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Research Paper

Salvianolic acid B synergized with Tanshinol shows a potent osteogenesis effect *in vitro*

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ABSTRACT

This study aims to discover a formula containing an appropriate proportion of SAB and TAN and to evaluate its effect on promoting the differentiation and mineralization of the Rat Osteoblasts Cells (ROB). Seven mixtures containing different proportions of SAB and TAN were acquired by hydrolyzed *Salvia militorrhiza* (Danshen); a recipe that contains nine molecules of SAB and one molecule of TAN was selected by MTT assay for further study. Its effects on the differentiation and mineralization of the ROB were determined by Alkaline Phosphatase (ALP) assay and Alizarin Red S staining assay. Additionally, its effect on oxidative stress induced by the hydrogen peroxide (H_2O_2) and dexamethasone (DEX) was analyzed. Finally, High-Performance Liquid Chromatography (HPLC) was applied to analyze the metabolism of the recipe in the ROB model. The recipe could increase the ALP activity and promote mineralization of ROB and can also reverse the deleterious effect induced by H_2O_2 and DEX on ROB through decreasing malondialdehyde (MDA) and cellular Reactive Oxygen Species (ROS) and increasing the catalase (CAT) and the superoxide dismutase (SOD) level. These effects of the recipe were better compared to SAB or TAN alone. The HPLC results indicate that SAB could metabolize into TAN, extending the half-life time of TAN. The result indicated that a combination of SAB and TAN might provide a novel therapeutic approach for osteoporosis.

Key words: Salvianolic acid B, tanshinol, osteoblast cells, osteoporosis.

Abbreviation: SAB: Salvianolic acid B; **TAN:** Tanshinol; **ROB:** Rat osteoblasts cells; **ALP:** Alkaline phosphatase; **H_2O_2:** Hydrogen peroxide; DEX: Dexamethasone; **HPLC:** High-performance liquid chromatography; **MDA:** Malondialdehyde; **ROS:** Reactive oxygen species; **CAT:** Catalase; **SOD:** Superoxide dismutase; **OP:** Osteoporosis.

INTRODUCTION

With the aging of population worldwide, osteoporosis (OP) has become a severe health problem (Black and Rosen, 2016). Despite the development of several therapeutic strategies for OP, Chinese herb may still be beneficial to osteoporosis and may be better than the placebo or other standard anti-osteoporotic drugs (Wang et al., 2013).

Salvia militorrhiza (DanShen), a well-known traditional Chinese herb, is used to treat postmenopausal, senile and secondary osteoporosis with high efficacy and low toxicity (Guo et al., 2014). Several individual constituents of this herb mainly including lipophilic compounds (Tanshinone I, IIA and IIB) and hydrophilic compounds (Tanshinol and Salvianolic acid B) have been identified (Zhou et al., 2005). Either lipophilic compounds or hydrophilic compounds have been reported possessing anti-osteoporosis effect.

Zhu et al. (2018) reported that Tanshinone IIA had

protective effects against oxidative stress in osteoblastic differentiation in mice with osteoporosis by regulating the NF-κB signaling pathway. Chen et al. (2017) investigated the effect of Tanshinol on glucocorticoid-induced osteoporosis rats and claimed that TAN could significantly reverse the glucocorticoid-induced loss of bone mineral density. Luo et al. (2016) observed that SAB could stimulate bone formation in larval zebrafish. In summary, Danshen is a potent agent for therapy of osteoporosis.

In animal study, TAN was rapidly absorbed after oral administrations while SAB was poorly absorbed. In other words, the half-life time of TAN is quite short. Structurally, SAB consists of three molecules of TAN and a fragment of caffeic acid and as such could metabolize to TAN (Guo et al., 2014; Zhou et al., 2005). Hence, we marvel if a combination of SAB and TAN could overcome the drawbacks thus, exhibiting a better anti-OP effect. The aim of this study is to explore a suitable proportion of SAB and TAN, investigate their effect on osteoblast cells, and determine their anti-oxidative stress capability. Finally, the pharmacokinetics of the formulation was studied.

MATERIALS AND METHODS

The minimum standards of reporting checklist contains details of the experimental design, statistics and resources used in this study.

Study design

Several recipes containing different rations of SAB and TAN were acquired from hydrolyzing the DanShen. We selected a promising mixture by MTT assay and SAB and TAN as control. The recipe's effect on the differentiation and mineralization of ROB and its anti-oxidative stress activity was further investigated; finally, the metabolism of the recipe in the supernatant of the ROB growth medium was evaluated using HPLC.

Preparation of SAB and TAN mixtures

SAB and TAN are well-known water-soluble components of Danshen, hence, hydrolysis is applied to acquire some recipes containing different proportions of SAB and TAN. The protocol was involved in our new-drug application which may be later disclosed in the near future.

Primary culture of ROB

The primary ROB was obtained for the calvaria of neonatal Sprague-Dawley (SD) rats by type 1 collagen digestion. Our previous literature described the protocol used (Cui et al., 2009).

Cell viability assay

Three thousand ROB were plated in 96 well plates in their growth medium for 24 h. Subsequently, the ROB was further exposed to different mixtures containing SAB and TAN in different concentrations for 48 h. After the incubation time, cells were cultured for further 4 h at 37°C in refreshed medium containing MTT (5 mg/ml). Thereafter, the formazan crystals were solubilized in dimethyl sulfoxide (DMSO), and the value of optical density of each well measured at a wavelength of 570 nm by a microplate reader (Thermo Fisher Scientific, Vantaa, Finland).

ALP activity assay

10^6 cells were plated in 6-well plate in their growth medium. After 24 h of plating, cells were treated with indicated reagents and cultured sequential for a specified period of time. For the quantitative determination of ALP activity at day 3, 5 and 7, cells were lysed in 100 nM glycine, 1 mM $MgCl_2$, and 1% Triton X-100 at PH 10. Cell lysates were further subjected to ALP activity using an ALP assay kit (Nanjing Jiancheng Biotech, China). For ALP staining, cells in the plate were stained by ALP staining kit (Sigma).

Alizarin red S staining

10^6 cells were plated in 6-well plate medium containing 10 mmol/L β-sodium glycerophosphate and 0.3 mmol/L vitamin C. To measure the mineralization activity, ROB were stained with alizarin red S after 21 days treatment. Red staining was visualized, and representative pictures photographed with a microscope. For the quantitative determination of the osteocalcin secretion in supernatants of the medium at day 21, an ELISA kit (Nanjing Jiancheng Biotech, China) was used.

Oxidative stress assay

After exposure to H_2O_2 and DEX, cells were treated with SAB and TAN as earlier indicated. The intracellular oxidative stress was a probe by DCFH-DA and quantified using flow cytometry, as previously described. MDA, CAT and SOD levels were determined by ELISA kit separately (Nanjing Jiancheng Biotech, China).

The metabolism of the recipe in ROB culture medium

After treatment with the indicated reagent, the supernatant

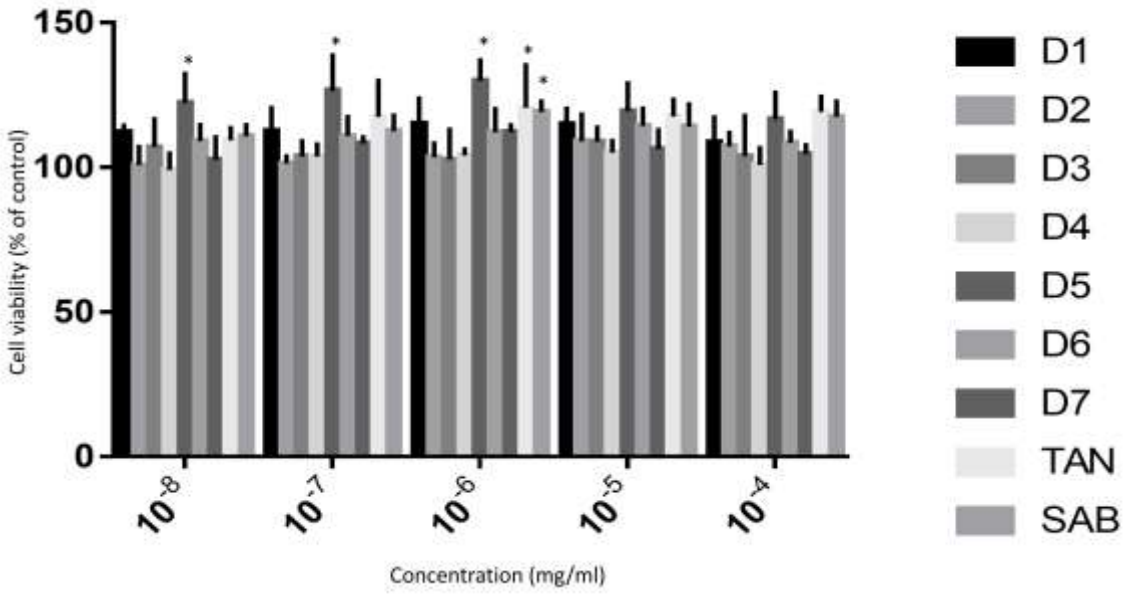

Figure S1: Seven extractions obtained from DanShen and the contents of SAB and TAN in the extraction determined by HPLC, including D1 (0.2:1), D2 (1:1), D3 (2:1), D4 (7:1), D5 (9:1), D6 (25:1) and D7 (67:1).

of the ROB was collected for HPLC in order to analyze SAB and TAN concentrations. The chromatographic evaluation was achieved at 40°C on UPLC ACQUITY BEHC18 (50 × 2.1 mm, 1.7 µm). The mobile phase was acetonitrile and 0.1% formic acid. The run-time was set at 40 min. The flow rate was 1.0 ml/min. The sample volume was ten microliters. SAB and TAN, as ingredients of the DanShen were purchased from the National Institutes for Food and Drug Control (Beijing, China).

Statistical analysis

Data were presented as a mean ± standard deviation and analyzed using SPSS version 20.0 software for Windows (SPSS, Lnc., Chicago, IL, USA). The statistical differences among groups were evaluated using variance with Fisher's protected least significant difference test. A p-value less than 0.05 was chosen to indicate the significance.

RESULTS

Combination of SAB/TAN

From the results of the analysis, SAB/TAN at a ratio of 9:1 and concentration of 10^{-6}mg/ml produced the best effect on the proliferation of ROB. Seven extractions were first obtained from DanShen and the contents of SAB and TAN in the extraction determined by HPLC, including D1 (0.2:1), D2 (1:1), D3 (2:1), D4 (7:1), D5 (9:1), D6 (25:1) and D7 (67:1). Thereafter, MTT assay was performed to determine the osteogenic effect on ROB with these extractions at different concentrations, and the results showed that D5 at a concentration of 10^{-6} mg/ml, SAB and TAN at a concentration of 10^{-6}mol/L showed a promising effect on the proliferation of ROB (Figure S1). Therefore, 10^{-6} mg/ml of D5, 10^{-6} mol/L SAB and TAN were selected for further study.

The effects of D5 on the differentiation and mineralization of ROB is better than SAB and TAN alone

Furthermore, the effect of D5 on the differentiation and mineralization of ROB by ALP assay and alizarine red S staining, and osteocalcin was determined. The ALP staining and activity results showed that D5 could increase the ALP activity of ROB and promote its differentiation (Figure 1A and C). The alizarine red S staining and osteocalcin (OCN) Elisa results indicated that D5 could promote osteocalcin secretion and promote ROB mineralization (Figure 1B and D). The effect of D5 is better compared to SAB and TAN alone.

D5 could reverse the deleterious effect of H_2O_2 and DEX on the ROB

Oxidative stress is a risk factor for OP. In this study, a cell model with H_2O_2 was first established and it was observed that H_2O_2 at a concentration of 800 µM could significantly decrease the cell viability (about 50%) of ROB by MTT assay (Figure S2A). Figure 2A showed that D5 and TAN could reverse the inhibited effect induced by 800 µM H_2O_2 on the proliferation of ROB. Thereafter, the oxidative stress

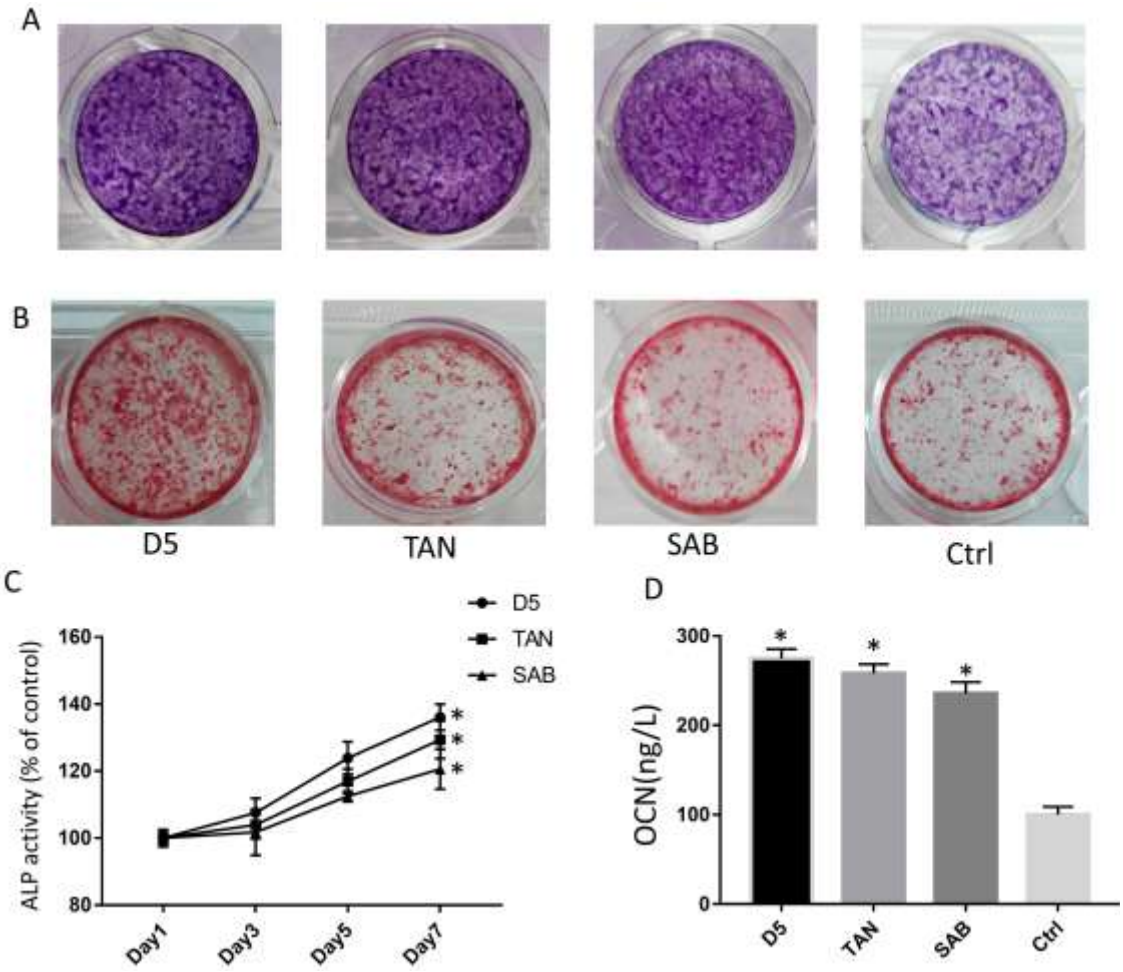

Figure 1: D5 promoted the differentiation and mineralization of ROB. A) ALP staining; B) alizarin red S staining; C) ALP activity and D) OCN level in growth medium at day 21. * means P<0.05 versus the control group.

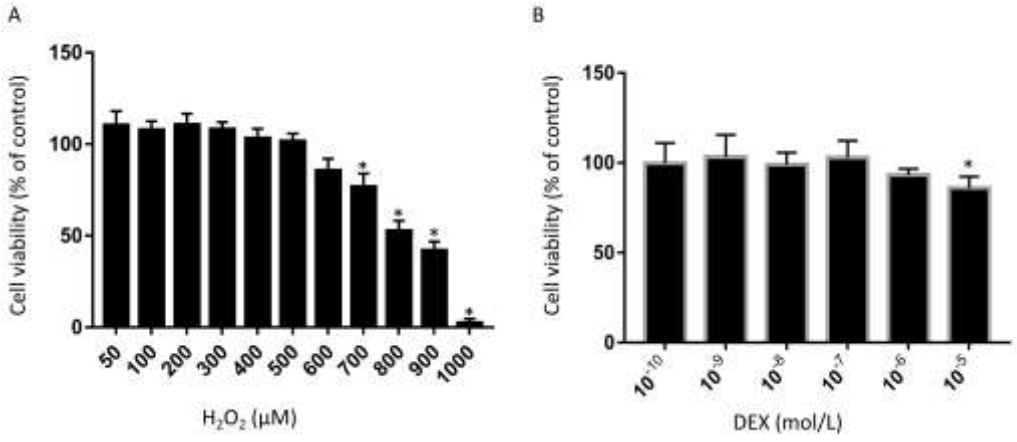

Figure S2: The deleterious effect of H_2O_2 and DEX on the ROB by MTT assay.

markers and cellular ROS level were determined. The results illustrated that H_2O_2 increased the MDA level and decreased CAT and SOD levels in the growth medium; D5 could reverse these effects significantly, showing a potent antioxidative stress activity and a better effect when compared to TAN and SAB alone (Figure 2). To further

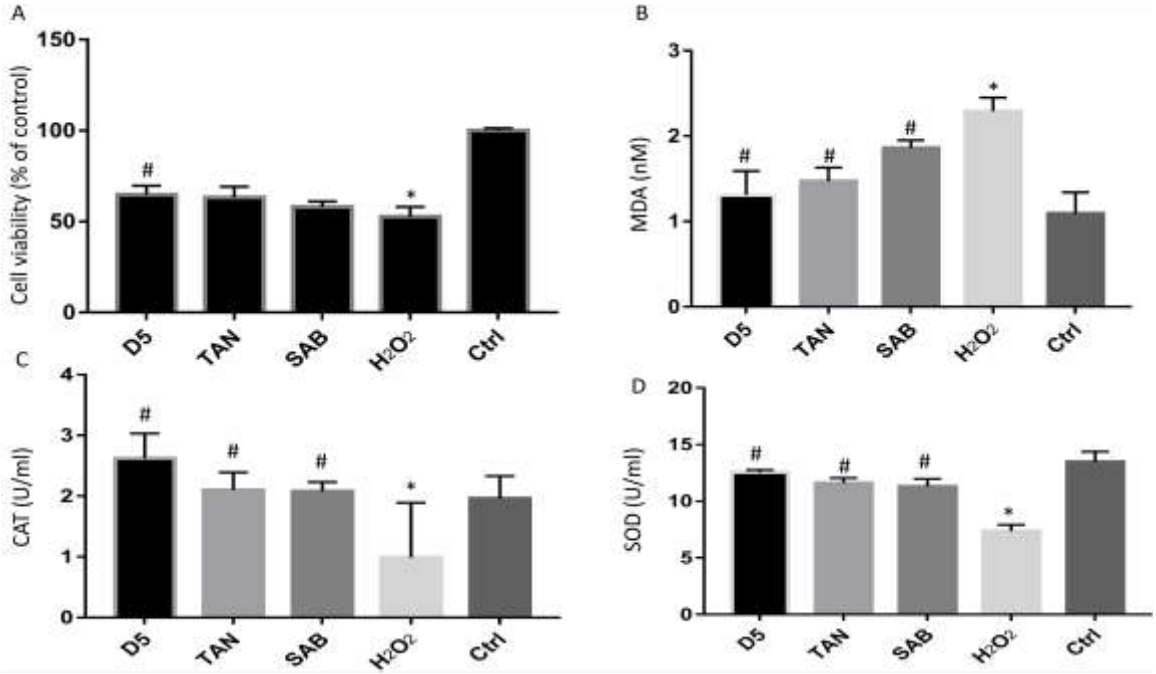

Figure 2: D5 reverse the deleterious effect induced by H_2O_2. A) MTT assay results. D5 could significantly increase the cell viability after H_2O_2 exposure; B) MDA level, D5, TAN and SAB could decrease the MDA level in the growth medium; C) CAT level and D) SOD level, D5, TAN and SAB could increase the CAT and SOD level in the growth medium. * means $P<0.05$ versus control, # means $P<0.05$ versus H_2O_2.

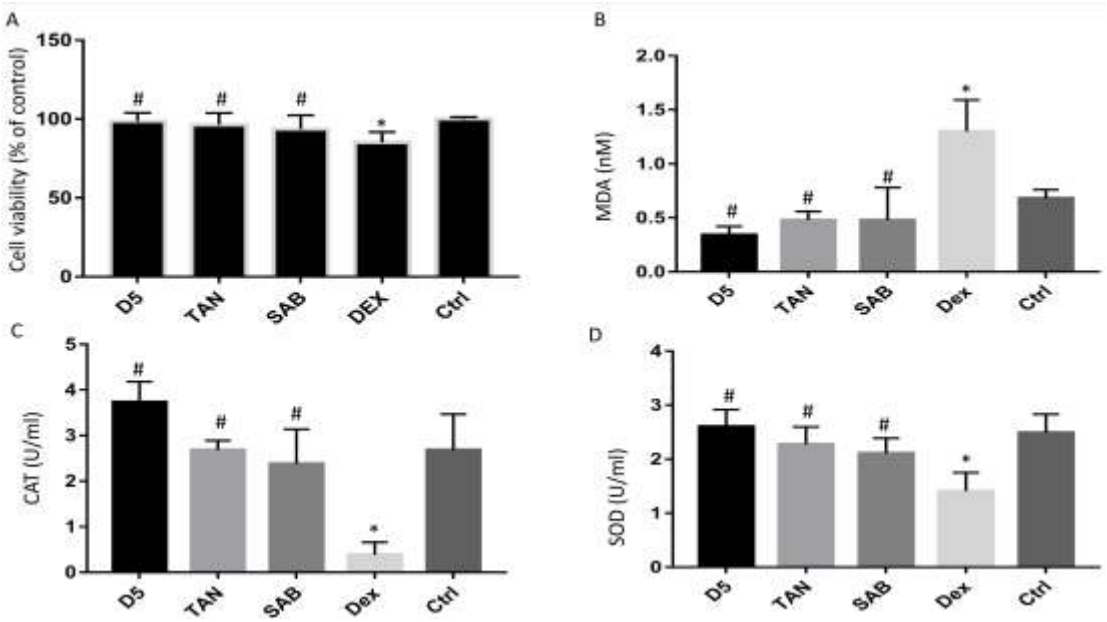

Figure 3: D5 reverse the deleterious effect induced by DEX. A) MTT assay results. D5 could significantly increase the cell viability after DEX exposure; B) MDA level, D5, TAN and SAB could decrease the MDA level in the growth medium; C) CAT level and D) SOD level, D5, TAN and SAB could increase the CAT and SOD level in the growth medium. * means $P<0.05$ versus control, # means $P<0.05$ versus DEX.

confirm the antioxidative stress effect of D5, DEX at a concentration of 10^{-5} mol/L was applied to induce the OS in ROB. The results obtained are similar to the H_2O_2 model (Figures S2B and 3).

Besides the determination of the oxidative stress markers in the growth medium, a flow cytometry was also applied to

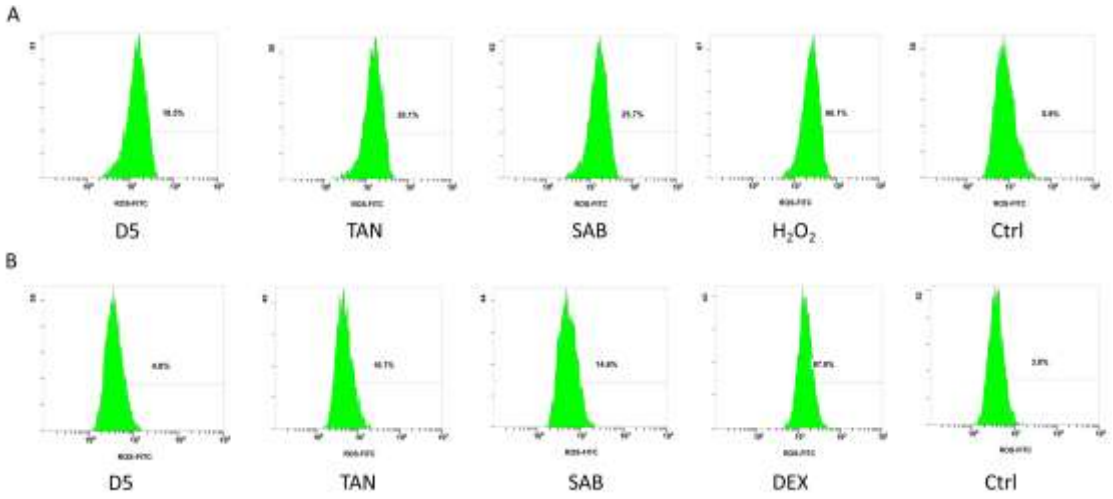

Figure 4: Intracellular ROS level determined by flow cytometry. (A) After exposure to H_2O_2 and (B) DEX, the intracellular ROS increased; D5, TAN and SAB could eliminate the ROS of the cell. D5 shows the best effect.

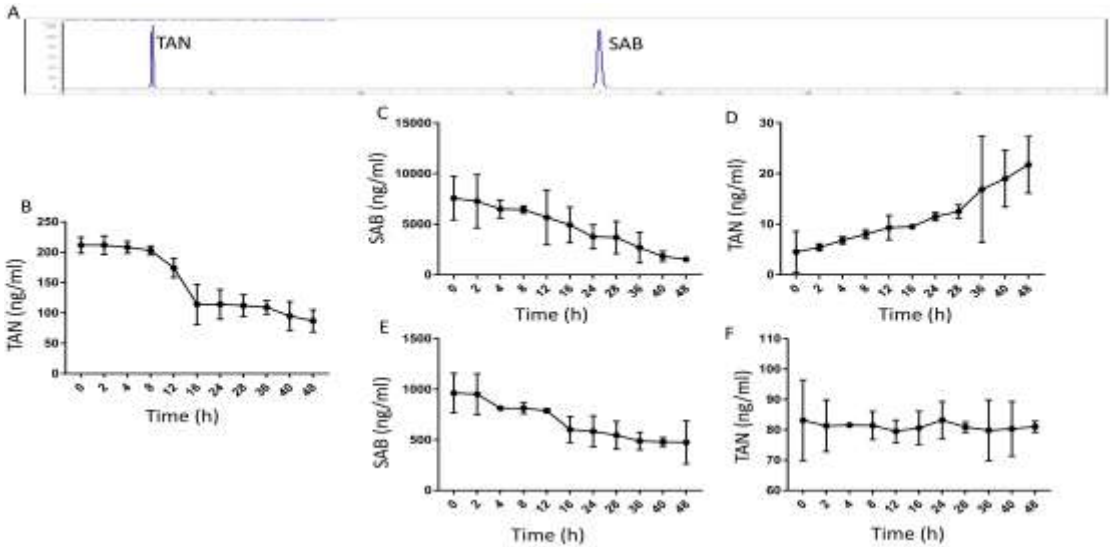

Figure 5: SAB could metabolize into TAN and extend its half-life time. A) TAN and SAB determined by HPLC; B) The pharmacokinetics of TAN; C and D) The pharmacokinetics of SAB, E and F) The pharmacokinetics of D5.

evaluate the intracellular ROS level of the ROB and the results showed that D5 could significantly decrease the intracellular ROS level induced by H_2O_2 and DEX (Figure 4). These results indicated that D5 can promote the differentiation and mineralization of ROB through antioxidative stress; hence, further investigation is needed to clarify its mechanism.

SAB could metabolize into TAN and extend its half-life time

Finally, HPLC was applied to investigate the pharmacokinetics of D5 in the growth medium. After treating with D5, ten microliters were collected from the supernatants at time points of 0, 2, 4, 8, 12, 16, 24, 28, 36, 40 and 48 h for HPLC analysis, respectively. The result indicated that SAB could metabolize into TAN and extend its half-life time (Figure 5).

DISCUSSION

TAN and SAB are the two main active compounds that have been documented (Zhao et al., 2008). Most of the publications studied their pharmacological effect alone (Luo et al., 2016; Yang et al., 2018; Zhang et al., 2017; Cui et al., 2012). However, most well-known Chinese medicine

herbs are applied in a mixture, like Fufang danshen tablet and Fufang danshen dripple pills (Ren-an et al., 2014). To the best of our knowledge, this is the first study as regards a combination of TAN and SAB on the osteogenesis of osteoblast cells.

Osteoporosis is characterized by low bone mineral density (BMD) and deteriorated microarchitecture of bone, leading to an increased risk of bone fracture (Gass and Dawson-Hughes, 2006). In simple terms, the healthy bone remains in a dynamic equilibrium of bone formation and bone absorption balance, which is mainly mediated by osteoblast and osteoclast cells. Once the homeostasis is interrupted, either the activity of osteoclasts cells will increase, or the activity of osteoblasts cells will be inhibited, which may likely result in OP. Osteoclasts play a central role in the pathogenesis of postmenopausal osteoporosis (Gruber et al., 1986). However, in some secondary osteoporosis, like glucocorticoid-OP, the bone formation activity of osteoblasts cells was inhibited (Hofbauer et al., 1999).

In this study, we investigated the role of the recipe on the osteoblast cells, aiming to provide a promising therapeutic strategy for secondary osteoporosis. Our results illustrated that SAB/TAN at a proportion of 9/1 exhibited satisfactory effects on the differentiation and mineralization of osteoblasts.

Oxidative stress is one of the risk factors for bone mass. How oxidative stress influence bone mass and leads to osteoporosis have been reviewed (Mohammad et al., 2005). Antioxidant may be helpful in the management of osteoporosis. In the present study, oxidative stress cell model using H_2O_2 and DEX was established. The intracellular ROS level rapidly increased, while cell viability significantly decreased after exposure to these two items. The recipe could reverse these deleterious effects significantly indicating its underlying mechanism through mediating oxidative stress. Further investigation is needed to clarify its molecular mechanism.

Finally, several researchers have carried out researches on the pharmacokinetics of TAN and SAB in an animal model (Chang et al., 2010; Zhou et al., 2009) and has been reviewed (Zhou et al., 2005). We at this moment applied HPLC method to determine the pharmacokinetics of the recipe in the growth medium at a series time point. We conclude that SAB could metabolize into TAN and extend its half-life time, thus, showing a promising osteogenesis effect. Even though the results were preliminary, this study provided evidence for developing a new therapeutic strategy for osteoporosis. Further investigation of the recipe in the animal model is necessary in obtaining a more detailed result.

Conclusion

From the result of the analysis carried out, we suggest a formula containing SAB and TAN at a proportion of 9/1 showing an excited osteogenesis effect *in vitro*, which provide an avenue to develop a new formula for osteoporosis and a new strategy to develop our traditional herb- *S. militorrhiza.*

ACKNOWLEDGMENTS

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