Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
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Attenuating effect of daidzein on polychlorinated biphenyls-induced oxidative toxicity in mouse testicular cells*

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Received Feb. 17, 2008; revision accepted Apr. 22, 2008

Abstract: The attenuating effect of daidzein (DAI) on oxidative toxicity induced by Aroclor 1254 (A1254) was investigated in mouse testicular cells. Cells were exposed to A1254 alone or with DAI. The oxidative damage was estimated by measuring malondialdehyde (MDA) formation, superoxide dismutase (SOD) activity and glutathione (GSH) content. Results show that A1254 induced a decrease of germ cell number, an elevation in thiobarbituric acid reactive substances (TBARS) but a decrease in SOD activity and GSH content. However, simultaneous supplementation with DAI decreased TBARS level and increased SOD activity and GSH content. Consequently, dietary DAI may restore the intracellular antioxidant system to attenuate the oxidative toxicity of A1254 in testicular cells.

Key words: Daidzein (DAI), Polychlorinated biphenyl (PCB), Oxidative damage, Germ cell
doi:10.1631/jzus.B0820048 **Document code:** A **CLC number:** Q95

INTRODUCTION

With the rapid development of industry and agriculture, environmental endocrine disrupters have drawn more and more concerns due to their potential hazard impacts on human and animal health. Polychlorinated biphenyls (PCBs), members of environmental contaminants, belong to the halogenated hydrocarbon class. Even though most countries had strictly prohibited their use, PCBs residues are still ubiquitous in the surface soils and animal products (Baars *et al*., 2004; Zhang *et al*., 2007). Furthermore, PCBs are biomagnified along food chains, increasing a risk of human exposure due to the ubiquitous, persistent and lipophilic characters.

PCBs potentially induce developmental abnormities and reproductive impairment, such as the inhibition of rat testicular androgenesis (Anderson *et al*., 1994; Andric *et al*., 2000) and interference of chicken gonadal development and spermatogenesis (Zhang and Qiao, 2004; Mi and Zhang, 2005a). In addition, Aroclor 1254 (A1254, a commercial mixture of PCBs) induced an increase of lipid peroxidation and free radicals and a decrease of the antioxidant defense system (Murugesan *et al*., 2005). Moreover, A1254 manifested oxidative stress in rat Sertoli cells by decreased superoxide dismutase (SOD), glutathione (GSH) peroxidase and GSH reductase activities. However, administration of antioxidant ascorbic acid or tocopherol ameliorated these effects (Senthil Kumar *et al*., 2004; Mi and Zhang, 2005b). Therefore, oxidative stress represents an important mechanism of PCBs in testicular toxicity.

Daidzein (DAI) belongs to the most common isoflavones and possesses multiple pharmacological effects on health. The most important biological action of DAI contributes to the antioxidant activity. In the present study, DAI was chosen to evaluate its protective effect against damage induced by A1254 in

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* Project supported by the Program for New Century Excellent Talents in University of the Ministry of Education of China (No. NCET-05-0514) and the Postdoctoral Science Foundation of China (No. 20060400325)

testicular germ cells. In order to reveal the antioxidant action of DAI, germ cells were exposed to the reactive oxygen species (ROS)-producing system hypoxanthine/xanthine oxidase (HX/XO) in the presence or absence of DAI. The malondialdehyde (MDA) formation, SOD activity and GSH content were determined to reflect the level of lipid peroxidation and intracellular antioxidant defense system. The results would facilitate understanding of the potential of flavonoids in preventing pollutants-induced oxidative damage to male reproduction.

MATERIALS AND METHODS

Isolation and culture of testicular cells

ICR (Institute of Cancer Research) male mice aged 3 weeks were obtained from Center of Laboratory Animals, Zhejiang University, China. The animals were kept at 20~22 °C with a 12-h light and 12-h dark photoperiod and free access to water and feed.

The seminiferous tubules were isolated from mice and minced into small fragments, and then digested in phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free) solution containing 1 mg/ml collagenase (GIBCO BRL, NY, USA). The dissociated cells were filtrated through a 150 μm mesh. Cell viability was always over 90% as determined by the trypan blue exclusion test. The cells were cultured in 24-well culture plates at a density of 2×10^5 per well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, and 3×10^{-8} mol/L selenium (Sigma, St. Louis, MO, USA). The cells were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2.

Treatments of cells with chemicals

A1254 (AccuStandard Inc., New Haven, CT, USA) was prepared in ethanol and diluted in medium. Cells were challenged with A1254 (10, 20, 30 μg/ml) and DAI (0.1, 1, 10 μg/ml) alone or in combinations for 24 h. The HX/XO system (a mixture of 10^{-5} mol/L hypoxanthine and 2.5×10^{-3} U/ml xanthine oxidase) alone or in combination with DAI (10 μg/ml) was added to the medium to study the antioxidant effect of DAI on ROS. The concentration of ethanol was ≤0.1% (v/v) in the medium. The control group received vehicle only.

Morphological analysis

Morphological changes of germ cells were observed under an IX70 phase contrast microscope (Olympus, Japan). Five different regions were selected randomly in each well and the image was captured with a video camera (Pixera Pro 150ES) connected to a computer. The number of germ cells was counted in each image by using Simple PCI Advanced Imaging Software (Compix, Inc., USA).

Determination of MDA formation, GSH content and SOD activity

MDA concentrations were calculated by the absorbance of thiobarbituric acid reactive substances at 532 nm as described in the kit instruction (Nanjing Jiancheng Bioengineering Institute, China). The GSH content was determined by measuring the rate of reduction of 5,5′-dithiobis-2-nitrobenzoate (DTNB) to 2-nitro-5-thiobenzoate with absorption maximum at 412 nm. Total SOD activity was evaluated by the inhibition in the rate of the superoxide radicals-dependent cytochrome C reduction and the absorbance at 550 nm was determined.

Statistical analysis

The experiment was repeated three times in quadruplicate. All data were expressed as the mean± *SD*. The statistical differences among the groups were determined by analysis of variance (ANOVA) and Duncan's multiple range test by using the SAS 8.1 software (SAS Inst. Inc., Cary, NC, USA). $P<0.05$ was considered significantly different.

RESULTS

Toxic effect of A1254 on testicular cells

After 24 h culture, germ cells in the control group showed normal integrated morphology similar to cells in the DAI-treated group. After exposure to A1254 at 20 and 30 μg/ml, the number of adherent germ cells was decreased by 13.0% and 22.4%, respectively, compared with the control. Vacuolated cytoplasm, cytolysis and disorganization were observed and many cell pieces were released into the medium (Fig.1). Furthermore, evaluation of testicular toxicity showed MDA formation was markedly increased after treatment with 30 μg/ml A1254 (Fig.2).

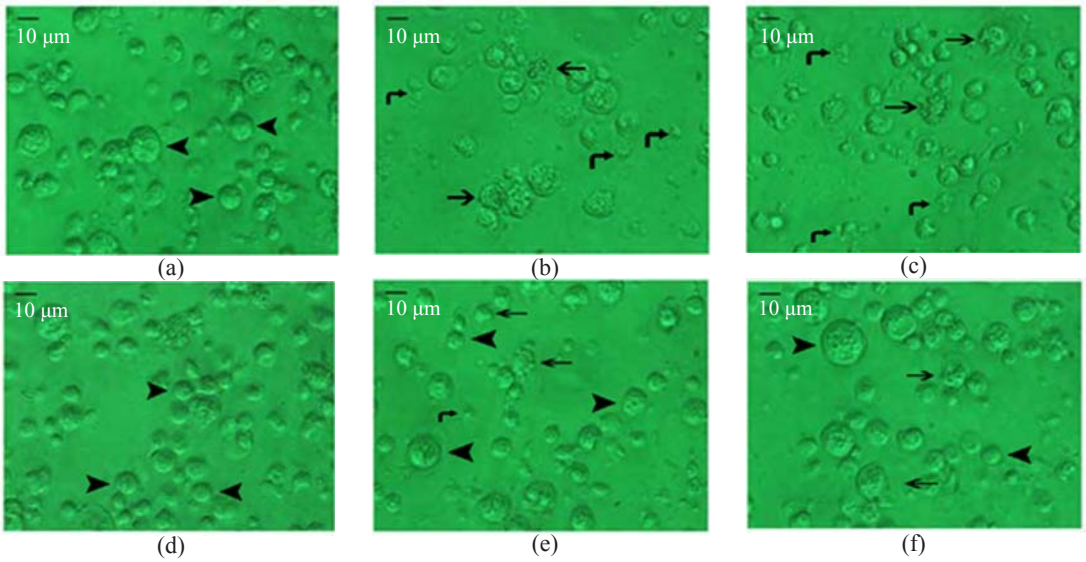

Fig.1 Morphological changes of testicular germ cells after treatments of chemicals for 24 h. (a)~(f) indicate cells of the control, HX/XO, A1254 (30 μg/ml), DAI (10 μg/ml), HX/XO+DAI (10 μg/ml) and A1254 (30 μg/ml)+DAI (10 μg/ml) groups, respectively. Arrowheads (➢), flexural arrows (↱) and arrows (→) indicate normal germ cells, vacuolated cytoplasm and cell pieces, respectively

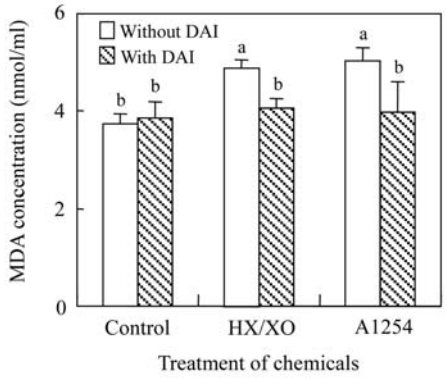

Fig.2 Assessment of cytotoxicity by MDA formation after 24 h culture. Values represent mean±*SD*. Bars with different superscripts were statistically different (*P*<0.05)

Effects of DAI on A1254-induced cell damage

After treatment with DAI for 24 h, there was no obvious difference between DAI-treated group and the control group. However, DAI (1~10 μg/ml) significantly attenuated A1254 (30 μg/ml)-induced germ cell death and the number of adherent germ cells was significantly increased (Figs.1 and 3). Meanwhile, A1254-induced germ cell damage and elevation of MDA formation were significantly reduced after combined treatment of 10 μg/ml DAI (Fig.2).

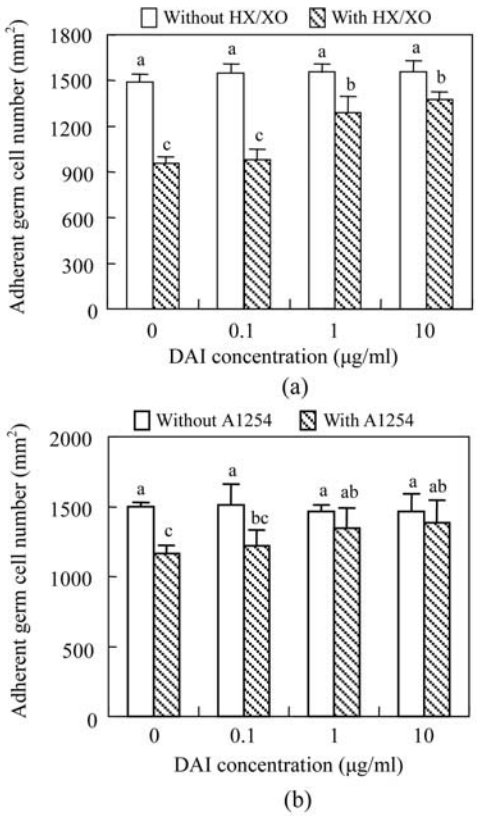

Fig.3 Assessments of testicular cell survival by adherent germ cell number after 24 h culture. (a) DAI treatment alone or combined with HX/XO; (b) DAI treatment alone or combined with A1254. Values represent mean±*SD*. Bars with different superscripts were statistically different (*P*<0.05)

Antioxidant activity of DAI on HX/XO-induced oxidative damage

HX/XO exerted cytotoxicity and notable damage was observed in testicular damage (Fig.1b). After incubation with HX/XO for 24 h, the number of adherent germ cells significantly decreased. DAI (1~10 μg/ml) significantly attenuated HX/XO-induced germ cell death and the number of adherent germ cells was significantly increased (Figs.1e and 3a). Compared with the control, MDA formation was increased significantly (Fig.2), and SOD activity and GSH level decreased significantly in HX/XO treatment. There were no obvious differences in MDA formation, SOD activity and GSH level between DAI and the control group. However, in combination with DAI (10 μg/ml), MDA production, SOD activity and GSH level were restored in the HX/XO group (Figs.2 and 4).

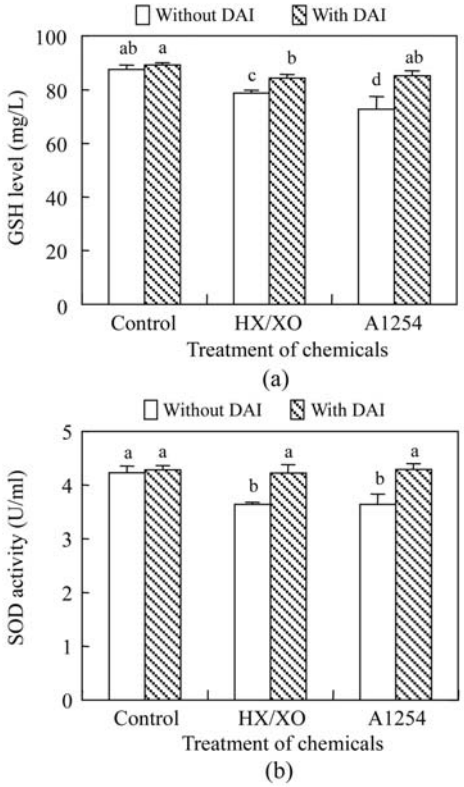

Fig.4 Protection of DAI against ROS or A1254-induced oxidative toxicity. (a) GSH level; (b) SOD activity. Values represent mean±*SD*. Bars with different superscripts were statistically different (*P*<0.05)

Changes of the intracellular antioxidant activity

After exposure to 30 μg/ml A1254 for 24 h, SOD activity and GSH content were decreased significantly compared with the control. There were no notable differences in SOD activity and GSH content between DAI and the control group. However, in the presence of 10 μg/ml DAI there were significant increases of the SOD activity and GSH level in the A1254 group (Fig.4).

DISCUSSION

ROS is generated by normal cellular metabolism and exogenous agents. Excessive ROS may damage cellular component, resulting in an increase in the concentration of lipid hydroperoxides and a loss of membrane polyunsaturated fatty acids (Griveau *et al*., 1995). The HX/XO system continuously generates superoxide radicals and H_2O_2 through electron transfer from the substrate hypoxanthine. In the present study, after incubation with HX/XO, obvious damaging effect on cell morphology was observed, cellular structure was disorganized, and many cell pieces were released. MDA is a breakdown product of the oxidative degradation of cell membrane lipids and is generally considered an indicator of lipid peroxidation. After exposure to HX/XO, MDA formation was increased significantly and thus HX/XO induced the lipid peroxidation. GSH is an important cellular non-enzymatic antioxidant and protects cellular constituents from oxidative damage. SOD is a scavenger of superoxide anion free radicals. HX/XO system decreased significantly SOD activity and GSH level; therefore, ROS induced by HX/XO resulted in damage of intracellular antioxidant defense system.

Reproductive abnormalities caused by PCBs were observed in many animal species (Andric *et al*., 2000; Mi and Zhang, 2005a). In the present study, A1254 damaged germ cell membrane integrity and induced cell death. These data show that exposure to A1254 resulted in damage of intracellular antioxidant defense system, increase of MDA formation, and decreases of SOD activity and GSH level. The findings in the present study are consistent with previous reports that A1254 interfered with the integrity of cell membrane and caused chicken embryo hepatocyte damage and induced decreased SOD activity and GSH concentration (Zhou and Zhang, 2005). Our results suggest that the cytotoxicity induced by A1254 related to oxidative stress and excessive pro-

duction of ROS, which caused lipid peroxidation, cellular structural impairment and cell death.

The antioxidant capacity of DAI was also revealed in the present study. In order to explore the protecting mechanism of DAI, antioxidant actions of DAI were estimated through production of ROS by HX/XO system, and the results show that DAI attenuated toxicity of PCBs and oxidative damage of ROS to germ cells. A1254 or HX/XO caused oxidative damage but after combined with DAI, cellular structure maintained integrity, and MDA formation, SOD activity and GSH level were restored to a normal range. Flavonoid quercetin attenuated oxidative damage induced by A1254 in embryonic chicken spermatogonial cells by inhibition of lipid peroxidation and protection of intracellular antioxidant system (Mi and Zhang, 2005b). In addition, α-tocopherol and ascorbic acid exhibited protective effect on sperm in adult rats by inhibiting PCB-induced ROS generation (Krishnamoorthy *et al*., 2007). These results indicated that DAI could scavenge excessive free radicals to protect germ cells from A1254-induced lipid peroxidation and membrane impairment through the antioxidant action.

CONCLUSION

Exposure to A1254 resulted in testicular cytotoxicity by production of oxidative stress and lipid peroxidation. DAI could protect germ cells from A1254-induced toxicity through a reduction in MDA formation and increases in SOD activity and GSH level to maintain germ cell viability. Therefore, dietary DAI may restore the antioxidant system and attenuate the negative effects of environmental endocrine disrupters in male reproduction.

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