

The therapeutic effects of WSY-0702 on benign prostatic hyperplasia in RWPE-1

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ABSTRACT

Benign prostatic hyperplasia (BPH) is one of the major diseases of the urinary system in older men. WSY-0702 is the extracted from the traditional medicinal plant; Seoritae, and it has effects of anti-obesity, chronic cervical pain, and anti-oxidant. The present study aimed to investigate the therapeutic potential of WSY-0702 in the prevention and treatment of BPH. Several parameters including inflammatory mediators, hormones, and oxidative stress (OS) have been considered to play a role in the development of BPH. Prostate tissue damage and OS may lead to compensatory cellular proliferation with resulting hyperplastic growth. An in vitro study showed that proliferation inhibited the human prostate epithelial cell line RWPE-1 in a dose-dependent manner. In cell line, the cell cycle at the G2/M and G0/G1 phase and downregulated the expression of CyclineB1 (CCNB1) and CyclineD1 (CCND1). In addition, we measured the H₂O₂-induced OS damage using RWPE-1 cells. We examined the relative expression of protein involved in the regulation of prostate apoptosis: transforming growth factor (TGF)- β , a negative growth factor able to induced prostate apoptosis under physiological conditions. These results suggest that WSY-0702 that can inhibit the growth of prostate epithelial cell by a mechanism that may involve arresting the cell cycle and downregulating CCNB1 and CCND1 expression. In addition, WSY-0702 exposure resulted in significant protective effects in H₂O₂-stressed PWPE-1 cells by reduction in TGF- β levels.

Keywords WSY-0702, benign prostatic hyperplasia, anti-oxidant, human prostatic epithelial cell line, cell cycle

INTRODUCTION

Benign prostatic hyperplasia (BPH), also called prostate adenoma, is one of the most common diseases of older men. Up to 80 percent of men have anatomic evidence of BPH by the 8th decade of life (Glynn et al., 1985). The increase in prostate volume causes the physical compression of the urethra and clinically manifests as lower urinary tract symptoms (LUTS), which seriously impact the quality of life of older men (Patel and Parsons, 2014). Several parameters including inflammatory mediators, hormones, dietary factors, inflammatory genes, and oxidative stress (OS) have been considered to play a role in the development of BPH, but there is no consensus as to which is the primary one (Bostanci et al., 2013).

Hormone imbalance plays an important in the development of BPH. Normal levels of androgens and oestrogens synergistically maintain the prostate (Kumar et al., 2012). The prostate is also a target tissue for oestrogens. As elderly men increase in age, the ratio of androgens to oestrogens decreases in both the serum and the prostate tissue (Suzuki et al., 1992). All these change enhance the effects of

oestrogen in the prostate of older men. A recent study has shown that an imbalance of the androgen level and enhanced oestrogenic effects were the main cause of BPH (Farnsworth, 1999).

Cyclin D1 (CCND1) gene plays an important role in the progress of the cell cycle (Moreno-Bueno et al., 2003). Cyclin E, dissimilar to the mitogen-dependent cyclin D, is a mitogen-independent activator of CDK and a critical regulator of G1/S transition (Sherr, 2000). More than in cell-cycle control genes have frequently discovered in many human malignant tumors (Agarwal et al., 2009; Barbieri et al., 1999). CCNB1, CCND1, and CCNE1 have distinct expressions in human prostatic stromal cell line (Nickel et al., 2007).

OS is considered to be one of the mechanisms that trigger the chain of reactions involved in the development and progression of BPH. OS is a condition in the cellular environment which occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of biological systems to repair oxidative damage or neutralize the effects of reactive intermediates including peroxides and free radicals. In addition, in the prostate, a high production of ROS has been linked with the development of adenoma and prostate cancers, where very high levels of ROS, especially H₂O₂ and the superoxide radical, were found (Khandrika et al., 2009).

Transforming growth factor (TGF)- β , is a member of a family of multifunctional regulators of cell growth, controlling diverse cellular processes, such as proliferation, differentiation, extracellular matrix formation, and morphogenesis (Massagué,

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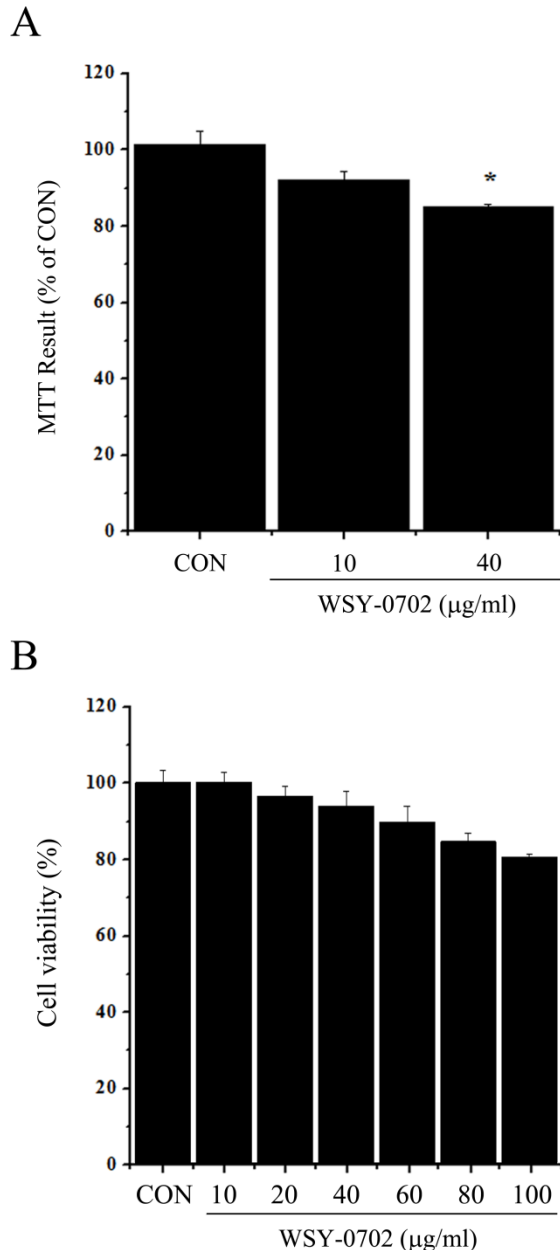


Fig. 1. WSY-0702 inhibits the proliferation of the human prostate epithelial cell line RWPE-1. (A) WSY-0702 inhibits the proliferation of RWPE-1 cells. RWPE-1 cells were treated for 24 h with a series of WSY-0702 concentration (10 and 40 µg/ml) and analysed by the MTT assay. (B) Cell viability was evaluated by an MTT assay (for 8 h). The values shown represent the mean \pm SD ($n = 3$). * $p < 0.05$ versus the vehicle treated control sample (CON)

1990). Recent evidence has linked the increase of expression TGF- β , with a concomitant induction of apoptosis in diverse cellular systems including the prostate (Kyprianou and Isaacs, 1989; Rotello et al., 1991; Yanagihara and Tsumuraya, 1992). Significantly enough, TGF- β has been shown to induce the apoptotic death of androgen-dependent prostatic epithelial cells even in the presence of physiological levels of androgens (Kyprianou and Isaacs, 1989; Martikainen et al., 1990), evidence that points to a role for TGF- β as a physiological regulator of epithelial cell apoptosis in the prostate.

Seoritae (*Glycine max* (L.) Merr.) is grown in Korea,

which gets its name from the fact that it is harvested early in October when the first frost occurs. It is kind of black soybean, unlike other black soybeans, the inside of Seoritae is of a bluish color. The description of black soybeans in 'food is medicine' section of the 'Ben Cao Gang Mu (本草綱目; compendium of materia medica in traditional medicine; UNESCO World Heritage Site), it shows effect of blood circulation and improving of kidney function. Recently, black soybean has been known effect of anti-obesity, chronic cervical pain and anti-oxidant (Lee et al., 2015; Li et al., 2015; Liu et al., 2015). In this study, we investigated a possible inhibitory effect of WSY-0702 on BPH through in vitro analyses. Our finding provides an experimental basis for developing health functional food for BPH prevention and treatment.

MATERIALS AND METHODS

Materials

Waters 996 Photodiode Array Detector (Waters corporation; Milford, MA, USA), keratinocyte serum free media (K-SFM; Gibco; Grand island, NY, USA), human recombinant epidermal growth factor (EGF) and bovine pituitary extract (BPE) were purchased from Invitrogen (Carlsbad, CA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St Louis, MO, USA). Human recombinant (r)TGF- β , anti-human TGF- β and biotinylated human TGF- β were purchased from BD pharmingen (San Diego, CA, USA). TMB substrate (Pharmingen, San Diego, CA, USA). The primary antibodies used are CCNB1 and CCND1 were purchased from Boster (Wu Han, China). The secondary antibody was goat anti-rabbit IgG (H + L)-HRP Conjugate (1/5000; Product No. #170-6515, Bio-Rad laboratories, CA, USA).

Preparation of WSY-0702

The WSY-0702 used in our experiment was produced using the following method: Seorite (150 g; Andong-si, Gyeongsangbuk-do, Korea) sample were extracted with 1,200 ml of 30% ethanol for 3 h at 90-100°C. The solution was then filtered twice through a 50 µm and a 1 µm filter and concentrated in a vacuum evaporator (60°C) to 70 brix% (700 mg/g). The residual solvent was removed from WSY-0702 by using a drying machine for 18 h at 60°C in a vacuum (HANPOONG PHARM & FOODS Co., Ltd.). The resulting powder was then stored in plastic bag until use.

Cell culture

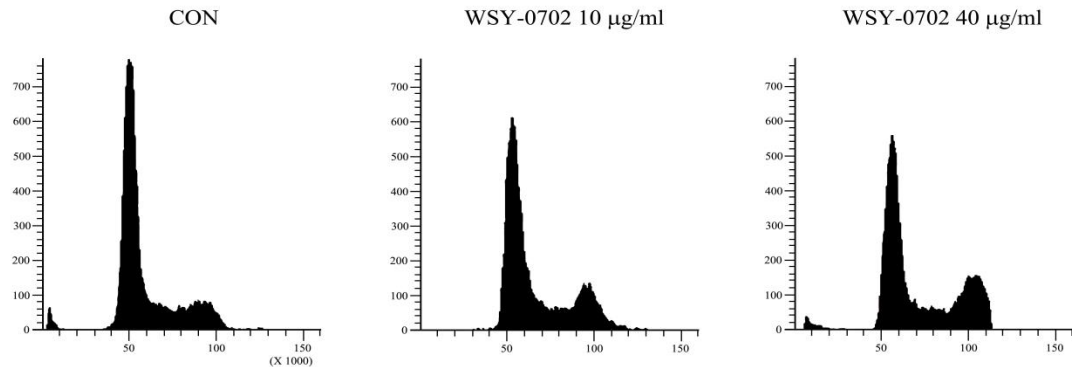
The normal human prostatic epithelial cell line, RWPE-1, respectively, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; cell passage number 4 or 5). RWPE-1 cells were cultured in K-SFM containing 5 ng/ml EGF and 0.05 mg/ml BPE.

Cell viability assay

RWPE-1 cell aliquots (1×10^5 cells/ml) were cultured in microplate wells for 8 h after treatment by WSY-0702 (10, 20, 40, 60, 80, or 100 µg/ml) and incubated with 20 µl of an MTT solution (5 mg/ml) for additional 4 h at 37 °C under 5% CO₂ and 95% air. Consecutively, 250 µl of DMSO was added to extract MTT formazan and the absorbance of each well at 540 nm was read by an automatic micro plate reader.

Cellular uptake of WSY-0702

A



B

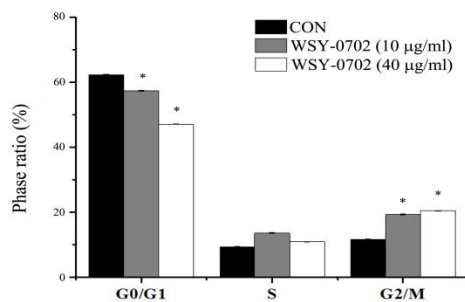


Fig. 2. The effects of WSY-0702 on cell cycle distribution in RWPE-1 cells. (A) Cell cycle analysis of RWPE-1 cells by flow cytometry. (B) The cell cycle phase distribution of RWPE-1 cells with or without WSY-0702 treatment. For cell cycle, the cells were harvested after 48 h of drug treatment. The values shown represent the mean \pm SD ($n = 3$). * $p < 0.05$ versus the vehicle treated control sample (CON)

RWPE-1 cells were seeded at a density of 1×10^5 cells/ml in 100 mm culture plates with three replicates for each incubation time point. After 24 h of seeding the culture medium was replaced with fresh medium containing WSY-0702 (10 or 40 $\mu\text{g/ml}$).

Reactive Oxygen Species measurement

RWPE-1 cells were plated at 1×10^5 cells per well in 96-well plates in appropriate culture medium. As cells reached to 75 - 80% confluence subsequently treated with different concentration of WSY-0702 for 24 h and 100 nM H_2O_2 for 2 h. The treatment medium was removed and cells were washed with phosphate buffered saline (PBS).

Cell proliferation assays

Cell proliferation was analyzed by the MTT assay and by cell counting in triplicate. RWPE-1 cells were seeded in 24-well plates in quadruplicates at a density of 2×10^4 cells/well and 3×10^4 cells/well, respectively. After adherence, WSY-0702 (10 or 40 $\mu\text{g/ml}$) or vehicle control, were added to the cells for the desired times. The MTT assays was conducted using the Cell Growth Determination kit according to the manufacturer's instructions.

Cell cycle analysis

For cell cycle analysis, WSY-0702 (10 or 40 $\mu\text{g/ml}$)-treated or untreated cells were harvested and washed with ice-cold PBS and then fixed overnight at -20°C in 70% ethanol. The cells were then washed twice, incubated for 30 min at 37°C with 0.5 ml PBS containing 1 mg/ml of RNase A, and stained with 0.5 ml of 50 $\mu\text{g/ml}$ propidium iodide for 20 min at 37°C . The

fluorescence intensity was quantified using a FACScan system (Becton Dickinson, Franklin Lake, NJ, USA)

Enzyme-linked immunosorbent assay (ELISA)

The amount of TGF- β was determined using a sandwich ELISA method. The ELISA was performed by coating 96-well plates with 1 $\mu\text{g/well}$ capture antibodies (Ab). Before the subsequent steps in the assay, the coated plates were washed twice with 1 x PBS containing 0.05% Tween 20. All reagents and coated wells used in this assay were incubated for 2 h at room temperature. The standard curve was generated from the known concentrations of cytokine, as provided by the manufacturer. After exposure to the medium, the assay plates were exposed sequentially to each of the biotin-conjugated secondary Ab was then added and again incubated at room temperature for 2 h. After washing the wells, avidin-peroxidase was added and the plate was incubated for 30 min at 37°C . The wells were again washed and TMB substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader.

Western blot analysis

After treatment with WSY-0702 (10 or 40 $\mu\text{g/ml}$) or vehicle control for 48 h, the cells were harvested for protein extraction. Western blot analysis was used for protein extracts were prepared by a detergent lysis procedure. Samples were heated at 95°C for 5 min, and briefly cooled on ice. Following the centrifugation at 15,000 g for 5 min, 50 μg aliquots were resolved by 10% SDS-PAGE. The resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerin, and 20% methanol at 25 V.

Blots were blocked for at least 2 h with PBST containing 5% nonfat dry milk and then incubated with primary Ab for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary Ab, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Statistical analysis

The experiments shown are a summary of the data from at least three experiments and are presented as the mean \pm SEM. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test or the paired t-test. All statistical analyses were performed using SPSS v12.00 (SPSS Inc.) statistical analysis software. The results were considered significant at a value of $p < 0.05$.

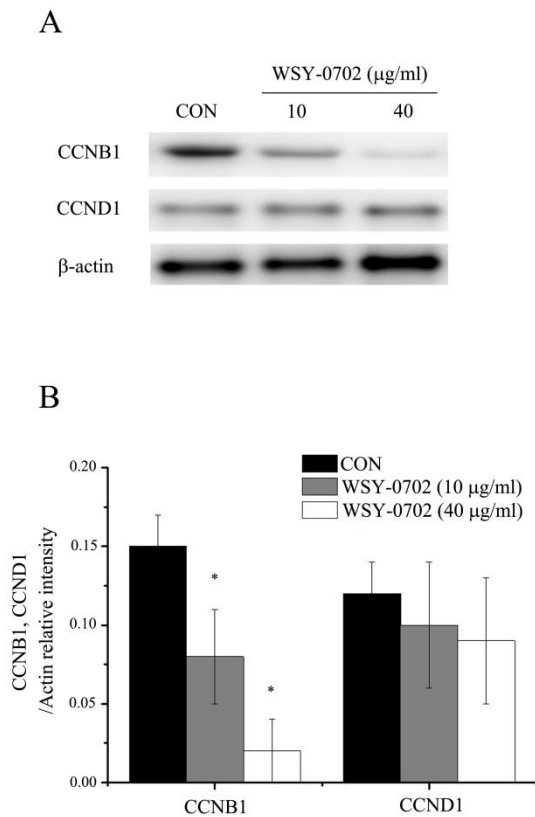


Fig. 3. The effects of WSY-0702 on cyclin expression in RWPE-1 cells. Western blot analysis of the CCNB1 and CCND1 proteins in RWPE-1 cells. RWPE-1 cells were seeded in 6-well plates at a density of 1×10^5 cells/well. Ten 10 or 40 $\mu\text{g/ml}$ WSY-0702 or the vehicle was added for the desired time. The values shown represent the mean \pm SD ($n = 3$). * $p < 0.05$ versus the vehicle treated control sample (CON)

RESULTS

WSY-0702 inhibited the proliferation of human prostate epithelial cells in vitro

After treatment with WSY-0702 for 48 h, the proliferation of the prostate epithelial cell line RWPE-1 was determined by the MTT assays. The MTT assay clearly showed that WSY-0702 suppressed the proliferation of RWPE-1 cells in a dose-dependent manner (Fig. 1A, $p < 0.05$). WSY-0702 had toxicity

at concentrations above 60 $\mu\text{g/ml}$ treated group (Fig. 1B).

WSY-0702 arrested the cell cycle of human prostate epithelial cells

To examine the effect of WSY-0702 on cellular apoptosis in prostate epithelial cells, we performed Hoechst 33,342 apoptosis staining. After treatment with WSY-0702 for 48 h, the apoptosis ratios of the RWPE-1 cells were increased by 2.0% and 0.8%, respectively (data not shown). These results suggest that apoptosis induction might not be a major mechanism by which WSY-0702 inhibits cell proliferation. A fluorescence activated cell sorting (FACS) assay was then conducted to test the effect of WSY-0702 treatment on the cell cycle of RWPE-1 cells. After treatment with WSY-0702 for 48 h, the proportion of RWPE-1 cells, percentage of cells in G0/G1 decreased and that of cells in G2/M increased significantly (Fig. 2, $p < 0.05$).

WSY-0702 arrested the expression of CCNB1 and CCND1 in human prostate epithelial cells

CCNB1 and CCND1 are important regulators of cell cycle checkpoints, which control the G2/M and G0/G1 transitions, respectively. In RWPE-1 cells, after WSY-0702 treatment, the CCNB1 and CCND1 protein was downregulated versus the compared to vehicle treated control sample (CON). These results suggest that the growth inhibition effects of WSY-0702 might be exerted by arresting prostate epithelial cells at the G2/M phase via decreasing the expression of CCNB1 (Fig. 3, $p < 0.05$).

Protection of human prostate epithelial cells from H₂O₂-induced cell death by WSY-0702

Next we examined whether WSY-0702 could decrease H₂O₂-mediated cellular injury and death of RWPE-1 cells. The cells were treated with 100 nM H₂O₂ for 2 h. As shown in Fig. 4, exposure of cells to H₂O₂ resulted in 47.4% increase of death rate in MTT assays demonstrating increase oxidative stress-mediated apoptosis (Fig. 4, * $p < 0.05$). To confirm the protective effect of WSY-0702, the cells were treated with 10 or 40 $\mu\text{g/ml}$ for 24 h and later exposed to 100 nM H₂O₂ for 2 h. Treatment of RWPE-1 cells with WSY-0702 resulted in a marked decrease in H₂O₂-mediated apoptotic cell death (Fig. 4, * $p < 0.05$). Treatment with WSY-0702 alone did not induce substantial apoptosis in these cells. Overall, these results suggest that WSY-0702 has the ability to protect prostate epithelial cells from H₂O₂-mediated cellular injury and apoptosis.

Reduction of oxidative stress and H₂O₂-mediated oxidative damage by WSY-0702

Next we determined the protective effect of WSY-0702 from oxidative stress. Exposure of RWPE-1 cells with H₂O₂ caused a significant increase in TGF- β generation as measured by culture medium, which converts to 405 nm color ELISA reader in the presence of intracellular TGF- β . Pretreatment of cells with 10 or 40 $\mu\text{g/ml}$ WSY-0702 caused significant decrease in TGF- β generation, compared to H₂O₂-treated cells (Fig. 5A). We also determined the levels of CCNB1 and CCND1, a hallmark of oxidative stress damage. As shown in Fig. 5B the levels of CCNB1 and CCND1 in protein expression were significantly lower in H₂O₂-treated cells than in untreated cells or in cells treated with WSY-0702. WSY-0702 significantly increases the protein expression levels of CCNB1 and CCND1 induced by H₂O₂ treatment. These results suggest that WSY-0702 has the ability to protect the cells from oxidative-mediated cellular injury.

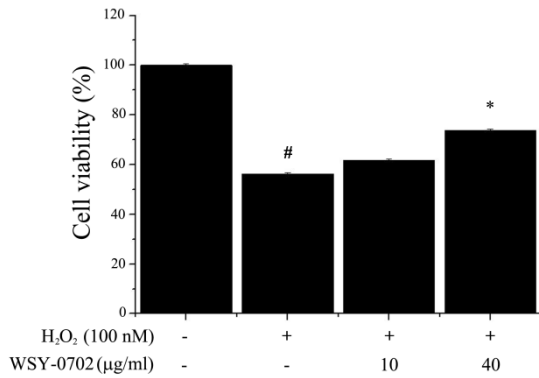
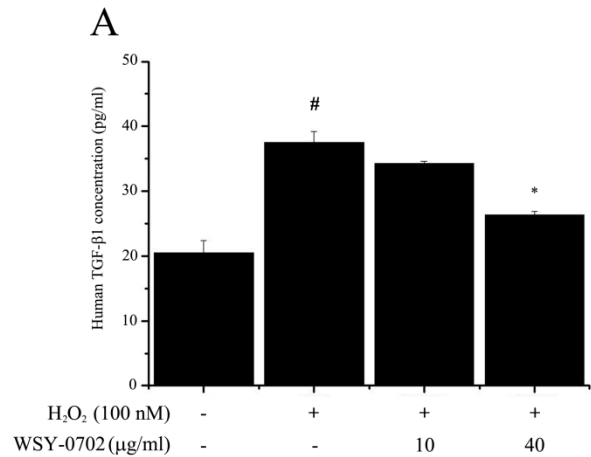


Fig. 4. Effect of WSY-0702 on H₂O₂-mediated RWPE-1 cell death. Effect of H₂O₂ on cell death in RWPE-1 cells. The cells were treated with 100 nM H₂O₂ for 2 h. RWPE-1 cells were treated for 24 h with a series of WSY-0702 concentration (10 and 40 μg/ml) and analyzed by the MTT assay. The values shown represent the mean ± SD (n = 3). #*p* < 0.05: significantly different from the unstimulated cells; **p* < 0.05: significantly different from the H₂O₂-stimulated cells.

DISCUSSION

It has been known that androgens and oestrogens regulate the expression of CCND1 via their receptors (Cai et al., 2011). In the current study, we have demonstrated that DHA downregulated the expression of ER, AR as well as CCNB1 simultaneously, however, the precise signaling cascade connecting ER, AR and CCNB1 requires further investigation. Our results showed that DHA arrests RWPE-1 in the G₂/M phase and decreases the expression of CCNB1, which is an important protein for the G₂/M phase transition, but not CCND1 comparing our results with what have been reported by other investigators, we speculate that the different response of the cells after DHA treatment are probably depending on the decrease characteristics of the cells used. Moreover, in this study we explored cellular uptake of WSY-0702 in transformed human prostate epithelial cells. Our results, for the first time, demonstrate that WSY-0702 preferentially regulates to reduce oxidative damage and apoptosis in prostate epithelial cells. We also have shown that increased effects of TGF-β on proliferation of prostate epithelial cells.

TGF-β is a pleiotropic cytokine whose signaling outcome is known to depend on the combination of available contributing factors and active pathways in each target tissue. Previous reports have shown that other intracellular proteins influence TGF-β effects (Hogan et al., 2013; Seystahl et al., 2015; Yamada et al., 2013). It has been well established that extensive interactions exist between TGF-β signaling pathways including Wnt, Notch, Hedgehog, MAPK and AKT/PI3K (Hirota et al., 2000; Javelaud et al., 2012; Letamendia et al., 2001; Nyhan et al., 2010; Qu et al., 2014). It is also becoming apparent that TGF-β signaling intersects with several transcription factors and regulators, such as GL1, SOX4, Tieg3/Klf11, Id and AP-1 proteins (Chakrabarti et al., 2013; Gohla et al., 2008; Lauth and Toftgård, 2007; Moreno, 2010; Walker et al., 2013). AP-1 proteins, there is growing evidence that Jun proteins play a major role in the control of cell proliferation and cell death by regulating the expression of cell cycle regulators (Güntherth et al., 2002; Shaulian and Karin, 2001; Shaulian and Karin, 2002; Vanhara et al., 2007).



B

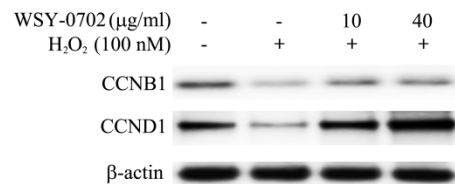


Fig. 5. Effect of WSY-0702 on reactive TGF-β and CCNB1 and CCND1 protein expressions with H₂O₂-mediated RWPE-1 cell oxidant stress. (A) ELISA assay with TGF-β on RWPE-1 cells treated with 10 or 40 μg/ml WSY-0702 for 24 h followed by 100 nM H₂O₂ incubation for 2 h. (B) Western blot analysis of the CCNB1 and CCND1 proteins in RWPE-1 cells. RWPE-1 cells were seeded in 6-well plates at a density of 1×10^5 cells/well. Then 10 or 40 μg/ml WSY-0702 or the vehicle was added for the 24 h followed by 100 nM H₂O₂ incubation for 2 h. The values shown represent the mean ± SD (n = 3). #*p* < 0.05: significantly different from the unstimulated cells; **p* < 0.05: significantly different from the H₂O₂-stimulated cells.

Reported studies to date indicate that frequent consumption of plant-based food products rich in flavones may be beneficial in reducing the risk of prostate cancer (Romagnolo and Selmin, 2012). Seoritae is known to have health-promoting effects due to its high isoflavone and anthocyanin contents. Isoflavones have variable effects on growth factor inhibition (Sarkar and Li, 2003), antioxidant properties (Patel et al., 2001; Yousef et al., 2004), cell adhesion (Bergan et al., 1996), 5α-reductase activity (Evans et al., 1995; Lewis et al., 2005), and angiogenesis (Schindler and Mentlein, 2006). Anthocyanin is a known antioxidant that has antiangiogenic, anticarcinogenic, and antioxidant effects (Bagchi et al., 2004; Liu et al., 2002; Liu et al., 2005). In particular, we hypothesized that the 5α-reductase inhibitory effect as well as the antioxidant properties of the isoflavones and anthocyanin in WSY-0702 would be helpful in preventing the occurrence and progression of BPH.

On the base of the results of the present study, we administered WSY-0702 to prostate epithelial cell during the induction of BPH. Our studies also show that WSY-0702 play minimum role in induction of cell proliferation in prostate epithelial cells. CCNB1 and CCND1 expression were then measured in order to investigate the mechanism by which

WSY-0702. Second, our results suggested that the antioxidant effect of WSY-0702 contributed to the reduction in prostate epithelial cells. WSY-0702 regulates oxidative stress and prostate cell proliferation so as to determine its potential in treating BPH.

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None.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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