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Original Article

Protective effect of *Mucuna pruriens* (L.) DC. var. pruriens seed extract on apoptotic germ cells in ethanolic male rats

Efeito protetor de *Mucuna pruriens* (L.) DC. var. extrato de semente de *pruriens*, em células germinativas apoptóticas em ratos machos etanólicos

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Abstract

Thai *Mucuna pruriens* (L.) DC. var pruriens (T-MP) seed containing levodopa (L-DOPA) and antioxidant capacity has been shown to improve sexual behavior and male reproductive parameters in rats treated with ethanol (Eth). However, its protective effect on testicular apoptotic germ cells has never been reported. This study aimed to investigate the potential effects of T-MP seed extract on expressions of caspase, proliferating cell nuclear antigen (PCNA), and dopamine D2 receptor (D2R) proteins in Eth rats. Thirty-six male Wistar rats were divided into four groups (9 animals/group), including control, Eth, T-MP150+Eth, and T-MP300+Eth, respectively. Control rats received distilled water, and Eth rats received Eth (3g/kg BW; 40%v/v). The T-MP groups were treated with T-MP seed extract at a dose of 150 or 300 mg/kg before Eth administration for 56 consecutive days. The results showed that the seminiferous tubule diameter and epithelial height were significantly increased in both T-MP treated groups compared to the Eth group. Additionally, the caspase-9 and -3, and PCNA expressions were decreased, but D2R expression was markedly increased in T-MP groups. It was concluded that T-MP seed extract could protect testicular apoptosis induced by Eth via changes in caspase, PCNA, and D2R protein expressions.

Keywords: *Mucuna pruriens*, ethanol, testis, apoptosis, dopamine receptor.

Resumo

Thai Mucuna pruriens (L.) DC. *var pruriens* (T-MP) contendo levodopa (L-DOPA) e capacidade antioxidante demonstrou melhorar o comportamento sexual e os parâmetros reprodutivos masculinos em ratos tratados com etanol (Eth). No entanto, seu efeito protetor sobre células germinativas apoptóticas testiculares nunca foi relatado. Este estudo teve como objetivo investigar os efeitos potenciais do extrato de semente de T-MP na expressão de proteínas de caspase, antígeno nuclear de proliferação celular (PCNA) e receptor de dopamina D2 (D2R) em ratos Eth. Trinta e seis ratos Wistar machos foram divididos em quatro grupos (9 animais/grupo), incluindo controle, Eth, T-MP150+Eth e T-MP300+Eth, respectivamente. Ratos controle receberam água destilada e ratos Eth receberam Eth (3g/kg PC; 40%v/v). Os grupos T-MP foram tratados com extrato de semente de T-MP na dose de 150 ou 300 mg/kg antes da administração de Eth por 56 dias consecutivos. Os resultados mostraram que o diâmetro dos túbulos seminíferos e a altura epitelial foram significativamente aumentados em ambos os grupos tratados com T-MP em comparação com o grupo Eth. Além disso, as expressões de caspase-9 e -3 e de PCNA diminuíram, mas a expressão de D2R aumentou acentuadamente nos grupos T-MP. Concluiu-se que o extrato de semente de T-MP pode proteger a apoptose testicular induzida por Eth através de alterações na expressão de proteínas caspase, PCNA e D2R.

Palavras-chave: *Mucuna pruriens*, etanol, testículo, apoptose, receptor de dopamina.

1. Introduction

It is well known that chronic ethanol abuse is a major cause of sexual dysfunction and male infertility in both men and animal models (Stutz et al., 2004; Ulanov, 2016; Tangsrisakda et al., 2022). Indeed, ethanol can cause reductions in gonadotropin and androgen levels, resulting in male subfertility (Maneesh et al., 2006; Akomolafe et al., 2017; Akbari et al., 2017). In rodents, many studies have shown that ethanol consumption decreased the male reproductive organ weight (Nishi et al., 2018), testosterone level (Uygur et al., 2014; Nishi et al., 2018; Yari et al., 2018; Tangsrisakda and Iamsaard, 2020), and sperm qualities including count, morphology, viability, motility, and acrosome reaction status (Siervo et al., 2015; Akomolafe et al., 2017; Tangsrisakda and Iamsaard, 2020;

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Tangsrisakda et al., 2022). It also could inhibit seminiferous germ cell proliferation (Koh and Kim, 2006). Additionally, ethanol was demonstrated to increase apoptotic germ cells (8, 13), which is corroborated by the increase of apoptotic markers such as caspase-3, -7, -8, and -9 (Eid et al., 2002; Koh and Kim, 2006; Jana et al., 2010; Nishi et al., 2018).

The seed of *Mucuna pruriens* (MP) has been used in traditional Ayurvedic Indian medicine to treat Parkinson's disease and male infertility (Katzenschlager et al., 2004; Suresh et al., 2009; Suresh et al., 2013; Rai et al., 2017). In addition, MP seed is reported to possess the levodopa (L-DOPA, levo-3,4-dihydroxyphenylalanine), the dopamine precursor, that can move across the blood-brain barrier (Lampariello et al., 2012; Camargo et al., 2014; Pulikkalpura et al., 2015). Moreover, L-DOPA in MP has been demonstrated to have an antioxidant capacity and could decrease apoptotic germ cells of male rats treated with ethinyl estradiol (Singh et al., 2013). In Thailand, there is a species of MP called Thai Mamui or *Mucuna pruriens* (L.) DC. var. *pruriens* (Fabaceae) (T-MP). The seed of T-MP has been believed and used in Thai folk medicine as a diuretic and aphrodisiac drug. A recent study has shown that the T-MP contained high antioxidant capacities without toxicity on vital organs in both male and female healthy rats (Iamsaard et al., 2020). T-MP seed extract has been further reported to improve sperm quality, testosterone level, testicular histopathology, and testicular protein expressions involved in spermatogenesis. These markers included androgen receptor, heat shock protein 70, steroidogenic acute regulatory protein, tyrosine-phosphorylated protein, and apoptotic markers, caspase-3 and -9 in chronic stress models (Choowong-In et al., 2021; Lapyuneyong et al., 2022). Although testicular damages caused by ethanol administration and prevented by T-MP treatments have been reported (Tangsrisakda and Iamsaard, 2020; Tangsrisakda et al., 2022), its protective effects on testicular apoptosis still need to be elucidated. Therefore, this study aimed to investigate the protective effect of T-MP seed extract on apoptotic germ cells induced by ethanol in male rats.

2. Materials and Methods

2.1. Plant extraction

Thai *Mucuna pruriens* (T-MP) seed extract used in this study that its plant authentication has been previously proven (voucher code: S. Iamsaard 01, the herbarium database, Khon Kaen University) was kindly obtained from Dr. Sitthichai Iamsaard's laboratory and was determined for L-DOPA content and antioxidant capacity with no toxicity on vital and reproductive organs (Iamsaard et al., 2020; Choowong-in et al., 2021; Tangsrisakda et al., 2022).

2.2. Animal administration and ethic

Thirty-six adult male Wistar rats (10 weeks old, 280-360 g) were purchased from Nomura Siam International (Bangkok, Thailand) before acclimatizing for 7 days in the Northeast Laboratory Animal Center (Khon Kaen University, Thailand). Animals were housed in the plastic cage (37.5x48x21 cm) within environmental control room (dark/light cycle at 12 hours, temperature at 23±2 ºC, light intensity at 350-400 Lux, relative humidity at 30-60%, and sound level < 85 decibel). The commercial food pellets and sterile water were provided ad libitum. After acclimatization, rats were randomly divided into 4 groups (n = 9/group [4-5 amimals/ case]) including control, ethanol (Eth), T-MP150+Eth, and T-MP300+Eth groups, respectively. The control rats received distilled water (DW, 4 ml) while Eth-treated animals were administed with Eth at 3 g/kgBW (40% v/v, diluted in distilled DW) via gavage (Uygur et al., 2014). In the T-MP treated groups, rats received T-MP seed extract dissolved in DW at 150 or 300 mg/kgBW for 1 hour before administered with Eth at 3 g/kgBW using gavage feeding needle (Iamsaard et al., 2020; Tangsrisakda et al., 2022). The interval time between T-MP and Eth treatments based on the previous studies (Tahir and Sultana, 2011; Nishi et al., 2018). The concentration of Eth used in this study was based on the intoxication of Eth in rat model, ranging between 2-5 g/kgBW as described previously (Aronowski et al., 2003). This study was performed for 56 consecutive days based on rat spermatogenic cycle and sperm transit into the epididymis (Adler, 1996; Creasy, 1997). The animal ethic of this study was proven by the Institutional Animal Care and Use Committee of Khon Kaen University based on the Ethic of Animal Experimentation of National Research Council of Thailand (Record No: IACUC-KKU-85/64).

2.3. Measurements of seminiferous tubule morphometry

At the end of the experiment, rats were anesthetized and euthanized by intraperitoneal injection with thiopental sodium (80 mg/kg BW, Sigma-Aldrich, Inc., USA). Then, the right testis was collected to remove surrounding fat pads before fixation with 10% buffer formalin solution for 3 days. Fixed testis was routinely processed for paraffin block before sectioning at 5 µm thickness by using semi-automatic microtome (ERM 3,100, Heston, Australia). Testicular sections were further stained with commercial Mayer's hematoxylin (Merck KgaA, Germany) and 1% eosin Y aqueous solution (Merck KgaA, Germany). Subsequently, the stained sections were observed under light microscope (Nikon ECLIPSE E200) equipped with a DXM1200 digital camera (Nikon, Japan). The stage IX of seminiferous tubules was considered to perform the morphometric analysis using four axes of diameter and epithelial height, analyzed by the ImageJ program (Version 1.50i, National Institutes of Health).

2.4. Immunofluorescence staining

After deparaffinization, the testicular sections were soaked in citrate buffer (10 mM citric acid, 0.05% Tween-20, pH 6.0) before heating in the microwave (560 Watts, 5 minutes, 3 times) to retrieve the antigens on the tissue. Then, tissues were cooled down at room temperature before washing with PBS. The cell membranes on the section were permeabilized by incubation with 0.2% Triton X-100 (Fluka Chemical Corp., USA) for 10 minutes and washed with PBS. The endogenous peroxidase activity on tissues was blocked by incubation with 30% hydrogen

peroxide (H_2O2, Cat. No. 822287, Merck, Germany) for 20 minutes. Subsequently, the non-specific binding proteins on the section were blocked with 3% bovine serum albumin (BSA, Merck, Germany) for 30 minutes. Then, they were probed with rabbit anti-proliferating cell nuclear antigen (PCNA) (Cat. No. ab92552, Abcam, UK) diluted in PBS (1:100), 4 ºC for overnight in a moist chamber while the negative control section was omitted for the primary antibody. All sections were washed with PBS and incubated with goat anti-rabbit IgG (H+L) secondary antibody Alexa Fluor® 488 conjugate (Invitrogen, CA, USA) diluted in PBS (1:300) for 30 minutes. The tissues were counterstained with Hoechst 33342 (Cat. No. ab228551, Abcam, UK) diluted in PBS (1:10,000) for 5 minutes. After that, the stained sections were mounted with glycerol (Thermo Fisher Scientific, Australia). The fluorescence emission was detected and captured under Nikon ECLIPSE 80i fluorescence microscope equipped with Nikon DS Fi1c Camera (Nikon, Japan).

2.5. Testicular protein extraction

The left testis of each animal was stored at -20 ºC before protein extraction. In brief, the 200 mg of testicular tissue without tunica albuginea from each rat was added with 400 µl of 1X radioimmunoprecipitation assay (RIPA) buffer (Cell signaling Technology Inc., USA) containing protease inhibitor cocktails (Sigma-Aldrich, Inc., USA). Then, the samples were homogenized using a glass tissue grinder and sonicated with a sonicator probe (Ultrasonic Processor, Cole-Parmer Instrument Company, Thailand) on ice. After that, all samples were pooled as the same group before incubation on the shaker for 10 minutes on ice. Afterward, the testicular samples were sonicated again before centrifugation at 14,000 rpm, 10 minutes at 4 ºC. The supernatants were collected for determination of the total protein concentration of representative testis by using the NANO drop ND-100 Spectrophotometer (NanoDrop ND-1000 Spectrophotometer V3.5 User's Manual, NanoDrop Technologes Inc., USA) at a wavelength of 280 nm.

2.6. Immuno-Western blot

Each group's total testicular protein (150 µg) was added with 2X loading buffer before separation on 10% SDS-PAGE gel. Then, the separated proteins were blotted onto the nitrocellulose membrane (Bio-Rad Laboratories, Inc., Germany) at 110 volts for 120 minutes. Subsequently, the non-specific proteins were blocked by incubating with 5% skimmed milk (for dopamine D2 receptor (D2R) antibody) or 5% bovine serum albumin (BSA) dissolved in 0.1% PBST (for caspase-3 and caspase-9 antibodies) for 10 minutes at room temperature. Then, the whole membranes were incubated with the specific primary antibody, including rabbit anti-dopamine D2 receptor (D2R; Cat. No. AB5084P, Merck Millipore Co., CA, USA) mouse anti-caspase-3 (Cat. No. sc-7272, Santa Cruz Biotechnology, Dallas, Texas, USA) , rabbit anti proliferating cell nuclear antigen (PCNA) (Cat. No. ab92552, Abcam, UK) diluted in PBS (1:100), 4 ºC , or mouse anti-caspase-9 (Cat. No. sc-56076, Santa Cruz Biotechnology, Dallas, Texas, USA) at 4 ºC for overnight. The unbound antibodies on membranes were washed with 0.05% PBST before incubation with anti-rabbit IgG secondary antibody peroxidase (Cat. No. AP132P, Merck, Germany) conjugated with horseradish peroxidase for anti-D2R or anti mouse (Cat. No. AP160P, Merck Millipore Co., USA; 1:2,000) for caspase-3 and caspase-9 for 2 hours at room temperature. After washing, the antigen-antibody complexes on membrane were detected for the specific immunoreactivity by using the enhance chemiluminescence (ECL) substrate reagent kit (GE Healthcare Life Science, USA) and visualized by using Gel Documentation 4 (ImageQuant 400, GH HealthCare, USA).

2.7. Statistical analysis

All statistical analyses were carried out with IBM SPSS version 19.0 software (Statistical Package for the Social Sciences, SPSS Inc, Armonk, New York, USA, downloaded and installed from KKU Software Center, Khon Kaen University). The one-way analysis of variance (ANOVA) and least significant difference (LSD) test were used to examine the significant difference between groups. The results were expressed as the mean ± SD and the significant differences were considered when p value < 0.05.

3. Results

3.1. Effect of T-MP seed on seminiferous tubule morphometry

The results showed that the seminiferous tubule diameter and epithelial height of Eth group were significantly shorter than that of the control group (Figures 1A and 1B). After administration with T-MP extract, it was found that the diameter and epithelial height of T-MP plus Eth-treated groups were improved compared to the Eth group (Figures 1A and 1B).

3.2. T-MP seed extract improved the expression of caspases 3 and 9

As revealed by SDS-PAGE (Figure 2A), all groups' protein profiles of testicular lysate were not different. However, the western blots showed an increase of caspase -9 and -3 expressions in the ethanolic group compared to the control group (Figure 2B). Those expressions were decreased in T-MP-treated rat testis, as shown in Figure 2B.

3.3. T-MP seed extract decreased proliferating cell nuclear antigen expression

The proliferating cell nuclear antigen (PCNA) expressions were observed and localized in the spermatogonial nuclei on the testicular section (revealed by green fluorescence) of all groups (Figure 3). Obviously. The expression of PCNA was increased in Eth testicular lysate compared to control but it was reduced in both doses of T-MP treated groups (Figure 2B).

3.4. T-MP seed extract enhanced the expression of D2-like receptors (D2R)

Figure 2B revealed that the D2R expression was increased in T-MP treated groups compared to that of

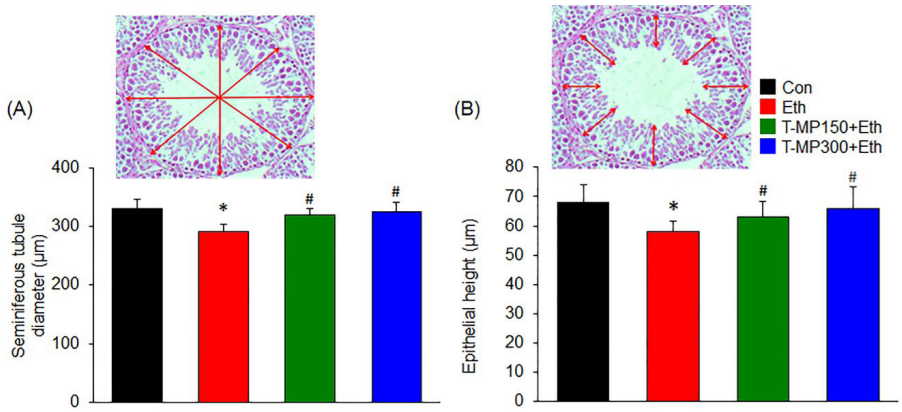

Figure 1. Showing measurement of seminiferous morphometry. The seminiferous tubule diameter (A) and epithelial height (B) compared among control, Eth, T-MP140+Eth, and T-MP300+Eth groups. *$p < 0.001$ compared to control group and the #$p < 0.001$ compared to Eth group.

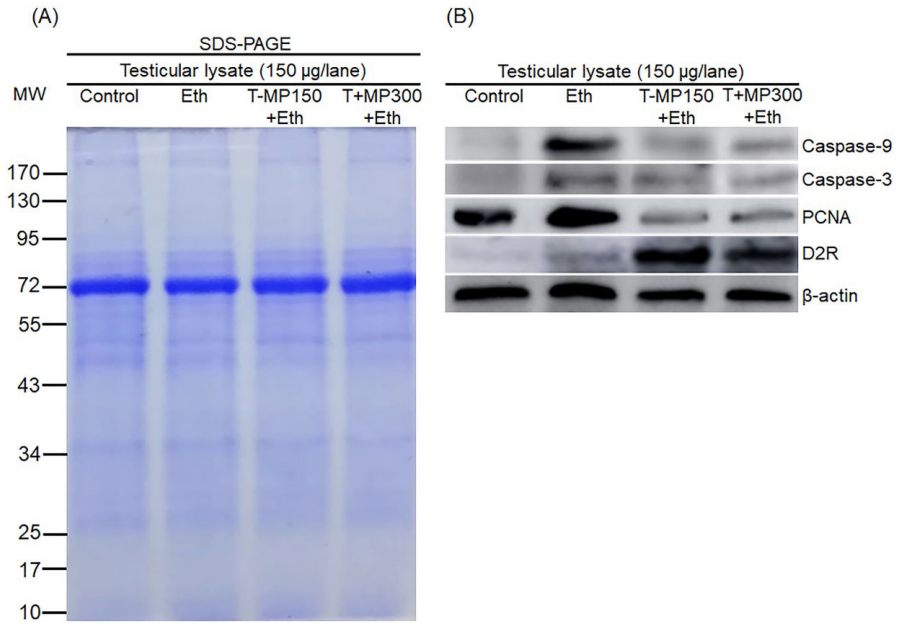

Figure 2. Comparison of testicular protein profiles among control and treated groups revealed by SDS-PAGE (A) and the expression of caspase -9 and -3, PCNA, and D2R (B). β- actin was used as an internal control; MW, molecular weight.

control and Eth rats. Interestingly, the T-MP150 group has a higher expression of D2R in the testis than the T-MP300 group (Figure 2B).

4. Discussion

In the intrinsic apoptotic pathway, ethanol metabolism can induce the formation of reactive oxygen species (ROS) (Tsedensodnom et al., 2013). Then, ROS mediates the translocation of Bcl-2 associated X (Bax), the pro-apoptotic proteins, to the outer mitochondria membrane to increase the permeability of cytochrome c. Subsequently, cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 in the cytoplasm to form apoptosome. Then, apoptosome recruits pro-caspase-9 to be cleaved form before activating the caspase-3 to stimulate apoptosis (Young et al., 2003; Krzyzanski et al., 2007; Muralidharan et al., 2013; Qin et al., 2019). In this study, the increased caspase-9 and -3 expressions in the testicular tissue of ethanolic rats implied that ethanol could induce apoptosis in germ cells. Our results were consistent with the previous studies demonstrating that the expression of caspase proteins was increased after

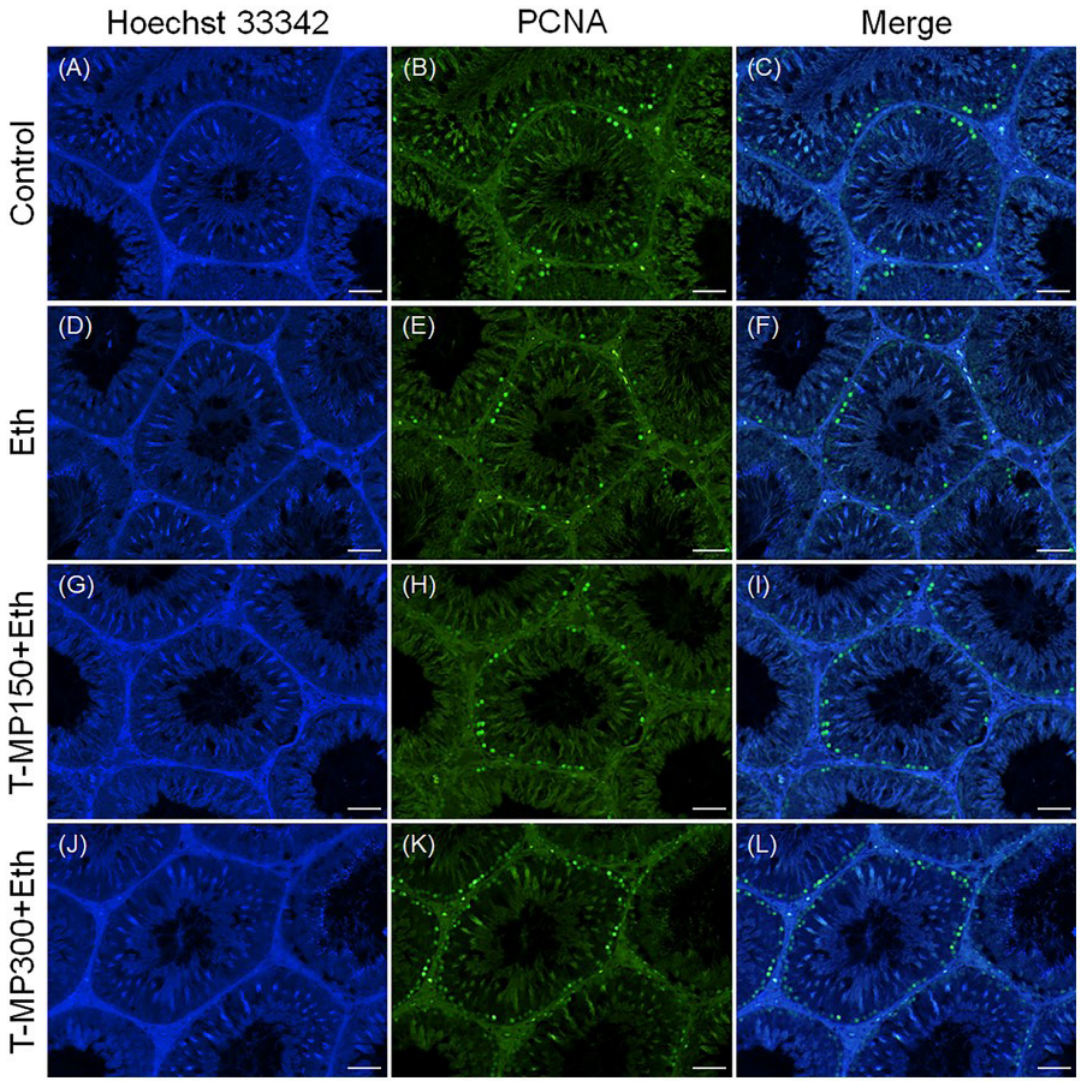

Figure 3. Immunofluorescence staining against proliferating cell nuclear antigen (PCNA, green fluorescence) in the nuclei of spermatogonia of control (B-C), Eth (E-F), T-MP150+Eth (H-I), and T-MP300+Eth (K-L). Hoechst 33342 (blue fluorescence) used as counterstaining (A-C, D-F, G-I, and J- L). Scale bar is 50 µm.

ethanol administration resulting in germ cell apoptosis (Zhu et al., 2000; Eid et al., 2002; Koh and Kim, 2006). Interestingly, male rats treated with T-MP showed a decrease of caspase-9 and -3, which corresponded to the previous studies revealed in stress models (Choowongin et al., 2021; Lapyuneyong et al., 2022). We assumed that the L-DOPA and other antioxidants contained in T-MP could potentially decrease ROS formation from ethanol metabolism, leading to suppressing the apoptotic process of germ cells. The increase of seminiferous tubule diameter confirmed it, and epithelial height in both T-MP treated groups. Also, a previous study demonstrated that sperm qualities, malondialdehyde (MDA) levels, spermatogenic proteins, and seminiferous tubule structure were improved after T-MP administration (Tangsrisakda et al., 2022). These findings indicated that the antioxidant activities of T-MP extract could prevent the apoptotic process in germ cells induced by ethanol.

Proliferating cell nuclear antigen (PCNA), playing a role in DNA replication, has been shown to increase at late G1 and S phases, while it is absent at the G0 phase of cell cycles (Altay et al., 2003; Koh and Kim, 2006; Zhao et al., 2018). It is commonly used as a marker of spermatogenic cell proliferation (Zhao et al., 2018). This study showed the presence of PCNA in the spermatogonial nuclei, which is consistent with the previous studies (Koh and Kim, 2006; D'Andrea et al., 2008). However, PCNA expression increased in ethanolic rats compared to the control and T-MP-treated groups. This result disagreed with the previous study demonstrating that acute ethanol administration at low doses (1.5 and 3.0 g/kg) for 10 days could suppress the expression

of PCNA (Koh and Kim, 2006). Chronic ethanolic administration in our study possibly caused apoptosis in germ cells as confirmed by the increase of testicular caspase-9 and -3 expressions. As a result, non-apoptotic spermatogonia might try to accelerate the proliferation to maintain the number of testicular germ cells, leading to increased PCNA expression in the ethanolic group. We demonstrated the decreased expression of PCNA in T-MP treated groups which was corroborated by the decrease of caspase-9 and -3. It was assumed that the ROS formation in the ethanolic testis was suppressed by the L-DOPA and other antioxidants found in T-MP. Therefore, as shown in our investigation, it leads to the suppression of the testicular apoptotic process. Moreover, our previous study showed that sperm quality and daily sperm production (DSP) in ethanolic testis were improved after treatment with T-MP extract (Tangsrisakda et al., 2022). A mechanism could explain such protection effects of T-MP extract in ethanol-treated rats via decreasing PCNA and apoptotic expressions.

Dopamine is known to play a role in the nervous system via D1-like (D1R) and D2-like receptors (D2R) (Martel and Gatti McArthur, 2020; Penedo et al., 2021). Moreover, previous studies demonstrated the presence of dopamine receptors in the heart, kidney, lymphocytes, and testis (Otth et al., 2007; Cavallotti et al., 2010; Olivares-Hernández et al., 2021; Penedo et al., 2021). Especially in rat testis, Otth and coworkers (2007) investigated that the D2R was expressed in the spermatocyte, spermatid, and spermatozoa (Otth et al., 2007). In addition, a previous study showed the reduction of the D2R expression in spermatocytes, round spermatids, and Sertoli cells but an increase in the elongated spermatids of methamphetamine-treated rats (Thanoi et al., 2020). It indicated that D2R might play a pivotal role in spermatogenesis. In this study, the increase of D2R and apoptotic markers (caspase-9 and -3) expressions in Eth testis might result from the overstimulation of germ cells to accelerate sperm production. After treatment with T-MP, the expression of D2R was markedly increased, whereas caspase-9 and 3 expressions were decreased compared to the Eth group. Indeed, the antioxidant capacity, including the L-DOPA component in T-MP, might improve such changes in D2R and caspase expressions as previously reported (Iamsaard et al., 2020; Choowong-In et al., 2021; Tangsrisakda et al., 2022). It could be explained that L-DOPA might be transformed to be dopamine before binding with the D2R in the germ cells to facilitate spermatogenesis, as confirmed by the improvement of the sperm quality, testicular morphometry, and essential proteins involved in spermatogenesis of rats treated with ethanol (Tangsrisakda et al., 2022).

5. Conclusion

Therefore, it was concluded that the T-MP seed extract containing L-DOPA and antioxidants could protect testicular apoptosis induced by ethanol via changes of caspase (3 and 9), PCNA, and D2R protein expressions.

6. Future Prospects

These investigations have provided the preventive effects of T-MP consumption on testicular apoptosis caused from ethanol via improvement of caspase, PCNA and D2R protein expressions. Possibly, the seed of T-MP can be used as a potential dietary supplement for testicular health of alcoholism men to protect apoptosis.

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