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Original Research Article

Evaluation of Embryotoxicity of *Radix scutellariae* Based on Embryonic Stem Test

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Abstract

Purpose: *To determine the potential embryotoxicity of Radix scutellariae (RS) extract using an embryonic stem cell test (EST) and to evaluate its effect on the differentiation of mouse embryonic stem (ES) cells.*
Methods: *All the test samples were obtained by water extraction method. The embryotoxicity of RS was assessed with cytotoxicity assays, namely, embryonic stem (ES) cells (IC_{50}ES) and 3T3 fibroblasts (IC_{50}3T3), as well as cardiac differentiation inhibition assay (ID_{50}ES). The expression of specific genes and proteins was analyzed by quantitative reverse transcription – polymerase chain reaction (RT-PCR) and Western blot.*
Results: *RS was weakly embryotoxic with IC_{50}ES, IC_{50}3T3 and ID_{50}ES of 0.1524, 0.1061, and 0.4169 mg/ml, respectively. Also RS had discordant effects on the expression of tissue-specific genes and proteins in three germ layers, promoting differentiation of the ectoderm (*$p < 0.05$; **$p < 0.01$) and endoderm (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$), while inhibiting mesoderm differentiation (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The effect of RS on the embryonic development of the three germ layers was concentration-dependent.*
Conclusion: *These results indicate that RS possesses weak embryotoxicity. The variability in the effects of RS may be responsible for its weak embryotoxicity.*

Keywords: *Embryonic stem test, Radix scutellariae, Embryotoxicity, Cardiac differentiation inhibition assay, Ectoderm, Endoderm, Mesoderm*

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INTRODUCTION

Women are frequent users of natural herbs worldwide [1]. It is well known that some herbs have powerful pharmacological effects, and significant side effects have been reported in recent years. For instance, ginsenoside Rb1, a major active agent of ginseng, was shown to have direct teratogenic effects in a whole embryo culture assay [2]. It is vital that risks of herbal therapy to pregnant women, including traditional Chinese medicine (TCM), are evaluated [3,4].

Radix scutellariae (RS), the dried root of *Scutellariae baicalensis* Georgi (labiatae), is commonly used in TCM to treat cardiovascular disease, inflammation, respiratory conditions, and gastrointestinal infections [5,6]. RS has additional therapeutic effects, such as preventing miscarriage [7]. Although RS is one of the most commonly used tocolytic agents, there has been little research on its toxicity [8].

In recent years, the embryonic stem test (EST) has become a widely used assay in the area of developmental toxicity [9,10]. The EST does not require experimental animals. The test is based on murine-derived embryonic stem (ES) cells from the blastocyst stage. Due to the pluripotency of ES cells, they differentiate in all lineages of the three germ layers: ectoderm, mesoderm, and endoderm [11]. The in vitro EST embryotoxicity assay requires a relatively simple procedure, which takes a shorter time than other assays used in developmental toxicological studies with experimental animals.

In the study we attempted to assess the embryotoxicity of RS using an EST and to analyze the expression levels of specific genes and proteins by quantitative RT-PCR and Western blot, respectively. The results are expected to shed light on the effects of RS on the three germ layers.

EXPERIMENTAL

Test material

RS was purchased from Mining Chi Lin Pharmacy of Guangzhou of China in July 2012 (produced in Anhui province, China; voucher no.05121802) and was authenticated by a specialist (Zhiguo Ma) in the Pharmacy of Jinan University. All the test samples were prepared by hot aqueous extraction. In brief, 100 g of the dried plant material RS were cut into small pieces and boiled in 1000 ml of distilled water for 1 h. The decoction was collected, while the residue was boiled again two times. The decoctions obtained from the three separate extractions were mixed, filtered, and lyophilized by freeze drying and then stored at –20 °C. The dried extracts were dissolved in double-distilled water to give an initial concentration of 1 g/ml and centrifuged at 14000 g for 5 min before filtration sterilization to obtain a clear, sterile supernatant for tests.

3T3 fibroblast and ES cell culture

Mouse ES cells (line OG2, purchased from Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences) were maintained in Dulbecco's modified Eagles medium (DMEM), supplemented with 15 % knockout serum replacement (KSR) (Gibco), 100 μg of streptomycin/ml, 100 U penicillin/ml, 2 mM of glutamine, 2 mM of sodium pyruvate, 0.1 % β-merca-ptoethanol, 1 % nonessential amino acids, and 1000 U of leukemia inhibitory factor (LIF) (ESG1-06)/ml. All cell culture reagents were purchased from Sigma-Aldrich (MO, USA) unless otherwise stated. The ES cell medium was refreshed daily, and the cells were subcultured every 2 – 3 days. A BALB/c 3T3 cell line was purchased from the cell bank of Zhongshan University of China. The BALB/c 3T3 cells were grown in DMEM, supplemented with 10% fetal calf serum (HyClone). The cells were cultured in 95 % humidity and 5 % CO_2 and at 37 °C.

Cytotoxicity assay

Followed the EST method as described previously [12], the cytotoxicity assay is a cell proliferation and tractviability test where the cells are exposed to a test compound. In brief, 500 cells were placed in each well of a 96-well tissue culture dish and incubated in a humidified atmosphere with 5 % CO_2 at 37 °C on day 0. Then, 200 μL of a culture medium containing the appropriate dilution of RS extract was added on day 1. On days 3 and 5, the same test medium was changed. Cytotoxicity was assessed on day 10. Cell viability was determined with the CCK8 assay, which quantifies the proliferation and viability of cells by a colorimetric method. In short, at the indicated time points, the medium in different experimental wells was exchanged for 110 μl of DMEM with CCK-8 reagent (10 μl CCK-8 reagent and 100 μl DMEM), and the cells were incubated for 2 h. Absorbance was measured for each well at a wavelength of 450 nm. An increase or decrease in absorbance values at 450 nm in different experimental wells relative to the initial value indicated cell viability [13]. The half-maximum inhibitory concentration of RS extracts on 3T3 cells and ES cell viability (IC_{50}3T3 and IC_{50}ES) were obtained through a curve fit analysis (concentration-response curve) using the program GraphPad Prism 5.0 (San Diego, CA, USA) [10,12].

Differentiation assay

To detect the effects of RS extract on ES cell differentiation, a differentiation assay was performed as described in detail previously [14,15]. In brief, 750 ES cells in 20 μL of differentiation medium (without mouse LIF) were placed on the lid of a petri dish filled with PBS on day 0. They were incubated for 2 days at 37 °C under 5 % CO_2 and 95 % humidity in the presence of a concentration range of the test compound. The hanging drop culture method was used to produce cell aggregates referred to as embryoid bodies (EBs). The EBs was grown in suspension in bacterial petri dishes containing the appropriate concentration of test compound for another 3 days. On day 5, the EBs were transferred singly into wells of a 6-well tissue

culture plate (containing the appropriate concentration of test compound) to allow adherence and outgrowth of the EBs and development of spontaneously beating myocardial cells. On day 10, the presence of the β-myosin heavy chain gene (β-MHC), which was used as a marker of cardiac cell differentiation, was determined by quantitative PCR. Inhibition of the differentiation (ID_{50}) of ES was expressed as the concentration of test compound inhibiting the development of contracting cardiomyocytes by 50 % (calculated from the dose-response curve). The sequences of the primers used are listed in Table 1.

Classification of the embryotoxic potential of RS

ES cell lines and BALB/c 3T3 cells were used to assess the potential embryotoxicity of RS [16]. The IC_{50} 3T3, IC_{50} ES, and ID_{50} ES embryotoxicity was assessed according to the classification criteria of the European Centre for the Validation of Alternative Methods (ECVAM) (Table 2). The embryotoxicity of test compounds is classified into three grades: nonembryotoxic (Class I), weakly embryotoxic (Class II), and strongly embryotoxic (Class III).

Analysis of differentiation of specific genes using RNA isolation, cDNA synthesis, and quantitative RT-PCR

Total RNA was extracted from the samples on day 10 of the differentiation assay using an E.Z.N.A.TM Total RNA Kit II (Omega, GA, USA). cDNA was synthesized with 1 µg of RNA, and 4 µL of PrimeScript™ RT Master Mix (Perfect Real Time; Takara, Shiga, Japan) in a total volume of 20 µL. The reaction system was subjected to 37 °C for 15 min for cDNA synthesis and 85° C for 5 s for enzyme inactivation.

Gene expression was detected by quantitative RT-PCR using a SoAdvanced™ SYBR® Green Supermix kit (Bio-Rad, CA, USA) in a final volume of 20 µL based on the manufacturer's instructions. The reaction mixtures contained 1 µL of template cDNA with 100 nM of forward and reverse primers and 10 µL of SsoAdvanced™ SYBR® Green Supermix in a total volume of 20 µL. Duplicate assays were run for each sample, and each included a negative control and a standard curve. Specific oligonucleotide primers were designed to produce 100–200 bp products.

Table 1: Primer sequences and product lengths of tissue-specific genes investigated

Gene	Primer sequence (from 5' to 3')		Product length (bp)
	Forward	*Reverse*	
Oct-4	GGTGGAGGAAGCCGACAAC	TTCGGGCACTTCAGAAACATG	141
Sox-2	AGATGCACAACTCGGAGATCAG	CCGCGGCCGGTATTTATAAT	146
Naong	CTCAAGTCCTGAGGCTGACA	TGAAACCTGTCCTTGAGTGC	120
GAPDH	GCCTTCTCCATGGTGGTGAA	GCACAGTCAAGGCCGAGAAT	150
Nkx2.5	CCATCCGTCTCGGCTTTGT	CCAAGTGCTCTCCTGCTTTCC	148
MyoD	ACGGCTCTCTCTGCTCCTTTG	CGTGCTCCTCCGGTTTCA	138
Tnni3	ACCTGTCCACGTTCCGCAAG	CTTGTTGACGTGGGACTCGG	100
β-MHC	GCCCTTTGACCTCAAGAAAG	CTTCACAGTCACCGTCTTGC	156
ALB	CAGCAATGGCAGGCAGATC	GGAACTTGCCAAGTACATGTGTGA	146
AFP	CAGCCATGAAGTGGATCACAC	GGAGGAATCTAACGTGGAAGCT	126
TTR	GTCCTCTGATGGTCAAAGTC	TCCAGTTCTACTCTGTACAC	193
GFAP	TGCCACGCTTCTCCTTGTCT	GCTAGCAAAGCGGTCATTGAG	146
Nestin	CTGCAGGCCACTGAAAAGTT	TCTGACTCTGTAGACCCTGCTTC	73
NFH	GCAGGAGGAGTGCGGCTAC	CCAACCTCACTCGGAACCACT	224

Table 2: ECVAM's validated prediction model of EST

A	Function I	$5.9157\lg(IC_{50}3T3) + 3.500\lg(IC_{50}ES) - \frac{5.307(IC_{50}3T3 - ID_{50}ES)}{IC_{50}3T3} - 15.72$	
	Function II	$3.6511\lg(IC_{50}3T3) + 2.3941\lg(IC_{50}ES) - \frac{2.033(IC_{50}3T3 - ID_{50}ES)}{IC_{50}3T3} - 6.85$	
	Function III	$-0.125\lg(IC_{50}3T3) - 1.917\lg(IC_{50}ES) + \frac{1.500(IC_{50}3T3 - ID_{50}ES)}{IC_{50}3T3} - 2.67$	
B	Class 1	Non-embryotoxic	If I > II and I > III
	Class 2	Weakly embryotoxic	If II > I and II > III
	Class 3	Strongly embryotoxic	If III > I and III > II

Linear functions discriminate Class I, II, and III and (B) embryotoxicity

The amplification protocol was as follows: 30 s at 95 °C, then 40 cycles at 95 °C for 5 s and 60 °C for 20 s, followed by a melt curve at 65 °C to 95 °C and an increment of 0.5 °C for 5 s. Specific amplification was verified by the melt curve analysis. The relative quantitative expression of tissue-specific markers after normalization with the ubiquitously expressed GAPDH housekeeping gene was calculated. All the primer sequences are shown in Table 1.

Western blot assay

Western blot assays were performed to analyze the expression levels of tissue-specific protein makers. In brief, proteins were separated by 8 % or 12 % SDS-PAGE and transferred to PVDF membranes (Bio-Rad, CA, USA). The blotted membrane was blocked in TBST containing 5 % non-fat milk for 1.5 h at room temperature. The primary antibody dilution was 1:1000 for α-fetoprotein (AFP) and neurofilament H (NFH) antibodies (CST, BSN, USA), and 1:800 for cardiac troponin I (cTnI) antibody (Merck Millipore, MA, USA). Antibody binding was detected with a secondary anti-antibody diluted at 1:2000 using a BM chemiluminiscence kit (Roche, IN, USA). Relative band intensity analysis was performed by densitometry scanning using Image J software. GAPDH was used as a cell fractionation control.

Statistical analysis

GraphPad Prism 5.0 (San Diego, CA, USA) was used for data plotting, non-linear regression, and statistical analysis. Data are given as the mean ± SEM (n=3). Data groups were compared using a one-way ANOVA and Dunnett's multiple comparison post-test for more than two groups. Results were considered statistically significant at $p < 0.05$.

RESULTS

EST classification of RS

Cytotoxicity

A cell viability assay was used to assess the cytotoxic effect of RS on the BALB/c 3T3 fibroblasts, representing adult tissues, and ES cells, representing embryonic tissues. In both cell lines, the RS extract inhibited the viability of cells, in a dose-dependent manner (Fig 1A). The IC50 value was 0.1061 mg/ml for the 3T3 cells and 0.1524 mg/ml for the ES cells. The significant difference in the cytotoxic sensitivities of the two cell lines to RS indicates that the 3T3 cells are more sensitive to RS than the ES cells. RS extract stimulated the proliferation of the ES cells at low concentration levels.

Differentiation inhibition

Inhibition of cardiomyocyte differentiation was determined by quantitative expression analysis of the β-MHC gene (a marker of cardiac cell differentiation) normalized to GAPDH expression at day 10. The RS extract restrained cardiomyocyte differentiation of the ES cells in a dose-dependent manner (Fig 1B).

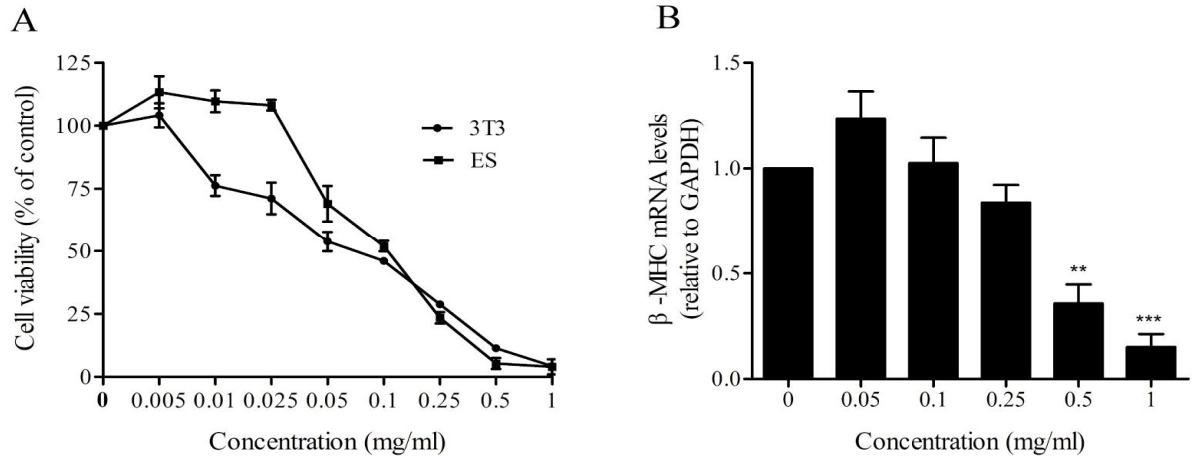

Figure 1: Cytotoxicity and differentiation assays of RS. (A) Cytotoxicity effects of RS on ES cells and BALB/c 3T3 cells. Values are mean ± SEM (n = 3). Cell viability (%) = (OD value of treated cells - OD value of blank control)/(OD value of control cells - OD value of blank control) ×100 %. Control cells refer to the cells untreated with RS. Values are means ± SEM (n = 3); (B) Results of differentiation assays. Expression level of the β-MHC marker gene of cardiomyocytes normalized to GAPDH expression and analyzed by quantitative PCR

As shown in Figure 1, RS stimulated cardiomyocyte differentiation of the ES cells at low concentrations and inhibited cardiomyocyte differentiation at high concentrations. The inhibiting effect became more evident as the concentrations increased. The ID_{50} ES value was 0.4169 mg/ml.

Based on these data and the ECVAM classification criteria (see Table 2), RS was classified as weakly embryotoxic.

Effect of RS on ES cell differentiation: undifferentiated specific markers

To assess the tissue-specific effects of RS (0 - 1 mg/ml) on ES differentiation at the molecular level, real-time quantitative PCR analysis of the expression levels of typical tissue-specific genes was performed on day 10 of the differentiation assay. The expression of Sox-2, Naong, and Oct-4 genes, markers of undifferentiated ES cells, all decreased first and then increased gradually in the presence of RS, with ES cell differentiation induced at a concentration range of 0 - 0.1 mg/ml and restrained at concentrations greater than 0.25 mg/ml (Fig 2A).

Effect of RS on ES cell differentiation: ectodermal lineages

The expression level of GFAP, an astrocyte-specific marker, gradually increased at all concentrations, Nestin, a primitive neural stem cell marker, continued to increase in a dose-dependent manner but precipitously declined at 1 mg/ml. NFH, a later-stage neuron-specific marker, increased first and then showed a decreasing trend (Fig 2B).

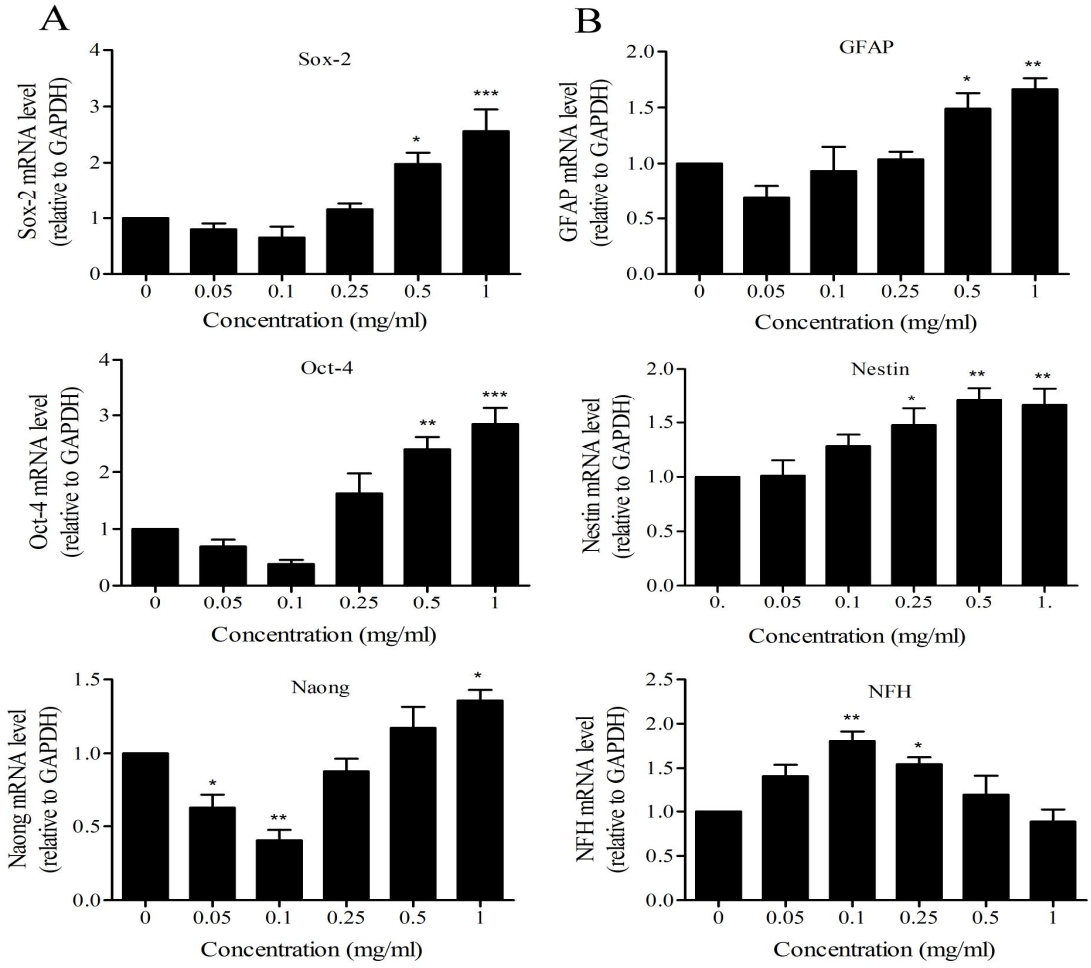

Figure 2: Analysis of expression levels of markers of undifferentiated ES cells and ectoderm differentiation. (A) Expression levels of the undifferentiated markers, *Sox-2*, *Oct-4*, and *Naong*; (B) Expression levels of the ectodermal markers GFAP, Nestin, and NFH. All the genes were quantified at appropriate concentrations of RS with real-time quantitative PCR. Values are mean ± SEM (n = 3). Comparisons with the control condition (0 mg/ml); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

Effects of RS on ES cell differentiation: mesodermal lineages

In the mesodermal lineage, the expression levels of Tnni3, a cardiac cell marker, and MyoD, a muscle cell marker, declined in a concentration-dependent manner. The expression level of Nkx2.5, a late-stage marker, showed a slight increase at a concentration of 0.1 mg/ml but was slightly down-regulated at the other concentrations tested (Fig 3A).

Effect of RS on ES cell differentiation: endodermal lineages

The expression level of a marker of late differentiation stages, transthyretin (TTR), slightly increased at low concentrations of RS and rapidly decreased at high concentrations. The expression of albumin (ALB), a definitive endodermal hepatic marker, showed an increasing trend, but declined at the maximum treatment concentrations. The expression of AFP increased gradually in a concentration-dependent manner (Fig 3B).

Effects of RS on the expression of AFP, cTnI, and NFH by Western blot analysis

The expression of AFP, cTnI, and NFH was assessed in ES cells treated with RS for 10 days to determine the effect of RS on ES cell differentiation in the ectoderm, mesoderm, and endoderm. Western blot analysis revealed that the protein expression level of AFP increased in accordance with a rise in the concentration of RS. However, the protein level of cTnI was effectively down-regulated by RS and not detected at an RS concentration of 1 mg/ml. The expression of NFH slightly increased at low concentrations of RS, then decreasing gradually as the concentration increased (Fig 4).

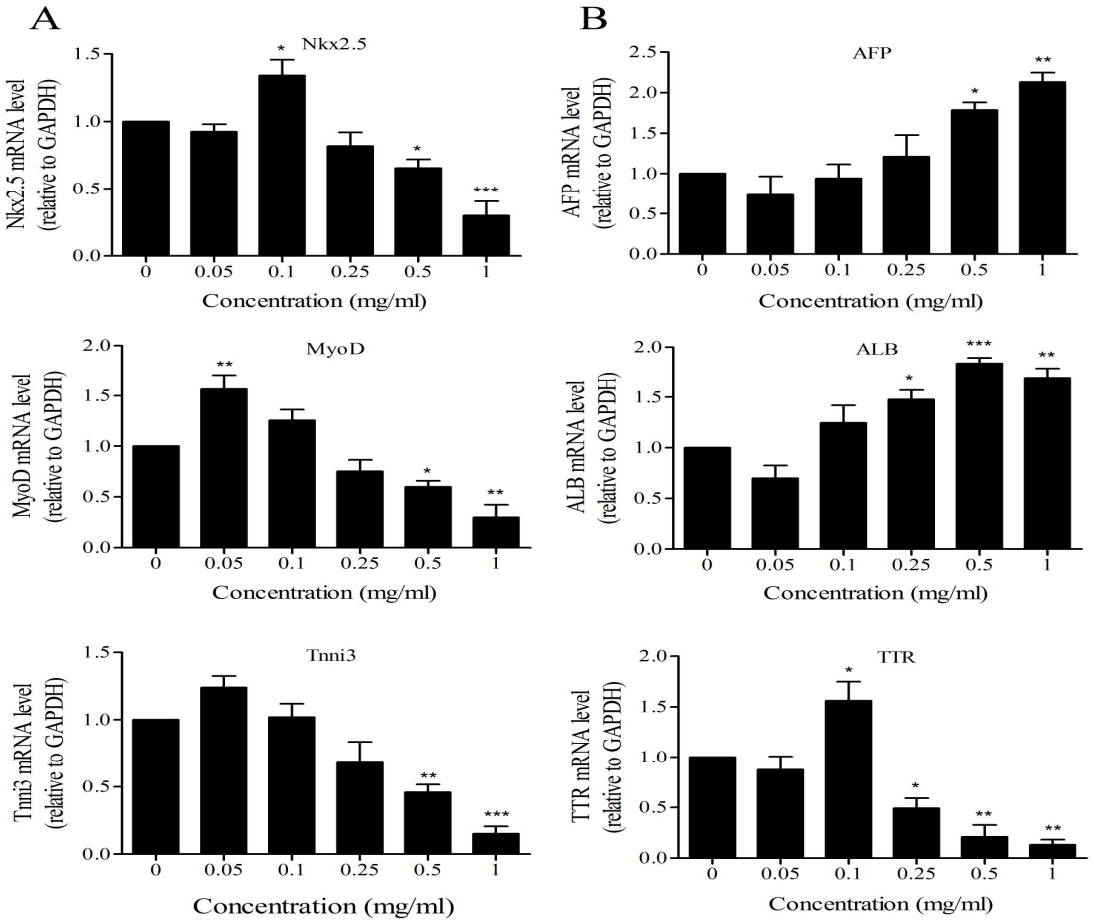

Figure 3: Analysis of expression levels for mesoderm and endoderm differentiation. (A) Expression levels of the mesodermal markers Nkx2.5, MyoD, and Tnni3; (B) Expression levels of the endodermal markers AFP, ALB, and TTR. All the genes were quantified at appropriate concentrations of RS with real-time quantitative PCR. Values are mean ± SEM (n = 3). Comparisons with the control condition (0 mg/ml); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

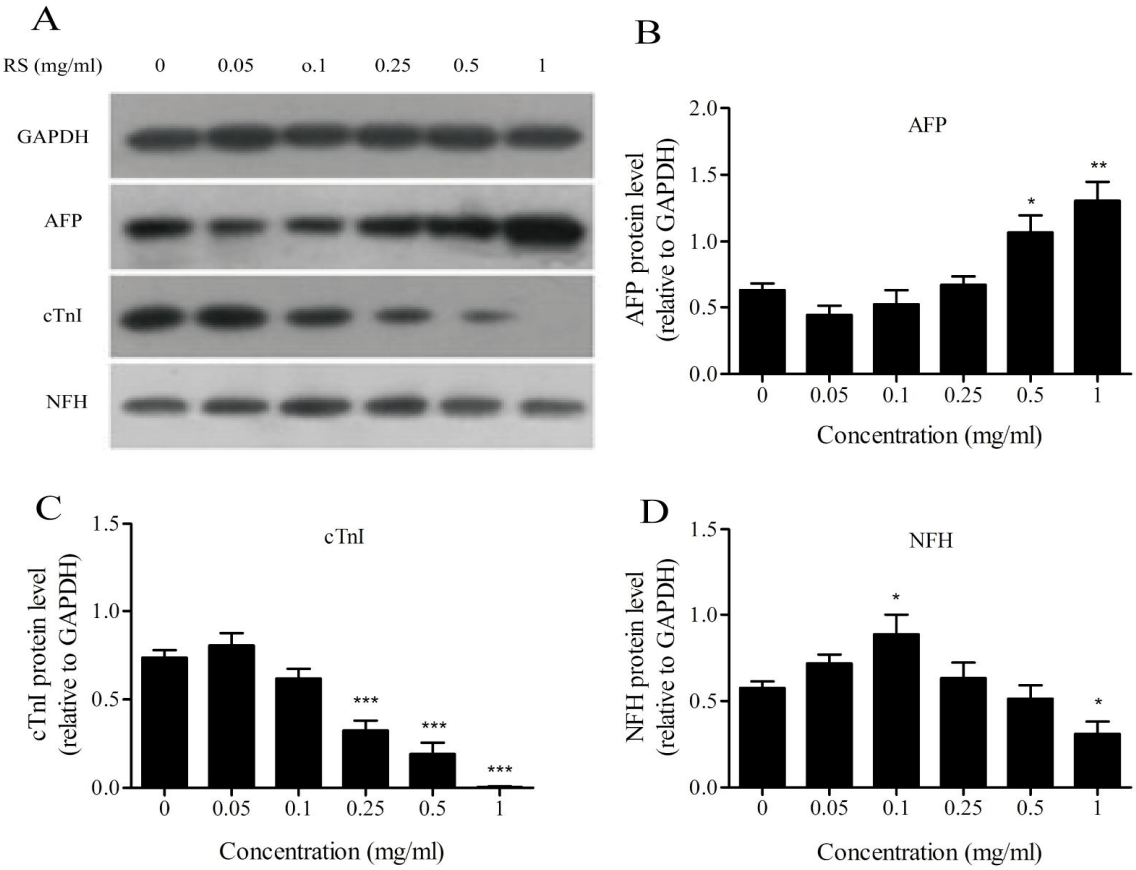

Figure 4: Western blot analysis of the expression of AFP, cTnI, and NFH after treatment with RS (*n*=3). Protein expression was assessed by quantitation of GAPDH.. Values are mean ± SEM. Comparisons with the control condition (0 mg/ml); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

DISCUSSION

The ECVAM designed the EST as a tool for classifying the embryotoxicity compounds [10,14]. The IC_{50}3T3 value of a number of chemical compounds is higher than the IC_{50}ES value [17,18] . In other words, ES cells, an embryonic tissue cell model, are more sensitive to the cytotoxic effect of compounds than 3T3 cells, an adult tissue cell model. However, in this study, we found that 3T3 cells were more sensitive than ES cells to the cytotoxic effect of RS. In fact, the survival of ES cells was higher than that of 3T3 cells at an RS concentration of 0.005–0.05 mg/ml, and RS inhibited cell proliferation. Many reports have shown that some Chinese medicines can stimulate cell proliferation [19-22]. In the present study, the cytotoxic effect of RS was apparent at concentrations greater than 0.005–0.05mg/ml, indicating that it shows weak embryotoxicity. Therefore, we should consider the safety of RS in pregnant women.

To further evaluate the effects of RS on embryonic development, we selected specific molecular markers of the three germ layers and then assessed their expression following RS treatment. Interestingly, the RS extract has discordant effects on the expression of tissue-specific genes within the three germ layers. For example, the expression levels of ALB and AFP, endoderm-specific genes, were elevated at increasing concentrations of RS, suggesting that it promotes differentiation in endodermal lineages. In contrast, the expression of TTR decreased in a concentration-dependent manner, possibly because it is a sensitive gene. Further investigation is required to determine whether TTR can serve as a toxicity marker for RS.

In addition, the results showed that RS seemed to promote differentiation in ectodermal lineages, with the expression of the ectoderm-specific genes Nestin and GFAP increasing and reaching the highest level at an RS concentration of 1 mg/ml. Nestin, a marker of early nerve cell differentiation, displayed an upward trend, except at the highest concentration. The expression of the NHF gene and protein increased first and then decreased at > 0.25 mg/ml. The expression levels of the mesoderm-specific markers MyoD and Tnni3 exhibited a downward trend at increased concentrations of RS, and Nkx2.5 was

slightly down-regulated at high concentrations. These data indicate that RS may inhibit ES cell differentiation in myocardial cells. The Western blot analysis of the specific protein markers of the three germ layers (AFP, cTnI, and NFH) in the present study following RS treatment demonstrated that the expression of all the proteins was similar to that of their corresponding genes.

In summary, these findings suggest that RS exhibits a tendency to promote differentiation in ectodermal and endodermal lineages and inhibit differentiation in mesodermal lineages. Thus, RS may exert different effects on germ layer differentiation. Comprehensive analysis of the effects of different concentrations of RS on tissue-specific genes and proteins in the three germ layers is important to evaluate the embryotoxicity of RS.

The pharmacodynamic effects of medicinal plants are significantly associated with their phytochemical components, and analysis of their toxic ingredients is a key step in understanding their adverse effects and mechanisms. In general, agents used in TCM are taken in oral form. The composition of remedies used in TCM can be complex, and the way in which one TCM may interact with another is unknown. Thus, it is difficult to evaluate the side effects of herbs used in TCM. Here, the EST was employed to detect the toxicity of RS to ES cells and the effect of RS on differentiation. The EST model is appropriate since it cannot be constrained by the complexity of the chemical constituents of TCM [8,23].

CONCLUSION

An *in vitro* EST has been used to characterize the embryotoxic effects of TCM, RS, and its effects on developmental processes in a mouse ES cell differentiation system. The results suggest that RS exhibits weak embryotoxicity based on assessment of IC_{50} and ID_{50} values. This study highlights the value of EST in identifying the potential developmental toxicity of herbal remedies at cellular levels. *In vivo* data and additional *in vitro* studies are needed to clarify the potential embryotoxicity of RS.

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