Alleviative Effects of Quercetin and Onion on Male Reproductive Toxicity Induced by Diesel Exhaust Particles

Hiromi Izawa,†‡; Machiko Kohara, Koichi Aizawa, Hiroyuki Suganuma, Takahiro Inakuma, Gen Watanabe, Kazuyoshi Taya, and Masaru Sagai

1Division of Human Sciences, Faculty of Health Sciences, Aomori University of Health and Welfare, Aomori 030-8505, Japan
2Department of Basic Veterinary Science, The United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan
3Department of Life Sciences, Graduate School of Health Sciences, Aomori University of Health and Welfare, Aomori 030-8505, Japan
4Research Institute, Kagome Co., Ltd., Tochigi 329-2762, Japan
5Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

Received October 30, 2007; Accepted February 17, 2008; Online Publication, May 7, 2008
[doi:10.1271/bbb.70705]

Diesel exhaust particles (DEPs) are particulate matter from diesel exhaust that contain many toxic compounds, such as polyaromatic hydrocarbons (PAHs). Some toxicities of PAH are thought to be expressed via aryl hydrocarbon receptors (AhRs). The male reproductive toxicity of DEPs might depend on AhR activation induced by PAHs. We hypothesized that AhR antagonists protect against the male reproductive toxicity of DEPs. Quercetin is a flavonoid and a well-known AhR antagonist, while onion contains many flavonoids, including quercetin. Hence, we examined whether quercetin and onion have alleviative effects against the male reproductive toxicity induced by DEPs. BALB/c male mice were fed quercetin- or onion-containing diets and received 10 injections of DEP suspension or vehicle into the dorsal subcutaneous layer over 5 weeks. The mice were euthanized at 2 weeks, after the last treatment, and their organs were collected. Daily sperm production and total incidence of sperm abnormalities were significantly affected in the DEP groups as compared with the vehicle group, but the total incidence of sperm abnormalities in the quercetin + DEP-treated mice was significantly reduced as compared with the DEP-treated mice. The numbers of Sertoli cells were significantly decreased in DEP-treated mice as compared with the vehicle-treated mice, but, the numbers of Sertoli cells were significantly increased in the quercetin and the onion + DEP-treated mice as compared with the DEP-treated mice. These results clearly indicate alleviative effects of quercetin and onion against the male reproductive toxicity induced by DEP.

Key words: diesel exhaust particles; onion; quercetin; Sertoli cell; sperm

Diesel exhaust particles are particulate matter from diesel exhaust (DE) and containing many toxic compounds that can cause pulmonary cancer, allergic rhinitis, and bronchial asthma.1,2) It has been reported that they also contain endocrine disrupters, such as polyaromatic hydrocarbons (PAHs),1) dioxin derivatives,3,4) and nitrophenols.5–9) DEPs and DE have also been reported to cause male reproductive toxicity. DEPs decrease sperm production in mice10) and rats,11) while DE increases serum concentrations of testosterone and the weights of the accessory glands in rats.12) Furthermore, the numbers of sperm and Sertoli cells are decreased in mature rats exposed to DE as fetuses.13) We have reported that DEP-treated mice showed decreased daily sperm production (DSP), increased plasma concentrations of testosterone, and hepatic ethoxyresorufin-O-deethylase (EROD) activity, an indirect index of aryl hydrocarbon receptor (AhR) activity.14) Moreover, DEP toxicity toward the male reproductive system can occur in an AhR-dependent manner, since the DSP/g of the testis and the incidence of morphologically abnormal sperm are correlated with AhR activity.15) In addition, DEP can activate AhR and quercetin can inhibit its activation in an in vitro system, as evaluated with an Ah-Immuno-assay kit.16) Quercetin is a flavonoid and an antagonist of AhRs activated by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in vitro.17–19)

† To whom correspondence should be addressed. Fax: +81-17-765-4214; E-mail: h.izawa@auhw.ac.jp

Abbreviations: DEP, diesel exhaust particles; PAH, polyaromatic hydrocarbon; AhR, aryl hydrocarbon receptor; DE, diesel exhaust; DSP, daily sperm production; EROD, ethoxyresorufin-O-deethylase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin
We hypothesized that quercetin reduces the male reproductive toxicity of DEPs. In the present study, we examined the effects of quercetin against the male reproductive toxicity induced by DEPs in mice. In addition, the effects of onion, a quercetin-rich vegetable, were also examined.

Materials and Methods

Chemicals and DEPs. Quercetin dehydrate and mononcional anti-tyrosine tubulin antibody clone TUB-1A2 were obtained from Sigma Chemical (St. Louis, MO). Biotinylated anti-mouse IgG and a Vectastain Elite ABC kit were obtained from Vector Laboratories (Burlingame, CA). Freeze-dried onion powder was donated by Research Institute, Kagome Co., Ltd. (Nasushiobara, Tochigi, Japan). The quercetin content in this onion powder was 32.2 mg/100 g of onion powder as measured by the Folin-Denis method. DEPs were collected and a suspension was prepared according to previously published methods. The other reagents purchased were of the purest grade commercially available.

Animals and DEP injection. Six-week-old BALB/c male mice (Clea Japan, Tokyo) were divided into six groups (n = 9 for each group): vehicle, DEP, 0.3% quercetin + DEP (0.3% Q + DEP), 0.1% quercetin + DEP (0.1% Q + DEP), 0.03% quercetin + DEP (0.03% Q + DEP); and 0.5% onion powder + DEP (0.5% Oni + DEP). The vehicle and DEP groups were fed CE-2 commercial diets alone (Clea Japan). The 0.3% Q + DEP, 0.1% Q + DEP, and 0.03% Q + DEP groups were fed the CE-2 diet containing 0.3, 0.1, and 0.03% w/w quercetin respectively. The 0.5% Oni + DEP group was fed the CE-2 diet containing 0.5% w/w freeze-dried onion powder. The quercetin in the CE-2 was in trace amounts as measured by HPLC. The composition of the quercetin-containing diet was similar to the CE-2 diet, except that 0.3, 0.1, or 0.03% of the CE-2 components was replaced with quercetin. The composition of the onion-containing diet was similar to the quercetin-containing diets. The mice were housed in an environmentally controlled room at 23 ± 2 °C at 55 ± 7% humidity under a 12-h/12-h light/dark cycle, and were allowed a 1-week adjustment period. Diet and water were provided ad libitum. After 6 d of the adjustment period, all DEP-treated mice received 10 injections of DEP suspension (220 µg/mouse; 0.2 ml of 1.11 mg DEP/ml) into the dorsal subcutaneous layer over 5 weeks. The mice in the vehicle group received 0.2 ml of phosphate-buffered saline containing 0.05% Tween 20 injected in the same manner. Sperm produced in the testis passes the epididymis within 5 to 12 d. Hence, samples were collected at 2 weeks after the last treatment. The mice were weighed and euthanized under deep ethyl ether anesthesia at 13 weeks of age. Testis and epididymis samples were collected from each animal and weighed. The testes from three mice in each group were fixed in Bouin’s solution and embedded in paraffin. The paraffin-embedded testicular tissues were serially sectioned at a thickness of 4 µm and placed on poly-L-lysine-coated glass slides for immunohistochemistry. The testes from the six other mice were stored at −80 °C until analysis of DSP. Five epididymides were immediately used in an analysis of sperm morphology.

The study was carried out in accordance with the “Guidelines for Animal Experimentation” of Aomori University of Health and Welfare.

Sperm analysis. DSP was determined by the procedures of Joyce et al. and Yoshida et al. Briefly, the left testis was homogenized for 30 s in 5 ml of 10 mm PBS containing 0.05% Triton X-100 using a Polytron homogenizer. Aliquots of each suspension were placed in a hemocytometer chamber, and the number of steps 14–16 spermatids per testis was counted. Developing spermatids spend 4.84 d in steps 14–16 during spermatogenesis in mice. Hence, the sperm count was divided by 4.84 to obtain the DSP as well as reports which were written about reproductive toxicity-induced DE. 10,11,13,27 Sperm morphology was evaluated by the procedures of Wyrobek et al. and Watanabe et al., with minor modifications. Sperm smears were created using a suspension collected immediately from the left cauda epididymis into 4 ml of physiological saline at 37 °C. The sperm preparations were stained with 1% eosin Y solution and examined under a light microscope. Morphological abnormalities of the sperm were classified according to the criteria of Watanabe et al., viz., head, neck, midpiece, and tail abnormalities. A total of 400 sperm from each mouse were examined.

Immunohistochemical staining of Sertoli cells. Sertoli cells were stained immunohistochemically with a monoclonal anti-tyrosinated tubulin antibody using a Vectastain Elite ABC kit in order to count the cell numbers. Briefly, after deparaffinization with xylene, the testicular tissue sections were incubated in 0.3% H2O2 in methanol at room temperature for 1 h, followed by blocking with serum contained in the kit at room temperature for 1 h to quench nonspecific staining. Next, the sections were incubated with the monoclonal anti-tyrosinated tubulin antibody at a dilution of 1:6,400 in blocking serum at room temperature overnight. After washing, the sections were treated with biotinylated anti-mouse IgG at a dilution of 1:300 in blocking serum at room temperature for 1 h. They were then incubated with the avidin-biotin complex solution contained in the kit at room temperature for 30 min. The reaction products were visualized after treatment with 0.025% (w/v) 3,3′-diaminobenzidine tetrachloride in 0.1 mm Tris-buffered saline containing 0.01% H2O2 for 1–
Results

Effects of quercetin and onion on body, testis, and epididymis weights in DEP-treated mice

The effects of quercetin and onion on the body, testis, and epididymis weights of DEP-treated mice are shown in Table 1. The body weights in the 0.3% Q + DEP, 0.1% Q + DEP and 0.03% Q + DEP groups were significantly increased compared with that in the vehicle group. The testis weights in the DEP and 0.3% Q + DEP groups were significantly increased compared with that in the vehicle group. The relative testis, epididymis, and relative epididymis weights did not differ significantly among the groups.

Effects of quercetin and onion on daily sperm production (DSP) in DEP-treated mice

The effects of quercetin and onion on DSP in DEP-treated mice are shown in Fig. 1. DSP in all the DEP-treated groups, except for the 0.3% Q + DEP group, was significantly decreased compared with that in the vehicle group.

Effects of quercetin and onion on sperm morphological abnormalities in DEP-treated mice

Sperm smears made using a suspension collected from the left cauda epididymis were used in the evaluation of sperm morphology. The effects of quercetin and onion on sperm morphological abnormalities in DEP-treated mice are shown in Fig. 2. The incidences of morphologically abnormal sperm in all the DEP-treated groups were significantly increased compared with the vehicle group. The 0.1% Q + DEP, 0.03% Q + DEP and 0.5% Oni + DEP groups showed significantly deceased incidences compared with the DEP group.
Effects of quercetin and onion on the numbers of stained Sertoli cells in DEP-treated mice

Images of immunohistochemically stained Sertoli cells in the DEP-treated mice are shown in Fig. 3. Many Sertoli cells were observed in the seminiferous tubules in the vehicle group (arrow), but the numbers of Sertoli cells in the DEP-treated group appeared to be lower than in the vehicle group. Scale bars; 50 μm.

**Effects of quercetin and onion on the numbers of stained Sertoli cells in DEP-treated mice**

Images of immunohistochemically stained Sertoli cells in the DEP-treated mice are shown in Fig. 3. Many Sertoli cells were observed in the seminiferous tubules in the vehicle group (arrow), but the numbers of Sertoli cells in the DEP-treated group appeared to be lower than in the vehicle group. Hence, the immunohistochemically stained Sertoli cells were counted and the numbers of Sertoli cells per cross-sectional area of seminiferous tubules (cells/mm²) were calculated. The results are shown in Fig. 4. The numbers of Sertoli cells in the DEP and 0.3% Q + DEP groups were significantly decreased compared with that in vehicle group, but the numbers of Sertoli cells in the 0.1% Q + DEP and 0.03% Q + DEP groups were significantly increased compared with that in the DEP group.

**Discussion**

In the present study, we examined to determine whether quercetin and onion alleviative effects against the male reproductive toxicity induced by DEPs. Our results clearly indicate that quercetin and onion ameliorated the increased incidence of abnormal sperm and decreased number of stained Sertoli cells induced by DEPs. DSP was not significantly changed, but showed a similar tendency.

DEPs are major air pollutants in large cities and are toxic when inhaled. Following inhalation, toxic compounds in DEPs can be transported to various organs through the blood vessels. In this study, DEP toxicity was examined by dorsal subcutaneous treatment with a DEP suspension. Following subcutaneous treatment, the toxic compounds can also be transported from dorsal subcutaneous tissue to various organs through the blood vessels. The subcutaneous route was an inevitable selection due to a technical problem in the present study.

In our previous study, DEP suspensions were injected at 24.7, 74.0, and 220 μg/mouse. In the mice injected with DEP suspensions at 74.0 and 220 μg/mouse, DSP decreased and the incidence of abnormal sperm increased. Hence, the DEP suspension was injected at 220 μg/mouse in this study. This group was given 444 μg/mouse of DEPs per week. There were minute ventilations between 1.06–1.108 ml/min in mice; that is, about 0.01 m³/week. The DEP concentration was calculated using the following formula: 444 (μg/mouse/week)/0.01 (m³/week) = 44,400 μg/mouse/m³ = 44.4 mg/m³. If this amount of DEPs...
had been given by inhalation, the mice would have been exposed to 44.4 mg/m$^3$ of DE for 5 weeks. This concentration is 300 times higher than that found in urban areas such as Tokyo, where levels sometimes exceed 0.135 mg/m$^3$.[27] However, the effects seen in this study appear in mice if they inhale a low concentration of diesel exhaust over a long period of time. The dose of quercetin was based on the dietary intake of Europeans,[31–33] and corrected by interspecies scaling principles[34] to 0.01%. However, higher doses were used in the present study in order to observe the effects of quercetin more clearly. In addition, the 0.5% onion-containing diet used in the present study is equivalent to an approximately 0.00016% quercetin-containing diet, but an approximately 0.005% polyphenol-containing diet. This dose of onion was chosen because polyphenols other than quercetin might have alleviative effects against the male reproductive toxicity induced by DEPs.

In the DEP-treated groups, significant changes in the body and testis weights were observed. In particular, the testis weights in the quercetin-fed groups were significantly increased as compared with that in the vehicle group. On the other hand, no changes in the relative testis, epididymis, and relative epididymis weights were observed. Inhalation of DE does not markedly alter testis weights in mice[10] or rats,[11,12] but significantly affects the weights of the accessory sex glands, such as the prostate, coagulating glands, and adrenal glands, in rats.[12] In a previous report, the body, testis, and relative testis weights did not change, but the epididymis and relative epididymis weights increased significantly in 220 μg/mouse DEP-treated groups relative to the corresponding values in a vehicle group.[14] Hence, changes in body and testis weights were not discussed in that study. Quercetin elevates the testis weight, but the influence of DEPs and/or quercetin on body and reproductive organ weights must be further examined in future studies.

Figure 4. Effects of Quercetin and Onion on Numbers of Stained Sertoli Cells in DEP-Treated Mice.

Data are expressed as means ± SEM (n = 9). *P < 0.05 vs. the vehicle group. #P < 0.05 and ##P < 0.01 vs. the DEP group.

DSP is a quantitative index of the ability to produce sperm.[35] Sperm morphologies are qualitative indexes of spermatogenesis.[28] In the present study, DEPs affected these two indexes of spermatogenesis in the same manner as in our previous studies.[14,15] DSP in all DEP-treated groups, except for the 0.3% Q + DEP group, were significantly decreased as compared with that in the vehicle group. These results suggest that a dose of 0.3% quercetin tends to inhibit the spermatogenesis dysfunction induced by DEPs. The sperm abnormalities observed in DEP-treated mice are a serious problem of reproductive function, since abnormal sperm cannot reach the oviduct after intravaginal ejaculation.[36] These results suggest that DEPs reduce fertility, but the incidences of morphologically abnormal sperm in the 0.1% Q + DEP and 0.03% Q + DEP groups were significantly decreased compared with that in the DEP group. These results indicate that a dose of < 0.1% quercetin can inhibit the male reproductive toxicity induced by DEPs. In addition, the incidence of abnormal sperm was also lower in the 0.5% Oni + DEP group. In this case, a multiplier effect of various flavonoids may have occurred, since the dose of quercetin in this diet was lower than those in the other diets.

Sertoli cells were counted, since these cells are very important in spermatogenesis. Tubulin, a cytoskeletal protein enriched in Sertoli cells, was stained immunohistochemically, since these cells are difficult to discriminate by conventional staining procedures.[37,38] It is also known that fluoranthene, a PAH, altered tubulin distribution in cultured Sertoli cells.[39] This can result in dysfunction of Sertoli cells. In the present study, the numbers of stained Sertoli cells in the DEP and 0.3% Q + DEP groups were significantly decreased as compared with that in the vehicle group. Furthermore, the numbers for the 0.1% Q + DEP and 0.03% Q + DEP groups were significantly higher than that for the DEP
group. These results indicate that DEPs affect Sertoli cells, and that doses of < 0.1% quercetin can inhibit the toxicity induced by DEPs. It is difficult to consider the disappearance of Sertoli cells, since the cell population is thought to remain relatively stable in adult mice. Thus tubulin might be degraded by DEPs, and as a result, the functions of Sertoli cells for spermatogenesis were reduced. Consequently, Sertoli cells were not stained, and DSP and/or sperm morphology was affected, while doses of < 0.1% quercetin protected against tubulin degradation.

In present study, the alleviative effects of quercetin and/or onion on the male reproductive toxicity induced by DEPs were not clear. However, we believe that these effects depend on the AhR antagonistic function, for the following reasons: First, in our previous study, hepatic EROD activity, which is widely used as an indirect index of AhR activity, was assessed in DEP treated mice. This activity of DEP-treated mice was significantly greater as compared with that in the vehicle group, and a clear negative correlation was observed between hepatic EROD activity and DSP, whereas a clear positive correlation was observed between hepatic EROD activity and the incidence of morphologically abnormal sperm. Second, quercetin inhibited AhR activation induced by DEP in vitro.

In the high-dose quercetin group, DSP (Fig. 1) and Sertoli cell (Fig. 4) were decreased, but the incidence of morphologically abnormal sperm (Fig. 2) was increased. DSP and Sertoli cells were measured in the testis. The incidence of morphologically abnormal sperm was measured in the epididymis. These results suggest that the expression level or responsiveness of AhR or a later mechanism of toxicity in the testis is different from that in the epididymis.

In conclusion, DEPs affected spermatogenesis, especially sperm morphology, and these effects depend on Sertoli cell dysfunction. Moreover, quercetin and onion alleviate male reproductive toxicity induced by DEPs. Further studies are needed to clarify the alleviative mechanism of quercetin and/or onion on the male reproductive toxicity induced by DEPs.

Acknowledgments

This study was supported in part by a Grant from the Special Research Fund for Academic Research at Aomori University of Health and Welfare, Japan, to H. I.

References

17) Ashida, H., Fukuda, I., Yamashita, T., and Kanazawa, K., Flavonoids and flavonols at dietary levels inhibit a


