Apigenin induces oxidative stress in mouse Sertoli TM4 cells

Sathaporn Jittapalapong, Thapanee Poompoung and Samak Sutjarit

Department of Veterinary Technology, Faculty of Veterinary Technology, Kasetsart University, Bangkok 10900, Thailand.

Corresponding author: Samak Sutjarit, e-mail: cvtms@ku.ac.th

Co-authors: SJ: fvejspj@ku.ac.th, TP: thapanee.baitoey91@gmail.com

Received: 04-08-2021, Accepted: 02-11-2021, Published online: 20-12-2021

do: www.doi.org/10.14202/vetworld.2021.3132-3137 How to cite this article: Jittapalapong S, Poompoung T, Sutjarit S (2021) Apigenin induces oxidative stress in mouse Sertoli TM4 cells, Veterinary World, 14(12): 3132-3137.

Abstract

Background and Aim: Apigenin (API) is an estrogenic compound found in many plants. Sertoli cells reside in the testis and are a key target of environmental toxicants. This study aimed to examine the cytotoxicity, especially oxidative stress of API in mouse Sertoli TM4 cells.

Materials and Methods: Mouse Sertoli TM4 cells were treated with 50 and 100 µM API for 48 h. Cell viability, lactate dehydrogenase (LDH) activities, glutathione reductase (GR) activities, production of reactive oxygen species (ROS), and malondialdehyde (MDA) levels were evaluated using various assays.

Results: Treatment with API at both 50 and 100 µM decreased viability and GR activity but increased LDH activity, ROS production, and MDA levels in mouse Sertoli TM4 cells.

Conclusion: Exposure to API induced oxidative stress in mouse Sertoli TM4 cells.

Keywords: apigenin, malondialdehyde, oxidative stress, reactive oxygen species, TM4 cells.

Introduction

Over the past few decades, bioactive compounds in plants have attracted increasing interest from the public and scientific community due to their vast therapeutic benefits [1-3]. Notably, pharmacological studies have discovered that 4',5,7-trihydroxyflavone or apigenin (API), an estrogenic flavonoid found abundantly in oranges, grapefruit, onions, celery, parsley, wheat sprouts, green tea, chamomile, spearmint, and thyme [4-13], possesses anti-inflammatory [9,14-21], antioxidant [8,9,17,19,21], antimicrobial [14-16,18,20], anticancer [9,14-18,20,22], hepatoprotective [23], renoprotective [8], and neuroprotective [24] properties.

In contrast, emerging evidence has shown that Sertoli cells are particularly sensitive to harmful substances in food [25], hormonal treatments [25-27], and environmental toxicants. These cells are located in the seminiferous tubules of the testis and provide nutrients and structural support for developing spermatids [28,29]; therefore, disrupting them through xenobiotic-induced toxicity might compromise male reproductive health and function [30].

To the best of our knowledge, some studies have reported the effects of phytoestrogen on male reproduction. However, information about cytotoxic effects, especially oxidative stress of API is scarce. This study aimed to examine the cytotoxicity, especially oxidative stress of API in mouse Sertoli TM4 cells.

Materials and Methods

Ethical approval

No experiments in this study were conducted on live animals, so ethical approval was unnecessary.

Study period and location

The study was conducted from March to December 2020 at the Department of Veterinary Technology, Kasetsart University, Thailand.

Cells and chemicals

Mouse Sertoli TM4 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). MTS cell proliferation assay kit, lactate dehydrogenase (LDH)-cytotoxicity assay kit II, 2′,7′-dichlorofluorescin diacetate (DCFDA) cellular reactive oxygen species (ROS) detection assay kit, lipid peroxidation assay kit (malondialdehyde [MDA]), and GR assay kit were purchased from Abcam (Cambridge, MA, USA). API was purchased from Sigma-Aldrich (St. Louis, MO, USA) and mixed with dimethyl sulfoxide solution (DMSO) before being filtered.

Cell culture

Mouse Sertoli TM4 cells were cultured in complete Dulbecco’s Modified Eagle Medium/F-12 supplemented with 5% horse serum, 2.5% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere with 5% CO₂ at 37°C.

MTS assay

Cell viability was determined using the MTS cell proliferation assay kit. Briefly, mouse Sertoli
TM4 cells were seeded into 96-well plates at a density of 5×10^3 cells/well at a final volume of 200 µL/well and incubated for 24 h. Next, the cells were treated with API at concentrations of 0, 20, 40, 60, 100, 120, 140, and 160 µM or DMSO. In this test, we used two controls: Zero concentration of API and DMSO. The result showed DMSO has no influence on cells. However, we calculated the results between the zero concentration and API treatment groups. Then, the cells were further incubated for 24, 48, and 72 h in a humidified atmosphere with 5% CO₂ at 37°C. After this incubation, 20 µL MTS reagent was added to each well, and the cells were further incubated for 1-2 h at 37°C in the dark. Finally, absorbance was measured using an automated microplate reader (Bio-Rad, USA) at 490 nm. The results were expressed as a percentage of viable cells compared with the control.

**Determination of intracellular ROS production**

ROS was measured using a DCFDA cellular ROS detection kit. Briefly, mouse Sertoli TM4 cells were seeded into 96-well plates at a density of 5×10^3 cells/well at a final volume of 200 µL/well. After the cells became attached to the well, the medium was replaced with 100 µL medium containing 50 and 100 µM API, and then, the cells were incubated for 48 h. Next, each well was washed with 100 µL 1×buffer and stained with 100 µL 25 µM DCFDA in 1×buffer for 45 min at 37°C in the dark. Finally, this solution was removed from the cells, and 100 µL 1×buffer was added to each well for measuring fluorescence at Ex/Em=485/535 nm in endpoint mode.

**Determination of lipid peroxidation**

Lipid peroxidation was measured using an MDA assay kit. Briefly, mouse Sertoli TM4 cells were seeded into 96-well plates at a density of 5×10^3 cells/well at a final volume of 200 µL/well. After the cells became attached to the well, the medium was replaced with 100 µL medium containing 50 and 100 µM API, and then, the cells were incubated for 48 h. Next, thiobarbituric acid was added to the wells, and the cells were incubated for 60 min at 95°C. Finally, the cells were cooled in an ice bath for 10 min, and the absorbance was measured using an automated microplate reader (Bio-Rad) at 532 nm.

**Determination of GR activity**

GR activity was measured using a GR assay kit. Briefly, mouse Sertoli TM4 cells were seeded at 1×10^5 cells/well and treated with 50 and 100 µM API or nothing for 48 h before being homogenized with 0.2 mL cold assay buffer. The cells were centrifuged at 10,000×g for 15 min at 4°C, and then, the supernatant was collected and stored at −80°C until further testing. Before performing the GR assay, the samples were treated with 3% H₂O₂ and catalase to destroy glutathione (GSH). Then, the samples were seeded into 96-well plates and mixed with 50 µL reaction mixture. The absorbance was measured using an automated microplate reader (Bio-Rad) at 405 nm.

**Statistical analysis**

All experiments were repeated at least thrice, and values are expressed as mean±standard deviation. Paired t-tests were employed to determine when differences between values were statistically significant, with p<0.05 regarded as statistically significant.

**Results**

**Effect of API on the viability of TM4 cells**

The results showed that the addition of 50 and 100 µM API to the medium significantly increased LDH activity in TM4 cells compared with no treatment (control). In addition, a striking increase in LDH activity was observed in cells treated with 50 and 100 µM API relative to DMSO (Figure-2).

**Effect of API on ROS production in TM4 cells**

The results showed that the addition of 50 and 100 µM API to the medium significantly increased ROS levels in TM4 cells compared with no treatment (control). In addition, a striking increase in LDH activity was observed in cells treated with 50 µM API relative to DMSO (Figure-3).

**Effect of API on GR activity in TM4 cells**

The findings indicated that the addition of 50 and 100 µM API to the medium significantly increased GR activity in TM4 cells compared with no treatment (control). In addition, a striking increase in LDH activity was observed in cells treated with 50 µM API relative to DMSO (Figure-4).
In this investigation, mouse Sertoli TM4 cells exposed to various concentrations of API experienced significant increases in oxidative stress. Cytotoxicity assays are advantageous for determining a compound’s potential to invoke apoptosis (i.e., cell death) [33]. Here, LDH leakage and MTS assays were used, which are superior to MTT assays because their reagents are added directly to the culturing medium without multiple steps. From these kits, analyses demonstrated that API was cytotoxic to TM4 cells in a time-dependent manner. Copious research has established that API increases LDH activity levels in TM4 cells while decreasing the proliferation of numerous cell lines, such as human hepatocarcinoma HepG2 cells [31], human leukemia [32], human hepatocarcinoma Hep3B and HepG2 [34], human cholangiocarcinoma [35], and human breast carcinoma [36].

Oxidative stress result from an imbalance between the production and accumulation of ROS inside cells and an individual’s inability to detoxify ROS. Hence, ROS generation and increased lipid peroxidation are signs of oxidative stress [37]. In this study, we found that the addition of API to the culturing medium significantly increased ROS levels in TM4 cells, which agrees with the findings from comparable investigations that exposure to API elevates ROS levels in human hepatocarcinoma HepG2 cells [31], human leukemia [32], human hepatocarcinoma Hep3B and HepG2 [34], and human breast carcinoma [36]. Furthermore, our data revealed that API increased MDA levels (the end product of oxidative injury and an indicator of lipid peroxidation) and decreased GR activity (an enzyme involved in the GSH redox cycle) in TM4 cells. According to our review of literature, we reported that API-induced

Discussion

In this investigation, mouse Sertoli TM4 cells exposed to various concentrations of API experienced significant increases in oxidative stress. Cytotoxicity assays are advantageous for determining a compound’s potential to invoke apoptosis (i.e., cell death) [33]. Here, LDH leakage and MTS assays were used, which are superior to MTT assays because their reagents are added directly to the culturing medium without multiple steps. From these kits, analyses demonstrated that API was cytotoxic to TM4 cells in a time-dependent manner. Copious research has established that API increases LDH activity levels in TM4 cells while decreasing the proliferation of numerous cell lines, such as human hepatocarcinoma HepG2 cells [31], human leukemia [32], human hepatocarcinoma Hep3B and HepG2 [34], and human breast carcinoma [36].

Oxidative stress result from an imbalance between the production and accumulation of ROS inside cells and an individual’s inability to detoxify ROS. Hence, ROS generation and increased lipid peroxidation are signs of oxidative stress [37]. In this study, we found that the addition of API to the culturing medium significantly increased ROS levels in TM4 cells, which agrees with the findings from comparable investigations that exposure to API elevates ROS levels in human hepatocarcinoma HepG2 cells [31], human leukemia [32], human hepatocarcinoma Hep3B and HepG2 [34], and human breast carcinoma [36]. Furthermore, our data revealed that API increased MDA levels (the end product of oxidative injury and an indicator of lipid peroxidation) and decreased GR activity (an enzyme involved in the GSH redox cycle) in TM4 cells. According to our review of literature, we reported that API-induced
GR activity inhibits ethanol-induced oxidative stress and lipopolysaccharide (LPS)-induced inflammatory cytokine production in cultured rat hepatocytes [38]; API-induced GR activity inhibits d-galactosamine/LPS-induced liver injury by upregulating hepatic Nrf-2 and peroxisome proliferator-activated receptor γ expressions in mice [39]; GR activity has antioxidant properties and superoxide dismutase and GR activities in HepG2 cells after the addition of a fungal endophyte producing API from pigeon pea (Cajanus cajan (L.) Millsp.) [40]. In this study, we used a GR assay kit because GR catalyzes the nicotinamide adenine dinucleotide phosphate-dependent reduction of the oxidized GSH (a non-enzymatic antioxidant) to reduce GSH ratio, which plays an important role in the GSH redox cycle. A high oxidized GSH/reduced GSH ratio is essential for protection against oxidative stress.

This study has strengths and limitations that should be mentioned. Regarding study strengths, mouse Sertoli TM4 cells were selected because they share features with human Sertoli cells [41] and are useful in evaluating the impact of API exposure in vitro. The main disadvantage of an in vitro study is the lack of systemic effects, while that of an in vivo study is the animal welfare legislation, which is currently applicable in most countries to prevent misuse of animals and decrease the number of animals required in toxicological testing. Hence, Sertoli cells are essential for spermatogenesis and are a major target for various toxicants; thus, they serve as an ideal model for toxicity studies on the male reproductive system.

Conclusion

This study demonstrated that exposure to API induces oxidative stress in mouse Sertoli TM4 cells, as evidenced by reductions in cell viability and GR activity and increases in LDH activity, ROS production, and MDA levels.

Authors’ Contributions

SS and SJ: Conceptualization. SS and TP: Methodology and laboratory experiments. SS, TP, and SJ: Interpretation of the data. SS: Writing of the manuscript. All authors have read and approved the final manuscript.

Acknowledgments

This study was funded by the Disease-Free Animals and People Safe from Rabies Project.
Thailand. The authors also received funding from Her Royal Highness Princess Chulabhorn Walailak Archarajkumari Krom Phra Sri Swangwattana Worakattiyarajnari, Thailand.

Competing Interests

The authors declare that they have no competing interests.

Publisher’s Note

Veterinary World remains neutral with regard to jurisdictional claims in published institutional affiliation.

References

1. Kalra, E.K. (2003) Neutreacutaceutical-definition and introduc
tion. AAPS PharmSci., 5(3): e25.
2. Arts, I.C.W., Hollman, P.C.H. and Kromhout, D. (1999) Chocolate as a source of tea flavonoids. Lancet, 354(9177): 488.
3. Mamede, M.E.O., Cardello, H.M.A. and Pastore, G.M. (2004) Evaluation of an aroma like that of sparkling wine: Sensory and gas chromatography analyses of fermented grape musts. Food Chem., 89(1): 63-68.
4. Bak, M.J., Das Gupta, S.D., Wahler, J. and Suh, N. (2016) Role of dietary bioactive natural products in estrogen receptor-positi
ve breast cancer. Semin. Cancer Biol., 40-41: 170-191.
5. Duthie, G. and Crozier, A. (2000) Plant-derived phenol
lic antioxidants. Curr. Opin. Clin. Nutr. Metab. Care, 3(6): 447-451.
6. Manach, C., Scalbert, A., Morand, C., Rémésy, C. and Jiménez, L. (2004) Polyphenols: Food sources and bioavail
ability. Am. J. Clin. Nutr., 79(5): 727-747.
7. Peterson, J. and Dwyer, J. (1998) Flavonoids: Dietary occurrence and biochemical activity. Nutr. Res., 18(12): 1995-2018.
8. Sharma, H., Kanwal, R., Bhaskaran, N. and Gupta, S. (2014) Plant flavone apigenin binds to nucleic acid bases and reduces oxidative DNA damage in prostate epithelial cells. PLoS One, 9(3): e91588.
9. Shukla, S. and Gupta, S. (2010) Apigenin: A promising mol
cule for cancer prevention. Pharm. Res., 27(6): 962-978.
10. Tsanova-Savova, S. and Ribarova, F. (2013) Flavonoids and flavones in some Bulgarian plant foods. Pol. J. Food Nutr.
Sci., 63(3): 173-177.
11. Zhang, Q., Zhou, M.M., Chen, P.L., Cao, Y.Y. and Tan, X.L. (2011) Optimization of ultrasonic assisted enzymatic hydrolysis for the extraction of luteolin and apigenin from celery. J. Food Sci., 76(5): C680-C685.
12. Zhou, Y., Zheng, J., Li, Y., Xu, D.P., Li, S., Chen, Y.M. and Li, H.B. (2016) Natural polyphenols for prevention and treatment of cancer. Nutrients, 8(8): 515.
13. Zhu, Y., Wu, J., Li, S., Wang, X., Li, S., Xu, X., Hu, Z., Lin, Y., Chen, H., Qin, J., Mao, Q. and Xie, L. (2015) Apigenin inhibits migration and invasion via modulation of epithelial mesenchymal transition in prostate cancer. Mol. Med. Rep., 11(2): 1004-1008.
14. Banerjee, K. and Mandal, M. (2015) Oxidative stress trig
gered by naturally occurring flavone apigenin results in senescence and chemotherapeutic effect in human colorec
tal cancer cells. Redox Biol., 5: 153-162.
15. Bian, M., Zhang, Y., Du, X., Xu, J., Cui, J., Gu, J., Zhu, W., Zhang, T. and Chen, Y. (2017) Apigenin-7-diglucuronide protects retinas against bright light-induced photoreceptor degeneration through inhibition of retinal oxidative stress and inflammation. Brain Res., 1663: 141-150.
16. Bao, Y.Y., Zhou, S.H., Fan, J. and Wang, Q.Y. (2013) Anticancer mechanism of apigenin and the implications of GULT1 expression in head and neck cancers. Future Oncol., 9(9): 1353-1364.
17. Birt, D.F., Walker, B., Tibbels, M.G. and Bresnick, E. (1986) Anti-mutagenesis and anti-promotion by apigenin, robe
tin and indole-3-carbinol. Carcinogenesis, 7(6): 959-963.
18. Hu, X.Y., Liang, J.Y., Guo, X.J., Liu, L. and Guo, Y.B. (2014) 5-Fluourouracil combined with apigenin enhances anticancer activity through mitochondrial membrane potential ([ΔΨe]-mediated apoptosis in hepatocellular carcinoma. Clin. Exp. Pharmacol. Physiol., 42(2): 146-153.
19. Kang, O.H., Lee, J.H. and Kwon, D.Y. (2011) Apigenin inhibits release of inflammatory mediators by blocking the NF-kappaB activation pathways in the HMC-1 cells. Immunopharmacol. Immunotoxicol., 33(3): 473-479.
20. Kaur, P., Shukla, S. and Gupta, S. (2008) Plant flavonoid apigenin inactivates AKT to trigger apoptosis in prostate cancer: An in vitro and in vivo study. Carcinogenesis, 29(11): 2210-2217.
21. Xu, X., Li, M., Chen, W., Yu, H., Yang, Y. and Hang, L. (2016) Apigenin attenuates oxidative injury in ARPE-19 cells through activation of Nrf2 pathway. Oxid. Med. Cell. Longev., 2016: 4378461.
22. Saeed, M., Kadioglu, O., Khalid, H., Sugimoto, Y. and Effert, T. (2015) Activity of dietary flavonoid, apigenin, against multidrug-resistant tumor cell as determined by pharmacogenomics and molecular docking. J. Nutr.
Biochem., 26(1): 44-56.
23. Yang, J., Wang, X.Y., Xue, J., Gu, Z.L. and Xie, M.L. (2013) Protective effect of apigenin on mouse acute liver injury induced by acetaminophen is associated with incre
ment of hepatic glutathione reductase activity. Food Funct., 4(6): 939-943.
24. Wang, C.N., Chi, C.W., Lin, Y.L., Chen, C.F. and Shiao, Y.J. (2001) The neuroprotective effects of phytoestrogen on amyloid beta protein-induced toxicity are mediated by aborting the activation of caspase cascade in rat cortical neurons. J. Biol. Chem., 276(7): 5287-5295.
25. Dias, T.R., Alves, M.G., Bernadino, R.L., Martin, A.D., Moreira, A.C., Saliva, J., Barros, A., Sousa, M., Saliva, B.M. and Oliveira, P.F. (2015) Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile: Relevance for male fertility. Toxicologist, 328: 12-20.
26. Qui, L., Quan, Y., Liu, Z., Wang, C., Qu, J. and Wang, S. (2016) Perfluorooctane-sulfonate (PFOS) disrupts blood-tests barrier by down-regulating junction proteins via p38 MAPK/ATF2/MMP9 signaling pathway. Toxicologist, 373: 1-12.
27. Rocha, C.S., Martins, A.D., Rato, L., Silva, B.M., Oliveira, P.F. and Alves, M.G. (2014) Melatonin alters the glycolytic profile of Sertoli cells: Implications for male fertili
ty. Mol. Hum. Reprod., 20(11): 1067-1076.
28. Guttenbach, M., Steinlein, C., Engel, W. and Schmid, M. (2001) Cyto
genetic characterization of the TM4 mouse sertoli cell line. I. Conventional banding techniques, FISH and SKY. Cyogenet. Genome Res., 94(1-2): 71-78.
29. Mruk, D.D. and Cheng, C.Y. (2004) Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocr. Rev., 25(5): 747-806.
30. Lanning, L.L., Cressy, D.M., Chapin, R.E., Mann, P.C., Barlow, N.J., Regan, K.S. and Goodman, D.G. (2002) Recommended approaches for the evaluation of testicular (∆Ѱe)-mediated apoptosis in hepatocellular carcinoma. Endocr. Rev., 23(3): 477-494.
31. Valdameri, G., Trombeta-Lima, M.T., Worrel, P.R., Pires, A.R.A., Martinez, G.R., Nolte, G.R., Cadena, S.M.S., Sogayar, M.C., Winnischofer, S.M.B. and Rocha, M.E.M. (2011) Involvement of catalase in the apoptotic mecha
nism of HepG2 human hepatoma cells. Chem. Biol. Interact., 193(2): 180-189.
32. Jayasooriya, R.G.P., Kang, S.H., Kang, C.H., Choi, Y.H., Moon, D.O., Hyun, J.W., Chang, W.Y. and Kim, G.Y. (2012) Apigenin decreases cell viability and telomerase activity in human leukemia cell lines. Food Chem. Toxicol., 50: 1863-1868.
33. Weyermann, J., Lochmann, D. and Zimmer, A. (2005) A practical note on the use of cytotoxicity assays. *Int. J. Pharm.*, 288(2): 369-376.

34. Kang, C.H., Molagoda, I.M.N., Choi, Y.H., Park, C., Moon, D.O. and Kim, G.Y. (2018) Apigenin promotes TRAIL-mediated apoptosis regardless of ROS generation. *Food Chem. Toxicol.*, 111: 623-630.

35. Subhasitanont, P., Chokchaichamnankit, D., Chiablaem, K., Keeratichamroen, S., Ngiwsara, L., Paricharttanakul, N.M., Lirdprapamongkol, K., Weeraphan, C., Svasti, J. and Srisomsap, C. (2017) Apigenin inhibits growth and induces apoptosis in human cholangiocarcinoma cells. *Oncol. Lett.*, 14(4): 4361-4371.

36. Harrison, M.E., Coombs, M.R.P., Delaney, L.M. and Hoskin, D.W. (2014) Exposure of breast cancer cells to a subcytotoxic dose of apigenin causes growth inhibition, oxidative stress, and hypophosphorylation of Akt. *Exp. Mol. Pathol.*, 97(2): 211-217.

37. Liu, X., Nie, S., Huang, D. and Xie, M. (2015) Mitogen-activated protein kinase and Akt pathways are involved in 4-nonylphenol induced apoptosis in mouse Sertoli TM4 cells. *Environ. Toxicol. Pharmacol.*, 39(2): 815-824.

38. Wang, F., Zhou, R.J., Zhao, X., Ye, H. and Xie, M.L. (2018) Apigenin inhibits ethanol-induced oxidative stress and LPS-induced inflammatory cytokine production in cultured rat hepatocytes *J. Appl. Biomed.*, 16: 75-80.

39. Zhou, R.J., Ye, H., Wang, F., Wang, J.L. and Xie, M.L. (2017) Apigenin inhibits d-galactosamine/LPS-induced liver injury through upregulation of hepatic Nrf-2 and PPARγ expressions in mice. *Biochem. Biophys. Res. Commun.*, 493(1): 625-630.

40. Gao, Y., Zhao, J., Zu, Y., Fu, Y., Liang, L., Luo, M., Wang, W. and Efferth, T. (2012) Antioxidant properties, superoxide dismutase and glutathione reductase activities in HepG2 cells with a fungal endophyte producing apigenin from pigeon pea [Cajanus cajan (L.) Millsp.] *Food Res. Int.*, 49(1): 147-152.

41. Sandig, M., Kalnins, V.I. and Siu, C.H. (1997) Role of NCAM, cadherins, and microfilaments in cell-cell contact formation in TM4 immature mouse Sertoli cells. *Cell Motil. Cytoskeleton*, 36(2): 149-163.

**********