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Repeated carbon nanotube administrations in male mice cause reversible testis damage without affecting fertility

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Abstract

Soluble carbon nanotubes are promising materials for *in vivo* delivery and imaging applications. Several reports have described the *in vivo* toxicity of carbon nanotubes, however, their effects on male reproduction have not been examined. Here we show that repeated intravenous injections of water-soluble multi-walled carbon nanotubes into male mice can cause reversible testis damage without affecting fertility. Nanotubes accumulated in the testes, generated oxidative stress, and decreased the thickness of the seminiferous epithelium in the testis at day 15, but the damage was repaired after 60 and 90 days. The quantity, quality, and integrity of the sperm and the levels of three major sex hormones were not significantly affected throughout the 90-day period. The fertility of treated male mice was unaffected; the pregnancy rate and delivery success of female mice that mated with the treated male mice did not differ from those that mated with untreated male mice.

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Author contributions

B. Y. and Y. B. conceived and designed the experiments. Y. B., Y. Z., J. Z., Q. M., W. Z., E. R. B., and S. E. S. performed experiments. B. Y., Y. B., and Y. Z. analyzed data. Y. B., Q. M., W. Z., and S. E. S. contributed materials and analysis tools. B. Y. and Y. B. co-wrote the paper.

Additional Information

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Keywords

carbon nanotube; nano-reproductive toxicity; nanotoxicity; nanotechnology; male fertility; mice

Biomedical applications of carbon nanotubes are promising^{1–4}, but their potential toxic effects remain a concern. Carbon nanotubes have been shown to accumulate in animal organs^{5,6}, generate oxidative stress^{7,8}, and damage cells^{9,10} and organs^{11,12}. Their *in vivo* effects include the induction of inflammation and the formation of epithelioid granuloma in the lung^{13,14}, increased percentage of aortic plaque and induction of atherosclerotic lesions in the brachiocephalic artery of the heart¹², and induction of mesothelioma in the abdominal cavity¹⁵.

Although the importance of reproductive toxicology of nanomaterials has been raised^{16,17} and the reproductive toxicity of carbon black nanoparticles has been reported^{18,19}, there is currently no comprehensive study on male reproductive toxicity of carbon nanotubes. The human male reproductive system has been known to be vulnerable to many exogenous materials and has continued to deteriorate in modern times^{20,21}. The causes of deterioration are complicated, but oxidative stress is known to be one of the main factors^{22,23}. Several studies have also attributed nanomaterial-induced cytotoxicity^{7,24} and organ damage^{6,11,13} to oxidative stress.

Here we evaluated the effects of amine (NH₂)- and carboxylate (COOH)-functionalized multiwalled carbon nanotubes on the male reproductive systems of mice. The amine-functionalized nanotube was derived from a carboxylated nanotube through amidation. The two have comparable shape and size distributions and the same density of functional groups (Table 1). Amine nanotubes were positively charged and carboxylated nanotubes were negatively charged at pH 7.0 and were therefore more water-soluble than pristine carbon nanotubes. Phosphate-buffered saline (PBS) suspensions of both nanotubes were stable for more than 24 h in the presence of plasma proteins or in 0.1% Tween 80.

To mimic the potential biomedical applications of the carbon nanotubes in terms of administration method and dose^{5,25}, we intravenously injected the nanotube suspension and vehicle control through the tail vein into healthy adult male BALB/c mice. The nanotubes and vehicle control were administered either as a single dose of 5 mg/kg (Scheme 1 in Fig. 1a) or in 5 doses over 13 days at 5 mg/kg per dose (Scheme 2, Fig. 1a). Reproductive toxicologic assessments were conducted on days 15, 60, and 90. Within 24 h, nanotubes were found in the testis, and accumulation resulted in oxidative stress and tissue damage. However, the damage was reversed after 2 months, and no effects on mating, fertility, delivery, or fetus viability were found under our experimental conditions.

Effects of nanotubes on mice after i.v. injection

The translocation and biodistribution of nanoparticles are key factors in their toxicity evaluation *in vivo*. Although other nanoparticles such as gold and magnetic nanoparticles have been reported to enter testes in small quantities^{26,27}, it is not known whether carbon nanotubes can enter or accumulate in the testis. Using ⁶⁴Cu-labeled carboxylated carbon

nanotubes, we examined the accumulation of nanotubes in the testes after a single dose. Approximately 41, 61, and 151 ng of nanotube were found in the testes 10 min, 60 min, and 24 h after the injection (Fig. 1b). Although the relative amount of nanotubes in the testes was small, the increasing trend suggests that with 5 repeated doses, more nanotubes are expected to accumulate in the testes.

After nanotube injection, none of the mice from any group showed stress or symptoms of abnormality, such as lethargy, anorexia, vomiting, or diarrhea during the entire experimental period. In the 5-dose experiment, we monitored the effects of nanotubes on the body weight, testis, and epididymis indices. It was reported that single-walled carbon nanotubes increased the lung and spleen indices due to pulmonary injury and induction of immunologic responses⁶. In our experiment, the average body weights (Fig. 1c&d), testis index, and epididymis index (Fig. 1e&f) of nanotube-treated mice showed no statistically significant difference from those of the control mice, suggesting that nanotubes' effect on the weight of male reproductive organs is negligible.

The tight junctions of Sertoli cells form the blood-testis barrier^{28,29}, a structure that partitions the interstitial blood compartment of the testis from the adluminal compartment of the seminiferous tubules. In addition to their role in blood-testis barrier formation, Sertoli cells also nurture the developing sperm cells through the stages of spermatogenesis. The accumulation of nanotubes in the testes raised the question of whether they could adversely affect Sertoli cells and seminiferous tubules.

Figure 2a-f shows the cross-sections of testes of mice treated with vehicle or carboxylated carbon nanotubes. In the seminiferous tubules, spermatozoa are generated and differentiated to sperm cells through meiosis. Micrographs of testes from control mice show normal structure (Fig. 2a&b). Histologic inspection of testes samples from administration scheme 1 and 2 showed quite different results on day 15. Testes showed little alterations after a single dose of nanotubes (Fig. S1A), while the testes from mice treated with 5 doses of carboxylated nanotubes (administration scheme 2) were characterized by partially damaged seminiferous tubules (Fig. 2g), significant reduction of the thickness of the germinative layer (Fig. 2c and h), and the reduction of the number of spermatogonia (Fig. 2j). Histologic studies also showed partial disappearance or vacuolization of Sertoli cells in the basal zones of some seminiferous tubules (Fig. 2d) and some necrotic and degenerative cells (Fig. S1B), vasodilatation, and hyperemia in the testes (Fig. S1C). No changes in Leydig cells (not shown) or spermatids (Fig. 2i) were observed in the samples examined. Amine nanotubes produced similar but slightly less severe alterations in the testes (Fig. S1D-F). These results suggested that the two types of water-miscible nanotubes affected testes integrity similarly despite their different surface charges. Comparing pathologic alterations resulting from these two administration schemes, we concluded that the total accumulated dose played an important role in reproductive nanotoxicity of carbon nanotubes. Because carbon nanotubes are highly persistent and their excretions in the body are not well known, this finding is particularly important in allowing us to understand their reproductive toxicity.

The above-mentioned alterations were observed only occasionally in the testes of mice examined on days 60 and 90, indicating that these alterations can be repaired over time

(Figure 2e,f,g-j). It is known that the testis itself has the ability to recover from some injuries^{30,31}. Because the administration schedule and the total amount of nanotubes administered in the 15-, 60-, and 90-day groups were the same, we assume that the extent of the alterations in testes may be compensated by an efficient recovery process, preventing a decrease in the testis index. The observed deleterious effects of nanotubes on testicular tissues (Fig. 2c,d,g,h) may be attributed to many possible mechanisms. Among them, carbon nanotube-generated free radicals may be involved in cell death and lipid peroxidation of unsaturated fatty acids in the plasma membrane. Since malondialdehyde (MDA) is a representative biomarker of oxidative stress and lipid peroxidation, we next examined the carbon nanotube-induced alterations in MDA levels in homogenates prepared from testes. Considering the key role of male sex hormones in spermatogenesis, we also measured their levels in blood.

Oxidative stress and change in hormone levels

Oxidative stress-mediated injury to the male reproductive system is a significant contributing factor in 30–80% of cases of male infertility^{22,23}. Oxidative stress is also the main mechanism of carbon nanotubes' toxicity to lung^{11,13} and liver^{6,32} tissues. Owing to some protective mechanisms⁶, such oxidative damage can be kept to a minimum in the liver. However, there is no such protective mechanism in the testes. A disturbed oxidative stress/antioxidant equilibrium caused by toxicants can elevate the oxidative stress level in the more vulnerable testes and reduce male fertility^{22,23}.

Detecting the accumulation of nanotubes in the testes, we speculated that nanotubes might cause oxidative stress and induce a chain of events in the testes. Fifteen days after administration of a single dose, neither type of nanotube had induced any change in MDA levels (Fig. 3a). There was no evident histologic alteration in the testes of mice from this group (Fig. S1A). Under the repeated administration regimen (administration scheme 2), carboxylated nanotubes induced an increase in the MDA level in testes at day 15 compared with the control group (Fig. 3a). However, MDA levels returned to the same level as in controls at days 60 and 90 while the measures in livers (as a control) remained elevated (Fig. S2).

Besides suitable oxidant/antioxidant equilibrium, a proper level of male sex hormones is also critical for spermatogenesis. Follicle stimulating hormone (FSH) stimulates both the production of androgen binding protein by Sertoli cells and the formation of the blood-testis barrier. Androgen binding protein is essential for concentrating testosterone to levels high enough for initiating and maintaining spermatogenesis, which can be 20–50 times higher than the concentration found in blood. FSH may initiate the sequestering of testosterone in the testes, but, once developed, only testosterone is required to maintain spermatogenesis. However, increasing the levels of FSH will increase the production of spermatozoa by preventing apoptosis of type A spermatogonia. Other hormones such as luteinizing hormone (LH) also play important roles in spermatogenesis. However, these functions can be affected by xenobiotics or foreign substances. Some endocrine disruptors, such as polychlorinated biphenyls³³ and inorganic lead³⁴ can alter the levels of these hormones, resulting in testicular injury, malfunction in spermatogenesis, and male infertility. It is noteworthy that

nanoparticle-rich diesel exhaust inhalation disrupts the endocrine activity of the male reproductive system by elevating the plasma testosterone concentration in male rats^{35,36}. In the present study, plasma levels of testosterone, LH, and FSH were determined on days 15, 60, and 90 by ELISA assays after 5 doses of nanotubes (administration scheme 2). Results (Fig. 3b-d) showed that nanotubes did not alter plasma sex hormone levels compared with those of controls under our exposure conditions. In contrast, more frequent administration of carbon black nanoparticles intratracheally caused changes in hormone levels, according to a recent report¹⁸.

Sperm health and fertility unaffected

Oxygen toxicity is an inherent challenge to aerobic life forms^{37,38} such as the spermatozoa³⁹. Although the hormone levels were not affected, the elevated oxidative stress level in the testes and the damage in the seminiferous tubules induced by nanotubes prompted us to examine their effects on spermatogenesis. Spermatozoa are generated in the testes and are transported to the epididymis for concentration and maturation. Spermatogenesis is a prolonged process spanning 40–50 days in rodents.

After administering 5 doses of nanotubes, we collected sperm from the cauda epididymis and examined the total sperm concentration, sperm motility, and percentage of abnormal sperm on days 15, 60, and 90 (Fig. 4a-c). No statistically significant alterations in these properties were observed. Because semen has a very weak antioxidant system³⁹, oxidative stress-mediated DNA fragmentation is common in spermatozoa of infertile men⁴⁰. We examined whether DNA fragmentation occurred in the nanotube-treated mice using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Our results showed that nanotubes did not induce detectable amounts of DNA damage in sperm examined on days 15, 60, and 90 (Fig. S3).

Another toxicant-mediated sperm damage is the loss of acrosome integrity⁴¹. In mammalian sperm, the acrosome contains digestive enzymes (including hyaluronidase and acrosin), which can break down the outer membrane of the ovum and allow the haploid nuclei in the sperm to join with the haploid nucleus in the ovum. By fluorescence-labeling of sperm nuclei and acrosomes (Fig. 4e-g), we examined the integrity of the sperm on days 15, 60, and 90 after administering 5 doses of nanotubes as shown in scheme 2 in Figure 1. At least 200 sperm were examined for each sample. The acrosome integrity of sperm was not affected by nanotubes throughout the 90-day period (Fig. 4d).

The initial histologic alterations and the increase in oxidative stress in the testes indicate that nanotubes may harm the male reproductive system. However, in a dosing schedule similar to typical biomedical applications, the extent of damage in the testes was much less than the damage caused by other toxicants⁴². Although carbon nanotubes induced initial pathologic alterations in the testes of mice (Fig. 2), these alterations showed signs of recovery over time. The lack of adverse effects on the quality and quantity of the sperm further support this observation (Fig. 4). To determine whether these initial alterations could affect fertility, mice treated with carboxylate and amine nanotubes (according to scheme 2 in Fig. 1a) were paired with healthy female mice. Fifteen and 60 days after the first treatment, the treated

mice showed no changes in mating behavior. The female mice successfully delivered healthy pups; the number of live pups per litter and live fetuses with visible abnormalities were the same as in the control group, in which untreated male mice mated with healthy female mice (Table 2).

Conclusions

Our pilot study investigated the effects of intravenous injection of single and multiple doses of water-soluble multi-walled carbon nanotubes on the reproductive system of male mice. Nanotubes accumulated in the testes 24 h after a single i.v. administration, and by day 15 the nanotubes caused oxidative stress and tissue damage that were repaired by days 60 and 90. We could not measure the cumulative dose in the testis after repeated injections due to the short lifetime of the radiolabels, but it is likely that 5 doses of nanotubes would cause a much higher accumulation in the testes. Sex hormones and sperm were unaffected by the nanotubes throughout the 90-day period, and treated mice continued mating with healthy female mice to produce healthy offspring. Although our study showed that carbon nanotubes have minor effects on the male reproductive system in mice, oxidative stress and the alterations in the testes raise concerns because it is possible that these materials may accumulate at higher quantities over a longer period and may have adverse effects on male fertility.

A recent paper reported that mice intratracheally administered with carbon black nanoparticles at 10 doses over 10 weeks (compared to 5 doses over 13 days in our study) showed partial vacuolation of the seminiferous tubules and elevated serum testosterone levels¹⁸. In that study, mice were sacrificed and examined one day after the last dose and so it is possible that the mice did not recover from the effect of carbon nanoparticles. Another report showed that these nanoparticles affected the fertility of male offspring¹⁹ when pregnant female mice were exposed to the particles. Considering the highly diverse structures and properties of nanomaterials and the multiple ways of exposure of nanomaterials to humans, further studies on reproductive toxicity of nanomaterials, especially with long-term and early-life exposure, are urgently needed.

Materials and Methods

Carboxylate- and amine-functionalized multiwalled carbon nanotubes

Carboxylate-functionalized multiwalled carbon nanotube were synthesized as previously reported⁴³. Amine-functionalized nanotubes were prepared by reacting carboxylate nanotubes with an 18-fold excess of 1,3-diaminopropane for 12 h at 40°C. The elemental analysis showed that nitrogen increased from <0.5% in the former to 2.8% in the latter. For animal experiments, both types of nanotubes were dispersed in PBS (pH 7.4) with 0.1% Tween 80 to a final concentration of 1.0 mg/ml. The suspensions were sonicated for 10 min before use.

Animal administration and sampling

Male BALB/c mice (20–25 g, from Charles River Laboratories Inc., Wilmington, MA, USA, or the Animal Center of Shandong University) were used. All animal experiments

were done in accordance with the NIH guidelines “Guide for the Care and Use of Laboratory Animals” and experimental protocols approved by an institutional animal care and use committee. After acclimation for 1 week, 96 mice were randomly divided into 12 groups (15-day single dose, 15-day multi-dose, 60-day multi-dose, and 90-day multi-dose groups for controls, carboxylate nanotubes and amine nanotubes) with 8 mice per group. Mice were given injections of vehicle and nanotubes via the tail vein once (single dose) or every 3 days for 5 times. For the fertility assessment, the administration according to administration scheme 2 was given to 3 groups (6 mice per group) for evaluation 15 and 60 days after the first dose. Mice were euthanized by CO₂ asphyxiation and blood/organ samples were collected. Plasma samples were obtained from blood by centrifugation (600 g for 10 min). Testes, liver, and epididymis were collected and weighted for organ indices (organ weight/body weight) calculations. Left testes were fixed in Bouin solution. The right testes and plasma samples were stored at -70 °C.

***In vivo* biodistribution studies**

To maintain the integrity of the carboxylate nanotubes, fewer than 5% of the nanotubes were first reacted with diaminopropane and then coupled with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), which chelated ⁶⁴Cu. Mice were given injections of 67–123 µCi of radiotracer via the tail vein. Animals (*n*=4 at each time) were euthanized, testes were weighed, and radioactivity of ⁶⁴Cu was counted using an automatic γ-counter (see SI for details).

Semen evaluation

Each left cauda epididymis were dissected, incised with a pin in 1.5 ml of DMEM with 10 mg/ml bovine serum albumin (BSA), and incubated for several minutes to allow sperm to release. To assess sperm concentration, an optical microscopy-based hemocytometer method was used with an appropriate counting chamber. For the sperm movement assessment, one drop of sperm suspension was placed on a microscopic slide, and the movement of 200 sperm cells was examined using a light microscope and 40× magnification. Sperm morphologic abnormalities were classified according to the criteria of Watanabe⁴⁴. At least 200 sperm from each mouse were examined. Acrosomal status was assessed according to the procedures of Ramalho-Santos⁴⁵ and Ozaki⁴⁶.

Histologic observations

The testes were fixed in Bouin solution for 24 h and embedded in paraffin, thin-sectioned, and mounted on glass microscope slides. The mounted sections were stained with hematoxylin-eosin and examined by light microscopy. To quantitatively analyze the pathologic alterations, the germinative layer thickness, counts of spermatogonia, Sertoli cells, spermatids, and the percentage of the damaged seminiferous tubules were determined. At least 10 histologic sections from each testis and a total of approximately 100 seminiferous tubules were examined for statistical analysis of alterations. A statistical evaluation of each parameter was performed by examining 100 randomly selected tubular profiles in each histologic section of testes.

Oxidative stress and hormone measurements

The homogenates prepared from testes were diluted to 2.5% with PBS for biochemical assays. The homogenates were centrifuged at $3,000 \times g$ for 15 min at 4°C to collect the supernatants for MDA assays. MDA was determined using a thiobarbituric acid reactive method⁶. Hormone levels were measured using a double-antibody sandwich ELISA assay according to the manufacturer's protocol (Yanji Biotech Limited Corporation, Shanghai, P.R. China).

Fertility evaluations

Mice were dosed according to administration scheme 2. On days 15 and 60, each male mouse was kept with two untreated virgin female BALB/c mice in an individual cage for 10 days or until copulation was found by vaginal plug or vaginal smear. Once insemination was confirmed, female mice were checked at least three times daily on days 19–21 of pregnancy to determine the time of delivery. The females were allowed to deliver spontaneously and nurse their pups until postnatal day 4 (PND 4). Total litter size and the numbers of live and dead pups were recorded and examined on PND 4.

Statistical analysis

The two-sided Student's *t*-test was used to analyze differences between experiments or groups of mice. Data are reported as mean values $\pm$ SD of multiple determinations. *P* values of <0.05 were considered statistically significant, and all statistical calculations were done using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Treatment of BALB/c mice with multiwalled carbon nanotubes (MWNT). a, Scheme 1 shows single administration and scheme 2 shows 5 doses over 13 days at 5mg/kg per dose. Reproductive toxicology was assessed on day 15, 60 and 90. b, Accumulation of ⁶⁴Cu-labeled MWCNT-COOH in testes (n=4). c,d Body weights of BALB/c mice intravenously injected with MWCNT-COOH, MWCNT-NH₂, or PBS with 0.1% Tween 80 in 60-day (c) and 90-day (d) experiments show no statistically significant differences. Data are mean $\pm$ standard deviation (n=8 per group). e,f Testes (e) and epididymis (f) indices determined on days 15, 60, and 90 after 5 doses of MWCNT-COOH, MWCNT-NH₂, or PBS with 0.1% Tween 80 show no statistically significant differences between the groups. Values represent mean $\pm$ SD (n=8) at each time point.

Figure 2.

Pathologic and morphometric analysis of testes treated with MWCNTs. a-f, Histology cross sections of seminiferous tubules (a) and an enlarged view (b) from testes of control mice show normal Sertoli cells. The decreased germinative layer thickness (c) and vacuolization of Sertoli cells (arrows in d) were observed on day 15 after 5 doses of MWCNT-COOH treatment. On days 60 (e) and 90 (f), most alterations disappeared, indicating a general recovery from early damage. All scale bars are 20 μm. g-j, Alterations in seminiferous tubules in the testes of mice were evaluated for abnormalities (g), average thickness of germinative layer (h), and average number of spermatid cells (i) and spermatogonia cells (j) per section (n=8 per group; about 100 seminiferous tubules were examined for each mouse).

* $p < 0.05$ indicates significant difference between treatment groups and control groups.

Figure 3.

MDA levels in testes homogenate and sex hormone levels in blood. a, The average MDA level in testes was determined from testes homogenate. MDA level in liver was also measured as control (see Fig. S2). b-d, Plasma testosterone (b), LH (c), and FSH (d) levels in the blood of control and MWCNT-treated mice were determined at day 15, 60, and 90. Carbon nanotubes were injected into mice according to administration scheme 2, except for the first group of data in (a), in which scheme 1 was followed. Each data point in the figure represents the mean $\pm$ SD from 8 mice. * $p < 0.05$ indicates MDA level of MWCNT-COOH treated group at 15 days was significantly different from the control group.

Figure 4.

Sperm concentration, motility, morphology, and acrosome integrity. a-d, Sperm concentration (a), percentage of motile sperm (b), percentage of abnormal sperm by morphologic examination (c), and acrosome integrity (d) of control and MWCNT-treated mice show no statistically significant changes between the groups or over time throughout the study. Data points represent mean $\pm$ SD (n=8). e-g, Acrosome integrity was examined by labeling the nuclei (e) with Hoechst 33258, and acrosomes (f) with FITC-PSA. Overlay of

acrosome and sperm nucleus (g) showed the integrity of acrosome formation. Red arrow points to an intact acrosome, and white arrow points to an incomplete acrosome.

Table 1

Characterization of Multiwalled Carbon Nanotubes (MWCNTs)

	MWCNT-COOH	MWCNT-NH ₂
Chemical structure
TEM
Suspension in plasma
Diameter (nm)	20~30	20~30
Length (μm)	0.5~2.0	0.5~2.0
Zeta potential (mV in H₂O)	-57	26
Zeta potential (mV in plasma)	-48	-35
Functional group loading (mmol/g)	0.4	0.4
Fe,Al,Cr,Mn (%) by ICPMS	0.3,0.02,0.002,0.00001	0.21,0.02,0.00058,0.00065
Other 19 metals (%) by ICPMS (see SI)	<0.00008	<0.00004

Table 2

Fertility of Male Mice 15 and 60 Days after Five Doses of MWCNT Treatment

	Copulation Index(%) ^a	Fertility Index(%) ^b	Gestation Index (%) ^c	Average no. of live pups/pregnant female	Viability index PND4 ^d
Control	100.0	91.6	100	7.4	95.6%
MWCNT-COOH-15 days	100.0	91.7	100	7.8	97.7%
MWCNT-NH ₂ -15 days	100.0	100.0	100	7.5	100.0%
MWCNT-COOH-60 days	100.0	83.3	100	7.2	95.8%
MWCNT-NH ₂ -60 days	100.0	91.7	100	6.8	97.3%

Note: One male was paired with two females. N (male) = 6/group.

^a Copulation index (%) = (no. of animals with successful copulation/no. of animals paired) × 100.

^b Fertility index (%) = (no. of pregnant females/no. of females with successful copulation) × 100.

^c Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.

^d Viability index on PND 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) × 100.

Control mice were injected the same amount of PBS with 0.1% Tween 80. No statistically significant changes were observed between groups or over time throughout the duration of the study.