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Berberine Disturbs the Expression of Sex-hormone Regulated Genes in β -naphthoflavone-induced Mice

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Abstract: Berberine is a major active constituent in several medicinal plants such as *Coscinium fenestratum*, *Berberis aristata*, *Coptis japonica* and *Coptis chinensis*. Berberine possesses a wide range of biochemical and pharmacological activities including anti-inflammation, anti-microorganism, anti-cancer activities and anti-diabetes. In this study, we determined the effect of berberine, in combination with β -naphthoflavone (BNF, a pro-carcinogen), on the expression of sex-hormone synthesis genes at mRNA level including CYP17, CYP19, 3 β -HSD, 17 β -HSD1, 17 β -HSD3 in mouse testes. The single treatment of berberine up-regulated the expression of testicular CYP17, 3 β -HSD and 17 β -HSD1 mRNA. BNF up-regulated only 17 β -HSD1 mRNA. Moreover, the combination of berberine and BNF up-regulated the expression of CYP17 and 3 β -HSD. These observations suggested that berberine might disturb sex-hormone synthesis pathway; consequently possibly resulted in modification of estrogen or testosterone synthesis. Therefore, a caution should be noted for the use of berberine as an alternative medicine, especially at high dose or long period.

Key words: Berberine, hydroxylase, 17 β -HSD, sex hormone, β -naphthoflavone

INTRODUCTION

In steroidogenesis (Fig. 1), the first step is converting cholesterol into pregnenolone by cytochrome P450 (CYP11 α) encodes cytochrome P450_{scc} (cholesterol side-chain cleavage enzyme) (Miller, 1988; Stocco, 2000). Afterwards, pregnenolone is converted to progesterone by 3 β -hydroxysteroid dehydrogenase (3 β -HSD). The human cytochrome P450_{c17 α} enzyme (CYP17) possesses two enzymatic actions namely 17 α -hydroxylase and 17,20-lyase which participate in two different catalytic steps in the steroid hormone synthesis pathway (Zuber *et al.*, 1986). Pregnenolone and progesterone are converted by 17 α -hydroxylase to 17-hydroxypregnenolone and 17-hydroxyprogesterone, respectively. After that, the 17,20-lyase turns both 17-hydroxypregnenolone and 17-hydroxyprogesterone into dehydroepiandrosterone (DHEA) and androstenedione, respectively. The expression of human CYP17 is constitutively expressed in female ovarian theca cells and adrenal cortex. Rare mutations in the coding region of CYP17 have been found in patients with 17 α -

hydroxylase/17,20-lyase deficiency, resulting in various clinical profiles such as congenital adrenal hyperplasia, abnormal sexual development, osteoporosis and irregular menstruation (Yanase *et al.*, 1991; Yanase, 1995). The 3 β -hydroxysteroid dehydrogenase-isomerase enzyme (3 β -HSD) functions by converting pregnenolone to progesterone. Moreover, 3 β -HSD can convert progesterone and DHEA to androstenedione (Bates *et al.*, 2005). The aromatase P450 enzyme (CYP19) is a catalyzer for the conversion of androstenedione to estrone, or modification of testosterone to estrogen in many tissues (Morishima *et al.*, 1995). The reproductive systems of rats, mice and human are affected by aromatase (CYP19) inhibition. Deficiency of CYP19 enzyme in male mice severely disturbed spermatogenesis resulted in infertility and consequently deteriorated ability to breed pups. In females, CYP19 deficiency likewise resulted in lack of estrogen followed by pseudohermaphroditism and masculinization in adolescence (Carami *et al.*, 1997). The latter pathway culminates in the formation of testosterone via conversion of androstenedione by 17 β -hydroxysteroid

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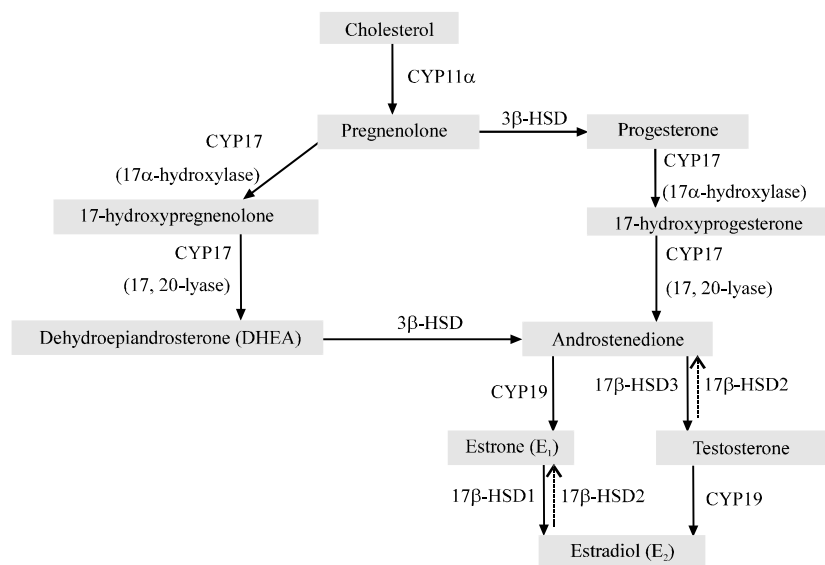


Fig. 1: Sex-hormone synthesis pathway

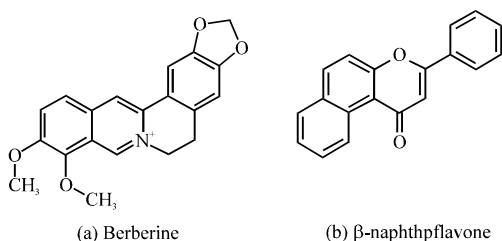


Fig. 2: Structure of (a) Berberine and (b) β-naphthoflavone

via conversion of androstenedione by 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) while 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) converts the formation of estrone to estradiol (Sha *et al.*, 1996).

Berberine (Fig. 2) is a major plant alkaloid present in several Indian and Chinese herbal plants; *Coscinium fenestratum*, *Berberis aristata*, *Berberis vulgaris*, *Hydrastis Canadensis*, *Tinospora cordifolia*, *Coptis japonica* and *Coptis chinensis* (Rojsanga *et al.*, 2006; Sato and Yamada, 1984). The extensive researches demonstrated a number of biological and pharmacological benefits of berberine, *i.e.*, anti-inflammation (Kuo *et al.*, 2004), anti-microorganism (Schmeller *et al.*, 1997), anti-cancer activities (Wu *et al.*, 1999; Letasiova *et al.*, 2006; Piyanuch *et al.*, 2007) and anti-diabetes (Zhang *et al.*, 2008; Zhang *et al.*, 2011).

Our previous study noted the effects of berberine on β-naphthoflavone (BNF)-induced CYP1A expression in both primary mouse hepatocytes and mouse livers (Chatuphonprasert *et al.*, 2011). The BNF-induced

expressions of mouse CYP1A mRNA and protein and the related enzyme activities were significantly suppressed by berberine. In addition, berberine significantly lowered the level of BNF-induced lipid peroxidation in mouse hepatic microsome (Chatuphonprasert *et al.*, 2011). The study suggested that the use of berberine as an alternative medicine might bring more advantages due to its ability to decrease the risk of carcinogenesis from induction of CYP1A expression and inhibitory effect on lipid peroxidation activity. In this study, we determined the effect of berberine, in combination with BNF (as pro-carcinogen), on the expression of sex-hormone synthesis genes at mRNA level including CYP17, CYP19, 3β-HSD, 17β-HSD1, 17β-HSD3 in mouse testes. Completeness of the study provided more detail of utilization of berberine. In contrast, if there is any negative effect found, precaution of berberine would be concerned.

MATERIALS AND METHODS

Chemicals: Berberine choride (Ber), β-Naphthoflavone (BNF) and corn oil were supplied by Sigma-Aldrich Chemical (St. Louis, MO). ReverTraAce was a product of Toyobo (Osaka, Japan). Trizol® reagent, random primers (hexadeoxyribonucleotide mixture), Taq DNA polymerase, RNase inhibitor and dNTP mixtures were products of Invitrogen™ (Carlsbad, CA). All other laboratory chemicals were of the highest purity available from commercial suppliers. Forward and reverse primers of mouse *CYP17*, *CYP19*, *3β-HSD*, *17β-HSD1*, *17β-HSD3* and *GAPDH* genes were synthesized by Bio Basic, Inc. (Markham Ontario, Canada). The primers of each gene are shown in Table 1.

Table 1: Primers sequences for PCR

Genes	Forward primers (5' - 3')	Reverse primers (5' - 3')	Size (bp)	References
<i>CYP17</i>	CAC CCT TCC AAG TGA CAG GA	AAA AAA GTA AAG TTC TAT GGG AA	120	Udomsuk <i>et al.</i> (2011)
<i>CYP19</i>	GCC TGA CAG ACA TTC TG	TCG TGA TGC AGT GCC CAG	420	Degawa <i>et al.</i> (2006)
<i>3β-HSD</i>	TGG TGA CAG GAG CAG GA	AGG AAG CTC ACA GTT TCC A	890	Degawa <i>et al.</i> (2006)
<i>17β-HSD3</i>	ATT TTA CCA GAG AAG ACA TCT	GGG GTC AGC ACC TGA ATA ATG	367	Sha <i>et al.</i> (1996)
<i>17β-HSD1</i>	ACT GTG CCA GCA AGT TTG CG	AAG CGG TTC GTG GAG AAG TAG	310	Udomsuk <i>et al.</i> (2011)
<i>GAPDH</i>	TCC ACT CAC GGC AAA TTC AAC G	TAG ACT CCA CGA CAT ACT CAG C	145	Chatuphonprasert <i>et al.</i> (2009)

Table 2: Conditions of PCR cycles

Genes	Hot start (°C min ⁻¹)	Denaturation (°C sec ⁻¹)	Annealing (°C sec ⁻¹)	Extension (°C min ⁻¹)	Finishing (°C min ⁻¹)	Cycles
<i>CYP17</i>	95/5	95/60	55/60	72/1	72/5	24
<i>CYP19</i>	95/10	95/60	60/60	72/1	72/5	27
<i>3β-HSD</i>	95/10	95/60	58/60	72/1	72/5	17
<i>17β-HSD3</i>	95/2	95/30	56/30	72/1	72/5	38
<i>17β-HSD1</i>	95/5	95/30	58/60	72/1	72/5	24
<i>GAPDH</i>	95/5	95/30	64/30	72/1	72/5	20

Animals and treatments: Male C57BL/6 mice were supplied by National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. All mice were housed in the Animal Unit of Faculty of Pharmaceutical Sciences, Khon Kaen University. Mice were housed in the Northeast Laboratory Animal Center (Khon Kaen University, Khon Kaen, Thailand) under the supervision of a certified laboratory veterinarian. They were treated according to a research protocol approved by the Animal Ethics Committee for Use and Care of Khon Kaen University, Khon Kaen, Thailand (AEKKU06/2553). At all times, the mice were housed on wood chip bedding in polysulfone cages with water and commercial animal diet supplied *ad libitum* and were acclimated for at least 7 days in housing with a 12 h dark/light cycle under controlled temperature (23±2°C) and humidity (45±2%) before dosing. Seven week-old mice were orally fed daily with 7.5 mg/kg/day of berberine (Ber) for 7 days and/or intraperitoneally given 30 mg/kg/day of BNF in the last 3 days. The control group was orally given corn oil daily for 7 days. The mice were sacrificed at the 24 h after the last treatment and the livers were excised immediately for preparation of total RNA from testes.

Semi-quantitative reverse transcription polymerase chain reaction: Mouse *CYP17*, *CYP19*, *3β-HSD*, *17β-HSD3*, *17β-HSD1* and *GAPDH* mRNAs were semi-quantified by RT-PCR. Testicular total RNA was reverse-transcribed using random primer and ReverTraAce[®], then cDNA was amplified. The conditions of PCR cycle were followed by the method of Degawa *et al.* (2006), Sha *et al.* (1996), Udomsuk *et al.* (2011) and Chatuphonprasert *et al.* (2009) with some modifications (Table 2). After separation of the PCR products by 2% agarose gel electrophoresis, the target cDNA bands were detected under ultraviolet light in the presence of ethidium bromide and semi-

quantified by Gel documentation (Ingenius syngene bio-imaging, model: IngeniusL) and Gene Tool Match program (Syngene, Lab Focus Co. Ltd., Cambridge, UK). The mRNA levels of the target genes were normalized to that of a house keeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

RESULTS AND DISCUSSION

Modification of sex-hormone related genes including *CYP17*, *3β-HSD*, *CYP19*, *17β-HSD3* and *17β-HSD1* and *GAPDH*, at the transcriptional level after treatment with berberine and/or BNF were examined in testes. The single treatment of berberine and the co-treatment with BNF significantly up-regulated expression of *CYP17* and *3β-HSD* mRNA (Fig. 3). Polymorphism of *CYP17* gene influences breast cancer in young women and up-regulated transcription of *CYP17* might affect the synthesis of estrogen and testosterone (Bergman-jungstrom *et al.*, 1999). In addition, the retinoids stimulated mRNA abundance and promoter function of *CYP17* was found in polycystic ovary syndrome theca cell (Wickenheisser *et al.*, 2005). Hence, the enhancing effect of berberine and the co-treatment with BNF might be aware due to *CYP17* induction effect. *3β-HSD* involves in several step of sex-hormone synthesis pathway (Zuber *et al.*, 1986; Bates *et al.*, 2005). Therefore, berberine might disturb sex-hormone synthesis, consequently possibly resulted in modification of circulating pattern of estrogen or testosterone in the body. Moreover, the expression of *17β-HSD1* mRNA was significantly up-regulated after single treatment with berberine or BNF but there was no change in co-treatment group (Fig. 4). The up-regulated expression of *17β-HSD1* might be involved in estradiol metabolism pathway by an increase the conversion of estradiol. Hepatic

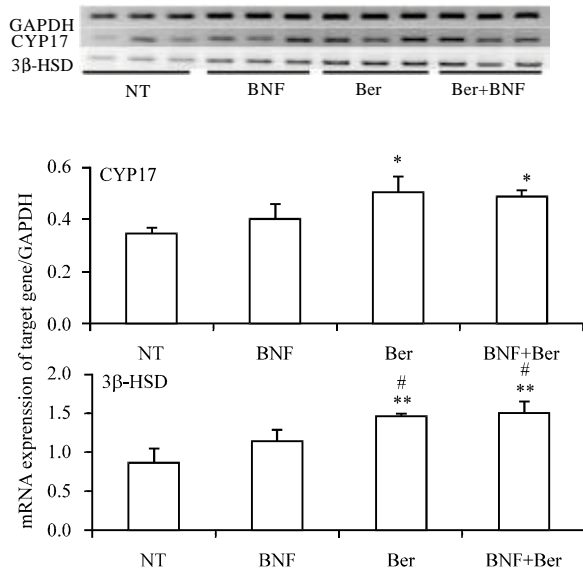


Fig. 3: Expression pattern of testicular CYP17 and 3β-HSD mRNA in male mice. Seven week-old mice were orally fed daily with 7.5 mg/kg/day of berberine (Ber) for 7 days and/or intraperitoneally given 30 mg/kg/day of β-naphthoflavone (BNF) in the last 3 days. The control group (NT) was orally given corn oil daily for 7 days. The relative mRNA expression levels were normalized by that of GAPDH. The data are presented as the Mean±SD (n = 3-4) from at least 2 independent experiments. A significant difference was examined by ANOVA with Tukey *post hoc* test. *p<0.05, **p<0.001 compared to NT group; #p<0.05 compared to BNF group

cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1B1 (CYP1B1) play key roles in catalyze estrogen to 2 and 4-hydroxyestradiol and convert to methoxyestradiol by catechol-O-methyltransferase (Liehr, 2000; Dawling *et al.*, 2003). The previous study showed that BNF induced CYP1A1 and CYP1B1 activities. Activations of these enzymes lead to increase estradiol metabolism and resulted in up-regulated expression of 17β-HSD1. The unchange of 17β-HSD1 by the co-treatment resulted from the feedback inhibition of estradiol metabolism by methoxyestradiol (Liehr, 2000; Dawling *et al.*, 2003). The results in estradiol accumulation accordance with 17β-HSD1 recovered to nearly the same level as the normals (Fig. 4). The expressions of CYP19 and 17β-HSD3 were not significantly modified by all treatments (Fig. 5).

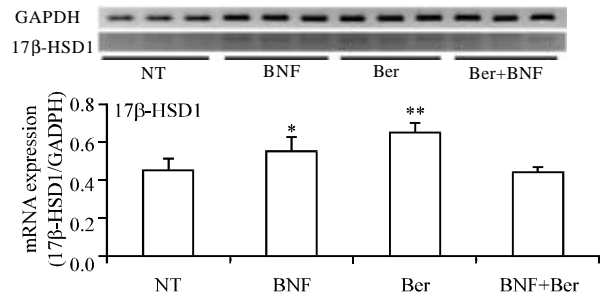


Fig. 4: Expression pattern of testicular 17β-HSD1 mRNA in male mice. Seven week-old mice were orally fed daily with 7.5 mg/kg/day of berberine (Ber) for 7 days and/or intraperitoneally given 30 mg/kg/day of β-naphthoflavone (BNF) in the last 3 days. The control group (NT) was orally given corn oil daily for 7 days. The relative mRNA expression levels were normalized by that of GAPDH. The data are presented as the Mean±SD (n = 3-4) from at least 2 independent experiments. A significant difference was examined by ANOVA with Tukey *post hoc* test. * p<0.05, ** p<0.001 compared to NT group; # p<0.05 compared to BNF group

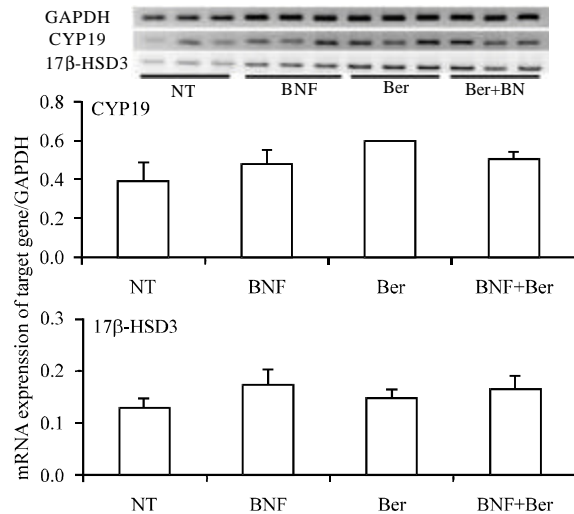


Fig. 5: Expression pattern of testicular CYP19 and 17β-HSD3 mRNA in male mice. Seven week-old mice were orally fed daily with 7.5 mg/kg/day of berberine (Ber) for 7 days and/or intraperitoneally given 30 mg/kg/day of β-naphthoflavone (BNF) in the last 3 days. The control group (NT) was orally given corn oil daily for 7 days. The relative mRNA expression levels were normalized by that of GAPDH. The data are presented as the Mean±SD (n = 3-4) from at least 2 independent experiments. A significant difference was examined by ANOVA with Tukey *post hoc* test. *p<0.05, **p<0.001 compared to NT group; #p<0.05 compared to BNF group

CONCLUSION

In the conclusion, the single treatment of berberine up-regulated the expression of testicular CYP17, 3 β -HSD and 17 β -HSD1 mRNA. The single treatment of BNF up-regulated only 17 β -HSD1 mRNA expression. While the combination of berberine and BNF up-regulated in expression of CYP17 and 3 β -HSD. These observations suggested that berberine disturbed sex hormone synthesis pathway; consequently possibly resulted in modification of circulating estrogen pattern in the body. Therefore, a caution should be noted for the use of berberine as an alternative medicine. However, a further study to affirm the evidence of sex hormone modification by berberine in illness is still required.

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