenticity. Cell lines were cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal serum of calf (Invitrogen, Carlsbad, CA), 1 U/mL of penicillin G, and 1 μg/mL of streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

**Sanjie Yiliu Formula (SJYLF) Preparation.** Herbs and beetle species used in the SJYLF formula were purchased from Pharmaceutical Market (Shanghai, China). SJYLF is a four-component decoction of Thunberg Fritillary bulb, Glabrous Sarcandra herb, Rhizoma Pinelliae Preparatum, and ground beetle species mixed in a ratio of 1:5:10:15. A fine powder of...
mixture was extracted with distilled water at 100 °C twice for 2 h and centrifuged at 4000g to obtain the supernatant that was further evaporated to a dried powder using a rotary evaporator. This powder was used as the SJYLF extract in further experiments by dissolving it in phosphate-buffered saline. Multiple extractions were performed to evaluate the SJYLF extract’s efficacy, and at least three different batches of SJYLF were used to replicate the results as statistically significant.

Cell Viability-MTT Assay. Cell viability was assessed using a modified MTT colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Calbiochem) as described elsewhere.34 Cells (100 μL, 4 × 10^5 cells) were seeded in 6-well plates and incubated in the presence of 5% carbon dioxide at 37 °C. Cell viability was examined after 24 and 48 h by adding the increased concentration of SJYLF extract (0.25–2 mg/mL in PBS). IC_{50} was determined for all cell lines, and one with potent IC_{50} was used in further experiments to elucidate the mechanism of action for SJYLF.

SJYLF was found to be selectively toxic to the CRC cell line and did not affect normal human cells (HK-2). For the subsequent experiments, including the suppression of proliferation, apoptosis stimulation, cell cycle progression, expression of apoptosis-related genes and proteins, HCT-8 cells (most affected by SJYLF) were used.

Trypan Blue and Colony Formation Assays. To determine the percentage of dead and live cells, the trypan blue assay was performed as described by Yao et al.,56 where “number of the trypan blue-stained cells divided by the total cell number”. HCT-8 cells were grown in the presence of increasing concentrations of SJYLF extract (0.5–1.5 mg/mL in PBS) for 24 h. Trypan blue (0.4%) was added to 100 μL of cells and mixed thoroughly before examining the cells under a light microscope. Stained cells were considered dead cells. For the formation of a cell colony, a 6-well plate was used to culture cells. After 2 weeks, crystal violet was used to stain the cell colony and count them.

Immunofluorescence Staining. Immunofluorescence staining was performed as described in a previous study.57 HCT-8 cells were treated with increasing SJYLF extract concentrations (0.5–1.5 mg/mL in PBS). Cells were harvested and washed with PBS. To avoid the heat shock, paraffin sections were used to fix the cells on glass coverslips, followed by a methanol treatment for permeabilization. Cells were incubated at 4 °C with a 1:800 dilution ratio of rabbit and antimouse CCR2 antibody (cat. no. ab203128, Abcam, London, U.K.). Secondary antibodies were labeled for fluorescent visualization using Texas Red-labeled antirabbit IgG (Zsbio, Beijing, China) for 1 h at 37 °C, whereas 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Beyotime, Beijing, China) was used to examine the nuclei. SPOT Flex camera (Tokyo, China) and Olympus BX600 microscope were used to evaluate all specimens.

Flow Cytometric Analysis. HCT-8 cell lines were cultured in DMEM containing 10% fetal calf serum in the presence of increasing concentrations of SJYLF extract (0.5–1.5 mg/mL in PBS). Harvested cells were washed with ice-cold PBS (Invitrogen, MA) having 0.2% ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, MA) and resuspended in the same buffer containing 3% fetal bovine serum to obtain a cell suspension (PBS, Gibco, MA). Annexin V-FITC/PI labeling was performed through BioLegend as fluorescent-conjugated antibodies, as described elsewhere.58 A minimum of 30,000 cells were analyzed on a NovoCyte flow cytometer (ACEA Biosciences), and NovoExpress software was implied to analyze the data. Annexin V and PI double-positive cells were considered late apoptotic or necrotic cells where PI fluorescence was observed at >625 nm.

Quantitative PCR. To assess the gene expression levels of cyclin D1, CDK4 (cyclin-dependent kinases), Bcl-2 (B cell leukemia/lymphoma 2), BAX in SJYLF-treated HCT-8 cells (0.5–1.5 mg/mL extract), quantitative PCR, and Western blot analyses were performed. Trizol reagent (Invitrogen, Carlsbad, CA) was used to extract the total RNA from HCT-8 cells according to the manufacturer’s instruction. Reverse transcription was performed using an RT kit (cat. no. RR014A; Takara, Dalian, China). Complementary DNA was employed

Figure 8. Quantitative PCR and Western blot analyses in SJYLF-treated HCT-8 cells. Determination of mRNA and protein expression levels of cyclin D1, CDK4, Bcl-2, and BAX in SJYLF-treated HCT-8 cells (0.5–1.5 mg/mL extract) shows significantly reduced levels of cyclin D1, CDK4, and Bcl-2 (a, b). Western blot analysis of four proteins, cyclin D1, CDK4, Bcl-2, and Bax, in SJYLF-treated HCT-8 cells shows that SJYLF has a significant induction effect on the expression of Bax. In contrast, Bcl-2, CDK4, and cyclin D1 protein expression were significantly reduced in the presence of 1.5 mg/mL of SJYLF (c).
for quantitative PCR analysis using a SYBR kit (cat. no. RR840A; Takara, Dalian, China) on a CFX96 machine (Bio-Rad, MA) with programmed cycles following a cycle of 90 s at 95 °C and after that 40 cycles of 95 °C for 10 s and 58 °C for 30 s. GAPDH was utilized as a control. Each experiment was conducted three times, and the 2−ΔΔCT method was used to analyze the relative expressions of mRNA.59 The primers for cyclin D1, CDK4, Bcl-2, and BAX are given in Table 2.

Table 2. Primers Synthesized for Amplification of Cyclin D1, CDK4, Bcl-2, and BAX Genes

| Gene  | Forward Primer                     | Reverse Primer                     |
|-------|-----------------------------------|-----------------------------------|
| cyclin D1 | 5′-ATGTTCTGTCAGCTCTAAGATGA-3′ | 5′-CATTTCCACATTTGAGCTCTTCTC-3′ |
| CDK4   | 5′-ATGCTACCTCCTCTGATAGGACC-3′   | 5′-CATTGGGAGCTCTCACACTCT-3′     |
| Bcl-2  | 5′-GGTGTGTCATTGGTTGGG-3′         | 5′-CGGGTCAGGCTACGTCATGACCC-3′   |
| BAX    | 5′-CCGGAGGGCTCCTTTTTCGAGG-3′     | 5′-CCACGCCCAGTATGGTCTCTGAT-3′   |

**Western Blotting.** SJYLF-treated HCT-8 cells (0.5−1.5 mg/mL extract) were lysed in an ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Beijing, China) having phosphatase inhibitors (Merck Millipore, MA) and protease inhibitor cocktails (Merck Millipore, MA) for about 30 min. Lysed cells were centrifuged (6000 × g, 15 min) at 4 °C, and the clear supernatant was obtained.

Bicinchoninic acid (BCA) kit was used to check protein concentrations. Proteins were denatured by adding a 5X loading buffer to 20 μL of sample followed by sample boiling for 5 min at 90 °C. Tris–glycine SDS-PAGE (12%, 120 V) was used to separate the protein. Proteins were then transferred to poly(vinylidene fluoride) (PVDF) membrane (Millipore, MA) by electroblotting. To avoid nonspecific binding, 5% bovine serum albumin (BSA) in TBS/T buffer was used as a blocking buffer. After washing with PBS, membranes were incubated overnight with primary antibodies of β-actin, Bcl-2, Bax, CDK4, and cyclin D1 at 4 °C. After primary incubation, horseradish peroxidase-conjugated secondary antibody (Zsbio, Beijing, China) was added, and membranes were again incubated for 1 h. Chemiluminescent substrate ECL kit (Merck Millipore, MA) was added to detect the protein bands that were visualized using X-ray film exposure.

**Statistical Analysis.** After three times the conduction of all experiments, the values were obtained as a mean ± standard deviation. Three batches of SJYLF were used to replicate the results as statistically significant. Student’s t test estimated group comparisons. Two-sided p-values were reported, and a p-value < 0.05 was designated as statistically significant. SPSS statistical software package (SPSS 19.; SPSS, Chicago, IL) was used to conduct all analyses.

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**Author Contributions**

R.Z.T., M.F.R., and C.B.Y. conceptualized the project. R.Z.T., F.K., and Z.Z.L performed the experiments. A.I.B. and M.M. analyzed the data. All authors were involved in the preparation of the manuscript. R.Z.T., Z.Z.L., and C.B.Y. provided the resources. F.K., M.F.R., and C.B.Y. analyzed the results and reviewed/editied the manuscript. All authors have read and agreed to the published version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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