



Quercetin protects embryonic chicken spermatogonial cells from oxidative damage intoxicated with 3-methyl-4-nitrophenol in primary culture

Yuling Mi^{a,b}, Caiqiao Zhang^{b,*}, Chunmei Li^{c,d}, Shinji Taneda^c, Gen Watanabe^{a,e}, Akira K. Suzuki^c, Kazuyoshi Taya^{a,e}

^a Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

^b Key Laboratory of Animal Epidemic Etiology & Immunological Prevention of the Ministry of Agriculture, College of Animal Sciences, Zhejiang University, No. 268 Kaixuan Road, Hangzhou, Zhejiang 310029, China

^c Environmental Nanotoxicology Section, Research Center for Environmental Risk, National Institute for Environmental Studies, Tsukuba 305-8506, Japan

^d Laboratory of Animal Reproduction, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

^e Department of Basic Veterinary Science, The United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan

ARTICLE INFO

Article history:

Received 1 May 2009

Received in revised form 30 June 2009

Accepted 1 July 2009

Available online 9 July 2009

Keywords:

Quercetin

3-Methyl-4-nitrophenol

Oxidative damage

Antioxidant

Spermatogonial cells

ABSTRACT

Diesel exhaust particles (DEP) are considered to be one of the most important air pollutants. In this study, the protective effect of quercetin, an antioxidant flavonoid, on oxidative damage of testicular cells was studied by analysis of the intracellular antioxidant system of embryonic chickens after treatment with 3-methyl-4-nitrophenol (PNMC) derived from DEP. Testicular cells from 18-day-old embryos were cultured in serum-free McCoys'5A medium and challenged with PNMC (10^{-7} to 10^{-5} M) alone or in combinations with quercetin (1.0 $\mu\text{g/ml}$) for 48 h. Results showed that exposure to PNMC (10^{-5} M) induced condensed nuclei and vacuolated cytoplasm, a decrease in testicular cell viability and spermatogonial cell number. Exposure to PNMC induced lipid peroxidation by an elevation of thiobarbituric acid reactive substances as well as decreasing glutathione peroxidation activity and superoxide dismutase activity. However, simultaneous supplementation with quercetin restored these parameters to the similar levels as the control. PNMC is therefore concluded to have induced the oxidative stress of the spermatogonial cells, which can

of the most commonly occurring flavonoids, is ingested in edible fruits (apples), vegetables (onions) and beverages (tea) at levels of up to 16 mg per day (Hertog et al., 1993). Therefore, quercetin attracted more attentions. Quercetin exhibits a wide range of physiological and pharmacological activities relevant to human health, such as anticarcinogenic, anti-inflammatory and antiviral actions (Brown, 1980; Read, 1995). Many of these effects are supposed to be related to its antioxidant property. Quercetin can act by scavenging free radicals, chelation of divalent cations, inhibition of some enzymes and protecting against DNA damage (Da Silva et al., 1998; Wilms et al., 2008). Therefore, quercetin may be considered as an effective attenuating factor for preventing various disorders caused by environmental contaminants (Kuriyama et al., 2005; Mi and Zhang, 2005; Kaindl et al., 2008).

In this study, the reproductive toxicity of PNMC was further investigated using a testicular germ-somatic cell co-culture model. Quercetin was chosen to examine its attenuating effect on cytotoxicity caused by PNMC. In order to show the mechanism of toxicity of PNMC, testicular cells were exposed to PNMC in the presence or absence of quercetin. In addition to the analysis of cytotoxicity, malondialdehyde (MDA) content was measured as an end-product of lipid peroxidation. The defense systems against free radical attack were assessed by the measurement of glutathione peroxidation (GSH-Px) activity and superoxide dismutase (SOD) activity. These results would help to identify dietary sources of protection against reproductive toxicity induced by environmental oxidative toxicants such as PNMC.

2. Materials and methods

2.1. Isolation and culture of testicular cells

Fertilized Hy-line chicken eggs were obtained from a commercial hatchery and incubated at 38.5 °C and 60% humidity until day 18. Testicular cells were prepared and cultured according to a previous study (Mi et al., 2004).

2.2. Treatments of cultured cells with chemicals

PNMC was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). The stock solution of PNMC and quercetin (Sigma, St. Louis, MO) was prepared in PBS and ethanol solution, respectively. For morphological observations, Cells were incubated in medium with PNMC (10^{-7} to 10^{-5} M) alone or in combinations with quercetin (1.0 µg/ml) at 5×10^4 cells/well in 96-well Falcon culture plates. After treatment for 48 h, we observed the morphological change in the cells. For biochemical analysis, we plated the cells at 2×10^5 cells/well in 24-well Falcon culture plates. Cells were incubated in medium supplemented with PNMC (10^{-5} M) with or without quercetin (1.0 µg/ml). The control received the vehicle only. After treatment for 48 h, the medium and cultured cells were used for determinations of MDA, SOD and GSH-Px.

2.3. Morphological studies of testicular cells

Morphological changes of testicular cells were visualized under an inverted phase-contrast microscope (Olympus, Japan) and the image was captured with a video camera (Pixera Pro 150ES, USA). The number of spermatogonial cells was counted in each image by using Simple PCI Advanced Imaging Software (Compix Inc., USA).

2.4. Biochemical analysis

After 48 h culture the medium and cells were used for biochemical analysis. Lipid peroxidation was evaluated by measurement of MDA concentrations using spectrophotometric measurement of the color produced during the reaction to thiobarbituric acid with MDA (Agostinho et al., 1997). MDA concentrations were calculated by the absorbance of thiobarbituric acid reactive substances (TBARS) at 532 nm and were expressed in nmol/ml. Total SOD activity was determined by inhibition of the rate of the superoxide radical-dependent cytochrome C reduction (Flohé and Otting, 1984). The absorbance at 550 nm was determined and the values were expressed as U/ml. The activity of GSH-Px was assessed according to established methods of Rotruck et al. (1973) which makes use of the reaction: $\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG}$ (oxidized glutathione). The absorbance was determined at 412 nm. The enzymatic activity was expressed as U/L.

2.5. Statistical analysis

The experiment was repeated three times with quadruplications. All data were expressed as the means \pm S.D. and analyzed by two-way ANOVA and Duncan's multiple range test using the GLM procedure of SAS 6.12 software. $P \leq 0.05$ was considered significantly different.

3. Results

3.1. Changes in morphology of testicular cells

After culture for 48 h, spermatogonial cells in the control group showed a rounded shape and were anchored on the overslip by the filopodia of somatic cells (Fig. 1A). Compared with the control group, there was no differentiating change in testicular cell morphology after exposure to quercetin at 0.1–1 µg/ml (Fig. 1B). Obvious toxic effects of PNMC (10^{-7} to 10^{-5} M) on cell morphology were observed. Many spermatogonial cells showed condensed nuclei and vacuolated cytoplasm in the PNMC-treated group at 10^{-5} M (Fig. 1C). However, in combination with quercetin, more spermatogonial cells survived with intact integrity and the cytotoxicity caused by PNMC was mitigated (Fig. 1D).

3.2. Change of spermatogonial cell number

The two-way ANOVA of the data showed that PNMC was a significant factor in determining spermatogonial cell numbers ($P=0.0001$) but quercetin was not a significant factor in determining this parameters ($P=0.06$). There was also an interaction between the PNMC and quercetin in determining spermatogonial cell number ($P=0.0001$). There were no significant changes in spermatogonial cell numbers between quercetin (1 µg/ml) and the control group (Fig. 2; $P>0.05$). However, the number of spermatogonial cell was significantly decreased in the group treated with PNMC at 10^{-5} M (Fig. 2; $P<0.001$). In combinations with PNMC, quercetin significantly increased spermatogonial cell number and remained more spermatogonial cells integrity (Fig. 2; $P<0.001$).

3.3. Assessment of intracellular antioxidative status

The two-way ANOVA for intracellular antioxidative parameters showed that MDA formation, GSH-Px activity and SOD activity were significantly affected by PNMC ($P=0.008$; $P=0.01$; $P=0.014$, respectively). However, quercetin was not a significant factor in determining these three parameters ($P=0.08$; $P=0.07$; $P=0.06$). There was also an interaction between the PNMC and quercetin in determining these three parameters ($P=0.01$; $P=0.02$; $P=0.04$). No significant differences in MDA formation, GSH-Px activity and SOD activity were observed between the quercetin-treated and the control group (Figs. 3–5; $P>0.05$). There was a significant increase in MDA production (Fig. 3; $P<0.01$) and a significant decrease in GSH-Px and SOD activity after exposure to PNMC for 48 h (Figs. 4 and 5; $P<0.01$; $P<0.05$, respectively). The increase in the MDA production was significantly reduced (Fig. 3; $P<0.01$) and the decrease in the GSH-Px activity and SOD activity was significantly restored by combined treatment with quercetin (Figs. 4 and 5; $P<0.05$).

4. Discussion

We have previously reported that PNMC, one nitrophenol derivatives isolated from DEP, possess estrogenic and anti-androgenic activity both *in vivo* and *in vitro* (Furuta et al., 2004, 2005; Li et al., 2006b). In contrast to previous findings, little is known about the mechanisms that govern the effects of PNMC exposure on male reproductive functions *in vitro*. In the present study, the number of spermatogonial cells was markedly decreased

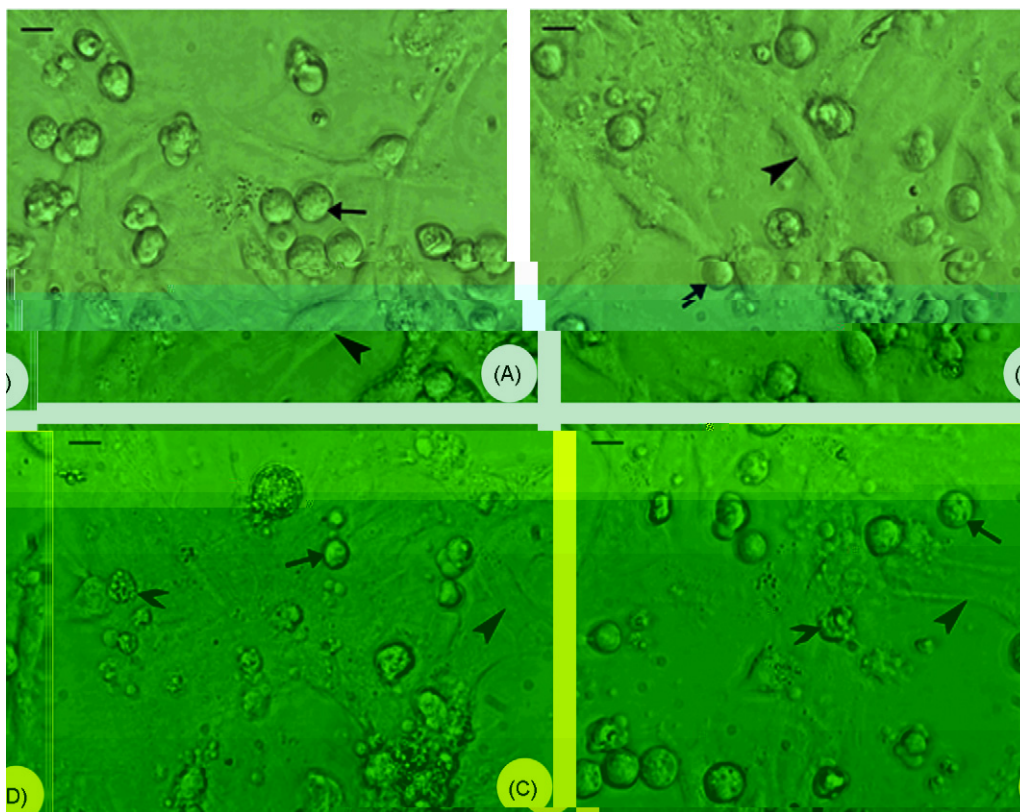


Fig. 1. Morphological changes of embryonic chicken testicular cells after treatment with quercetin alone and in combined with PNMC for 48 h. (A)–(D) indicated testicular cells of the control, quercetin (1 µg/ml), PNMC (10^{-5} M), PNMC+quercetin-treated groups, respectively. Note somatic cells (▶), normal spermatogonial cells (→) and spermatogonial cells with condensed nuclei and vacuolated cytoplasm (▶•). Scale bar: 10 µm.

by PNMC exposure. PNMC induced lipid peroxidation, decreased SOD activities and GSH-Px activities. Tsukue et al. (2002) reported that DEP adversely impacted on female reproductive functions. Exposure to diesel exhaust significantly decreased the numbers of sperm, spermatids and Sertoli cells produced daily in adult rats and mice (Watanabe, 2005; Izawa et al., 2007c). Li et al. (2006a) reported that seminiferous tubules of PNMC-treated adult male quail were devoid of all cells except spermatogonia and Sertoli cells and PNMC induces reproductive toxicity at both the central and testicular levels in the adult male quail. *In vitro*, we used spermatogonial and somatic cells to reveal that PNMC exposure markedly decreased the number of spermatogonial cells and had detrimental effects on male reproductive functions *in vitro*.

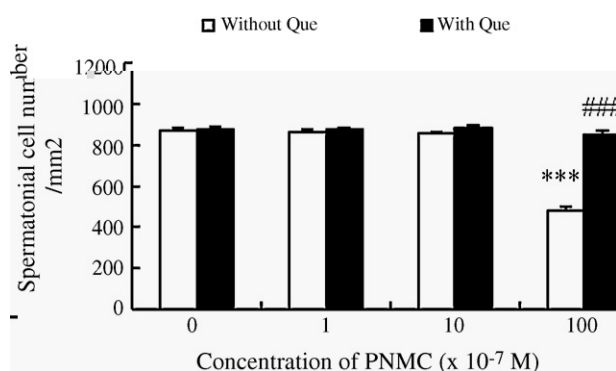


Fig. 2. Changes in the number of spermatogonial cells in cell cultures treated with PNMC (10^{-7} to 10^{-5} M) and quercetin (Que, 1.0 µg/ml) for 48 h in culture. Values represent means \pm S.D. ($n=4$). Multiple symbols indicate different significance levels: *** $P < 0.001$ when compared to the control; ### $P < 0.001$ when compared to 10^{-5} M PNMC treatment.

Among the possible mechanisms of PNMC-induced injury, ROS generation remained an important consideration. Study by Donaldson et al. (2003) showed that DEP exposure generated ROS in the lungs of exposed mice, and the generated ROS are responsible for protein oxidation, lipid peroxidation, and DNA damage. Additionally, Danielsen et al. (2008) and Park et al. (2006) reported DEP exposure increased the TBARS generation and induced DNA damage. Our results were consistent with these other studies. Treatment of testicular cells with PNMC at the concentration of 10^{-5} M for

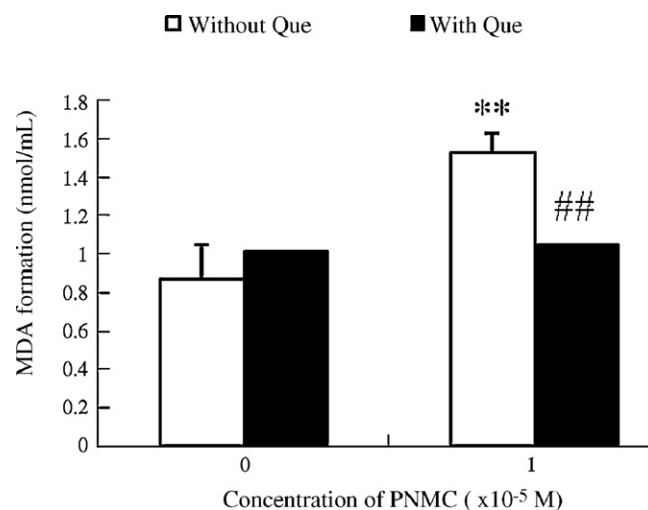


Fig. 3. Results of lipid peroxidation in spermatogonial cells. The culture medium was collected for analysis of MDA formation. Values represent means \pm S.D. ($n=4$). Multiple symbols indicate different significance levels: ** $P < 0.01$ when compared to the control; ## $P < 0.01$ when compared to 10^{-5} M PNMC treatment.

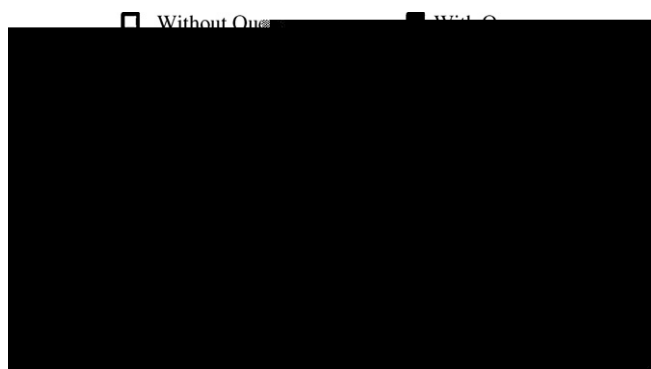


Fig. 4. Changes in GSH-Px activity in chicken testicular cells after 48 h culture. The cultured cells were collected for analysis of GSH-Px activity. Values represent means \pm S.D. ($n=4$). Multiple symbols indicate different significance levels: ** $P < 0.01$ when compared to the control; # $P < 0.05$ when compared to 10^{-5} M PNMC treatment.

48 h significantly increased MDA production. In the present study, PNMC cause lipid peroxidation, which is quantified by MDA generation. Furthermore, the PNMC treatment significantly decreased SOD activities and GSH-Px activities. Under physiological conditions, the normal production of ROS is efficiently scavenged by enzymatic antioxidant system (such as SOD, GSH-Px, catalase) and nonenzymatic antioxidants (such as glutathione, vitamin A, C, and E). SOD is a scavenger of superoxide. The decreases of SOD activities suggested accumulation of excess superoxide. The decreases of GSH-Px activities suggested accumulation of excess H_2O_2 . GSH-Px converts H_2O_2 or other lipid peroxides to water or hydroxyl lipids and in the process, GSH is converted to oxidized glutathione. Therefore, the toxic influence of PNMC from DEP was concluded to most likely be due to the formation of excessive free radicals that caused oxidative stress, leading to cell damage.

Natural compounds and dietary components as antioxidants against diseases and injuries attracted a growing number of scientific attention. Quercetin, an important flavonoid, and provides beneficial effects on health due to its antioxidant function. Little is known about the protective effect of quercetin on the toxicity caused by DEP. The present study demonstrated that quercetin showed no deleterious effects on spermatogonial cells and quercetin's antioxidant capacity was also revealed. The formation of MDA was significantly elevated but SOD activity and GSH-Px activity decreased in testicular cells after exposure to PNMC. However, these parameters were restored by combined treatment with quercetin. These data on the protective ability of quercetin against oxidative damage were in agreement with other

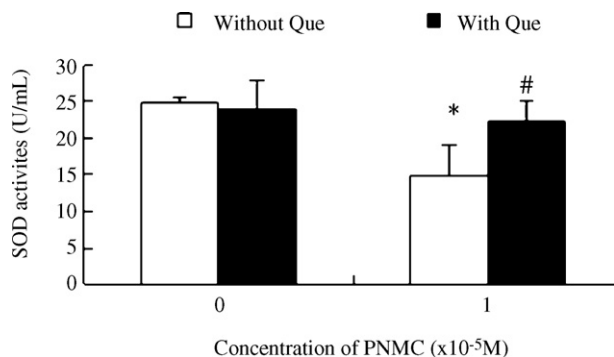


Fig. 5. Changes in SOD activity in chicken testicular cells after 48 h culture. The cultured cells were collected for analysis of SOD activity. Values represent means \pm S.D. ($n=4$). Multiple symbols indicate different significance levels: * $P < 0.05$ when compared to the control; # $P < 0.05$ when compared to 10^{-5} M PNMC treatment.

results using different cell lines. Studies by Mi and Zhang (2005) and Robaszekiewicz et al. (2007) showed that quercetin decreased production of reactive oxygen species in human non-small lung cancer cells A549 and chicken testicular germ cells. Lima et al. (2006) reported that quercetin significantly decreased lipid peroxidation and prevented glutathione depletion induced by tert-butyl hydroperoxide in HepG2 cells, and significantly decreased DNA damage. The present results showed no evidence of toxicity of quercetin at the indicated dose range.

Quercetin is a dietary antioxidant as a result of a suitable structure for free radical scavenging and ion chelation with the phenolic hydroxyl groups at the B-ring and the 3-position providing its free radical-scavenging activity (Cao et al., 1997). Additionally, quercetin was an effective alimentary antioxidant because of not only its structural characteristics but also its ability to interact with and penetrate lipid bilayers. Therefore, the structure of quercetin plays a vital role in attenuating the oxidative damage caused by PNMC. Furthermore, quercetin can inhibit cytotoxicity of oxidant agents like PNMC by maintaining normal cell morphology.

Exposure to PNMC caused cytotoxicity in embryonic chicken testicular cells by increasing lipid peroxidation and reducing intracellular antioxidant system. However, as an effective antioxidant, quercetin could block these deleterious effects through a reduction in MDA, and an increase in SOD and GSH-Px to maintain the membrane integrity. To our knowledge, this is the first study that indicates that the dietary antioxidant quercetin could attenuate the toxic effects of PNMC and provide protection against testicular toxicity caused by oxidative environmental contaminants.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was supported by a grant in Aid for Scientific Research (B-18310044, 19.07445) from the Japan Society for the Promotion of Sciences and the National Natural Science Foundation of China (30871843). We thank Mr. Weidong Zeng for help in the experiment.

References

- Agostinho, P., Uarte, C.B., Oliveira, C.R., 1997. Impairment of excitatory amino acid transporter activity by oxidative stress conditions in retinal cells: effect of antioxidants. *FASEB J.* 11, 154–163.
- Aherne, S.A., O'Brien, N.M., 2002. Dietary flavonols: chemistry, food content, and metabolism. *Nutrition* 18, 75–81.
- Bhushan, B., Samanta, S.K., Chauhan, A., Chakraborti, A.K., Jain, R.K., 2000. Chemotaxis and biodegradation of 3-methyl-4-nitrophenol by *Ralstonia* sp. *SJ98*. *Biochem. Biophys. Res. Commun.* 275, 129–133.
- Brown, J.P., 1980. A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. *Mutat. Res.* 75, 243–277.
- Cao, G., Sofic, E., Prior, R.L., 1997. Antioxidant and prooxidant behavior of flavonoids: structure–activity relationships. *Free Radic. Biol. Med.* 22, 749–760.
- Crozier, A., Burns, J., Aziz, A.A., Stewart, A.J., Rabiasz, H.S., Jenkins, G.L., et al., 2000. Antioxidant flavonols from fruits, vegetables and beverages: measurements and bioavailability. *Biol. Res.* 33 (2), 79–88.
- Da Silva, E.L., Piskula, M.K., Yamamoto, N., Moon, J.-H., Terao, J., 1998. Quercetin metabolites inhibit copper ion-induced lipid peroxidation in rat plasma. *FEBS Lett.* 430, 405–408.
- Danielsen, P.H., Risom, L., Wallin, H., Autrup, H., Vogel, U., Loft, S., Møller, P., 2008. DNA damage in rats after a single oral exposure to diesel exhaust particles. *Mutat. Res.* 637, 49–55.
- Donaldson, K., Stone, V., Borm, P.J., Jimenez, L.A., Gilmour, P.S., Schins, R.P., Knaapen, A.M., Rahman, I., Faux, S.P., Brown, D.M., MacNee, W., 2003. Oxidative stress and calcium signaling in the adverse effects of environmental particles (PM(10)). *Free Radic. Biol. Med.* 34, 1369–1382.
- Flohé, L., Otting, F., 1984. Superoxide dismutase assays. *Methods Enzymol.* 105, 93–104.
- Furuta, C., Suzuki, A.K., Taneda, S., Kamata, K., Hayashi, H., Mori, Y., Li, C., Watanabe, G., Taya, K., 2004. Estrogenic activities of nitrophenols in diesel exhaust particles. *Biol. Reprod.* 70, 1527–1533.

- Furuta, C., Li, C., Taneda, S., Suzuki, A.K., Kamata, K., Watanabe, G., Taya, K., 2005. Immunohistological study for estrogenic activities of nitrophenols in diesel exhaust particles. *Endocrine* 27, 33–36.
- Hertog, M.G., Hollman, P.C., Katan, M.B., Kromhout, D., 1993. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr. Cancer* 20, 21–29.
- Izawa, H., Kohara, M., Watanabe, G., Taya, K., Sagai, M., 2007a. Diesel exhaust particle toxicity on spermatogenesis in the mouse in aryl hydrocarbon receptor dependent. *J. Reprod. Dev.* 53, 1069–1078.
- Izawa, H., Watanabe, G., Taya, K., Sagai, M., 2007b. Inhibitory effects of foods and phyphenols on activation of aryl hydrocarbon receptor induced by diesel exhaust particles. *Environ. Sci.* 14, 149–156.
- Izawa, H., Kohara, M., Watanabe, G., Taya, K., Sagai, M., 2007c. Effects of diesel exhaust particles on the male reproductive system in strains of mice with different aryl hydrocarbon receptor responsiveness. *J. Reprod. Dev.* 53, 1191–1197.
- Izawa, H., Kohara, M., Aizawa, K., Suganuma, H., Inakuma, T., Watanabe, G., Taya, K., Sagai, M., 2008. Alleviative effects of quercetin and onion on male reproductive toxicity induced by diesel exhaust particles. *Biosci. Biotechnol. Biochem.* 72, 1235–1241.
- Justesen, U., Knuthsen, P., Leth, T., 1998. Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by highperformance liquid chromatography with photo-diode array and mass spectrometric detection. *J. Chromatogr. A* 799, 101–110.
- Kaindl, U., Eyberg, I., Rohr-Udilova, N., Heinzle, C., Marian, B., 2008. The dietary antioxidants resveratrol and quercetin protect cells from exogenous oxidative damage. *Food Chem. Toxicol.* 46, 1320–1326.
- Kuriyama, K., Kitamura, T., Yokoi, R., Hayashi, M., Kobayashi, K., Kuroda, J., Tsujii, H., 2005. Evaluation of testicular toxicity and sperm morphology in rats treated with methyl methanesulphonate (MMS). *J. Reprod. Dev.* 51, 657–667.
- Li, C., Takahashi, S., Taneda, S., Furuta, C., Watanabe, G., Suzuki, A.K., Taya, K., 2006a. Impairment of testicular function in adult male Japanese quail (*Coturnix japonica*) after a single administration of 3-methyl-4-nitrophenol in diesel exhaust particles. *J. Endocrinol.* 189, 555–564.
- Li, C., Taneda, S., Suzuki, A.K., Furuta, C., Watanabe, G., Taya, K., 2006b. Anti-androgenic activity of 3-methyl-4-nitrophenol in diesel exhaust particles. *Eur. J. Endocrinol.* 155, 555–564.