Effects of Exposure to Nanoparticle-rich Diesel Exhaust on Pregnancy in Rats

ChunMei LI1,2, Xuezheng LI3,4, Akira K. SUZUKI2, Yonghui ZHANG1, Yuji FUJITANI2, Kentaro NAGAOKA4,5, Gen WATANABE4,5 and Kazuyoshi TAYA4,5

1) College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, 210095, China
2) Research Center for Environmental Risk, National Institute for Environmental Studies, Ibaraki 305-8506, Japan
3) Department of Pharmacy, Affiliated Hospital of Yanbian University, Yanji 133000, China
4) Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8538, Japan
5) Department of Basic Veterinary Science, The United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan

Abstract. Pollutants from burning of diesel fuel are hazardous to human health. Nanoparticles in diesel exhaust potentially have profound impact on fetal development and maternal endocrine function during pregnancy due to their ability to penetrate deeply into the body. To investigate the effects of nanoparticle-rich diesel exhaust (NR-DE) on pregnancy, pregnant rats were exposed to NR-DE, filtered diesel exhaust (F-DE) or clean air for 19 days of gestation. Relative weights of maternal liver and spleen to body weight were significantly lower in the NR-DE and F-DE groups than those in the control group. The serum concentration of maternal progesterone was significantly lower, while those of luteinizing hormone (LH) and corticosterone were significantly higher in the NR-DE and F-DE groups than those in the control group. The serum concentration of estradiol-17β was significantly higher in the F-DE group than that in the control group. The levels of cytochrome P450 side-chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase and LH receptor mRNA in the corpus luteum were significantly lower in the NR-DE and F-DE groups than those in the control group. In fetuses, body weight and crown-rump length were significantly greater and shorter, respectively, in both males and females in the NR-DE and F-DE groups than those in the control group. These results demonstrate that exposure of pregnant rats to NR-DE and F-DE suppresses the function of corpora lutea and stimulates the function of the adrenal cortex, suggesting a risk of spontaneous abortion associated with maternal hormonal changes.

Key words: Diesel exhaust, Hormonal regulation, Luteal function, Nanoparticle, Pregnancy, Rat

Diesel exhaust particles (DEP) emitted from diesel engines are the major component of ambient particulate matter (PM), which contains small particles with diameters ranging from nanometers [nanoparticles (NPs)] to micrometers (course particles) [1, 2]. People living near roadsides and underground mines are commonly exposed to diesel-derived NPs, and may be impacted by their toxicity [3]. Hazardous effects of NPs have been observed in laboratory animals [4, 5]. NPs are thought to be able to penetrate deeply into the respiratory tract because of their relative large surface area [6]. They can cross the pulmonary epithelium and reach the interstitium, causing widespread damage in the cardiopulmonary system [7]. Furthermore, NPs can enter the blood stream and be transported to liver, kidney, spleen, brain and heart [5, 8, 9]. Recently, the potential reproductive toxicity of NPs has been recognized. Our previous studies have shown that nanoparticle-rich diesel exhaust (NR-DE) increases testosterone levels in the serum and testis of adult male rats [10, 11] and mice [12] and disrupts adrenocortical function in adult male mice [13]. We have also shown that prenatal exposure to NR-DE leads to endocrine disruption in offspring and suppresses testicular function in immature male rats [14].

During pregnancy, the corpus luteum is the major source of progesterone in the rat. Other sources of progesterone include the ovary, adrenal gland and placenta [15]. In the corpus luteum, both the small and large luteal cells possess steroidogenic capability, with the large luteal cells being the major steroid producer [16]. During luteal steroidogenesis, cholesterol precursor is taken up by cells from circulating cholesterol-rich lipoproteins and transported into mitochondria by steroidogenic acute regulatory protein (StAR), which controls the translocation from the outer to inner mitochondrial membrane. Cholesterol is converted into pregnenolone by cytochrome P450 side-chain cleavage enzyme (P450scC) and then further transformed into progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) [17].

Emerging evidence has suggested potential adverse effects of NPs on fetal and child development [18]. However, the underlying mechanism remains to be delineated. The aim of the present study was to address these questions using established NR-DE and filtered diesel exhaust (F-DE) exposure systems in a rat model.
Materials and Methods

Animals

Pregnant Fischer rats (F344/DuCrlCrlj; day 0 of pregnancy = day of sperm positivity) were obtained from Charles River Laboratories Japan (Tokyo, Japan) and housed individually in wire-mesh cages in whole-body exposure chambers (2.25 m³; Sibata Science Technology, Tokyo, Japan) for histology. Other ovaries were fixed in 10% neutral phosphate-buffered formalin (pH 7.4; Wako Pure Chemical Industries, Osaka, Japan) for 5 h daily from day 1 to 19 of gestation. Body weight was measured on days 8, 15 and 20. On day 20, the rats were euthanized under pentobarbital anesthesia. Blood samples were collected for assessing the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), testosterone, estradiol-17β, progesterone and corticosterone. Total RNA was isolated from the corpora lutea at day 20 of gestation using an RNeasy Mini kit (Qiagen, Tokyo, Japan). cDNA was prepared using a PrimeScript First-Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). Briefly, 1 μg total RNA was mixed with 50 μM oligo-dT primer and 10 μM dNTPs in DNase- and RNase-free water to a final volume of 10 μl. RNA and primer were denatured at 65 C and then cooled immediately on ice. Reverse transcription was performed by using 10 μl of a master mixture comprised of 5× PrimeScript buffer (Takara Bio), RNase inhibitor (40 U/μl), PrimeScript reverse transcriptase (200 U/μl; Takara Bio) and DNase- and RNase-free water. The reaction was carried out at 42 C for 60 min and terminated by incubation at 75 C for 15 min.

Polymerase chain reaction (PCR) primers (Table 1) were designed using the Primer Express 1.0 software (Applied Biosystems, Singapore). Quantitative real-time PCR was performed using a Thermal Cycler Dice Real Time System (TP800, Takara Bio) according to the manufacturer’s instructions. The PCR reaction was programmed with an initial 10 sec at 95 C followed by 40 cycles of 95 C for 5 sec and 60 C for 30 sec. Melting curves were optimized to gain desired amplicons and eliminate any primer dimers or products from DNA contamination. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control for data analysis. Levels of mRNA in the samples transcribed from all genes of interest were normalized to that of GAPDH.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Statistical analysis was
performed using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when the P value was less than 0.05.

Results

Maternal organ and body weights

No deaths, miscarriages or other signs of general toxicity were observed in rats exposed to NR-DE or F-DE. Body weights showed no significant difference between the NR-DE or F-DE group and the control group (Fig. 1).

The relative weights of the liver and spleen, measured at day 20, were significantly lower (P<0.001 and P<0.05) in the NR-DE and F-DE groups than those in the control group (Table 2). There were no differences in relative weight for other organs, including the kidney, adrenal gland, uterus and ovary.

Table 1. Primers used in real-time PCR

| Gene      | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) |
|-----------|---------------------------|---------------------------|
| GAPDH     | GGCACTGCGATGCAAGGGCTAGG   | ATGGTGCTGAAGACGCAGTA      |
| StAR      | CTGCAAGCAAGCTGCTGGG       | GGGTAACACGCTAGCAGGTAAG    |
| P450scc   | GGAAGGATGCGACGGAGCCCTGA   | TGGAGGATGCTGGTGGCATGG     |
| 3β-HSD    | AGCAGAGGTAGGCGGAGAA       | GGCAAGTATGCAATGTGCC       |
| LHR       | CTGGGTGCTTGCTGCCC         | CACCTTAAGTTCGCTGGAA       |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side-chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; LHR, luteinizing hormone receptor.

Table 2. Body weights and relative organ weights on gestation day 20 in pregnant rats exposed to clean air, NR-DE or F-DE from day 1 to day 19 of pregnancy

| Exposure groups                          | Clean air (Control) | Nanoparticle-rich diesel exhaust (NR-DE) | Filtered diesel exhaust (F-DE) |
|------------------------------------------|---------------------|------------------------------------------|-------------------------------|
| Number of animals                        | 6                   | 6                                        | 6                             |
| Body weight (g)                          | 221.0 ± 2.8         | 206.8 ± 3.3                              | 204.3 ± 5.1                  |
| Weight gain (g)                          | 59.7 ± 2.6          | 55.7 ± 2.2                               | 52.4 ± 3.2                   |
| Liver weight (g)                         | 8.5 ± 0.1           | 7.3 ± 0.1***                             | 7.1 ± 0.2***                 |
| Liver/body weight (mg/g)                 | 40.2 ± 0.5          | 31.5 ± 0.4***                            | 34.6 ± 0.2***                |
| Spleen weight (g)                        | 0.55 ± 0.01         | 0.50 ± 0.02***                           | 0.48 ± 0.01**                |
| Spleen/body weight (mg/g)                | 2.60 ± 0.04         | 2.43 ± 0.05*                             | 2.35 ± 0.02**                |
| Kidney weight (g)                        | 1.21 ± 0.02         | 1.18 ± 0.02                              | 1.17 ± 0.03                  |
| Kidney/body weight (mg/g)                | 5.75 ± 0.12         | 5.72 ± 0.06                              | 5.76 ± 0.11                  |
| Adrenal weight (mg)                      | 53.52 ± 0.89        | 50.32 ± 1.12                             | 52.27 ± 1.51                 |
| Adrenal/body weight (mg/g)               | 0.254 ± 0.004       | 0.243 ± 0.004                            | 0.256 ± 0.006                |
| Uterus weight (g)                        | 2.53 ± 0.14         | 2.51 ± 0.10                              | 2.48 ± 0.15                  |
| Uterus/body weight (mg/g)                | 12.1 ± 0.6          | 12.2 ± 0.5                               | 12.1 ± 0.6                   |
| Ovary weight (g)                         | 0.10 ± 0.01         | 0.11 ± 0.01                              | 0.10 ± 0.01                  |
| Ovary/body weight (mg/g)                 | 0.48 ± 0.06         | 0.54 ± 0.02                              | 0.51 ± 0.03                  |

*P<0.05, **P<0.01 and ***P<0.001 compared with the value for the control group (Tukey’s multiple comparison test). Values are expressed as the mean ± SEM (n = 6).
While serum concentrations of FSH (Fig. 2B), PRL (Fig. 2C), ir-inhibin (Fig. 2D), testosterone (Fig. 2F), estradiol-17β (G) and corticosterone (H) at gestation day 20 in pregnant rats exposed to nanoparticle-rich diesel exhaust (NR-DE), filtered exhaust (F-DE) or clean air from day 1 to day 19 of pregnancy, each bar represents the mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 compared with the control group (n=6).

mRNA expression of StAR, P450scc, 3β-HSD and LHR in the corpus luteum

Expression levels of P450scc, 3β-HSD and LHR mRNA were significantly lower in the NR-DE and F-DE groups than those in the control group (Fig. 2C, 2D and 2H). The mRNA levels of StAR showed no difference between the control and the exposed groups (Fig. 2A).

Litter parameters

Fetal body weights were significantly greater in both males and females of the NR-DE and F-DE groups than in the control group (Table 3). Fetal crown-rump lengths were significantly lower in both males and females of the NR-DE and F-DE groups than in the control group (Table 3). There were no significant differences in fetal morphology and sex ratio between control and treated litters (Table 3).

Discussion

The present study clearly demonstrates that exposure to NR-DE or F-DE disrupts hormonal regulation in pregnant rats, as indicated by decreased progesterone and increased LH, estradiol-17β and corticosterone. In pregnant rats, the corpus luteum, but not the placenta, is the major site of progesterone production [15], indicating that it is essential for pregnancy. Similarly, the corpus luteum, but not the placenta, also secretes estradiol-17β, primarily in the middle of gestation [15]. At the end of gestation, the granulosa cells of growing antral follicles produce estradiol-17β for postpartum ovulation [15]. Pituitary prolactin maintains progesterone secretion in the corpus luteum during the first half of pregnancy, whereas placental lactogens maintain it during the second half of gestation [27]. LH is also an important luteotropic hormone in the rat. In addition, at the end of pregnancy, high levels of circulating prolactin cause structural luteolysis in the corpus luteum [28].

In rodents, luteolysis is induced by 20α-hydroxysteroid dehydrogenase (20α-HSD) [15]. This enzyme converts progesterone to 20α-dihydroprogesterone, an inactive metabolite of progesterone. It is well known that prolactin maintains secretion of progesterone in the corpora lutea by inhibiting 20α-HSD in female rats [29]. At the end of pregnancy, activity of 20α-HSD and circulating levels of 20α-dihydroprogesterone increase with a concomitant decrease in progesterone [15]. The decline in circulating progesterone in
conjunction with an increase in estradiol-17β induces delivery. The key role of 20α-HSD as a critical enzyme of luteolysis at the end of pregnancy has been clearly demonstrated using a 20α-HSD knockout mouse model, in which the circulating progesterone level remains elevated and parturition is delayed [30].

In the present study, the decrease in mRNA levels of 3β-HSD, P450scc and LH receptor and increase in estradiol-17β mRNA in the corpus luteum suggest an impairment in luteal function. These results strongly suggest that the mode of action of NR-DE or F-DE in the disruption of secretion of progesterone is due to suppression of the activity of steroid enzymes and production of LH receptors in corpora lutea. In addition to these hormone-producing enzymes, an elevation of estrogen was observed in late gestation. Estrogen upregulates expression of oxytocin receptors [31] and relaxin [32] in the uterus, which are required for delivery. The present results suggest that exposure of pregnant animals to NR-DE or F-DE increases the risk of spontaneous abortion.

The higher levels of corticosterone observed in animals exposed to NR-DE and F-DE in this study suggest that androgenization of the sexually dimorphic nucleus of the preoptic area (SDN-POA) may be disrupted in the male fetus. This structure has been shown to be susceptible to stress in pregnancy, resulting in suppression of testosterone secretion from fetal testes [33].

The present study also suggests that maternal liver function is affected by NR-DE and F-DE, as the liver weight was significantly decreased. Future work is warranted to unravel the underlying mechanisms, for example, whether hepatic inflammation and dyslipidemia are involved [34]. The present study also showed a significant decrease in weight of the spleen in the NR-DE- and F-DE-treated rats. However, the circulating level of corticosterone was increased, suggesting that corticosterone influences cytolysis of spleen cells.

It is an important to point out that no difference was observed in the levels of progesterone and corticosterone between the NR-DE and F-DE treatments. These results suggest that NPs exert no significant effect on the function of the corpora luteum and adrenal cortex. Diesel exhaust contains thousands of chemicals, including nitrophenols, nitrogen oxide, dioxin-like compounds and polycyclic aromatic hydrocarbons [35]. In our previous studies, nitrophenols from DEP have been shown to disrupt gonadal and adrenal function in rats and Japanese quails [36-47]. Taken together, it is suggested that toxic chemicals in F-DE may be responsible for dysfunction of the corpora lutea and the adrenal cortex.

In conclusion, the present study shows, for the first time, that inhalation of NR-DE or F-DE disrupts steroid hormone production in the corpus luteum and adrenal cortex in pregnant rats. Such adverse effects may induce abortion and suppress androgenization of the SDN-POA in the brain of male fetuses. This study, therefore, suggests that exposure to NR-DE in the environment may have detrimental effects on pregnancy and fetal growth in wildlife and humans.

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### Table 3. Parameters of gestation day 20 fetuses from mother rats exposed to clean air, NR-DE or F-DE from day 1 to day 19 of pregnancy

| Exposure groups | Clean air | Nanoparticle-rich diesel exhaust | Filtered diesel exhaust |
|-----------------|-----------|---------------------------------|------------------------|
| Control         | NR-DE     | F-DE                            |
| Number of fetuses |          |                                 |                        |
| Males           | 23        | 30                              | 19                     |
| Females         | 22        | 23                              | 28                     |
| Body weight of fetuses (g) |          |                                 |                        |
| Males           | 3.39 ± 0.06 | 3.58 ± 0.05*                    | 3.72 ± 0.05***          |
| Females         | 3.14 ± 0.06 | 3.30 ± 0.03*                    | 3.52 ± 0.03***          |
| Crown-rump length (mm) |          |                                 |                        |
| Males           | 37.6 ± 0.3 | 35.9 ± 0.5**                    | 36.0 ± 0.4*            |
| Females         | 37.3 ± 0.3 | 34.8 ± 0.3***                   | 35.2 ± 0.5***          |
| Anogenital distance (mm) |          |                                 |                        |
| Males           | 5.7 ± 0.2 | 6.0 ± 0.1                       | 6.0 ± 0.2              |
| Females         | 4.1 ± 0.2 | 4.0 ± 0.1                       | 4.5 ± 0.1              |
| Anogenital index (mm/g^{1/3}) |          |                                 |                        |
| Males           | 3.77 ± 0.21 | 3.81 ± 0.15                     | 3.89 ± 0.11           |
| Females         | 2.79 ± 0.11 | 2.72 ± 0.08                     | 2.98 ± 0.08           |

*P<0.05, **P<0.01 and ***P<0.001 compared with the value for the control group; #P<0.01 compared with the value for the NR-DE group (Tukey’s Multiple Comparison Test). Values are expressed as the mean ± SEM (n = 19 to 30 per group). Anogenital index (mm/g^{1/3}) represents the ratio of the anogenital distance to the cube root of the body weight.
