

Protective effect of gomisin N on BBP-induced dysfunction of testosterone production in TM3 Leydig cells

Seung Ju Lee

Kyungsung University

Min Ju Park

Kyungsung University

Dong Hyeok Jeong

Kyungsung University

Rohith Arunachalam

North Carolina A&T State University: North Carolina Agricultural and Technical State University

Eunsoo Yoo

North Carolina A&T State University: North Carolina Agricultural and Technical State University

Hye Kyung Kim

Kyungsung University

Min Hi Park (✉ parkmh@ks.ac.kr)

Kyungsung University <https://orcid.org/0000-0002-6191-993X>

Research Article

Keywords: Benzyl butyl phthalate, Testosterone, Oxidative stress, TM3 Leydig cell, Schisandra chinensis

Posted Date: June 6th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2995007/v1>

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Version of Record: A version of this preprint was published at Molecular Biology Reports on January 16th, 2024. See the published version at <https://doi.org/10.1007/s11033-023-09060-6>.

Abstract

Background

Exposure to benzyl butyl phthalate (BBP) may induce disorders in the male reproductive system. However, the molecular mechanisms remain unknown. Here we investigated the effect of BBP on testosterone production and its molecular mechanisms. Furthermore, we also investigated the role of gomisins N (GN) from *Schisandra chinensis* (*S. chinensis*) in testosterone synthesis in TM3 Leydig cells.

Method and results

First, we examined the effects of BBP on expression levels of testosterone biosynthesis-related genes (StAR, CYP11 α 1, CYP17 α 1, 3 β HSD, and 17 β HSD) and attenuation-related genes (CYP11 β 1, CYP19 α 1, and Srd5 α 1-3). Although testosterone biosynthesis-related genes did not change, attenuation-related genes such as CYP11 β 1 and CYP19 α 1 were upregulated with ROS generation and testosterone level attenuation in the presence of 50 μ M of BBP. However, the compound with the highest ROS and ONOO⁻ scavenging activity from *S. chinensis*, GN, significantly reversed the expression of BBP-induced testosterone attenuation-related gene to normal levels. Subsequently, GN improved the testosterone production levels in TM3 Leydig cells. These events may be regulated by the antioxidant effect of GN.

Conclusions

In conclusion, our study suggests, for the first time, that BBP impairs testosterone synthesis by the modulation of CYP11 β 1 and CYP19 α 1 expression in TM3 cells; GN could potentially minimize the BBP-induced dysfunction of TM3 cells to produce testosterone by suppressing CYP19 α 1 expression.

Introduction

Testosterone, which is primarily produced in testes and adrenal glands, is a crucial sex hormone in men that has diverse effects on various physiological functions, including libido, strength, and depressed mood [1]. Testosterone deficiency (TD) is a common condition with an estimated prevalence of 20–50% among American men [2, 3]. The age of around 50 years marks the onset of declining testosterone levels, significantly impacting an individual's overall quality of life [2–4]. Additionally, metabolic disorders and exposure to endocrine-disrupting chemicals (EDCs) are known to lower testosterone levels [5, 6]. However, the precise molecular mechanism underlying the association between low testosterone and EDCs remains unclear.

Low testosterone levels are called testosterone deficiency syndrome (TDS), which may result from dysfunction of Leydig cells, where synthesized testosterone, lacking luteinizing hormone (LH), or a reduction in testosterone bioavailability because of elevated sex hormone-binding globulin (SHBG) levels [7–9]. Testosterone is synthesized by acute steroidogenic regulation (StAR) protein, cholesterol side-chain cleavage enzyme (P450_{scc}), and several testosterone synthesis-related enzymes [10]. Conversely, the

aromatase enzyme CYP19 α 1 converts testosterone to estradiol, and the breakdown enzyme CYP1 β 1 catalyzes 6-hydroxylation of testosterone, which affects decreasing testosterone levels [11–14].

EDCs are exogenous substances that alter the physiology of the endocrine system by interfering with the effect, such as biosynthesis, transport, and metabolism of hormones [15]. EDCs are ubiquitous and can be found in various sources, including pesticides, plastic items, personal care products, hydraulic and electronic devices, clothing chemicals, flame retardants, solvents, and numerous other substances [16]. We are constantly exposed to EDCs throughout our lifetime via various routes, such as food, skin contact, and inhalation of air, among others, in our daily lives [16, 17]. Benzyl butyl phthalate (BBP) is one of the most common EDCs found in the environment [18]. The metabolism of BBP by lipase within our bodies results in the activation of inflammatory responses, an upsurge in fatty acid oxidation, and a decrease in the capacity to handle increased oxidative stress [19, 20]. Hence, BBP is associated with compromised male reproductive health along with inhibition of testosterone synthesis [21, 22].

Schisandra chinensis (*S. chinensis*) has been used in traditional herbal medicine in South Korea, Japan, and China. Several studies have shown that *S. chinensis* exhibits diverse pharmacological effects such as antioxidant, anti-tumor, anti-obesity, and anti-inflammatory properties [23, 24]. Also, *S. chinensis* contains various bioactive constituents such as lignans, sterols, polysaccharides, and triterpenoids. Gomisin N (GN) is a lignan from *S. chinensis* and was identified to possess hepatoprotective, anticancer, and anti-inflammatory effects [24]. The research on the relevance of gene enzymes related to testosterone attenuation by oxidation stress is still insufficient.

Here, we found that exposure to BBP-induced reactive oxygen species (ROS) generation leads to increased CYP1 β and CYP19 α 1. Consequently, the testosterone level was decreased. However, GN was able to recover testosterone levels through preventing CYP19 α 1 repression. The protective effects of GN were dependent on antioxidation in Leydig cells. These studies suggest that GN can prevent BBP-induced testosterone deficiency syndrome by reducing ROS generation. This preclinical evidence suggests that GN has the potential to be developed as an effective therapeutic agent.

Materials and methods

Chemical and reagents

Benzyl butyl phthalate (BBP), dimethyl sulfoxide (DMSO), and penicillin-streptomycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's medium (DMEM), Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12), horse serum, fetal bovine serum (FBS), and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) was purchased from Invitrogen (Camarillo, CA, USA). GN was obtained from ChemFaces (Wuhan, China).

Measurement of ROS and ONOO⁻ scavenging activity

The ROS scavenging ability of the *Schisandra chinensis* extract (SCE) and its compound were investigated using H₂DCFDA. SCE was prepared according to the recent studies [25] and dissolved in DMSO at concentrations of 10, 100, and 1000 µg/mL. For GN, which has a common high ROS and ONOO⁻ scavenging activity among SCE compounds, we used it at final concentrations of 1, 10, and 100 µM. 12.5 mM H₂DCFDA was mixed with 600 unit esterase in a 15 mL conical tube, and the mixture was incubated for 20 min in the dark. Measurement of samples (SCE and GN), 50 µL of H₂DCFDA mixture and 190 µL of SIN-1 were mixed in a 96-well plate, and the samples were incubated for 30 min. Fluorescence of the resulting mixture was recorded using Varioskan™ LUX spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with excitation and emission wavelength of 485 nm and 530 nm, respectively.

Peroxynitrite (ONOO⁻) scavenging activity was measured using the oxidation of dihydrorhodamine 123 (DHR123). 10 µL of samples (SCE and GN) were loaded into a 96-well, and 10 µM SIN-1, 10 µM DTPA, and 50 µM DHR123 were treated to each well. The ONOO⁻ scavenging activity was determined using the spectrophotometer (Thermo Fisher Scientific) at room temperature. DMSO was used as a control, and penicillamine (Sigma Aldrich) was used as a positive control.

Cell culture

The mouse Leydig cell line (TM3 cell) was purchased from Korea Cell Line Bank (Seoul, Korea). TM3 cells were cultured in a 1:1 (v:v) mixture of DMEM:DMEM/F12 media supplemented with 5% horse serum, 2.5% FBS, 100 units penicillin, and 100 µg/mL streptomycin in an incubator (37 °C and 5% CO₂). The cell culture media was replaced every 2–3 days as needed, and cells were sub-cultured using 0.25% trypsin-EDTA when the cells reached 80% confluency.

Measurement of cell viability

The cell viability was evaluated using an EZ-Cytox assay kit (DoGenBio, Seoul, Korea), which included the water-soluble tetrazolium salt following the manufacturer's instruction. For this purpose, the TM3 Leydig cells were plated in 96-well plates at a density of 2×10^4 cells/well and cultured for 24 h. After 24 h, cells were treated with BBP (6.25, 12.5, 25, 50, and 100 µM), SCE (62.5, 125, 250, and 500 µg/mL), and GN (0.1, 1, 10, and 100 µM) for 24 h. After adding the 10 µL of EZ-Cytox solution to each well, the cells were incubated for 2 h at 37°C. Absorbance was measured using a microplate reader at 450 nm.

Testosterone ELISA assay

The TM3 cells were cultured in a 48-well plate at a 1.6×10^5 cells/well density and incubated for 24 h. Then wells were divided into four groups, including vehicle, 0.5, 5, and 50 µM BBP-treated cells with or without different concentrations of SCE (10 and 100 µg/mL) and GN (1 and 10 µM) for 24 h. The supernatants were collected, and testosterone concentration was measured by Testosterone ELISA Kit (Enzo Biochem, Inc., Farmingdale, NY, USA) following the manufacturer's instructions.

Detection of intracellular ROS levels

TM3 cells were seeded in a 96-well plate at a concentration of 5×10^4 cells/well until 80% confluence. After 24 h, cells were exposed to BBP (0.5, 5, and 50 μM) for 1 h. Subsequently, the cells were treated with 100 μM H₂DCFDA and incubated for 1 h. Then, the H₂DCFDA-containing medium was removed and washed with PBS. The fluorescence intensity was analyzed using Varioskan™ LUX spectrophotometer (Thermo Fisher) at 485 nm excitation and 535 nm emission.

To investigate whether SCE and GN treatment inhibited BBP-induced ROS levels, cells were plated in the other 96-well plates (5×10^4 cells/well) and incubated for 24 h. The medium was removed, and the cells were treated with SCE (10 and 100 $\mu\text{g/mL}$) and GN (1 and 10 μM). About 100 μL of 50 μM BBP was added to each well, and the cells were incubated at 37 °C for 1 h. The cells were treated with 100 μM H₂DCFDA for 1 h, and the fluorescence was measured using a spectrophotometer (Thermo Fisher).

RNA isolation and qPCR

qPCR was conducted to assess the effects of BBP on mRNA expression in the TM3 cells. TM3 cells were cultured in a 12-well plate at 3×10^5 cells/well density and incubated for 24 h. BBP (vehicle, 0.5, 5, and 50 μM) were treated in each well and incubated at 37°C for 24 h.

To evaluate the effects of SCE and GN treated on BBP-induced cell, cells were plated in the 96-well plates (2×10^5 cells/well) and incubated for 24 h. After 24 h, cells were treated with SCE (10 and 100 $\mu\text{g/mL}$) and GN (1 and 10 μM) for 24 h at 37°C.

Total RNA isolation was conducted using miRNeasy Tissue/Cells Advanced Mini Kit (QIAGEN, Hilden, Germany), and RNA concentration was determined with Nano-300 Micro-Spectrophotometer (Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, China). About 1–2 μL of extracted total RNA was used for cDNA synthesis using a Compact cDNA Synthesis Kit (SJ Bio Science). Gene expressions were performed with SYBR Green PCR Master Mix and specific primers, and gene amplification was carried out with QuantStudio™ 1 Real-Time PCR Instrument (96-Well 0.2 mL Block) (Thermo Fisher). Primers used for the qPCR are listed in Table 1. The relative expression of the mRNA level of each gene was normalized to that of 18S and quantified using $2^{-(\Delta\Delta\text{Ct})}$ method.

Table 1
Sequences of testosterone biosynthesis relative genes for quantitative real time PCR

Target mRNA	Forward primer (5'→3')	Reverse primer (5'→3')
StAR	TGCCCATCATTTTCATTCATCCTT	AAAAGCGGTTTCTCACTCTCC
3 β -HSD	TGGACAAAGTATTCCGACCAGA	GGCACACTTGCTTGAACACAG
17 β -HSD	ACTTGGCTGTTCGCCTAGC	GAGGGCATCCTTGAGTCCTG
CYP11 α 1	AGGTCCTTCAATGAGATCCCTT	TCCCTGTAAATGGGGCCATAC
CYP17 α 1	GCCCAAGTCAAAGACACCTAAT	GTACCCAGGCGAAGAGAATAGA
CYP1 β 1	CACCAGCCTTAGTGCAGACAG	GAGGACCACGGTTTCCGTTG
CYP19 α 1	ATGTTCTTGGAATGCTGAACCC	AGGACCTGGTATTGAAGACGAG
Srd5 α 1	GAGTTGGATGAGTTGCGCCTA	GGACCACTGCGAGGAGTAG
Srd5 α 2	GATCCTGTGCTTTGGGAAACC	GCATCCCTACCGACACCAC
Srd5 α 3	CTACGTCATCTCAGTTGTGTGG	GAGCAGAGCACTAAGCCAGT
18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG

Western blot analysis

TM3 cells were washed twice with PBS. Once the PBS was completely removed, the cells were lysed with 200 μ L of lysis buffer containing 50 mM phosphate, 1 mM DTPA, and 50 mM NEM (pH 7.4) for 30 min on ice. The lysates were centrifuged for 10 min at 13,000 rpm and the supernatants were used by western blot assay. The concentrations of proteins were quantified by Bradford protein assay with standard protein curve (Bio-Rad, Hercules, CA), and each sample was diluted to the same protein concentration. Protein samples were separated with 10% sodium dodecyl sulfate-polyacrylamide gels, and proteins were transblotted to the membrane. Then, the membranes were blocked with membrane blocking solution (Translab, Daejeon, Korea), and the membranes were incubated with CYP1 β 1 (1:1,000 dilution, Abclonal, MA, USA) or CYP19 α 1 (1:1,000 dilution, Abclonal) antibody at 3 °C overnight. After the membrane was washed with TBS-T buffer for three times, the membrane was added HRP Rabbit Anti-Goat IgG (H + L) (1:10,000 dilution, Abclonal) and the incubation for 1 hr 30 min. The protein band was detected using ECL Ottimo (Translab, Daejeon, Korea), and the protein concentrations were quantified by Image J.

Statistical Analysis

Experimental data were subjected to a statistical analysis using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). One-way ANOVA was used to determine differences among three or more groups, and $P < 0.05$ is considered statistically significant.

Results

GN from the SCE scavenges ROS and ONOO⁻

To investigate whether SCE has an antioxidant capacity, ROS and ONOO⁻ scavenging assays were determined. As shown in Fig. 1A and D, SCE enhanced ROS and ONOO⁻ scavenging activities in a dose-dependent manner compared to SIN-1-treated group ($P < 0.001$). To find the most active compound from SCE, ROS and ONOO⁻ scavenging assays of individual lignans were performed. Schisandrol A did not have ROS and ONOO⁻ scavenging activities (Fig. 1B and E). On the other hand, other lignans, schisandrin A, GN, and schisandrin C, significantly decreased ROS and ONOO⁻ generation. The most active compound from SCE against ROS and ONOO⁻ generation was schisandrin C and GN, respectively. Interestingly, GN attenuated ROS and ONOO⁻ generation in a dose-dependent manner ($P < 0.001$; Fig. 1C and F).

BBP, SCE, and GN do not alter cell viability in TM3 cells

To investigate whether BBP, SCE, and GN affect cell viability, we exposed varying concentrations of BBP (6.25, 12.5, 25, 50, and 100 μ M), SCE (62.5, 125, 250, and 500 μ g/ml), and GN (0.1, 1, 10, and 100 μ M) for 24 h on TM3 cells. MTT assay showed BBP, SCE, and GN did not have an effect on cell viability significantly (Fig. 2A-C). SCE and GN were treated with concentrations of 10, 100 μ g/mL and 1, 10 μ M individually for further studies.

GN recovered BBP-induced testosterone production impairment

Evidence has shown phthalate exposure impairs testosterone secretion levels [26]. Consistent with previous studies, the concentration of testosterone was decreased with BBP treatment on TM3 cells. (Fig. 3A); therefore, the effect of SCE and GN on testosterone secretion in TM3 cells was investigated. As shown in Fig. 3B, SCE and GN significantly increased the concentration of testosterone in the BBP-induced TM3 cells in a dose-dependent manner ($P < 0.05$, $P < 0.01$, and $P < 0.001$).

GN attenuates cellular oxidative stress induced by BBP in TM3 cells

As GN provoked strong ROS and ONOO⁻ scavenging activities, subsequently, the cellular ROS levels, triggered by BBP, which has shown a generator of ROS ($P < 0.001$; Fig. 4A), were measured. BBP significantly increased ROS levels at 50 μ M concentrations, while SCE and GN inhibited the generation of ROS on BBP-induced TM3 cells ($P < 0.01$ and $P < 0.001$; Fig. 4B).

GN decreased CYP19 α 1 RNA and protein expression levels

To investigate the pathways involved in the impairment of testosterone secretion, TM3 cells were exposed to 0.5, 5, and 50 μM of BBP for 24 h, and testosterone biosynthesis-related genes, including StAR, CYP11 α 1, CYP17 α 1, 3 β HSD, and 17 β HSD, and testosterone attenuation-related genes, including CYP1 β 1, CYP19 α 1, and Srd5 α 1–3. Although testosterone biosynthesis-related genes' expression levels were not significantly changed (data not shown), testosterone attenuation-related genes, especially CYP1 β 1 and CYP19 α 1, were increased dramatically under 50 μM of BBP exposure ($P < 0.01$ and $P < 0.001$; Fig. 5A and B). Consistent with the gene expression, BBP treatment markedly increased the protein levels of CYP1 β 1 and CYP19 α 1 ($P < 0.05$ and $P < 0.01$; Fig. 5C-E). However, SCE and GN significantly inhibited the expression levels of CYP1 β 1 ($P < 0.01$ and $P < 0.001$; Fig. 6A) compared to 50 μM of the BBP group. Interestingly, SCE did not alter the expression levels CYP19 α 1 (Fig. 6B); GN was significantly suppressed in a dose-dependently manner ($P < 0.05$ and $P < 0.01$; Fig. 6B). Indeed, the protein expression levels of CYP1 β 1 did not alter by SCE and GN under 50 μM of BBP exposure; GN significantly attenuated CYP19 α 1 in a dose-dependent manner when compared to 50 μM of the BBP group ($P < 0.01$ and $P < 0.001$; Fig. 6C-E).

Discussion

In the aging male, environmental factors and accumulated oxidative stress contribute to the reduction of systemic testosterone. Increasing lines of evidence have shown the link between BBP-induced oxidative stress and testosterone deficiency, yet the association with BBP exposure on the aromatase gene is still unknown. Previous studies have demonstrated the antioxidant properties of *S. chinensis* and its ability to improve TDS. However, the molecular mechanism and its bioactive compounds of SCE remain unknown.

There appears to be a negative link between testosterone synthesis and oxidative stress. For example, pro-inflammatory cytokines such as TNF α and IL-6, induced by oxidative stress, were significantly higher in the testicular-feminized mice compared to the normal group after 28 weeks [27]. In the case of humans, it is difficult to provide evidence, but several studies have shown that tuberculosis patients had lower testosterone levels and higher levels of pro-inflammatory cytokines such as TNF α , IL-1 β , IL-6, and TGF β [28]. Indeed, the activation of oxidative stress leads to an increase in inflammation triggers, which are associated with TDS. Compelling evidence from both *in vivo* and *in vitro* studies have shown that EDCs are related to alterations in testosterone biosynthesis [29, 5]. However, the molecular mechanisms of declining testosterone biosynthesis caused by EDCs remain unclear. BBP, one of the most widely used phthalates, is abundant in several everyday household items. Several studies have proved that BBP has toxic effects on the kidneys, lungs, and reproductive system [30, 31, 26]. The most significant concern regarding the adverse effects of BBP is its potential impact on developmental health and reproductive toxicity [26, 32, 33]. In line with previous reports, in this current study, testosterone production was downregulated but had no significant change; however, SCE and its bioactive compounds recovered testosterone levels in Leydig cells. Among the five compounds, GN and Sch C are higher ROS-scavenging compounds.

Testosterone is synthesized in Leydig cells of the testis by StAR, CYP11a1, and several testosterone synthesis-related enzymes [10]. On the other hand, synthesized testosterone was reduced by CYP19a1, CYP17 β 1, Srd5a1, 2, and 3 [11–14]. Interestingly, our result showed that although BBP did not significantly change the expression of enzymes related to the testosterone biosynthesis-related gene, the testosterone attenuation-related genes were markedly upregulated under BBP treatment. Also, the protein expression level of CYP17 β 1 and CYP19a1 was increased. These results suggest that BBP impairs expression levels of CYP17 β 1 and CYP19a1, consequently, testosterone levels reduction. Consistent with our results, CYP17 β 1 and CYP19a1 expression levels are stimulated by oxidative stress through the p38 MAP kinase signaling pathway [34, 35]. However, SCE and GN contributed to inhibiting ROS generation, thus might be improving testosterone deficiency. Consistent with these results, several antioxidants like vitamin C and E have been observed to suppress oxidative stress, stimulating testosterone synthesis. Because GN can be dramatically reversed testosterone attenuation-related genes rather than Sch C, GN is a primary active compound from *S. chinensis* for improving the TDS.

In conclusion, we have determined for the first time that BBP can alter ROS levels, leading to increased expression levels of genes related to testosterone attenuation, such as CYP17 β 1 and CYP19a1. And SCE and its single compounds, especially GN, could be a possible compound for improving BBP-induced hypogonadism by decreasing ROS levels.

Declarations

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (NO. 2021R1F1A104572011 and 2021R1I1A3061377).

Funding This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (NO. 2021R1F1A104572011 and NRF-2021R1I1A3061377).

Competing interest The authors have no competing interests to declare that are relevant to the content of this article.

Author contributions Conceptualization: MHP, HKK; Methodology: MHP, HKK; Formal analysis: SJL, MJP, DHJ; Investigation: SJL, MJP, DHJ; Writing - original draft: SJL, MJP, DHJ, RA, EY, MHP, HKK; Writing-review & editing: RA, EY, MHP, HKK; Funding Acquisition: MHP, HKK; Supervision: MHP, HKK. All authors read and approved the final manuscript.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval The research did not involve any studies with human participants or animal as experimental model.

Consent for publication All authors approved the final version of the manuscript and consent for publication.

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36. Statements & Declarations

Figures

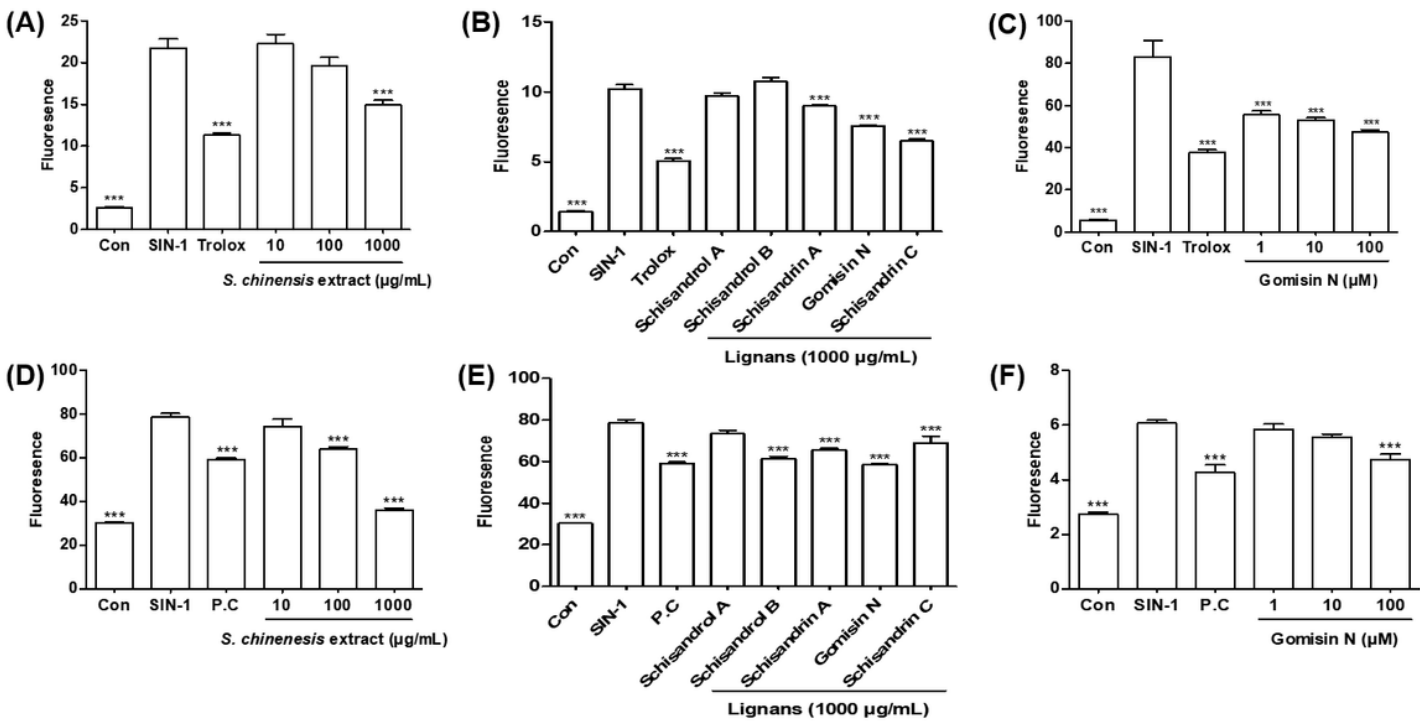


Figure 1

ROS and ONOO⁻ scavenging effects of SCE, GN. ROS scavenging activity of SCE (A), ROS scavenging activity of lignans from SCE (B), ROS scavenging activity of GN (C), ONOO⁻ scavenging activity of SCE (D), ONOO⁻ scavenging activity of lignans from SCE (E) and ONOO⁻ scavenging activity of GN (F). SIN-1-induced ROS and ONOO⁻ levels were reduced by SCE and GN treatment. Trolox, 100 μM Trolox; P.C, 1 mM penicillamine. The data are expressed as mean ± SEM, ****P* < 0.001, compared to SIN-1-treated group. The comparisons were analyzed by one-way ANOVA.

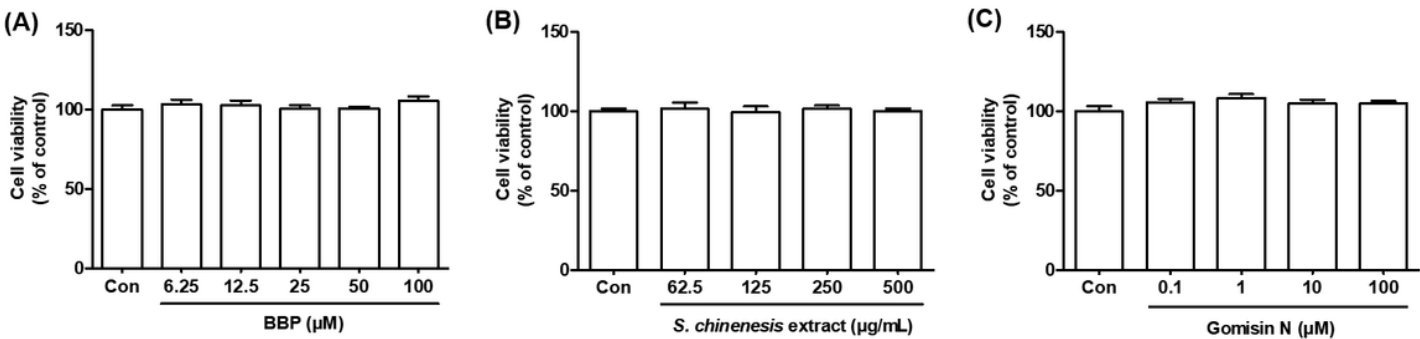


Figure 2

Cell viability effect of BBP, SCE and GN in TM3 cells. BBP (A), SCE (B) and GN (C). The data are expressed as mean $\pm$ SEM compared to control group. The comparisons were analyzed by one-way ANOVA.

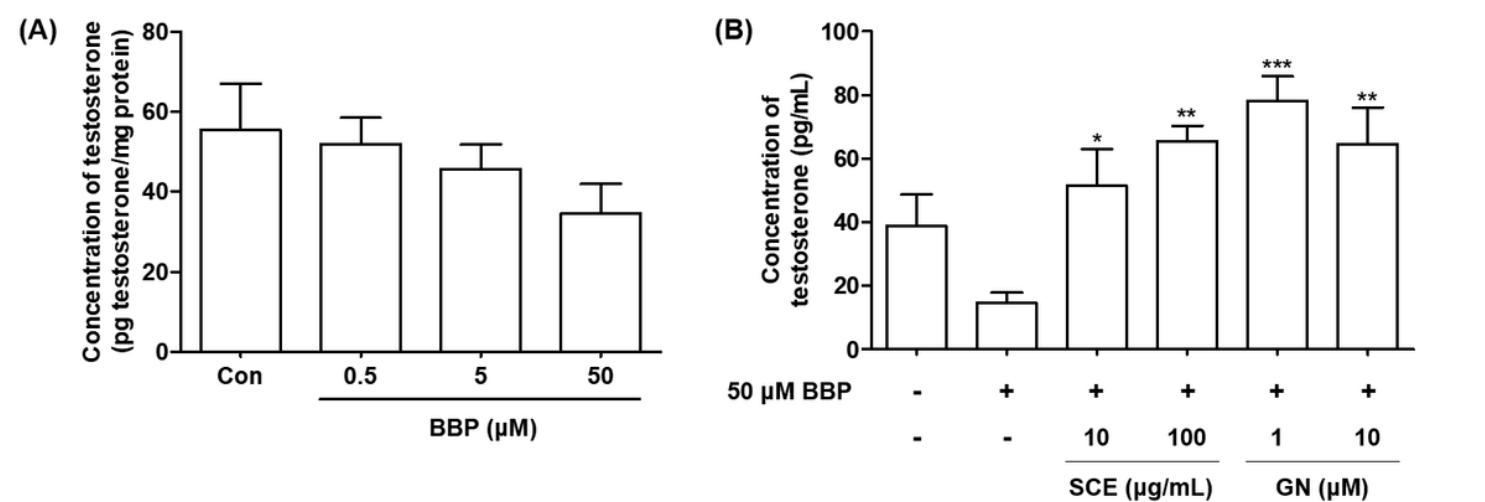


Figure 3

Changes in testosterone concentration of TM3 cells. Effects of BBP on testosterone concentration (A) and effects of SCE and GN on BBP-reduced testosterone concentration (B). The data are expressed as mean $\pm$ SEM, * P < 0.05, ** P < 0.01 and *** P < 0.001, compared to 50 μM BBP-treated group. The comparisons were analyzed by one-way ANOVA.

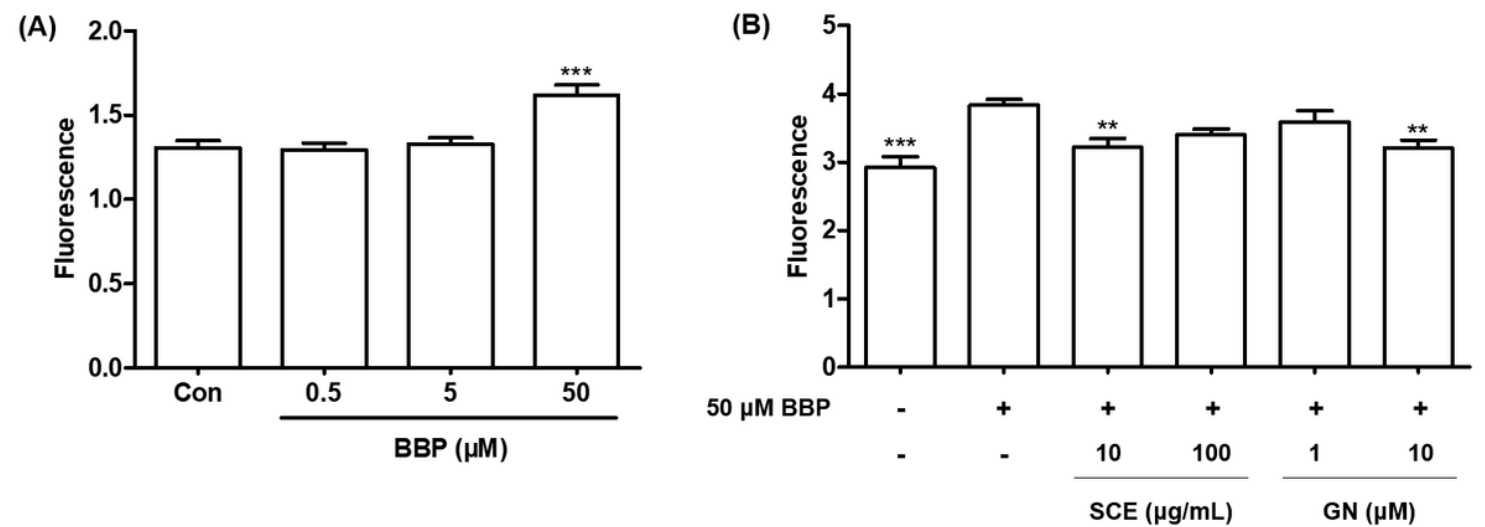


Figure 4

Changes in intracellular ROS level on TM3 cells. Effects of BBP on intracellular ROS level (*** P < 0.001, compared to control group) (A). Effects of SCE and GN on BBP-induced intracellular ROS level (** P < 0.01 and *** P < 0.001, compared to 50 μM BBP-treated group) (B). The data are expressed as mean $\pm$ SEM. The comparisons were analyzed by one-way ANOVA.

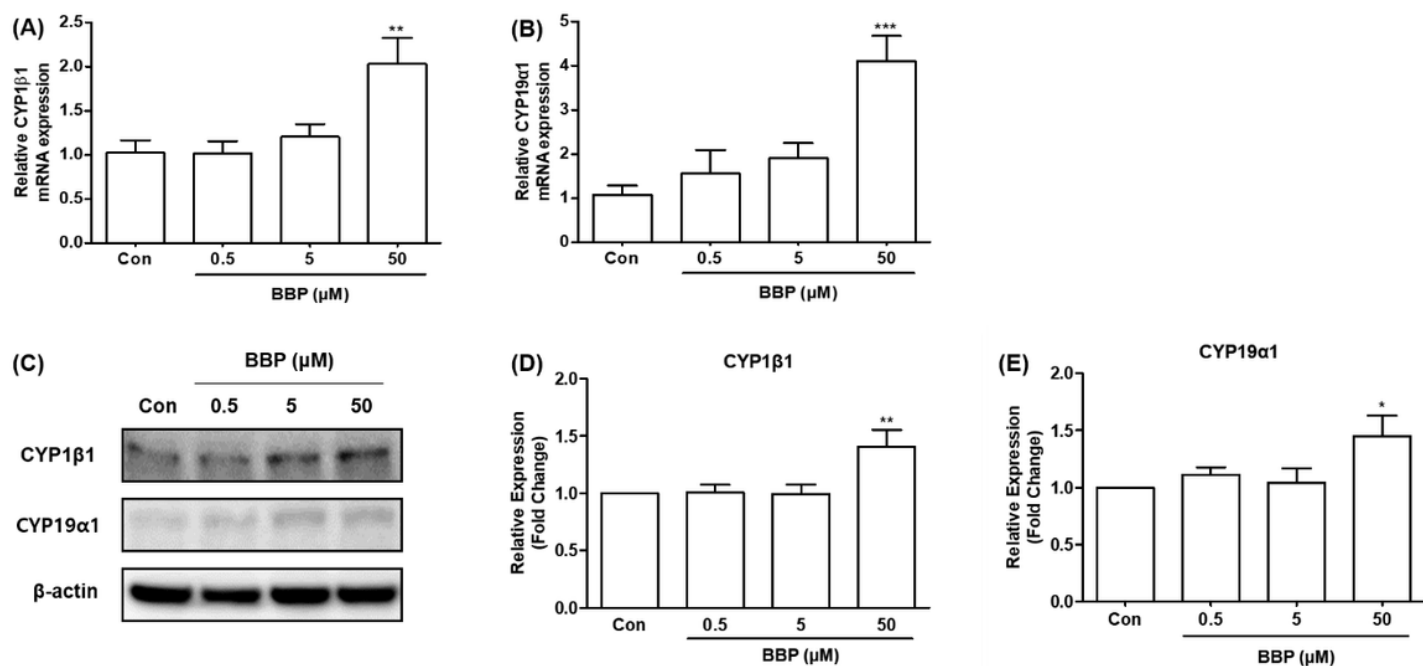


Figure 5

The effect of BBP on mRNA and protein expression levels of CYP1β1 and CYP19α1 in TM3 cells. Changes in mRNA expression levels (A, B). Changes in protein expression levels (C-E). The data are expressed as mean ± SEM, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared to control group. The comparisons were analyzed by one-way ANOVA.

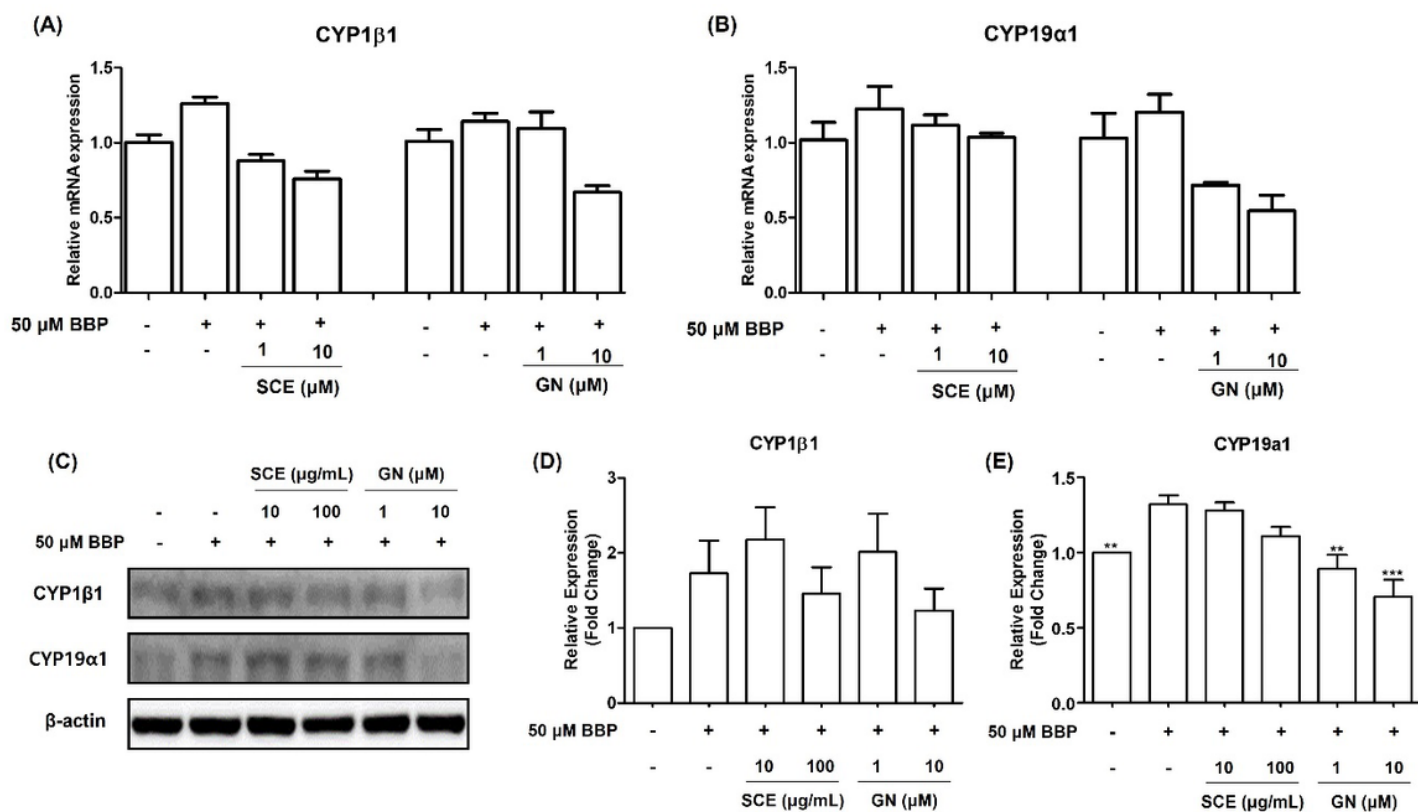


Figure 6

The effect of SCE and GN on BBP-induced mRNA and protein expression levels of CYP1 β 1 and CYP19 α 1 in TM3 cells. Changes in mRNA expression levels (A and B). Changes in protein expression levels (C-E). The data are expressed as mean $\pm$ SEM, * P < 0.05, ** P < 0.01 and *** P < 0.001, compared to 50 μ M BBP-treated group. The comparisons were analyzed by one-way ANOVA.