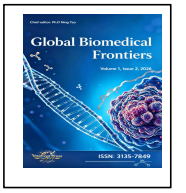
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Inhibitory Effects of Ganoderma lucidum Spore Oil on TS/A Breast Cancer Cell Growth

Chenge Gao

Beijing Normal University Affiliated High School, Beijing, China

Information

Keywords

Ganoderma lucidum spore oil

TS/A murine breast cancer cells

Apoptosis

Stat1

Tumor microenvironment

Abstract

Objective: To determine whether Ganoderma lucidum spore oil selectively inhibits the growth of TS/A murine breast cancer cells, induces TS/A apoptosis, and modulates apoptosis-related genes. Methods: MTT assays were used to examine the growth responses of normal LX-2 fibroblast-like hepatic stellate cells and TS/A cells. Flow cytometry with propidium iodide staining was used to estimate apoptosis, and real-time quantitative PCR was used to measure the expression of apoptosis-related transcripts. Results: Ganoderma lucidum spore oil markedly inhibited TS/A growth in a concentration-dependent manner. Low concentrations promoted LX-2 growth, whereas high concentrations inhibited it. The expression of Casp8, Casp3, and Stat1 changed after treatment, consistent with induction of apoptosis. Significance: Ganoderma lucidum spore oil may induce apoptosis in TS/A breast cancer cells by altering apoptosis-related gene expression and could provide candidate components for further antitumor drug development.

1. Introduction

1.1 Background

The GLOBOCAN 2020 cancer statistics indicated that breast cancer had become the most commonly diagnosed cancer worldwide. Other studies have shown that both the incidence and mortality of female breast cancer increase markedly with age. Effective inhibition of breast cancer is therefore important for improving women's health.

Current breast cancer treatments include cytotoxic chemotherapy and molecularly targeted therapy. As interest in traditional Chinese medicine has increased, herbal antitumor strategies have also been investigated. For example, vitamin E succinate combined with chemotherapeutic agents has been reported to increase Fas expression on breast cancer cells and significantly inhibit proliferation. However, radiotherapy, chemotherapy, and targeted treatments all have limitations and adverse effects, supporting the search for therapies with greater efficacy, higher selectivity, and lower toxicity.

1.2 Study Materials and Rationale

The TS/A cell line is a metastatic murine mammary carcinoma line established by Patrizia Nanni and colleagues in 1983. It was derived from a spontaneous mammary adenocarcinoma in a 20-month-old female BALB/c mouse and

Email: Chenge Gao , sumiself@outlook.com.

exhibits marked morphological heterogeneity [1]. LX-2 hepatic stellate cells are normal fibroblast-like cells of hepatic origin.

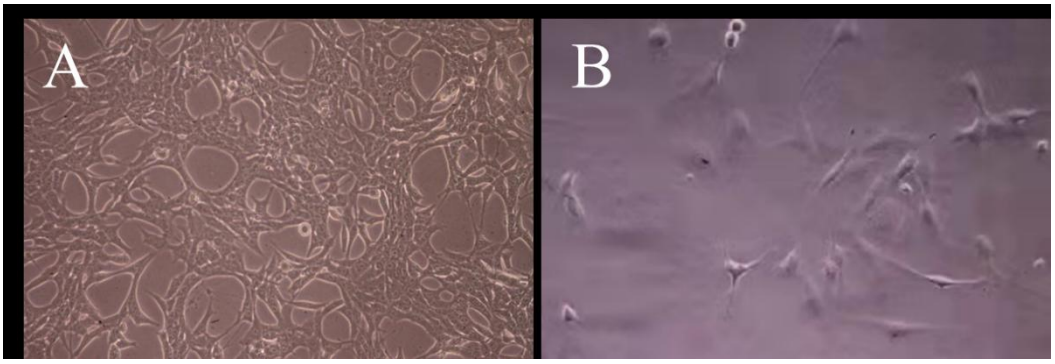


Fig. 1 Cell morphology. (A) TS/A murine breast cancer cells, 100x. (B) LX-2 hepatic stellate cells, 400x.

Ganoderma lucidum is described in the Compendium of Materia Medica as bitter and neutral in nature, nontoxic, beneficial to heart qi, and able to promote blood circulation. A growing market of Ganoderma-based health products has prompted debate about their efficacy. Ganoderma triterpenes are considered major active constituents of Ganoderma lucidum spore oil, and prior studies have reported inhibitory effects on prostate cancer as well as on the growth and metastasis of liver cancer. The product examined here was manufactured by Nutrition House (Chengdu) Biomedicine Co., Ltd.

1.3 Research Question and Experimental Design

Compared with drugs, health products generally undergo less stringent approval procedures and are marketed to subhealthy populations or people seeking supportive health care. The efficacy of herbal health products therefore requires experimental evaluation. Motivated by research on traditional Chinese medicines with antitumor activity, this study tested whether a Ganoderma-based health product could affect tumor-cell growth.

Ganoderma lucidum spore oil was selected as the test preparation after reviewing studies of its active constituents and biological effects. TS/A murine breast cancer cells were selected because breast cancer is particularly relevant to women's health and this line has been studied less frequently in China. LX-2 cells were used as a normal-cell comparison to estimate the concentration range in which TS/A inhibition might show selectivity.

The study first used an MTT assay to characterize the concentration-response relationship for TS/A proliferation and to compare the growth response of LX-2 cells. MTT and flow cytometry were then used to evaluate TS/A apoptosis. Finally, RT-qPCR was used to measure apoptosis-related transcripts and explore possible signaling mechanisms.

2. Materials and Methods

2.1 Cell Lines

TS/A murine breast cancer cells and LX-2 hepatic stellate cells were provided by the laboratory of Zhihai Qin.

2.2 Reagents

Table 1. Major experimental reagents.

Reagent	Manufacturer	Country/Region
Ganoderma lucidum spore oil	Nutrition House Biomedicine Co., Ltd.	Chengdu, China
Fetal bovine serum	Aidebach	Germany
Penicillin/streptomycin	Invitrogen	USA
Propidium iodide apoptosis assay kit	GenStar	China
PrimeScript RT Master Mix	Takara	Japan
DMEM	Gibco	USA

Reagent	Manufacturer	Country/Region
MTT reagent	Amresco	USA
TRIzol reagent	Invitrogen	USA

2.3 Instruments

Table 2. Major experimental instruments.

Instrument	Manufacturer	Country/Region
Microplate reader	Bio-Rad	USA
PCR instrument	Bio-Rad	USA
Flow cytometer	Bio-Rad	USA
Microvolume spectrophotometer	NanoDrop Technologies	USA
Centrifuge	Beckman	USA
Microscope	Olympus	Japan
Pipettes	Eppendorf	Germany
CO_2 incubator	SANYO	Japan
Vortex mixer	Kylin Medical Instruments	Jiangsu, China
qPCR instrument	Applied Biosystems	USA

2.4 Cell Culture

2.4.1 Complete Medium

TS/A cells were cultured in complete DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. A tray of distilled water was placed at the bottom of the incubator to maintain humidity.

2.4.2 Cell Passage

Culture medium was aspirated and the dish was rinsed with 2 mL PBS. After removing the PBS, 500 μL trypsin was added and the cells were incubated for 1 min. DMEM (500 μL) was then added, the cells were gently dispersed, and the suspension containing trypsin was transferred to a centrifuge tube. The tube was balanced with PBS and centrifuged at 1,500 rpm for 1 min. The supernatant was removed, 1 mL DMEM was added, and the cells were resuspended. A 500-μL aliquot of cell suspension and 4 mL DMEM were placed in a new medium-sized culture dish and returned to the 37 °C, 5% CO_2 incubator.

2.5 MTT Assays

2.5.1 Growth Effects after 24 h

Control and treatment groups were established with three replicate wells per group. TS/A and LX-2 cells were seeded into flat-bottom 96-well plates at 1×10^4 cells per well. Ganoderma lucidum spore oil concentrations in treatment groups 1-4 were 2.5%, 5%, 7.5%, and 10%, respectively, and DMEM was added to a total volume of 100 μL. Control wells received an equal volume of complete DMEM. Edge wells received 200 μL of 1× PBS to reduce edge effects. A matched background system without cells was included below each group.

After incubation for 24 h at 37 °C and 5% CO_2, 10 μL MTT reagent was added to each well in the dark. The plate was returned to the incubator for 4 h, after which 100 μL triple-solubilization solution (10% SDS, 5% isobutanol, 0.012 mol/L HCl in distilled water) was added. After 24 h of solubilization, absorbance at 570 nm was measured with a microplate reader. Optical-density data were analyzed in GraphPad Prism.

2.5.2 TS/A Apoptosis-Related Growth Inhibition

The same MTT procedure was used with Ganoderma lucidum spore oil concentrations of 2%, 4%, 6%, and 8%. For this dose-response experiment, TS/A cells were exposed for 48 h before viability was evaluated.

2.6 Flow Cytometry

Four groups were prepared in six-well plates, each containing 1.25×10^5 cells. Three wells received Ganoderma lucidum spore oil at 0%, 4%, or 6%; the remaining blank group was used to adjust instrument voltage. After incubation for 7 h at 37 °C and 5% CO_2, the cells were collected and stained with propidium iodide. Each sample was analyzed by flow cytometry, and the data were processed in FlowJo.

2.7 Real-Time Quantitative PCR

TS/A cells treated for 24 h with 0%, 2%, or 4% Ganoderma lucidum spore oil were collected. The supernatant was discarded, the cells were washed once with PBS, and 1 mL TRIzol was added per well. Lysates were transferred to RNase-free 1.5-mL tubes, mixed with 200 μL chloroform, vigorously shaken for 15 s, left at room temperature for 3 min, and centrifuged at 12,000 rpm for 15 min at 4 °C. A 400-μL volume of the upper aqueous phase was mixed with an equal volume of isopropanol. After vigorous mixing and precipitation for 3 min, samples were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded, and RNA was washed with 1 mL 75% ethanol and centrifuged at 12,000 rpm for 5 min at 4 °C. Pellets were air-dried at room temperature and dissolved in approximately 10 μL DEPC-treated double-distilled water.

RNA was quantified with a NanoDrop microvolume spectrophotometer. Total RNA was reverse-transcribed to cDNA with PrimeScript RT Master Mix using the reaction shown in Table 3. The reverse-transcription program was 25 °C for 10 min, 42 °C for 15 min, 85 °C for 5 min, and then 4 °C indefinitely.

Table 3. Reverse-transcription reaction mixture.

Component	Amount
Total RNA	1,000 ng
5× All-in-One RT Master Mix	4 μL
RNase-free water	To 20 μL

The mRNA levels of GAPDH, Casp8, Casp9, Casp3, and Stat1 were measured with the PrimeScript RT Master qPCR system. The qPCR mixture is shown in Table 4. Cycling conditions were 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 58 °C for 20 s, and 72 °C for 20 s; followed by 72 °C for 5 min.

Table 4. Quantitative PCR reaction mixture.

Component	Amount
2× Taq Mix	10 μL
DEPC-treated water	8 μL
Forward primer	0.5 μL
Reverse primer	0.5 μL
cDNA	1 μL

Data were processed with Rotor-Gene Series software. Relative mRNA levels were calculated by the 2^(-ΔΔCt) method with GAPDH as the internal reference. Primer sequences are listed in Table 5.

Table 5. Primer sequences used for real-time quantitative PCR.

Gene	Primer	Sequence (5'-3')
GAPDH	Forward	GGTCGGTGTGAACGGATTTG
GAPDH	Reverse	GGGGTCGTTGATGGCAACA

Gene	Primer	Sequence (5'-3')
Casp8	Forward	TGCTTGGACTACATCCCACAC
Casp8	Reverse	GTTGCAGTCTAGGAAGTTGACC
Casp9	Forward	GGCTGTTAAACCCCTAGACCA
Casp9	Reverse	TGACGGGTCCAGCTTCACTA
Casp3	Forward	CTCGCTCTGGTACGGATGTG
Casp3	Reverse	TCCCATAAATGACCCCTTCATCA
Stat1	Forward	TCACAGTGGTTCGAGCTTCAG
Stat1	Reverse	CGAGACATCATAGGCAGCGTG

2.8 Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5.0 using a t-test. A p value below 0.05 was considered statistically significant, and a p value below 0.01 was considered highly significant.

3. Results and Analysis

3.1 Growth Effects on TS/A and LX-2 Cells after 24 h

MTT results showed that after 24 h, TS/A viability decreased while LX-2 viability increased at low concentrations. Thus, 2.5%-5% Ganoderma lucidum spore oil inhibited TS/A cells while promoting the growth of normal LX-2 cells. At concentrations of 5%-10%, the TS/A and LX-2 viability curves converged, indicating that the inhibitory effects of high concentrations on the two cell types were broadly similar.

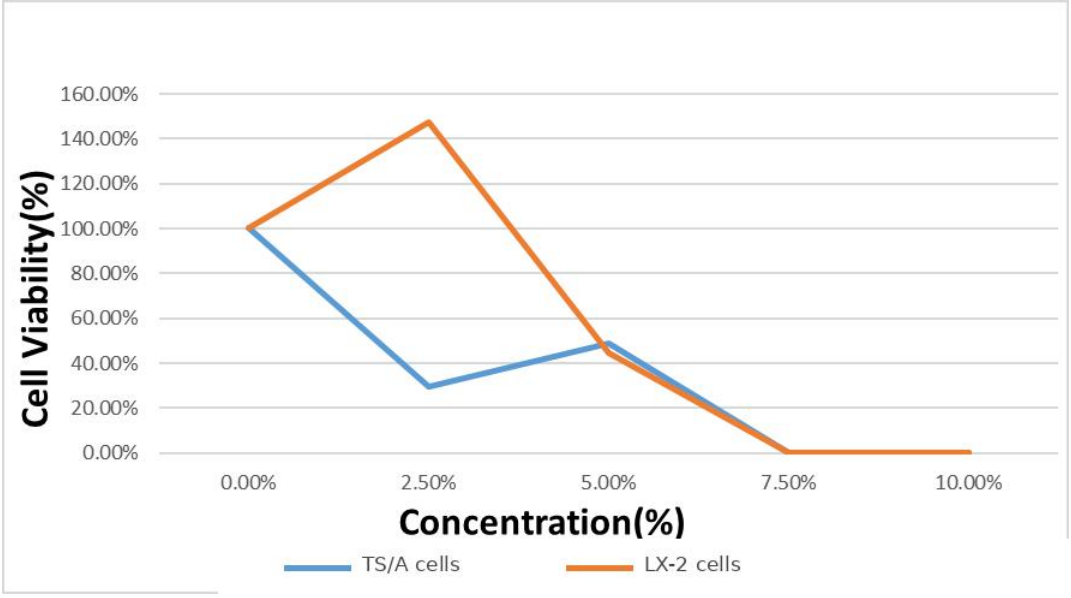


Fig. 2 Viability of TS/A and LX-2 cells after 24 h exposure to Ganoderma lucidum spore oil.

3.2 Dose-Dependent Inhibition of TS/A Cells

After 48 h of exposure, Ganoderma lucidum spore oil inhibited TS/A murine breast cancer cells. The inhibitory effect became stronger as the concentration increased, demonstrating a dose-response relationship.

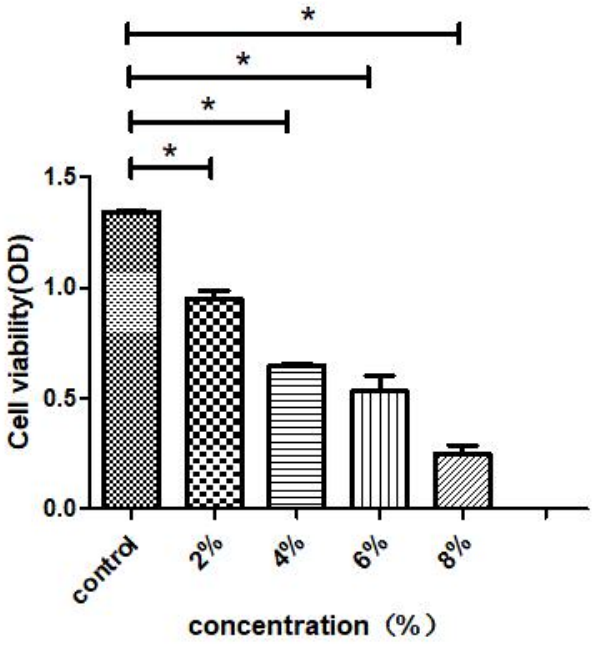


Fig. 3 Ganoderma lucidum spore oil inhibited TS/A cell growth in a dose-dependent manner after 48 h. *p < 0.001, indicating statistical significance.

3.3 Flow-Cytometric Analysis

PI single-staining flow cytometry was used to evaluate the inhibitory effect of Ganoderma lucidum spore oil on TS/A cells. Compared with the control group, the proportion of PI-positive TS/A cells increased in the treated groups. The overall PI-positive fraction increased from approximately 4.02% at 0% to 4.32% at 4% and 8.55% at 6%, supporting a concentration-related increase in cell death.

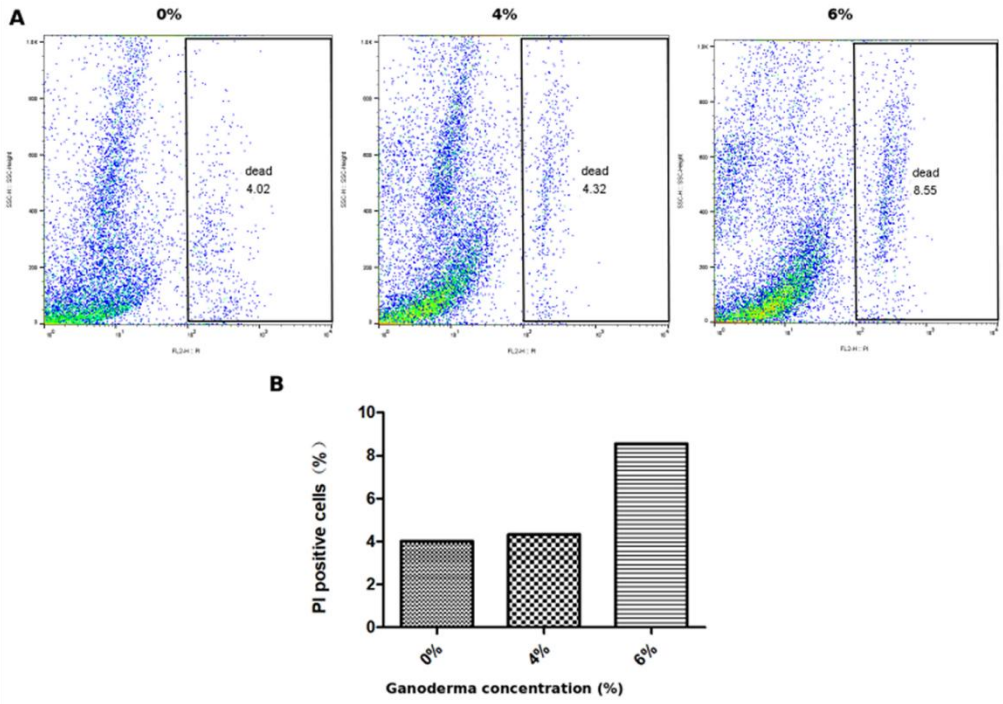


Fig. 4 Ganoderma lucidum spore oil induced death of TS/A murine breast cancer cells. (A) PI-stained flow-cytometry plots at 0%, 4%, and 6%. (B) Percentage of PI-positive cells.

3.4 Apoptosis-Related Transcriptional Responses

3.4.1 Caspase-Family Transcripts

To investigate the mechanism underlying TS/A apoptosis, RNA was extracted from cells treated with different concentrations of Ganoderma lucidum spore oil and analyzed by RT-qPCR. At 2%, Casp8 and Casp3 mRNA levels were upregulated, suggesting that caspase-family genes participated in the apoptotic response. Both transcripts declined again at 4%, indicating a concentration-dependent change rather than a simple monotonic response.

3.4.2 Casp9 Was Not Significantly Altered

Casp9 mRNA did not change significantly as the drug concentration increased ($p > 0.05$), suggesting that Casp9 was not a decisive component of the response under the conditions tested.

3.4.3 Stat1 Downregulation

Stat1 mRNA declined as the treatment concentration increased. At 4%, Stat1 expression was substantially lower than in the control group, suggesting that Ganoderma lucidum spore oil may promote TS/A apoptosis partly by reducing Stat1 expression.

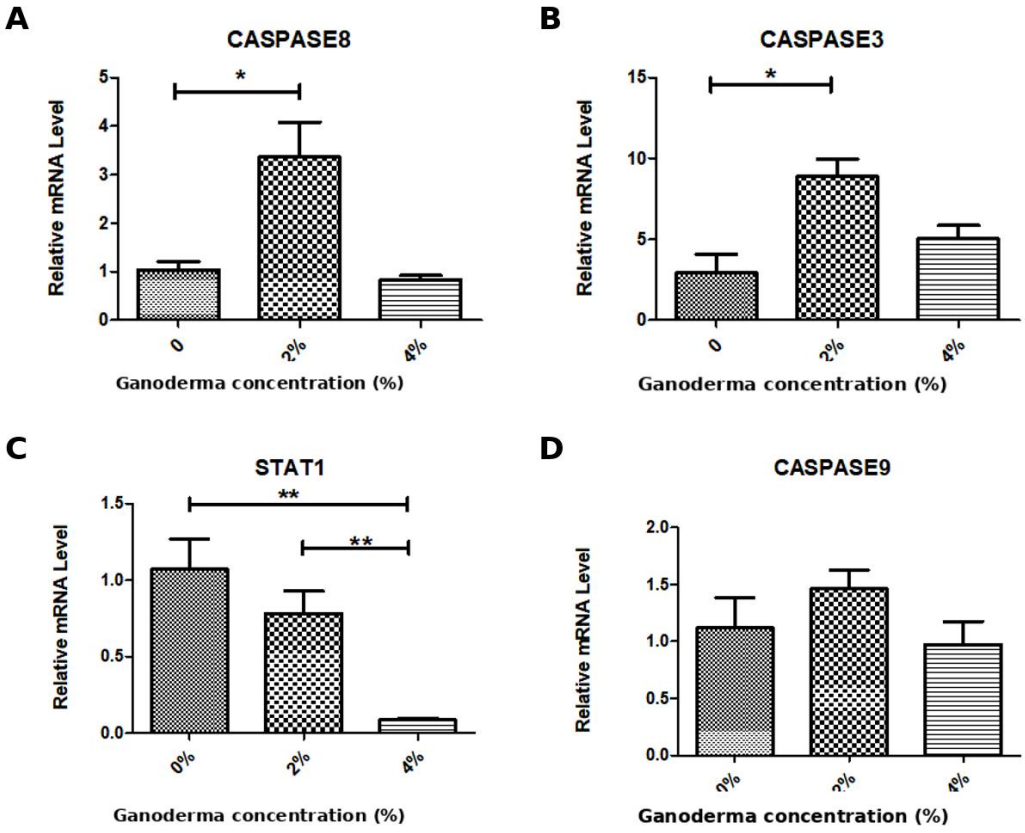


Fig. 5 RT-qPCR measurement of apoptosis-related transcripts in TS/A cells. (A) Casp8 expression increased at 2% and decreased at 4%. (B) Casp3 expression increased at 2% and decreased at 4%. (C) Stat1 expression decreased as the treatment concentration increased. (D) Casp9 did not change significantly ($p > 0.05$). *$p < 0.05$; **$p < 0.001$.

4. Discussion

4.1 Inhibition of TS/A Cells and Induction of Apoptosis

Traditional Chinese medicines have received broad attention because of their reported potential for antitumor activity with relatively low toxicity. During preliminary cell culture, TS/A cells displayed vigorous growth, supporting their use as a model for breast cancer research.

In initial experiments, direct exposure of TS/A cells to Ganoderma lucidum spore oil for less than 24 h did not produce a pronounced inhibitory effect. After the exposure time was extended to 48 h, TS/A growth was inhibited, and the effect increased with concentration. Flow-cytometric analysis further showed that the PI-positive fraction increased as the concentration rose, supporting the conclusion that the preparation induced TS/A cell death.

The mechanistic experiments indicated that Casp8 and Casp3 were responsive to treatment and may have contributed to apoptosis, whereas Casp9 did not change significantly. The increase in Casp8 and Casp3 at 2%, followed by a decline at 4%, suggests that high-concentration cell death may involve additional pathways or reduced transcriptional activity in severely damaged cells. Protein-level assays will be required to determine whether the corresponding caspases were activated.

4.2 Potential Role of Stat1 Downregulation

RT-qPCR showed a progressive reduction in Stat1 mRNA with increasing treatment concentration. STAT1 is activated through Janus kinases, which phosphorylate conserved tyrosine and serine residues in its C-terminal activation region. The resulting dimers translocate to the nucleus and regulate target genes. Prior research suggests that STAT1 activity can suppress, rather than induce, apoptosis in some tumor contexts. Stat1 downregulation in this

experiment therefore raises the possibility that Ganoderma lucidum spore oil promoted apoptosis by attenuating JAK/STAT signaling. This interpretation remains a hypothesis and requires direct pathway validation.

4.3 Potential Application in Breast Cancer

TS/A is a murine primary mammary carcinoma line introduced from abroad and has been studied relatively little in China. Studies have reported that ligustilide and curcumin induce apoptosis in prostate cancer-associated fibroblasts [2,3], while bitter melon extract inhibits breast cancer cell proliferation and promotes apoptosis [4]. Increasing numbers of researchers are investigating traditional Chinese medicines as potential sources of anticancer agents. Compared with conventional radiotherapy and chemotherapy, appropriately characterized natural products may offer lower toxicity, although their active compounds, selectivity, and safety require rigorous study.

In the present work, Ganoderma lucidum spore oil inhibited TS/A breast cancer cells. Future studies should isolate and identify the active constituents and assess their potential in cancer therapy and other medical applications.

5. Conclusion

Ganoderma lucidum spore oil induced death of TS/A breast cancer cells, and the effect increased with treatment concentration. The preparation modulated Casp8 and Casp3 transcription, with both genes upregulated at 2%, supporting involvement of caspase-related apoptosis. It also reduced Stat1 mRNA in a concentration-dependent manner, suggesting that suppression of Stat1-associated JAK/STAT signaling may contribute to the apoptotic response.

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CRediT authorship contribution statement

Chenge Gao: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing.

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