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Apoptotic Effects and Involvement of TRPM7 Channels of the Traditional Herbal Medicine, Dangkwisoo-San in Gastric Cancer Cells

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Abstract: Dangkwisoo-San (DS) is an herbal formula that has been traditionally used for the treatment of pain and blood stagnation caused by physical trauma in Korea. In the present study, the effects of DS in the growth and survival of AGS gastric adenocarcinoma cells and the involvement of Transient Receptor Potential Melastatin (TRPM) 7 channels were investigated. The AGS cells were treated with varying concentrations of DS. Analyses of the sub G1, caspase-3 and -9 activity and the mitochondrial depolarization were conducted to determine whether AGS cell death occured by apoptosis. Also, to identify the involvement of TRPM7 channels in AGS cell growth and survival, we used Human Embryonic Kidney (HEK) 293 cells overexpressed with TRPM7 channels. The sub G1, caspase-3 and 9 activity and the mitochondrial depolarization was increased by DS and this apoptosis was inhibited by SB203580 (a p38 Mitogen Activated Protein Kinase (MAPK) inhibitor) and by a c-jun NH_2-terminal kinase (JNK) II inhibitor. Additionally, DS inhibited TRPM7 like currents in AGS cells and in TRPM7 overexpressed HEK 293 cells. Furthermore, tetracycline induced TRPM7 channel overexpressions in HEK 293 cells increased DS-induced cell death. These results suggested that DS inhibits the growth and survival of gastric cancer cells and TRPM7 channel activity and MAPK signaling is involved in the apoptosis of gastric cancer cells. Therefore, DS may be have an anti-cancer effects and a potential drug for treatment of gastric cancer. Both TRPM7 channel and MAPK signaling may play an important role in proliferation of gastric cancer cells.

Key words: Dangkwisoo-San, transient receptor potential melastatin 7 channel, mitogen activated protein kinase inhibitor, AGS cells, apoptosis

INTRODUCTION

Gastric cancer is the second most common cause of cancer-related death in the world and it remains difficult to cure in Korea, primarily due to its frequency, poor prognosis and limited treatment options (Tang *et al*., 2013). Gastric cancer is rare before the age of 40, but its incidence steadily climbs thereafter and peaks in the seventh decade of life (Gore, 1997). Comparative studies between Asian and Western countries demonstrate striking differences in the incidence and overall survival of gastric cancer, which suggest ethnic origin as a possible risk factor (Curado *et al*., 2007; Davis and Sano, 2001; Gore, 1997). Incidence is highest in Japan (>40 per 100,000), Eastern Asia, South America and Eastern Europe; whereas Canada (10 per 100,000), Northern Europe, Africa and the United States have the lowest incidences (Dicken *et al*., 2005). Gastric carcinoma often produces no specific symptoms when it is superficial and potentially surgically curable, although up to 50% of patients may have nonspecific gastrointestinal complaints such as dyspepsia (Gore, 1997). Physical examination of early gastric cancer is usually uninformative. Patients with advanced tumors may present with a palpable abdominal mass, cachexia, bowel obstruction, ascites, hepatomegaly and lower extremity edema (Dicken *et al*., 2005; Gore, 1997). Additionally, endoscopy is regarded as the most sensitive and specific diagnostic method in patients suspected of harboring gastric cancer (Karpeh and Brennan, 1998).

In previous studies, it was suggested that human gastric adenocarcinoma cells expressed the Transient Receptor Potential Melastatin 7 (TRPM7) channel, which is essential for cell survival and is a potential target for

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pharmacological gastric cancer treatment (Kim *et al*., 2008, 2012). Recently, Zierler *et al*. (2011) found that waixenicin A (a xenicane diterpenoid isolated from the Hawaiian soft coral *S. edmondsoni*) is a potent and relatively specific inhibitor of TRPM7 ion channels and waixenicin A was found to inhibit the growth and survival of gastric and breast cancer cells (Kim *et al*., 2013). Additionally, ginsenoside Rd and quercetin inhibited the proliferation of gastric and breast cancer cells through TRPM7 channel activation and MAPK signaling (Kim, 2013; Kim *et al*., 2014). The TRPM7 is endogenously expressed in a wide variety of tissues (Minke and Cook, 2002; Montell, 2005; Montell *et al*., 2002; Runnels *et al*., 2001) and has many pathophysiological functions (Aarts *et al*., 2003; Clark *et al*., 2006; Elizondo *et al*., 2005; Hanano *et al*., 2004; He *et al*., 2005; Krapivinsky *et al*., 2006; Kim *et al*., 2005; Nadler *et al*., 2001; Schmitz *et al*., 2003; Su *et al*., 2006). Furthermore, TRPM7 has the function of regulation the survical in various cancer cells (Abed and Moreau, 2007; Jiang *et al*., 2007; Wykes *et al*., 2007). However, the role of the TRPM7 channel in the survival of gastric cancer cells after incubation with DS is unknown.

Traditional herbal medicine is based on natural plants and has many herbal prescriptions for treating cancer (Li *et al*., 2013), but its therapeutic efficacies as well as its mechanisms are unclear. Traditional Korean medications usually contain many compounds that affect multiple targets (Qiu, 2007; Wang *et al*., 2008). The combination of multiple drugs is thought to maximize therapeutic efficacy by facilitating synergistic actions and preventing potential adverse effects. DS contains nine species of herbal plants that have various pharmacological effects on the body (Li *et al*., 2006; Wang *et al*., 2004). However, there is no reports about the effects of DS on gastric cancer cell and its involvement of TRPM7 channels. In this study, the effects of DS and the role of TRPM7 channels in DS-inhibited apoptosis of AGS cells, (the most common human gastric adenocarcinoma cell lines) were examined.

MATERIALS AND METHODS

Water extraction of DS: The DS is composed of 9 species of herbal plants, each of which were purchased from Kwangmyungdang Natural Pharmaceutical Co., Ulsan, Korea. The formula of DS is described in Table 1 and 60 g of DS were boiled in 1 L of distilled water in an Herb Extractor (Dae-Woong Co, Korea) for 2 h, yielding final 200 mL of DS extract. The supernatant was harvested in sterile condition by centrifugation and lyophilized through evaporation at -80°C, yielding final 4.6 g. The lyophilized DS extract was dissolved in an appropriate volume of sterile PBS prior to administrating to cells. The water extract of DS (Voucher No. MH2014-0001) has been deposited at the Division of Longevity and Biofunctional Medicine, School of Korean Medicine, Pusan National University (Gao *et al*., 2013).

Table 1: Composition of dagkwisoo-san

Scientific name	Herbal name	Amount (g)
Angelica gigas nakai	Angelicae gigantis radix	5.625
Paeonia iactiflora pall	Paeoniae radix	3.750
Lindera strichnifoli fern andez-villar	Lindera e radix	3.750
Caesalpinia sappan L.	Sappan lignum	3.750
Cyperus rotundus L.	Cyperi rhizoma	3.750
Carthamus tinctorious L.	Carthami flos	3.000
Prunus persica batsch	Persicae semen	2.655
Cinnamoum cassia	Presl cinnamomi cortex	2.250
Glycyrrhiza uralensis fisch	Glycyrrhizae radix et rhizoma	1.875
Total		30.405

Cell: The AGS lines were used. The AGS cell lines were established at the Cancer Research Center, College of Medicine, Seoul National University, Korea. The cell lines were propagated in RPMI-1640 medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum and 20 μg mL^{-1} penicillin and streptomycin in an atmosphere of 5% CO_2 at 37°C.

Flow cytometric analysis: In order to investigate whether the cell cycle of AGS cells was redistributed, flow cytometric analysis was used with Propidium Iodine (PI) stain (Hellein *et al*., 2012; Nicoletti *et al*., 1991). The 1×10^6 cells were placed in an e-tube. About 700 μL of a ice-cold fixation buffer (ethyl alcohol) was slowly added with vortexing. Tubes were sealed with parafilm and incubated at 4°C overnight. Samples were spun for 3 min at 106×g at 4°C and the supernatant was aspirated and discarded. The cell pellet was resuspended by 200 μL of PI staining solution (PI [5 mg mL^{-1}] 2 μL and RNase 2 μL in PBS 196 μL) at 20817 g for 5 sec. After 30 min in the dark at room temperature, samples were analyzed in a fluorescence activated cell sorter (FACScan; Becton-Dickinson, Moutain View, CA, USA) at λ = 488 nm using Cell-Quest software (Becton-Dickinson). The DNA content distribution of normal growing cells is characterized by two peaks, the G1/G0 and G2/M phases. The G1/G0 phase comprises the normal functioning and resting state of the cell cycle with the most diploid DNA content, while the DNA content in the G2/M phase is more than diploid. Cells in the sub-G1 phase have the least DNA content in cell cycle distribution; this is termed hypodiploid. The hypoploid DNA contents represent the DNA fragmentation (Wang *et al*., 2005).

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay: Cell viability was assessed by using a MTT assay. The AGS cells were seeded into each well of 12-well culture plates and then cultured in Roswell Park Memorial Institute medium (RPMI)-1640 supplemented with other reagents for 72 h. After incubation, 100 μL of MTT solution (5 mg mL^{-1} in Phosphate-Buffered Saline (PBS)) was added to each well and the plates were incubated at 37°C for 4 h. After the supernatant had been removed and shaken with 200 μL of dimethyl sulfoxide (Jersey Lab Supply, Livingston, NJ, USA) for 30 min, absorbance was measured at 570 nm. All experiments were repeated at least 5 times.

Caspase assay: Caspase-3 and 9 assay kits (Cellular Activity Assay Kit Plus) were purchased from BioMol (Plymouth, PA, USA). After experimental treatment, cells were centrifuged (10000×g, 4°C, 10 min) and washed with PBS. Cells were re-suspended in ice-cold cell lysis buffer and incubated on ice for 10 min. Sample were centrifuged at 10000×g (4°C, 10 min) and the supernatant was removed. Supernatant samples (10 μL) were incubated with 50 μL of substrate (400 μM Ac-DEVD-pNA) in 40 μL of assay buffer at 37°C. The absorbance at 405 nm was read at several time points. The pNA concentrations in samples were extrapolated from a standard created using the absorbances of sequential pNA concentrations.

Assessment of mitochondrial membrane depolarization: Mitochondrial membrane depolarization was evaluated using a JC-1 fluorescence probe according to the manufacturer's instructions (Santa Cruz). The AGS cells were labeled with 2 μM JC-1 for 30 min at 37°C and then analyzed by using flow cytometry with 488 nm excitation and 530/30 or 585/42 nm bypass emission filters. The cells without red fluorescence were regarded as the cells manifesting mitochondrial membrane depolarization.

Patch-clamp experiments: A whole-cell configuration of the patch-clamp technique experiment was performed at room temperature (22-25°C). The AGS cells were transferred to a small chamber on an inverted microscope stage (IX70, Olympus, Japan) and were constantly perfused with a solution containing 2.8 mmol L^{-1} KCl, 145 mmol L^{-1} NaCl, 2 mmol L^{-1} $CaCl_2$, 10 mmol L^{-1} glucose, 1.2 mmol L^{-1} $MgCl_2$ and 10 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adjusted to a pH of 7.4 with NaOH. The pipette solution contained 145 mmol L^{-1} Cs-glutamate, 8 mmol L^{-1} NaCl, 10 mmol L^{-1} Cs-2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid and 10 mmol L^{-1} HEPES-CsOH, adjusted to a pH of 7.2 with CsOH. Axopatch I-D (Axon Instruments, Foster City, CA, USA) was used to amplify the membrane currents and potentials. For data acquisition and the application of command pulses, pCLAMP software v.9.2 and a Digidata 1322A units (Axon Instruments) were used. Results were analyzed using pClamp and Origin software (Microcal Origin version 6.0).

TRPM7 expression in human embryonic kidney 293 cells: Human Embryonic Kidney (HEK)-293 cells were transfected with the Flag-murine long transient receptor potential channel 7 (LTRPC7)/pCDNA4-TO construct and grown on glass coverslips in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, blasticidin (5 μg mL^{-1}) and zeocin (0.4 mg mL^{-1}). TRPM7 (LTRPC7) expression was induced by adding 1 μg mL^{-1} of tetracycline to the culture medium. Whole-cell patch-clamp experiments were performed at 21-25°C with cells that had been grown on the glass coverslips.

Statistical analysis: Data is expressed as Mean±Standard error of the mean (SEM). Differences between the data were evaluated by using the student's t-test. A p-value of 0.05 was taken to indicate a statistically significant difference.

RESULTS

DS induced cell death in AGS cells: To ascertain whether DS kills AGS cells, the MTT assays were performed. The viable cell population was gradually reduced with increasing concentrations of DS for 72 h in AGS cells (Fig. 1). Thus, the present results demonstrate that DS induces cell death in AGS cells.

DS triggered apoptosis in AGS cells: To determine whether AGS cell death occurs by apoptosis, sub-G1 analysis was conducted (Hotz *et al*., 1994; Vermes *et al*., 2000). In this protocol, cells were incubated with DS and stained with a fluorescent DNA stain (PI). The action of endogenous endonucleases in apoptotic cells cleaves DNA into endonucleosomal fragments of typical size, which are extracted from the cells. The loss of DNA is detected by FACS analysis, as the reduced nuclear staining in apoptotic cells, which results in a novel (sub-G1) fluorescence peak to the left of the regular fluorescence peak. Flow cytometric analysis showed that the percentage of sub-G1 phase cells markedly increases in the cells treated with DS in a dose-dependent manner in AGS cells (Fig. 2). In addition, DS elevated mitochondrial membrane depolarization, an early event of an intrinsic apoptosis signaling (Fig. 3). Thus, the present

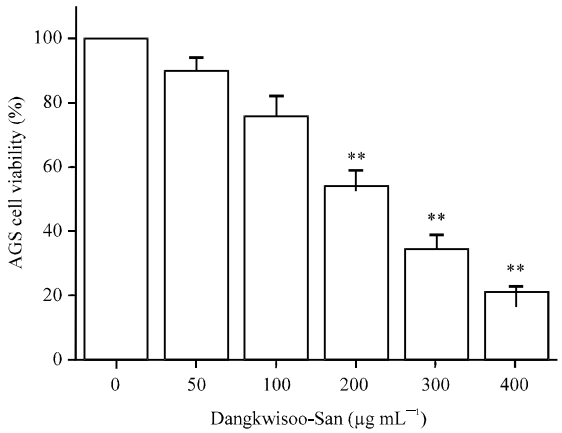

Fig. 1: DS induces cell death in AGS cells, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)-based viability assay. The AGS cells were treated with increasing concentrations of DS for 72 h. Distilled water was used as a vehicle. Cell viability is expressed as a value relative to that of the untreated cells which is set to 100%. The figures show Mean±SEM, **p<0.01

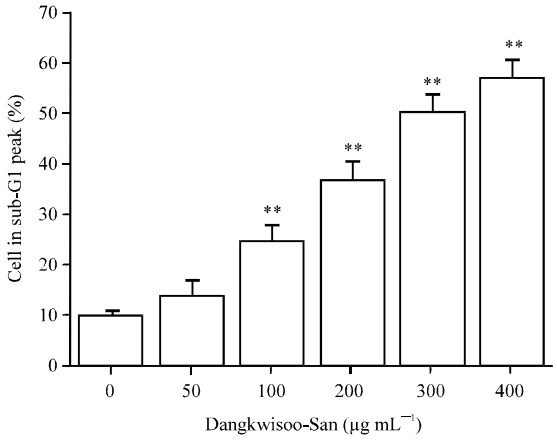

Fig. 2: Increases in activity of sub G1 peak in AGS cells due to DS. Sub-G1 peak measured by FACScan. The figures show Mean±SEM, **p<0.01

findings suggest that DS induces apoptosis via intrinsic apoptotic mechanisms. Caspase-3 and -9 activation is one of the hallmarks of apoptotic cell death. It was also measured that the enzyme activity in AGS cells after DS incubation. Using a synthetic substrate, the caspase-3 and 9 activity in AGS cells can be detected. DS increased the activity of caspase-3 and 9 (Fig. 4).

Involvement of mitogen-activated protein kinases (MAPKs) in DS-induced apoptosis in AGS cells: To investigate the signaling pathway of DS-induced

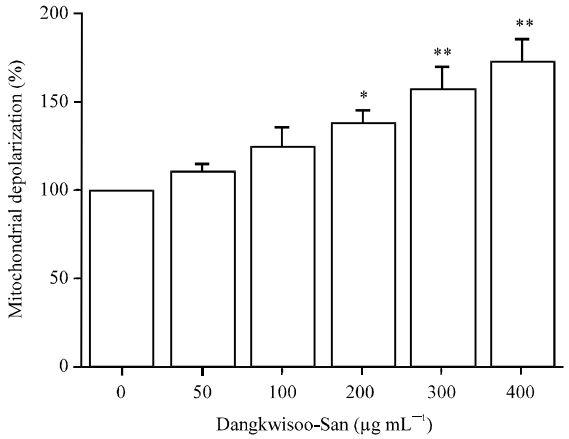

Fig. 3: Increases in mitochondrial membrane depolarization potentials in AGS cells due to DS. Mitochondria membrane depolarization is expressed as a value relative to that of untreated cells which is set to 100%. The figures show Mean±SEM, *p<0.05, **p<0.01

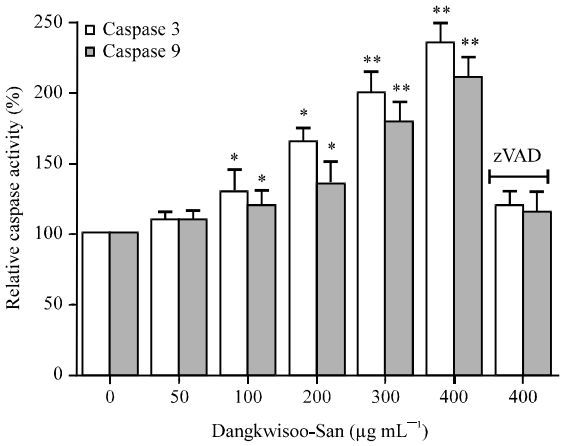

Fig. 4: Increases in caspase activity in AGS cells due to DS. The AGS cells were cultured with DS at the indicated concentrations for 24 h prior to caspase assays. Caspase activity from untreated cells is expressed as 100%. Pan-caspase inhibitor zVAD-fmk (zVAD) at 20 μM was used to validate the analytical method employed. The figures show Mean±SEM, *p<0.05, **p<0.01

apoptosis in AGS cells, we assessed the effect of DS on MAPKs, because they play critical roles in the apoptosis-related signaling pathway. As shown in Fig. 5, exposure to DS with c-jun NH_2-terminal kinase (JNK) II inhibitor (Fig. 5a) or SB203580 (a p38 MAPK inhibitor) (Fig. 5b) resulted in increases in viable cell populations.

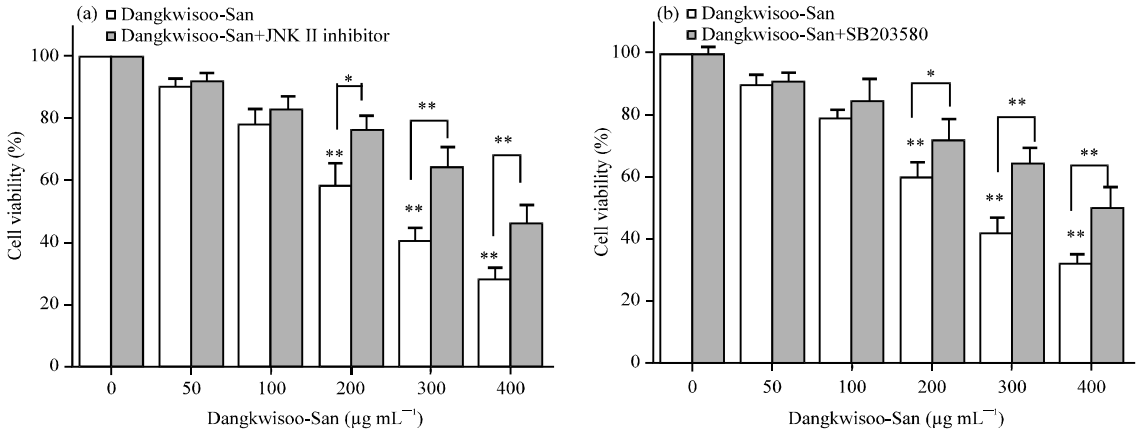

Fig. 5(a-b): Effects of JNK II inhibitor and SB203580 on DS-induced AGS cells. MTT-based viability assay, (a) C-jun NH_2-terminal kinase (JNK) II inhibitor or (b) SB203580 (a p38 MAPK inhibitor) were administered to the AGS cells pretreated by DS. The figures show Mean±SEM, *p<0.05, **p<0.01

Effects of DS in TRPM7 currents in AGS and TRPM7 overexpressed HEK293 cells: TRPM7 has been proposed to be required for cell survival on the basis of experiments on genetically-engineered DT-40 B-cells (Nadler *et al*., 2001). Also, recently it was suggested that as in previous reports, that AGS cells express the TRPM7 channel and that suppression of the TRPM7 channel induced cell death (Kim *et al*., 2008). Therefore, it as investigated that whether DS influences TRPM7 currents in AGS cells. To confirm the effect of DS in TRPM7 currents, the effects of DS in AGS cells using patch-clamp techniques were investigated. Whole cell voltage-clamp recordings was performed to investigate the effect of DS in TRPM7-like current in AGS cells. A voltage ramp of from +100 mV to -100 mV evoked small inward currents at negative potentials, whereas larger outward currents were evoked at positive potentials, showing that they were outward-rectifying cation currents (n = 4; Fig. 6a). However, in the presence of 500 μg mL^{-1} DS, the amplitudes of these currents were inhibited outwardly by 92.3±2.4% and inwardly by 95.1±2.1% (n = 7; Fig. 6a). Also similar results were obtained in HEK293 cells overexpressing TRPM7 (Fig. 6b). To provide additional evidence that supports the contribution of the TRPM7 channel to DS toxicity, the changing expression levels of TRPM7 channel and its influences on DS-mediated cell death was investigated. We used HEK293 cells with inducible TRPM7 channel expression (Nadler *et al*., 2001). In the absence of induced TRPM7 channel expression [TRPM7(-) cells, Tet(-)], HEK293 cells incubation with DS-induced cell death in the MTT assay (n = 5; Fig. 6c). However, when TRPM7 channel overexpression was induced by adding tetracycline [TRPM7(+) cells, Tet(+)], HEK293 cells incubation with DS induced cell death at an increased rate in the MTT assay, which suggests that increased expression of TRPM7 channels leads to increased rate of DS-induced cell death.

DISCUSSION

DS, an herbal extract, is widely used in traditional herbal medicine in Korea to treat traumatic ecchymosis and pain by promoting blood circulation and relieving blood stasis. However, the effects of DS in cancer has not been examined. In this study, it was demonstrated that DS suppresses AGS cell proliferation and shows sub G1 increase and mitochondrial membrane depolarization. Additionally, DS-induced apoptosis is inhibited by MAPK inhibitors and TRPM7 channels is involved in these effects. These results suggested that DS may be a useful agent for pharmacological approaches for future development of anticancer drugs. Therefore, DS would be a useful drug tool for approaches to identify novel therapeutic targets for gastric cancer.

Sophorae radix (SR), *Orostachys japonicas* (OJ) and quercetin inhibited the growth and survival of gastric and breast cancer cells due to a blockade of the TRPM7 channel activity (Hwang *et al*., 2012; Kim, 2012; Kim *et al*., 2014). Many ion channels are involved in regulation of pathophysiological role in cancer cells. Voltage-gated potassium ion channels were overexpressed in colon cancer (Abdul and Hoosein, 2002a) and voltage-gated sodium ion channels were involved in the growth of prostate cancer (Abdul and Hoosein, 2002b). Volume-regulated Cl-channels were found in a human prostate cancer cell line and in lung cancer cells (Jirsch *et al*., 1993; Shuba *et al*., 2000). Additionally, TRP channels also might have an important role in apoptosis

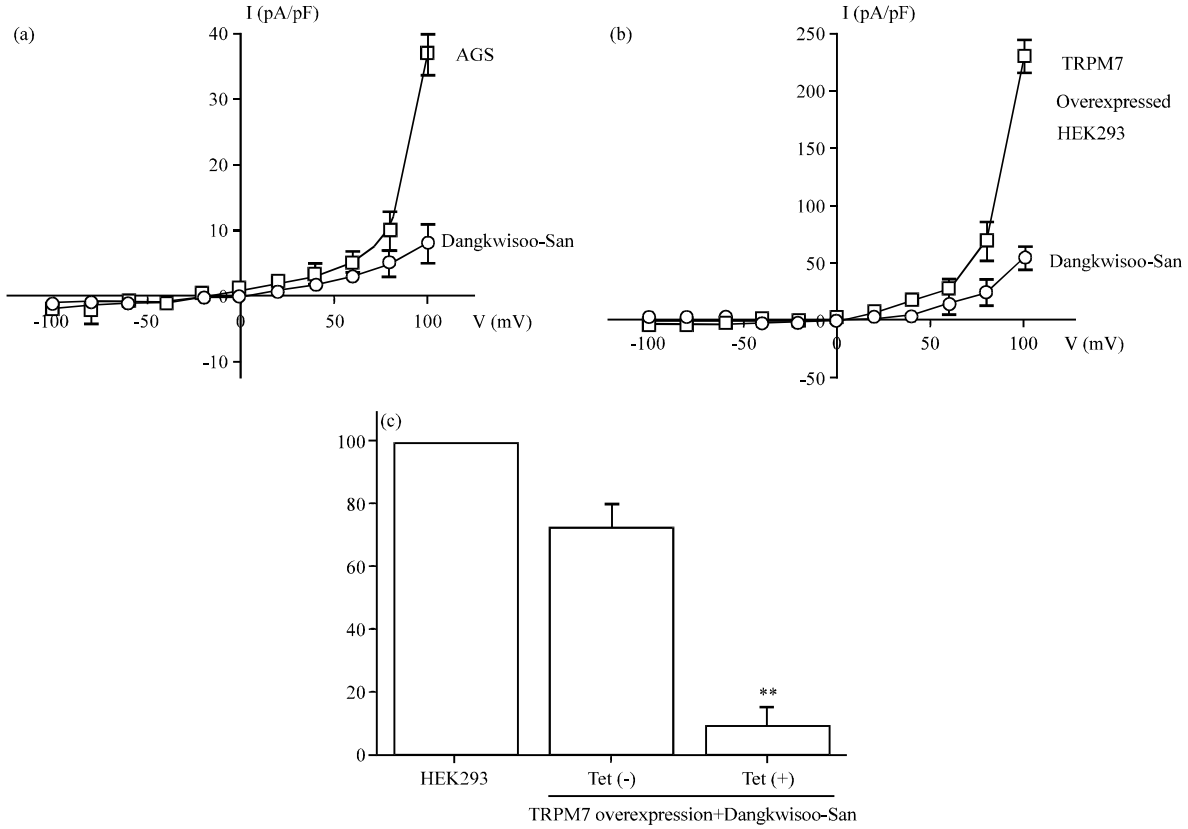

Fig. 6(a-c): Effects of DS on AGS and TRPM7 overexpressed HEK293 cells, representative I-V relationships of the effect of DS on TRPM7 currents in (a) AGS and (b) HEK293 cells while (c) TRPM7 channels were treated or not treated with tetracycline for 1 days. Cells were incubated with DS, followed by MTT assay. A voltage ramp from +100 to -100 mV was applied from a holding potential of -60 mV. The figures show Mean±SEM, $^{**}p<0.01$

and the involvement of TRP channel in cancer cells was also investigated. Among the TRP families, TRPC, TRPV and TRPM are mainly related to the growth and progression in cancer cells. Depending on the type of the cancer, regulation of TRP mRNA and protein expression have been changed. These ion channel changes are related with cell growth and apoptotic-induced cell death in cancer cells. Therefore, the regulations of ion channels in cancer cells are the most promising strategy and considerable efforts should be done to fight cancer cells (Santoni and Farfariello, 2011). In line with these studies, the present studies show that DS induces apoptosis in gastric adenocarcinoma cells, which may be due to a blocking of the TRPM7 channel activity and MAPK signaling.

DS represents a mixture of nine herbal medicines, consisting of Angelicae gigantis Radix, Paeoniae Radix, Linderae Radix, Sappan Lignum, Cyperi Rhizoma, Carthami Flos, Persicae Semen, Cinnamomi Cortex and Glycyrrhizae Radix et Rhizoma. Most traditional therapeutic formulations consist of a combination of several drugs. Bioactivity from each drug may collectively act to block multiple targets underlying apoptosis, although little is known about the mechanisms for their pharmacological activities (Lyu *et al*., 2012; Kim *et al*., 2011; Qiu, 2007; Wang *et al*., 2008). The combination of multiple drugs is thought to maximize therapeutic efficacy by facilitating synergistic actions and preventing potential adverse effects. However, little is known concerning the compounds responsible for the apoptotic effect of DS. In future, to perform additional experiments to identify the efficient compounds from DS need.

Taken together, DS induced the apoptosis in AGS cells and both MAPK signaling and TRPM7 channel activity are involved in DS-induced effects. Therefore, DS may be a potential drug for treatment of gastric cancer.

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