Original Article

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

Hyun-A Oh, Eun Bi Kwon, Yun Gyeong Hwang, Soon Eung Park, Ji Ye Mok, Sung Yeoun Hwang[*]

Department of Research and development, KEMIMEDI, Gangnam Nonhyeonno to 652, Seoul, 06106, Republic of Korea.

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 H_2O_2-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 H_2O_2-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 andtogens 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 mitrogen-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 H_2O_2 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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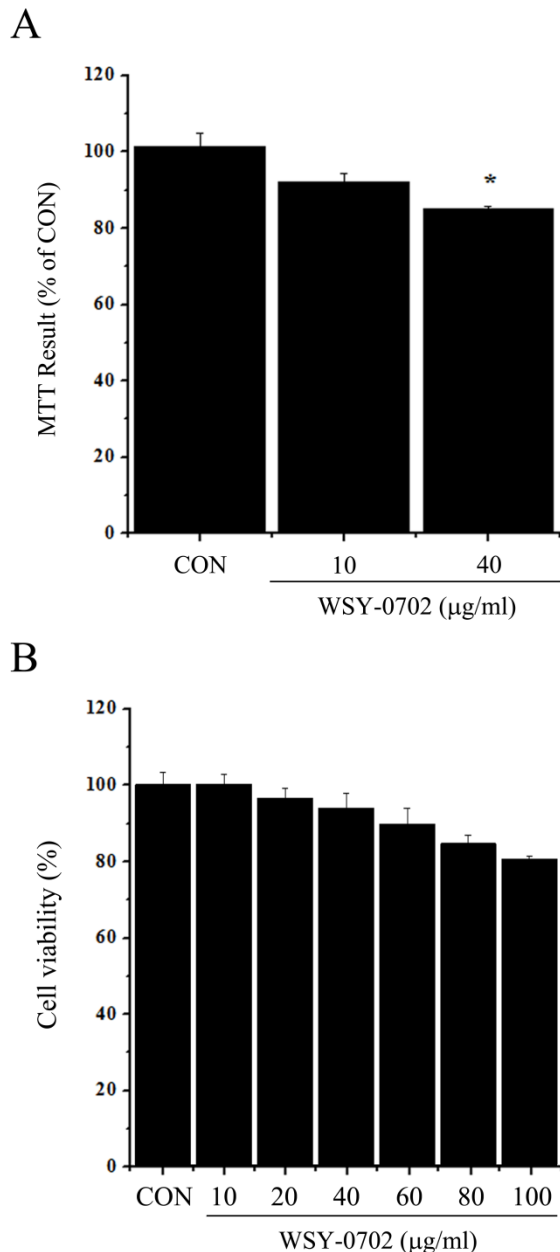
Fig. 1. WSY-0702 inhibits the proliferation of the human prostate epithelial cell line RWPE-1. (A) WSY-0702 inhibits the proliferation of RWPE-1 cells. RWPE-1 cells were treated for 24 h with a series of WSY-0702 concentration (10 and 40 μg/ml) and analysed by the MTT assay. (B) Cell viability was evaluated by an MTT assay (for 8 h). The values shown represent the mean ± 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 (本草纲目; compendium of materia medica in traditional medicine; UNESCO World Heritage Sit), 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

Meterials
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-dimethylth-iazol-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-Red 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℃. The solution was then filtered twice through a 50 μm and a 1 μm filter and concentrated in a vacuum evaporator (60℃) to 70 brix% (700 mg/g). The residual solvent was removed from WSY-0702 by using a drying machine for 18 h at 60℃ 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 ℃ under 5% CO_2 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

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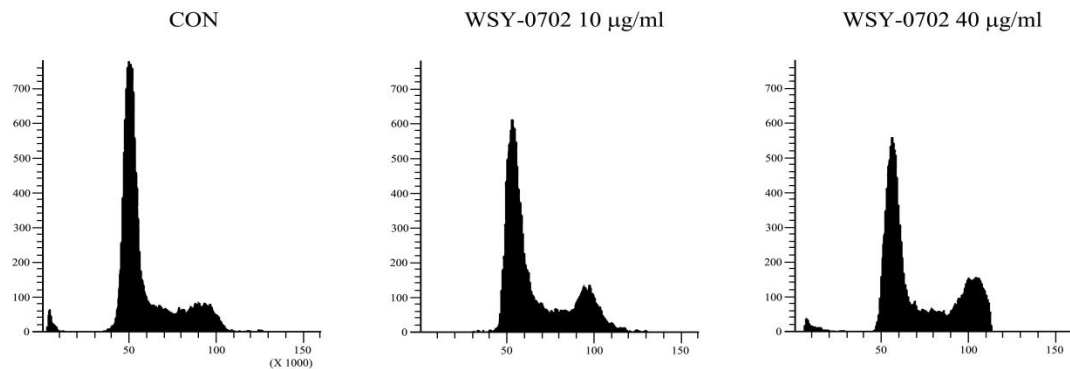
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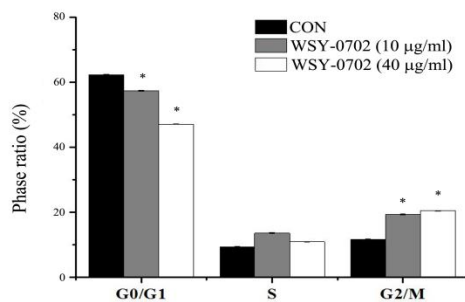
Fig. 2. The effects of WSY-0702 on cell cycle distribution in RWPE-1 cells. (A) Cell cycle analysis of RWPE-1 cells by flow cytometry. (B) The cell cycle phase distribution of RWPE-1 cells with or without WSY-0702 treatment. For cell cycle, the cells were harvested after 48 h of drug treatment. The values shown represent the mean ± 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 μg/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 μg/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 μg/ml)-treated or untreated cells were harvested and washed with ice-cold PBS and then fixed overnight at -20℃ in 70% ethanol. The cells were then washed twice, incubated for 30 min at 37℃ with 0.5 ml PBS containing 1 mg/ml of RNase A, and stained with 0.5 ml of 50 μg/ml propidium iodide for 20 min at 37℃. 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 μg/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℃. The wells were again washed and TMB substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader.

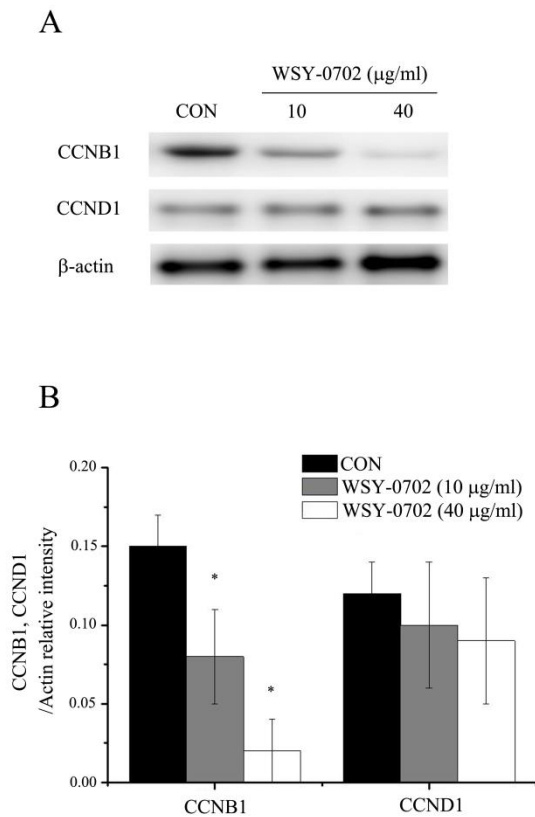**Western blot analysis**
After treatment with WSY-0702 (10 or 40 μg/ml) or vehicle control for 48 h, the cells were harvested for protein extraction. Western blot analysis was used for protein extracts were prepared by a detergent lysis procedure. Samples were heated at 95℃ for 5 min, and briefly cooled 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 Bioseciences, 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 ± 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 $p < 0.05$.

A

B

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 µg/ml WSY-0702 or the vehicle was added for the desired time. The values shown represent the mean ± 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 µg/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 H_2O_2-induced cell death by WSY-0702
Next we examined whether WSY-0702 could decrease H_2O_2-mediated cellular injury and death of RWPE-1 cells. The cells were treated with 100 nM H_2O_2 for 2 h. As shown in Fig. 4, exposure of cells to H_2O_2 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 µg/ml for 24 h and later exposed to 100 nM H_2O_2 for 2 h. Treatment of RWPE-1 cells with WSY-0702 resulted in a marked decrease in H_2O_2-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 H_2O_2-mediated cellular injury and apoptosis.

Reduction of oxidative stress and H_2O_2-mediated oxidative damage by WSY-0702
Next we determined the protective effect of WSY-0702 from oxidative stress. Exposure of RWPE-1 cells with H_2O_2 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 µg/ml WSY-0702 caused significant decrease in TGF-β generation, compared to H_2O_2-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 H_2O_2-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 H_2O_2 treatment. These results suggest that WSY-0702 has the ability to protect the cells from oxidative-mediated cellular injury.
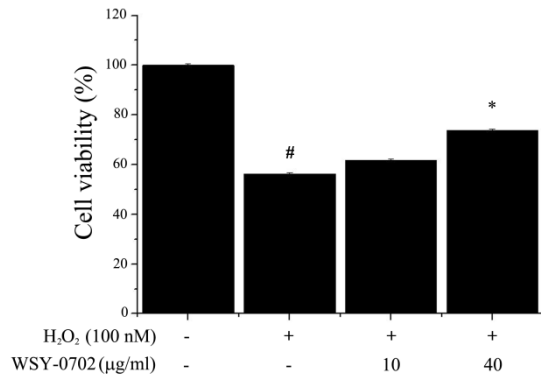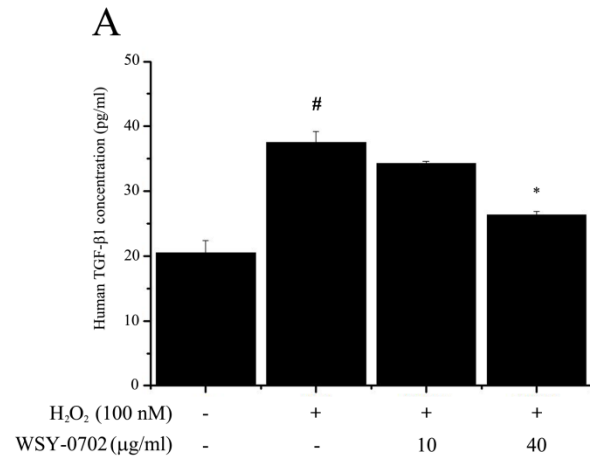
Fig. 4. Effect of WSY-0702 on H_2O_2-mediated RWPE-1 cell death. Effect of H_2O_2 on cell death in RWPE-1 cells. The cells were treated with 100 nM H_2O_2 for 2 h. RWPE-1 cells were treated for 24 h with a series of WSY-0702 concentration (10 and 40 μg/ml) and analyzed by the MTT assay. The values shown represent the mean ± SD (n = 3). #*p* < 0.05: signigicantly different from the unstimulated cells; **p* < 0.05: significantly different from the H_2O_2-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 decease 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 shoes 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/K1f11, 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 (Günthert et al., 2002; Shaulian and Karin, 2001; Shaulian and Karin, 2002; Vanhara et al., 2007).
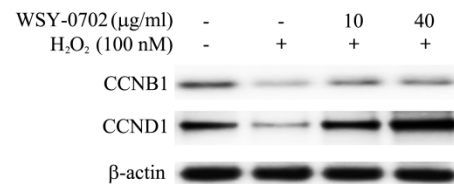
Fig. 5. Effect of WSY-0702 on reactive TGF-β and CCNB1 and CCND1 protein expressions with H_2O_2-mediated RWPE-1 cell oxidant stress. (A) ELISA assay with TGF-β on RWPE-1 cells treated with 10 or 40 μg /ml WSY-0702 for 24 h followed by 100 nM H_2O_2 incubation for 2 h. (B) Western blot analysis of the 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 H_2O_2 incubation for 2 h. The values shown represent the mean ± SD (n = 3). #*p* < 0.05: signigicantly different from the unstimulated cells; **p* < 0.05: significantly different from the H_2O_2-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.

ACKNOWLEDGEMENTS

None.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Agarwal R, Gonzalez-Angulo AM, Myhre S, Carey M, Lee JS, Overgaard J, Alsner J, Stemke-Hale K, Lluch A, Neve RM, Kuo WL, Sorlie T, Sahin A, Valero V, Keyomarsi K, Gray JW, Borresen-Dale AL, Mills GB, Hennessy BT. Integrative analysis of cyclin protein levels identifies cyclin B1 as a classifier and predictor of outcomes in breast cancer. Clin Cancer Res. 2009;15:3654–3662.

Bagchi D, Sen CK, Bagchi M, Atalay M. Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula. Biochemistry. 2004;69:75-80.

Barbieri F, Cagnoli M, Ragni N, Foglia G, Bruzzo C, Pedullà F, Alama A. Increased cyclin D1 expression is associated with features of malignancy and disease recurrence in ovarian tumors. Clin Cancer Res. 1999;5:1837–1842.

Bergan R, Kyle E, Nguyen P, Trepel J, Ingui C, Neckers L. Genistein-stimulated adherence of prostate cancer cells is associated with binding of focal adhesion kinase to beta-1-intergrin. Clin Exp Metastasis. 1996;14:389-398.

Bostanci Y, Kazzazi A, Momtahen S, Laze J, Djavan B. Correlation between benign prostatic hyperplasia and inflammation. Curr Opin Urol. 2013;23:5-10.

Cai J, Hong Y, Weng C, Tan C, Imperato-McGinley J, Zhu YS. Androgen stimulates endothelial cell proliferation via an androgen receptor/VEGF/cyclin A-mediated mechanism. Am J Physiol Heart Circ Physiol. 2011;300:H1210-H1221.

Chakrabarti L, Wang BD, Lee NH, Sandler AD. A mechanism linking Id2-TGF-beta crosstalk to reversible adaptive plasticity in neuroblastoma. PLos One. 2013;8:e83521.

Evans BA, Griffiths K, Morton MS. Inhibition of 5 -reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavonoids. J Endocrinol. 1995;147:295-302.

Farnsworth WE. Estrogen in the etiopathogenesis of BPH. Prostate. 1999;41:263-274.

Glynn RJ, Campion EW, Bouchard GR, Silbert JE. The development of benign prostatic hyperplasia among volunteers in the Normative Aging Study. Am J Epidemiol. 1985;121:78-90.

Gohla G, Krieglstein K, Spittau B. Tieg3/K1f11 induces apoptosis in OLI-neu cells and enhances the TGF-beta signaling pathways by transcriptional repression of Smad7. J Cell Biochem. 2008;104:850-861.

Günthert AR, Gründker C, Hollmann K, Emons G. Luteinizing hormone-releasing hormone induces JunD-DNA binding and extends cell cycle in human ovarian cancer cells. Biochem Biophy Res Commun. 2002;294:11-15.

Hirota Y, Tsukazaki T, Yonekura A, Miyazaki Y, Osaki M, Shindo H, Yamashita S. Activation of specific MEK-ERK cascade is necessary for TGF-beta signaling and crosstalk with PKA and PKC pathways in cultured rat articular chondrocytes. Osteoarthritis Cartilage. 2000;8:241-247.

Hogan KA, Ravindran A, Podolsky MA, Glick AB. The TGFbeta1 pathway is required for NFkappaB dependent gene expression in mouse keratinocytes. Cytokine. 2013;64:652-659.

Javelaud D, Pierrat MJ, Mauviel A. Crosstalk between TGF-beta and hedgehog signaling in cancer. FEBS Lett. 2012;586:2016-2025.

Khandrika L, Kumar B, Koul S, Maroni P, Koul HK. Role of oxidative stress in prostate cancer. Cancer lett. 2009;282:125-136.

Kumar R, Verma V, Sarswat A, Maikhuri JP, Jain A, Jain RK, Sharma VL, Dalela D, Gupta G. Selective estrogen modulators regulate stromal proliferation in human benign prostatic hyperplasia by multiple beneficial mechanisms-action of two new agents. Invest New Drugs. 2012;30:582-593.

Kyprianou N, Isaacs JT. Expression of transforming growth factor- in rat ventral prostate during castration-induced programmed cell death. Mol Endocrinol. 1989;3:1515-1522.

Lauth M, Toftgård R. Non-canonical activation of GLI transcription factors: implications for targeted anti-cancer therapy. Cell Cycle. 2007;6:2458-2463.

Lee YS, Choi BK, Lee HJ, Lee DR, Cheng J, Lee WK, Yang SH, Suh JW. Monascus pilosus-fermented black soybean inhibits lipid accumulation in adipocytes and in high-fat diet-induced obese mice. Asian Pac J Trop Med. 2015;8:276-282.

Letamendia A, Labbé E, Attisano L. Transcriptional regulation by Smads: crosstalk vetween the TGF-beta and Wnt pathways. J Bone Joint Surg Am. 2001;83-A Suppl 1:S31-39.

Lewis JG, Nakajin S, Ohno S, Warnock A, Florkowski CM, Elder PA. Circulating levels of isoflavones and markers of 5 -reductase activity are higher in Japanese compared with New Zealand males: what is the role of curcylating steroids in prostate disease? Steroids.2005;70:974-979.

Liu J, Wen XY, Zhang XQ, Pu HM, Kan J, Jin CH. Extraction, characterization and in vitro antioxidant activity of polysaccharides from black soybean. Int J Biol Macromol. 2015;72:1182-1190.

Liu M, Li XQ, Weber C, Lee CY, Brown J, Liu RH. Antioxidant and antiproliferative activities of raspberries. J Agric Food Chem. 2002;50:2926-2930.

Liu Z, Schwimer J, Liu D, Greenway FL, Anthony CT, Woltering EA. Black raspberry extract and fractions contain angiogenesis inhibitors. J Agric Food Chem. 2005;53:3909-3915.

Li X, Tian F, Wang F. The effects of black soybean (Glycine max var.) on chronic cervical pain of sedentary office workers in a northern Chinese population. Food Funct. 2015;6:3500-3511.

Martikainen P, Kyprianou N, Isaacs JT. Effect of transforming growth factor-beta 1 on proliferation and death of rat prostatic cells. Endocrinology. 1990;127:2963-2968.

Massagué J. The transforming growth factor- family. Annu Rew Cell Biol. 1990;6:597-641.

Moreno-Bueno G, Rodrı́guez-Perales S, Sa ́nchez-Este ́vez C, Hardisson D, Sarrió D, Prat J, Cigudosa JC, Matias-Guiu X, Palacios J. Cyclin D1 gene (CCND1) mutations in endometrial cancer. Oncogene. 2003;22:6115–6118.

Moreno CS. The Sex-determining region Y-box 4 and homeobox C6 transcriptional networks in prostate cancer progression: crosstalk with the Wnt, Notch and PI3K pathways. Am J Pathol. 2010;176:518-527.

Nickel JC, Roehrborn CG, O'leary MP, Bostwick DG, Somerville MC, Rittmaster RS. Examination of the relationship between symptoms of chemopevention trial. J Urol. 2007;1:896-900.

Nyhan KC, Faherty N, Murray G, Cooey LB, Godson C, Crean JK, Brazil DP. Jagged/Nach signaling is required for a subset of TGF-beta1 responses in human kidney epithelial cells. Biochim Biophy Acta. 2010;1803:1386-1395.

Patel ND, Parsons JK. Epidermiology and etiology of benign prostate htperplasia and bladder outlet obstruction. Indian J Urol. 2014;30:170-176.

Patel RP, Boersma BJ, Crawford JH, Hogg N, Kirk M, Kalyanaraman B, Parks DA, Barnes S, Darley-Usmar V. Antioxidant mechanisms of isoflavones in lipid systems: paradoxical effects of peroxyl radical scavenging. Free Radic Biol Med. 2001;12:1570-1581.

Qu X, Shen L, Zheng Y, Cui Y, Feng Z, Liu F, Liu J. A signal transduction pathway from TFG-beta1 to SKP2 via Akt1 and c-Myc and its correlation with progression in human melanoma. J Invest Dermatol. 2014;134:159-167.

Romagnolo DF, Selmin OI. Flavonoids and cancer prevention: a review of the evidence. J Nutr Gerontol Geriatr. 2012;31:206-238.

Rotello RJ, Lieberman RC, Purchio AF, Gerschenson LE. Coordinated regulation of apoptosis and cell proliferation by transforming factor beta in cultured uterine epithelial cells. Proc Natl Acad Sci USA. 1991;88:3412-3415.

Sarkar FH, Li Y. Soy isoflavones and cancer prevention. Cancer Invest. 2003;21:744-757.

Schindler R, Mentlein R. Rlavonoids and vitamin E reduce the release of the angiogenic peptide vascular endothelial growth factor from human tumor cells. J Nutr. 2006;136:1477-1482.

Seystahl K, Tritschler I, Szabo E, Tabatabai G, Weller M. Differential regulation of TGF-beta-induced, ALK-5-mediated VEGF release by SMAD2/3 versus SMAD1/5/8 signaling in glioblastoma. Neuro oncol. 2015;17:254-265.

Shaulian E, Karin M. AP-1 in cell proliferation and survival. Oncogene. 2001;20:2390-2400.

Shaulian E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol. 2002;4:E131-136.

Sherr CJ. The Pezcoller lecture: cancer cell cycles revisited. Cancer Res. 2000;60:3689–3695.

Suzuki K, Inaba S, Takeuchi H, Takezawa Y, Fukabori Y, Suzuki T, Imai K, Yamanaka H, Honma S. Endocrinal environment of benign prostatic hyperplasia-relationships of sex steroid hormone levels with age and the size of the prostate. Nihon Hinyokika Gakkai Zasshi. 1992;83:664-671.

Vanhara P, Bryja V, Horváth V, Kozubík A, Hampl A, Smarda J. c-Jun induces apoptosis of starved BM2 monoblasts by activating cyclin A-CDK2. Biochem Biophys Res Commun. 2007;353:92-97.

Walker L, Millena AC, Strong N, Khan SA. Expression of TGF-beta3 and its effects on migratory and invasive behavior of prostate cancer cells: involvement oh PI3-kinase/AKT signaling pathways. Clin Exp Metastasis. 2013;30:13-23.

Yamada D, Kobayashi S, Wada H, Kawamoto K, Marubashi S, Eguchi H, Ishii H, Nagano H, Doki Y, Mori M. Role of crosstalk between interleukin-6 and transforming growth factor-beta 1 in epithelial-mesenchymal transition and chemoresistance in biliary tract cancer. Eur J Cancer. 2013;49:1725-1740.

Yanagihara K, Tsumuraya M. Transforming growth factor beta induces apoptotic cell death in cultured human gastric carcinoma cells. Cancer Res. 1992;52:4042-4045.

Yousef MI, Kamel KI, Esmail AM, Baghdadi HH. Antioxidant activities and lipid lowering effects of isoflavone in male rebbits. Food Chem Toxicol. 2004;42:1497-1503.