Published in final edited form as:

J Appl Toxicol. 2015 December ; 35(12): 1438–1451. doi:10.1002/jat.3177.

Distribution and Biomarker of Carbon-14 Labeled Fullerene C₆₀ ([¹⁴C(U)]C₆₀) in Pregnant and Lactating Rats and their Offspring after Maternal Intravenous Exposure

Rodney W. Snyder^a, Timothy R. Fennell^a, Christopher J. Wingard^b, Ninell P. Mortensen^a, Nathan A. Holland^b, Jonathan H. Shannahan^b, Wimal Pathmasiri^a, Anita H. Lewin^a, and Susan C. J. Sumner^{a,*}

^aDiscovery Sciences, RTI International, 3040 Cornwallis Drive, Research Triangle Park, NC 27709, USA

^bDepartment of Physiology, Brody School of Medicine, East Carolina University, Greenville, NC 27834, USA

Abstract

A comprehensive distribution study was conducted in pregnant and lactating rats exposed to a suspension of uniformly carbon-14 labeled C₆₀ ([¹⁴C(U)]C₆₀). Rats were administered [¹⁴C(U)]C₆₀ (~0.2 mg [¹⁴C(U)]C₆₀/kg body weight) or 5% polyvinylpyrrolidone (PVP)-saline vehicle via a single tail vein injection. Pregnant rats were injected on gestation day (GD) 11 (terminated with fetuses after either 24h or 8d), GD15 (terminated after 24h or 4d), or GD18 (terminated after 24h). Lactating rats were injected on postnatal day 8 and terminated after 24h, 3d or 11d. The distribution of radioactivity in pregnant dams was influenced by both the state of pregnancy and time of termination after exposure. The percentage of recovered radioactivity in pregnant and lactating rats was highest in liver and lungs. Radioactivity was quantitated in over 20 tissues. Radioactivity was found in placenta and in fetuses of pregnant dams, and in the milk of lactating rats and in pups. Elimination of radioactivity was <2% in urine and feces at each time point. Radioactivity remained in blood circulation up to 11 days after [¹⁴C(U)]C₆₀ exposure. Biomarkers of inflammation, cardiovascular injury and oxidative stress were measured to study the biological impacts of [¹⁴C(U)]C₆₀ exposure. Oxidative stress were elevated in female pups of exposed dams. Metabolomics analysis of urine showed that [¹⁴C(U)]C₆₀ exposure to pregnant rats impacted the pathways of vitamin B, regulation of lipid and sugar metabolism and aminoacyl-tRNA biosynthesis. This study demonstrated that [¹⁴C(U)]C₆₀ crosses the placenta at all stages of pregnancy examined, and is transferred to pups via milk.

Keywords

Carbon-14 labeled C₆₀ ([¹⁴C(U)]C₆₀); *in vivo* disposition; pregnant dams; fetus; lactating rats; pups; maternal-fetal transfer; biomarkers; metabolomics analysis

*Corresponding author: Susan Sumner, Discovery Sciences, RTI International, 3040 Cornwallis Drive, Research Triangle Park, NC 27709, USA, ssumner@rti.org.

INTRODUCTION

Applications of engineered nanomaterials (ENMs) in nanomedicine include their use in contrast agents, cancer therapeutics, vaccines, and drug delivery, resulting in intentional human exposure as the list of consumer products containing ENMs is rapidly growing. One of the ENMs that is being used in biomedical and consumer products is fullerene, C₆₀ (Aschberger, et al. 2010, Yamashita, et al. 2013). Studies of the biological impacts and responses to C₆₀ exposure have been limited and, in some cases, contradictory. Only a few have focused on the reproductive biology and potential translocation from mother to offspring (Sumner, et al. 2010a, Tsuchiya, et al. 1996, Vidanapathirana, et al. 2014a). A basic understanding of the distribution of C₆₀ during pregnancy and maternal transfer is needed to assess its safety and potential health impact on early development.

Evaluation of the biological impact of exposure to ENMs during pregnancy and early development is essential to the access the safety of their use in consumer products. Reports that *in utero* exposure to nanoparticle(NP)-rich diesel exhaust by maternal inhalation during pregnancy has been found to impact testicular function in immature male offspring (Li, et al. 2009) underscore the significance. The mechanism appears to be disruption of testosterone biosynthesis and metabolism, mediated through growth hormone gene expression in the testes (Ramdhan, et al. 2009). There is also evidence that nanosized particles cross the human placenta *in vitro* (Wick, et al. 2010) and rat placenta *in vivo* (Sumner, et al. 2010a). The gestational/lactational translocation of a chemical from the mother to fetuses and offspring has been shown for many chemicals, such as polyaromatic hydrocarbons in humans (Perera, et al. 1998) and in mice (Detmar, et al. 2008), titanium dioxide in mice (Shimizu, et al. 2009), diesel exhaust particles in mice (Hemmingsen, et al. 2009, Hougaard, et al. 2008), and pharmaco-therapeutic agents in pregnant and neonatal animals (Langston 2001). The mechanisms and consequences of this translocational exposure are not yet well understood.

As previously mentioned, ENM exposure on early embryonic development has been reported, but only a few studies have focused on C₆₀. The effect of *in vivo* administration of C₆₀ (purity reported as >99.9%, formulated with polyvinylpyrrolidone [PVP]) solubilized into media (Yamakoshi, et al. 1994) to pregnant SLC mice on gestational day (GD) 10 by intraperitoneal (i.p.) injection is one such study (Tsuchiya, et al. 1996). The administered doses ranged from 25 to 137 mg/kg, and effects were monitored at 18 hours following administration. At a dose of 137 mg/kg, all the embryos died and showed “severe abnormalities.” At a dose of 50 mg/kg, C₆₀ was “clearly distributed into the embryos based on the characteristic color development of C₆₀, and caused abnormalities, especially around the head region and tail.” At 25 mg/kg, abnormal enlargement of the head was reported in one embryo (Tsuchiya, et al. 1996). More recently, the distribution of [¹⁴C(U)]C₆₀ (formulated in a 5% PVP-saline suspension), after intravenously (i.v.) administered to pregnant (GD 15) and lactating rats (postnatal day [PND] 8), demonstrated that radioactivity crossed the placenta and was transferred to fetuses during pregnancy, and to the pups via milk, with systemic absorption by the pup (Sumner, et al. 2010a). Differences in the distribution of radioactivity to the lung were observed between the physiological state of pregnancy, where <1% of administered dose was detected in the lungs at 24 hours after

dosing, and lactation, where ~25% of administered dose was detected in the lungs at 24 hours after dosing. Because C₆₀ has been shown to affect the cardiovascular system in non-pregnant rats, causing susceptibility to ischemia/reperfusion injury 24 hours following exposure (Thompson, et al. 2014), the difference in distribution to the lung could have a profound effect on the cardiovascular system. In addition, it has been reported that in rats i.v. exposure to C₆₀ during pregnancy increase vascular tissue contractility of the uterine artery through elements of Rho-Rho-kinase signaling during late stages in pregnancy (Vidanapathirana, et al. 2014a).

Since rat is a commonly used and well-characterized model for embryology and reproductive toxicology (de Rijk, et al. 2002) we have carried out an investigation of the distribution of [¹⁴C(U)]C₆₀ (a 5% PVP-saline suspension) in pregnant and lactating rat exposed at different developmental time points, and at different time points post administration, to study the distribution of radioactivity to the fetuses and pups. To gain insight into tissue distribution and elimination profiles, [¹⁴C(U)]C₆₀ was administered via a single tail vein injection to the adult animals. Biomarkers of inflammation, cardiovascular injury and oxidative stress were measured to study the biological impact of C₆₀ exposure. Based on these results we provide a basic understanding of the distribution, elimination and biological impacts after [¹⁴C(U)]C₆₀ administration to pregnant and lactating rats.

METHODS

Preparation of [¹⁴C(U)]C₆₀ and dose solution

Following the protocol from our previous published work (Sumner, et al. 2015), [¹⁴C(U)]C₆₀ was prepared using the Kratschmer and co-workers (Kratschmer, et al. 1990) plasma arc discharge method as modified by Scrivens and Tour (Scrivens, et al. 1994). [¹⁴C(U)]C₆₀ was isolated by exhaustive extraction of the soot with toluene, followed by column chromatography on alumina, eluting with hexane. Final purification was by preparative high-performance liquid chromatography (HPLC) (Cosmosil Buckyprep [Nacalai USA, Inc., San Diego, CA], toluene 1 mL/min, λ 340 nm). HPLC with UV and radiochemical detection were used to determine the chemical purity and radiochemical purity and were both >99%. The specific activity was calculated using a calibration curve (HPLC) generated using sublimed C₆₀ (#379646, Sigma Aldrich, St. Louis, MO) to determine the C₆₀ content of a counted aliquot.

The dose formulation was prepared using a modification of the of Yamakoshi and co-workers procedure (Yamakoshi, et al. 1994). Specifically, a solution was prepared by sonicating [¹⁴C(U)]C₆₀ (2.52. mg) in toluene (3.15 mL) until dissolved, adding a solution of polyvinylpyrrolidone (PVP, Cat. #: 234257, Lot: MKBD2366V, Aldrich, St. Louis, MO) (314.6 mg) in CHCl₃ (Cat #: 048-4L, Lot AX767, B&J, Morristown, NJ) (6.292 mL) and stirring for 30 min. The volatiles were evaporated under N₂ and the residual solid was kept in a vacuum desiccator under high vacuum overnight (Edwards Model RV5, Crawley Sussex, England). Blank (no C₆₀) suspensions were likewise prepared by adding a solution of PVP (314.6 mg) in CHCl₃ (6.292 mL) to toluene (3.15 mL), stirring for 30 min, and evaporating the volatiles.

Dosing solution—All glassware was silanized prior to use. The dose formulation was prepared by the addition of saline (0.9% injectable, Lot J7H025, B. Braun Medical Inc., Irvine, CA) (18.0 mL) to the dried solid, and sonicating for 2 minutes to produce a suspension of 0.14 mg/mL of C₆₀ (with specific activity 5.5 mCi/mmol or 7.23 µCi/mg) in 5% PVP-saline. Rats were dosed with ~0.2 mg [¹⁴C(U)]C₆₀/kg body weight. Dosing of rodents was started within 30 min of preparation of the dosing solution; prior to dosing each animal the suspensions were sonicated 5 sec and vortexed 5 sec to ensure uniformity.

Characterization of dosing solution—Characterization of the dosing solution was done as previously described (Sumner, et al. 2015). Briefly, the suspensions, were analyzed for hydrodynamic diameter using a Malvern Zetasizer NanoZS (Malvern Instruments, Worcestershire, UK) with a 633 nm laser source, 173° detection angle. To mimic the dosing conditions measurements were made 30 min after preparation of the saline suspension and each suspension was sonicated 5 sec and vortexed 5 sec prior to transfer to the cell. Three independent measurements were conducted. The hydrodynamic diameter and the polydispersity index (PDI) of the particles in the suspension were obtained using CONTIN algorithm. The hydrodynamic diameter (Z-average) of the C₆₀ formulated in 5% PVP-saline suspensions, obtained using CONTIN algorithm, was 1,091nm with a PDI of 0.29; the particles were uniformly dispersed with a normal size distribution in the range of 615–1,718 nm. Further characterization by transmission electron microscopy (TEM) was obtained using an FEI Tecnai G2 Twin (Hillsboro, OR, USA). The sample was prepared by dipping TEM copper grids (prod#01822, Ted Pella, Redding, CA) into C₆₀ in 5% PVP-saline suspensions (prepared as described above) followed by thorough drying in a well-ventilated fume hood before imaging. A count of 22 separate C₆₀ in 5% PVP-saline suspensions agglomerates showed an average particle size of 26±7 nm.

Housing and Dosing of Pregnant and Lactating Rats

Sprague Dawley rats were obtained from Charles River, Raleigh, NC. Timed-mated pregnant dams arrived at either gestational day (GD) 5, 8 or 13. Lactating rats arrived at postnatal day (PND) 3. Pregnant dams were housed in groups of 2–3 in microisolator cages until dosed, and lactating rats were housed individually with their litters. Animals were provided food (Purina rodent chow 5002) and water ad libitum.

Pregnant dams were dosed to evaluate the distribution of radioactivity in dam and fetuses in five different groups covering different stages of pregnancy and time after exposure (Table 1). Pregnant rats received a single tail vein injection on either GD 11 (terminated after 24 hours [GD11–24h] or after 8 days [GD11–8d]), GD15 (terminated after 24 hours [GD15–24h] or after 4 days [GD15–4d]), or GD18 (terminated after 24 hours [GD18–24h]). A separate group of pregnant rats received vehicle control on GD11 (terminated 24 hours and 8 days after exposure), or PND8 (terminated 24 hours or 11 days post exposure). Pregnant dams were placed individually in all glass metabolism cages for 24 hours for collection of urine and feces (over dry ice) before termination. Urine and feces were stored at –80°C. Lactating rats were injected at PND 8 and tissue collected after 24 hours, 3 days or 11 days (PND8–24h, PND8–3d or PND8–11d).

Necropsy and Sample Collection and Processing

Rats were euthanized under CO₂ and blood was drawn by cardiac puncture. An aliquot of whole blood was reserved and the remainder was processed to plasma. During necropsy, utensils were wiped following each tissue removal. The liver, spleen, kidney, adipose near kidney (hereafter referred to as adipose), lung, heart, brown fat, brain, lymph nodes, uterus (with cervix and uterine horns), ovaries, bladder, bladder urine (lactating rats), stomach, cecum, small intestine, large intestine, digestive content, muscle (hindleg), pancreas, skin, bone, bone marrow, mammary tissue/gland, and residual carcass were collected. Wet weights of all samples were recorded at the time of collection.

Radioactivity Distribution

Data for animal weights, dose administered, sample weights and radioactivity were collected using the Debra™ system (Lablogic Systems Ltd, Sheffield, England). Radioactivity was determined using a Packard 1900CA Liquid Scintillation Counter (PerkinElmer, Waltham, MA). Urine aliquots were weighed into scintillation vials containing Ultima Gold™ scintillation cocktail (PerkinElmer, Waltham, MA). Feces, carcass and digestive tract were homogenized in 2N ethanolic sodium hydroxide or water (feces only). Tissues were dissolved with Soluene-350™, bleached by addition of perchloric acid and hydrogen peroxide and counted after addition of scintillation cocktail. Control tissue was analyzed as described above to determine background counts. Radioactivity limit of quantitation (LOQ) was designated at 3-fold above background.

The distribution of [¹⁴C(U)]C₆₀ was calculated as percentage of total recovered radioactivity and as tissue concentration, (ng equivalents/g tissue). Blood volume in rats was estimated to be 7.4% of body weight based on the literature, muscles to be 40.43%, bones 7.3%, and skin 19.03% (Brown, et al. 1997). Adipose tissue changes over the course of pregnancy and lactation in adult rats, and was estimated as; GD12=8.5271%, GD16=9.0362%, GD19=9.4179%, PND9=9.97%, PND11=8.80%, and PND19=7.21% the same as healthy adult non-pregnant rats (Brown, et al. 1997, Loccisano, et al. 2012, Naismith, et al. 1982, Yoon, et al. 2009).

Analysis of cytokines, chemokines and markers of cardiovascular injury

Levels of cytokines, chemokines and markers of cardiovascular injury were determined using plasma samples collected from rats following exposure to the formulated [¹⁴C(U)]C₆₀ or vehicle. Plasma concentrations of eotaxin, interleukins; IL-1β, IL-4, IL-5, IL-6, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), were quantified utilizing Milliplex MAP Cytokine/Chemokine and Cardiovascular Immunoassay panels (RECYTMAG-65K and MPXMCYTO70K, EMD Millipore, Billerica, MA, USA). Plasma concentrations of plasminogen activator inhibitor (PAI)-1, von Willebrand factor (vWF), soluble intercellular adhesion molecule 1 (ICAM-1) and soluble e-selectin were measured quantitatively with a Milliplex MAP Cardiovascular Immunoassay Panel (RCVD1-89kMCVD77K and MCVD177AK, EMD Millipore, Billerica, MA, USA). PAI-1, ICAM-1 and e-selectin were measured in pg/mL, and vWF in ng/mL. All assay plates were run according to the manufacturer's protocol with samples run in duplicate. Assays were analyzed using a

Luminex 200 (Luminex, Austin, TX, USA) and results are reported using Luminex xPONENT® software version 3.1. Results are reported as mean $\pm$ standard deviation and statistical comparison between experimental groups was assessed by student t-test for matched time-points or one-way ANOVA with a Bonferroni post hoc test for multiple comparisons using Prism 6.0 (San Diego, Ca); difference were considered significant if $p < 0.05$.

Detection of 8-Oxo-2'-deoxyguanosine (8-OH-dG) in Urine

A commercially available "New 8-OH-dG Check" ELISA kit from JAICA (Shizuoka, Japan, Supplier: Genox, Baltimore, MD, USA) was used in the study to determine the levels of 8-OH-dG in rat urine samples obtained from rats administered the formulated [$^{14}\text{C}(\text{U})$]C₆₀ or vehicle. Assays were performed following the instructions of the manufacturer (JAICA, Japan). Urine samples were centrifuged for 10 min at 5000xg and the clarified urine samples were diluted five-fold. 50 μL of the diluted samples was used in the assay. Results are reported as mean $\pm$ standard deviation, and statistical analysis were done using a Wilcoxon matched pairs t-test using GraphPad Prism 5.0 (San Diego, CA) and difference were considered significant if $p < 0.05$.

Metabolomics Analysis of Urine

NMR metabolomics analysis were done on 24 hours urine samples collected from pregnant rats receiving [$^{14}\text{C}(\text{U})$]C₆₀ or vehicle 24 hours (GD11–24h) or 8 days (GD11–8days) post administration. Samples for NMR metabolomics analysis were prepared by mixing an aliquot (540 μL) of rat urine with 60 μL of Chenomx ISTD solution (Chenomx, Edmonton, Alberta, Canada) containing two internal standards [4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) and imidazole] used for line shape analysis and chemical shift referencing, pH determination, and quantification using the library in NMR Suite 7.1 Professional software (Chenomx, Edmonton, Alberta, Canada). After vortexing for 30 sec, 500 μL of the NMR sample was transferred into 5 mm NMR tubes. ^1H NMR spectra were acquired using a Bruker Avance III 950 MHz NMR spectrometer (located at the David H. Murdock Research Institute at Kannapolis, NC, USA) using the first increment of a NOESY sequence (Beckonert, et al. 2007), with a 100 ms mixing time, 2 sec relaxation delay, a spectral width of 16 ppm, 32k data points, and 128 transients. The water resonance was suppressed using resonance irradiation during the relaxation delay and mixing time. All spectra were acquired at 25 °C, zero filled, and Fourier transformed after exponential multiplication with line broadening factor of 0.5. Phase and baseline of the spectra were manually corrected for each spectrum. The quality of each NMR spectrum was assessed for the level of noise and alignment of identified markers.

Metabolomics analysis was conducted as previously described (Church, et al. 2014, Pathmasiri, et al. 2012, Sumner, et al. 2009, Sumner, et al. 2010a, Sumner, et al. 2015, Sumner, et al. 2010f). Briefly, NMR data were processed by automated integration (increments of 0.04 ppm) over the spectral window (binning, using ACD 1D NMR Processor 12.0, ACD Labs, Toronto, Canada), excluding the region of water suppression, urea, and the DSS signal and each of the bins was normalized to the total integral of the bins of each spectrum. NMR binned data (bin region and integral value) were transported for data

reduction and visualization using SIMCA-P+ 12.0 (Umetrics, Umeå, Sweden). Principal component analysis (PCA) and orthogonal partial least squares projection to latent structures discriminant analysis (OPLS-DA) were conducted using SIMCA-P+ 12.0 for the binned data. Data were preprocessed by mean centering and Pareto scaling prior to multivariate data analysis. Loadings plots and variable importance for projection plots (VIP) were examined to determine the bins that best define the separation of study groups. Metabolites in bins (VIP = 1) were matched to the Chenomx NMR library, and directional changes identified using loadings plots in OPLS-DA. Marker metabolites associated with specific bins, that best differentiate the group were entered into a pathway mapping software package, GeneGo 5.0, MetaCore module (St. Joseph, MI).

RESULTS

Distribution of Radioactivity in the Pregnant Dam and Fetuses

Percentage of Recovered Radioactivity (Table 2)—Pregnant rats received a dose of $0.76 \pm 0.07 \mu\text{Ci}$, with $0.212 \pm 0.003 \text{ mg } [^{14}\text{C}(\text{U})]\text{C}_{60}/\text{kg}$. In pregnant rats, mean recoveries of radioactivity ranged from 84–95%. The elimination in urine and feces, was less than 2% at all time points. The vast majority of the administered radioactivity was recovered in the tissues and carcass digests. Of the collected tissues, the most significant distribution of radioactivity was to liver and lungs (Table 2). Both stage of pregnancy and time of termination after exposure played a role in distribution of radioactivity. Dams dosed on GD11 had higher distribution to the liver (39% at GD11–24h), compared with the dams exposed later in pregnancy (23% at GD18–24h). The opposite effect was observed in the lungs (24% at GD11–24h, and 42% at GD18–24h). The percentage of radioactivity recovered in placenta was higher in the later stage of pregnancy (2% was recovered at GD18–24h, compared to 0.05% at GD11–24h). At the same time radioactivity in the pooled fetuses was lower at the later stages compared with the earlier stages (0.2% at GD11–24h and 0.04% at GD18–24h), suggesting that at later stages in pregnancy the placenta becomes a more efficient barrier, diminishing maternal-fetal transfer. Levels at least 3-fold above background were also detected in perirenal adipose, bladder, muscle, spleen, kidney brain, heart, digestive tract, bone, ovaries, skin, uterus/vagina/cervix, lymph nodes, brown fat, pancreas, and fetuses. Because plasma constitutes approximately half of the blood volume, the potential for $[^{14}\text{C}(\text{U})]\text{C}_{60}$ binding to or uptake into red blood cells is considered negligible.

Tissue Concentrations (ng/g) (Table 3)—The distribution of the radioactivity in the pregnant tissues and fetuses expressed as tissue concentration (ng/g) was calculated assuming that all the radioactivity was due to $[^{14}\text{C}(\text{U})]\text{C}_{60}$ (Table 3). Lung had the highest concentration, followed by spleen and liver, with lower concentrations in the lymph nodes, bone marrow, digestive tract and kidney. The concentration in brown fat (43–62 ng/g) was higher than adipose (14–20 ng/g). The concentration of radioactivity was higher in the placenta and fetus at 24 hours post exposure, compared with later time points. The concentration of radioactivity in blood was highest at 24 hours post injection; while radioactivity was still in circulation up to 8 days after exposure.

Distribution of Radioactivity in the Lactating Rats and Pups

Percentage of Recovered Radioactivity (Table 4)—Lactating rats received a dose of $0.79 \pm 0.11 \mu\text{Ci}$, with $0.167 \pm 0.004 \text{ mg } [^{14}\text{C}(\text{U})]\text{C}_{60}/\text{kg}$. In lactating rats, mean recoveries of radioactivity administered ranged from 90–102 %. Recovery was primarily in the tissue and carcass digests obtained from lactating rats. The tissue recovery, expressed as percent of recovered radioactivity is reported in Table 4. The highest distribution of radioactivity was to the liver followed by the lungs, spleen and muscle. The percent recovered radioactivity in liver (69–71%), lung (8–11%) and spleen (5–6%) were similar for all three time points. While the percentage of recovered radioactivity in the liver was 2-fold higher in lactating rats compared to pregnant dams, the percentage in lungs was 2–5 fold lower. Levels at least 3-fold above background were also detected in perirenal adipose, bladder, muscle, kidney, brain, heart, digestive tract, bone, ovaries, marrow, skin, uterus/vagina/cervix, lymph nodes, brown fat, and mammary tissue. Percentage of recovered radioactivity was low, but at least 3-fold above background, in the milk at 24 hours (0.0006%) and 3 days (0.0004%) post administration and in the pups' liver (0.003–0.02%), spleen (0.0007–0.004%), lungs (0.002–0.02%), digestive tract (0.02–0.08%) and blood (0.0002–0.007%) at all three time points. As with pregnant dams, the plasma constitutes approximately half of the blood volume in lactating rats, and the potential for $[^{14}\text{C}(\text{U})]\text{C}_{60}$ binding to or uptake into red blood cells is considered negligible.

Tissue Concentrations (ng/g) (Table 5)—The distribution of radioactivity expressed as concentration (ng/g) in lactating rats were highest in spleen, followed by lung and liver, with lower concentrations in skin, lymph nodes, kidney and bone marrow (Table 5). While the pregnant dam had 2–6 fold higher concentration in the lungs compared to spleen (Table 3), the lactating dam had similar spleen:lung ratios of radioactivity concentration (Table 5). The concentration in blood, heart and bone decreased with time after exposure. As with the pregnant dams, the concentration of radioactivity in blood was highest in lactating rats at 24 hours and decreased with time, while the blood concentration in pups increase 10-fold in both genders. In the lactating rats, the blood concentrations of radioactivity were 48 ng/g at 24 h, 12 ng/g at 3 days, and 10 ng/g at 11 days. C_{60} was detected in milk at 24 h (2 ng/g), and 3 days (0.6 ng/g), but was below LOQ at 11 days after dosing. For male pups, the concentrations in blood were 0.4 ng/g at 24 hours, 5 ng/g at 3 days and 4 ng/g at 11 days, and for female pups the concentrations at 24 hours was 0.6 ng/g increasing to 5 ng/g at 3 days and 7ng/g at 11 days. The delay in radioactivity uptake after oral exposure may be attributed to the transition of the C_{60} through the dam to the pup via lactational transfer (secretion of radioactivity into milk), and a lag in absorption from the gastrointestinal tract in the pup.

Biomarkers of Inflammation, Cardiovascular Injury and Oxidative Stress after $[^{14}\text{C}(\text{U})]\text{C}_{60}$ Exposure

Cytokine and chemokine levels (Tables 6 and 7)—Eight cytokines and chemokines were evaluated in plasma from pregnant dams (Table 6) and lactating rats and pups (Table 7) post i.v. administration of $[^{14}\text{C}(\text{U})]\text{C}_{60}$ or vehicle. While the standard deviations were large for cytokines and chemokines levels for pregnant and lactating rats, an overall average decrease was measured for levels of Eotaxin, IL-4, IL-5, IL-6, and MCP-1 at

all time points. IL-1 β , TNF- α and VEGF were not observed. The cytokine and chemokine levels in both female and male pups were, below background at most time points.

Markers of cardiovascular injury (Table 8 and 9)—Comparing [$^{14}\text{C}(\text{U})$] C_{60} exposed pregnant dams with time matched vehicle groups showed no significant difference in the levels of PAI-1 and vWF (Table 8). The levels of PAI-1 increased when pregnant dams were exposed to [$^{14}\text{C}(\text{U})$] C_{60} later in pregnancy. For lactating rats, PAI-1 levels were higher than time matched vehicle groups 24 hours post exposure, but were similar to background level 11 days post exposure (Table 9). While PAI-1 had low levels in pups compared to the dam, the opposite was the case for vWF, where levels, on average, were higher in the pups. The vWF levels were similar in plasma from pups collected 24h after [$^{14}\text{C}(\text{U})$] C_{60} exposure compared with time matched vehicle groups. The vWF levels, on average were lower in plasma from pups collected 11 days after [$^{14}\text{C}(\text{U})$] C_{60} exposure compared with time matched vehicle groups, the decrease was significant for female pups.

Oxidative stress (Figure 1)—Comparison between the [$^{14}\text{C}(\text{U})$] C_{60} dosed pregnant rats, lactating rats and pups with the time match vehicle groups did not indicate significant elevation in 8-oxo-2'-deoxyguanosine (8-OH-dG).

Metabolomics Analysis of [$^{14}\text{C}(\text{U})$] C_{60} Exposure

Urine collected from pregnant dams at 24 hours (GD11–24h) or 8 days (GD11–8d) post administration of vehicle or [$^{14}\text{C}(\text{U})$] C_{60} was analyzed using NMR spectroscopy, which is robust and highly reproducible (Beckonert, et al. 2007). Multivariate analysis was used to determine metabolites that best differentiated the vehicle and [$^{14}\text{C}(\text{U})$] C_{60} animal groups. Metabolites were matched to the Chenomx library, and directional changes identified (Table 10). The VIP values and fold changes are available in Supplemental Tables 1 and 2. These selected metabolites were used in GeneGo pathway analysis. The three most significant pathways at GD11–24h were nicotine signaling in cholinergic neurons (including the metabolites choline and acetylcholine), N-acylethanolamines, HSRL5-transacylation pathway (including the metabolite choline), and muscle contraction_nNOS signaling in skeletal muscle (including the metabolite acetylcholine) (Figure 2a). The three most significant pathways at GD11–8d were vitamin B6 (including the metabolites 4-pyridoxate and pyridoxine), regulation of lipid metabolism (glycerol) and aminoacyl-tRNA biosynthesis (leucine and methionine) (Figure 2b). We have previously shown that i.v. administration of C_{60} formulated in 5% PVP-saline in pregnant Sprague Dawley dams at 24 hours post exposure caused increases in vascular tissue contractility by increasing stress generation in uterine artery, thoracic aorta and umbilical vein (Vidanapathirana, et al. 2014a). Real-Time PCR of aortic tissue homogenate showed a trend in increase of mRNA expression, but we found an increase in RhoA, ROCK2 and endothelial nitric oxide synthase (eNOS) protein expression in rat aortic endothelial cells at 12 hours exposure to C_{60} *in vitro* (Vidanapathirana, et al. 2014a). Based on these findings we explored the possible metabolic perturbations in the pathways involved with RhoA, RhoA-Rho kinase (ROCK), and eNOS. A GeneGo network analysis (2-steps) using the marker metabolites that are deemed to be important for distinguishing [$^{14}\text{C}(\text{U})$] C_{60} administered rats from those administered vehicle at GD11–24h. The network analysis mapped choline and acetylcholine (both increased) into

the ROCK and eNOS pathways (Figure 3). The results from this exploratory analysis warrants further investigations using larger sample size and target approach to understand mechanisms involving [$^{14}\text{C}(\text{U})$] C_{60} exposure and ROCK and eNOS pathways.

DISCUSSION

To provide data for modeling of potential risks associated with C_{60} exposure during pregnancy and lactation, the distribution of C_{60} was studied in pregnant dams and fetuses, and in lactating rats and pups. Pregnant and lactating rats were exposed to ~0.2 mg [$^{14}\text{C}(\text{U})$] C_{60} (formulated as a 5% PVP suspension in saline) or to 5% PVP saline vehicle via a single tail vein injection. The doses were similar to our previous study, were pregnant and lactating rats were exposed to a single intravenous injection of ~0.3 mg [$^{14}\text{C}(\text{U})$] C_{60} /kg and tissue distribution analyses after 24 or 48 hour. Samples in the study here were collected for distribution analysis at different stages of pregnancy and for up to 8 days post exposure as well as for lactating rats and pups for up to 11 days after exposure. We have previously shown maternal-fetal transfer of [$^{14}\text{C}(\text{U})$] C_{60} at GD15–24h, and transfer to pup via milk after 24 hours (Sumner, et al. 2010e). Distribution of [$^{14}\text{C}(\text{U})$] C_{60} in non-pregnant female rats and mice showed that radioactivity were detectable in over 20 tissues at 30 days post a single i.v. dose (Sumner, et al. 2015). The results presented here showed a maternal-fetal transfer of [$^{14}\text{C}(\text{U})$] C_{60} at all gestational phases, and a continuous transfer via milk to the pups over 11 days.

Examining the distribution of radioactivity over a time course in pregnant and lactating rats after a single dose, demonstrated that the distribution is primarily influenced by the gestational age in pregnant rats, and by the time between injection and termination in lactating rats. Pregnant dams had the highest percentage of total recovered radioactivity in liver, followed by lungs, when exposed early in pregnancy (GD11). However, when dams were exposed later in pregnancy (GD15) the highest percentage of radioactivity was recovered in lungs followed by liver. Following liver and lungs, the highest percentages of recovered radioactivity were found in bones, muscles, spleen, and skin. For lactating rats the rank order for distribution expressed as a percent of total recovered radioactivity was liver followed by lungs, and then spleen, muscles, bones and skin. The combination of the filtering capacity of alveolar macrophages and increased blood flow to the lungs during the later stages of pregnancy (Abduljalil, et al. 2012, Clark, et al. 1989, Costantine 2014) could explain the increased percentage of radioactivity recovered in lung at these time points. Elimination through urine and feces of radioactivity was limited in pregnant dams, and accounted for less than 2% of the administrated dose at any time point.

During the course of pregnancy in the rat, there are complex changes that occur, with the changes in the volume of the placenta, mammary glands, and developing fetus and blood flow associated with these organs and liver, heart, and kidneys (Buelke-Sam, et al. 1982a, Buelke-Sam, et al. 1982b). Comparison of the current distribution data with an earlier study of the distribution of [$^{14}\text{C}(\text{U})$] C_{60} formulated as a 5% PVP suspension in saline in non-pregnant female rats (Sumner, et al. 2015), showed that lung distribution of radioactivity at 24 hours post exposure was similar in non-pregnant rats and lactating rats (17% and 11%, respectively), but was much higher in pregnant dams (24% for GD11–24h, 41% for GD15–

24h and 42% for GD18–24h). The percentage of recovered radioactivity in liver at 24 hours after exposure in non-pregnant rats (55%) and in lactating rats (70%) was higher than in pregnant dams regardless of GD (39% for GD11–24h, 30% for GD15–24h, and 23% for GD18–24h). The recovered radioactivity at 24 hours in the spleen was 2-fold higher in non-pregnant rats (10%) than in pregnant rats (4–6%) or lactating rats (5%). These results indicate that some aspects (liver and lungs), but not all (spleen), of the distribution in pregnant female rats shortly after delivery is similar to the non-pregnant state. These findings indicate the importance of investigating distribution, not only during pregnancy, but also during lactation to study potential transfer from mother to off spring, but also to understand the unique physique of the lactating mother.

Transfer of radioactivity from mother to fetuses and offspring was observed in both pregnant and lactating rats. The results suggest that [$^{14}\text{C}(\text{U})\text{C}_{60}$] is transferred to fetuses through the placenta during pregnancy and to pups via the lactating rats' milk. The dose of a toxicant reaching the embryo or fetus is a critical consideration in developmental toxicity and will be important in the consideration of interspecies variability in response. The timing of exposure can lead to differences in the extraembryonic environment (Carney, et al. 2004). The timepoints used in this study correspond to the development of the chorioallantoic placenta in the rat, which aligns with the human placental development. Moreover it appears that radioactivity had been transferred to the pups within 24 hours, and was continually secreted into the dam's milk 3 days post exposure, resulting in accumulation of radioactivity in pups' liver, spleen and lungs. No radioactivity was detected in the milk at 11 day post exposure, but the radioactivity levels in pup spleen and lungs were increased over time, with decreased levels in the digestive tract, illustrating the delay in absorption of ingested [$^{14}\text{C}(\text{U})\text{C}_{60}$] from the gastrointestinal tract.

In an effort to understand how circulating biomarkers may be related to i.v. administration of [$^{14}\text{C}(\text{U})\text{C}_{60}$], we examined the circulating concentration of selected pro and anti-inflammatory cytokines and chemokines in the plasma of pregnant and lactating rats and their offspring. Cytokine levels were noted in [$^{14}\text{C}(\text{U})\text{C}_{60}$] exposed pregnant and lactating rats or offspring, consistent with previous reports of limited toxicity of EMNs such as gold nanoparticles (Rattanapinyopituk, et al. 2014, Yang, et al. 2012). For [$^{14}\text{C}(\text{U})\text{C}_{60}$] exposed dams, the highest cytokine levels were at 24 hours post exposure, gradually declining over time. The waning of cytokine levels associated with vehicle administration 1 and 8 day post administration was interesting but not unexpected. The observed reduction of the circulating cytokines measured between GD 12 and GD 19 pregnant dams is like associated with an overall suppressed immune responses mediated by change hormonal signaling with continued duration of the pregnancy (Kraus, et al. 2012, Vidanapathirana, et al. 2014a). The temporal pattern of reduction in cytokine levels were also evident in the results from the [$^{14}\text{C}(\text{U})\text{C}_{60}$] exposure which may suggest that the variances observed are largely due to physiological differences of individuals and their life stage rather than the exposure to the nanomaterial. Markers of cardiovascular injury, PAI-1 demonstrated a pattern of increase during pregnancy and reduction following delivery, a pattern normal with progression of pregnancy, while vWF plasma levels remained relatively unchanged with the pregnancy. It is interesting to note that the mean serum level of vWF are nearly twice as high as the lactating dam, but the relevance of this to any potential vascular function in the pups is unexplored in

this model. Furthermore i.v. exposure to C₆₀ formulated in PVP has been demonstrated to exacerbate cardiac ischemia/reperfusion injury in both male and non-pregnant female Sprague-Dawley rats (Thompson, et al. 2014). Potential cardiovascular toxicity was not detected in this study using PAI-1 and vWF as circulating biomarkers for deleterious cardiac impact (Vasan 2006). It is possible that circulating biomarkers such as PAI-1 and vWF are not sensitive enough for detecting possible cardiovascular stress as a result of [¹⁴C(U)]C₆₀ exposure. However it is also possible that exposure to [¹⁴C(U)]C₆₀ does not result in profound cardiovascular stress or increases in circulating cytokines and chemokines in pregnant Sprague-Dawley rats.

In this study we also found that [¹⁴C]C₆₀ exposure on average, but not significantly, caused elevated 8-OH-dG urine levels in pups indicating systemic oxidative stress. The level detected in female pups after 11 days of lactation post exposure of the dam was, on average, higher than that due to exposure to vehicle. While the literature contains numerous examples of γ -radiation induced oxidative stress (Inano and Onoda 2002, Kasai, et al. 1986, Mori, et al. 1993), no such examples were found for β -radiation. The soft β -irradiation by ¹⁴C in our study was more than a magnitude lower than what has been reported to cause elevated 8-OH-dG levels for γ -irradiation, and the biological responses observed here appears to be caused by [¹⁴C(U)]C₆₀ administration.

Metabolomics analysis of urine from pregnant rats injected at GD11 and collected 24 hours or 8 days (GD11–24h and GD11-8d, respectively) were conducted as a discovery tool to determine metabolites that were most important to differentiate between [¹⁴C(U)]C₆₀ exposure in early and late pregnancy. Interestingly, using metabolomics analysis, and GeneGo pathway mapping, it was found that two metabolites that differentiate the [¹⁴C(U)]C₆₀ and vehicle groups, choline and acetylcholine, are involved in a pathway related to Rho-Rho kinase signaling (RhoA, ROCK, eNOS), supporting our previous finding of vascular response to [¹⁴C(U)]C₆₀ in pregnant rats (Vidanapathirana, et al. 2014a).

Prior investigations of C₆₀ in pregnant rodents are few, and the possible transfer of ENMs between mother and offspring is, in general, not well understood. The presence of ENMs in the placenta might affect normal placenta development. Normal placental and fetal development requires effective maternal circulation, an element of which is the conversion of the spiral arteries from highly tortuous and thick-walled vessels to flaccid sinusoidal conduits of low resistance. At 10 to 12 weeks of gestation in a normal human pregnancy, the onset of maternal circulation in the placenta results in an increase in oxygen tension in the placenta, which has been associated with potentially increased oxidative stress (Jauniaux, et al. 2000). A distribution study of gold nanoparticles has shown that distribution had a strong dependence on the embryonic and placental maturation stage, so the earlier exposure resulted in higher percentage recovered in fetuses (Yang, et al. 2012). This supports our finding that the placenta develops into a more efficient barrier later in pregnancy and diminishes maternal-fetal transfer of ENMs. Also, other studies of gold nanoparticles demonstrated no toxicological responses in the maternal or fetal organs (Rattanapinyopituk, et al. 2014, Yang, et al. 2012). The reproductive development and distribution of silver nanoparticles and zinc oxide nanoparticles were studied in rats, and both materials showed transfer from mother to offspring (Jo, et al. 2013, Melnik, et al. 2013). Zinc oxide

nanoparticles exposure started at 2 weeks before mating and was continued until PND 4, resulting in reduced number of born/live pups, decreased body weights of pups and increase fatal resorption; nanoparticles were detected in the liver and kidneys of pups (Jo, et al. 2013). Carbon nanotubes (CNT), like C₆₀, are composed entirely of carbon, and can either be single walled or multi walled (SWCNT or MWCNT, respectively). Pegylated SWCNT with a length of 86±38 nm present in the placenta of pregnant mice, but not in the embryo were found to produce teratogenic effects (Campagnolo, et al. 2013). Oxidized MWCNT with a length of 1–2 µm and a width of 10–30 nm present in low levels in the fetuses, led to fewer and narrower blood vessels in the placenta (Qi, et al. 2014). SWCNT and MWCNT, regardless of formulation, were detected in the placenta in mice and caused damaging effect on the placenta development as well as teratogenic effects (Campagnolo, et al. 2013, Qi, et al. 2014). MWCNTs administered to pregnant rats i.t. (suspended in 10% clinical grade surfactant) or i.v. (suspended in dispersion media [rat serum albumin and 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) in phosphate buffered saline]) caused a reduction in mean fetal weight gain (Vidanapathirana, et al. 2014g). The translocation of nanoparticles across the placenta is not well understood. As review by Kulvietis et al., in vivo and ex vivo studies has demonstrated that NP passage through the placenta depends on the size and the surface coating of NP (Kulvietis, et al. 2011) Investigations of particles uptake in non-phagocytizing cells have indicated that NP enter the cells through endocytosis, in a size-dependent pathway manner, so particles of ~60 nm is transported via caveolin-mediated endocytosis, ~90 nm clathrin- and caveolin-independent endocytosis, and ~120 nm via clathrin-mediated endocytosis (Conner and Schmid 2003). One study showed that 20 and 50 nm gold nanoparticle administered i.v. to pregnant mice at GD16 and GD17 showed upregulation of clathrin- and caveolin-mediated endocytosis at the maternal-fetal barrier in mouse placenta (Rattanapinyopituk, et al. 2014). Since agglomeration is likely to happen in biological systems, the study indicates endocytosis involving both caveolin- and clathrin-mediated uptake. However, since studies have shown that 50, 80 and 240 nm polystyrene nanoparticles cross the ex-vivo placenta, but not 500 nm nanoparticles (Wick, et al. 2010), and 35 nm titanium dioxide nanoparticles and 70 nm silica, crosses the placenta through, whereas 300 nm silica nanoparticles does not (Yamashita, et al. 2011). [¹⁴C(U)]C₆₀ administrated i.v. in this study at different time of gestation, had a diameter of 26±7 nm in the dosing solution, crossed the placenta and radioactivity was detected in fetuses and fetal liver in all collected samples, supporting cellular uptake via endocytosis in the placenta of pregnant rats. Despite the variations in the observed impact and toxicity of EMN exposure in pregnant rodents, the data clearly indicate that reproductive development can be strongly affected by ENM exposure and that maternal-fetal transfer often takes place. These observations emphasize the need to understand the consequences of exposure to EMN during pregnancy and lactation.

This study demonstrates that [¹⁴C(U)]C₆₀ is transferred from female rats to their offspring both during pregnancy through the placenta to fetuses, and via milk to lactating pups. The distribution and organ specific distribution were different between pregnant and lactating rats. In the case of pregnant dams maternal-fetal transfer depends on both the stage of gestation and the elapsed time between exposure and termination. This study shows the

importance of understanding the distribution of ENMs both during pregnancy and lactating to health aspects of human exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by National Institute of Environmental Health Sciences (U19 ES019525), and National Institute of Diabetes and Digestive and Kidney Diseases (1U24DK097193-01). The authors are grateful to Zacher Acuff and Susan McRitchie for providing statistical assistance with the metabolomics data analysis.

Reference

- Abduljalil K, Furness P, Johnson TN, Rostami-Hodjegan A, Soltani H. Anatomical, physiological and metabolic changes with gestational age during normal pregnancy: a database for parameters required in physiologically based pharmacokinetic modelling. *Clinical pharmacokinetics*. 2012; 51:365–396. [PubMed: 22515555]
- Aschberger K, Johnston HJ, Stone V, Aitken RJ, Tran CL, Hankin SM, Peters SA, Christensen FM. Review of fullerene toxicity and exposure--appraisal of a human health risk assessment, based on open literature. *Regulatory toxicology and pharmacology* : RTP. 2010; 58:455–473. [PubMed: 20800639]
- Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, Nicholson JK. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature protocols*. 2007; 2:2692–2703. [PubMed: 18007604]
- Brown RP, Delp MD, Lindstedt SL, Rhomberg LR, Beliles RP. Physiological Parameter Values for Physiologically Based Pharmacokinetic Models. *Toxicology and industrial health*. 1997; 13:407–484. [PubMed: 9249929]
- Buelke-Sam J, Holson JF, Nelson CJ. Blood flow during pregnancy in the rat: II. Dynamics of and litter variability in uterine flow. *Teratology*. 1982a; 26:279–288. [PubMed: 7163977]
- Buelke-Sam J, Nelson CJ, Byrd RA, Holson JF. Blood flow during pregnancy in the rat: I. Flow patterns to maternal organs. *Teratology*. 1982b; 26:269–277. [PubMed: 7163976]
- Campagnolo L, Massimiani M, Palmieri G, Bernardini R, Sacchetti C, Bergamaschi A, Vecchione L, Magrini A, Bottini M, Pietroiusti A. Biodistribution and toxicity of pegylated single wall carbon nanotubes in pregnant mice. *Particle and fibre toxicology*. 2013; 10:21. [PubMed: 23742083]
- Carney EW, Scialli AR, Watson RE, DeSesso JM. Mechanisms regulating toxicant disposition to the embryo during early pregnancy: an interspecies comparison. *Birth defects research* . Part C, Embryo today : reviews. 2004; 72:345–360.
- Church RJ, Wu H, Mosedale M, Sumner SJ, Pathmasiri W, Kurtz CL, Pletcher MT, Eaddy JS, Pandher K, Singer M, Batheja A, Watkins PB, Adkins K, Harrill AH. A systems biology approach utilizing a mouse diversity panel identifies genetic differences influencing isoniazid-induced microvesicular steatosis. *Toxicological sciences : an official journal of the Society of Toxicology*. 2014; 140:481–492. [PubMed: 24848797]
- Clark SL, Cotton DB, Lee W, Bishop C, Hill T, Southwick J, Pivarnik J, Spillman T, DeVore GR, Phelan J, et al. Central hemodynamic assessment of normal term pregnancy. *American journal of obstetrics and gynecology*. 1989; 161:1439–1442. [PubMed: 2603895]
- Conner SD, Schmid SL. Regulated portals of entry into the cell. *Nature*. 2003; 422:37–44. [PubMed: 12621426]
- Costantine MM. Physiologic and pharmacokinetic changes in pregnancy. *Frontiers in pharmacology*. 2014; 5:65. [PubMed: 24772083]
- de Rijk EP, van Esch E, Flik G. Pregnancy dating in the rat: placental morphology and maternal blood parameters. *Toxicologic pathology*. 2002; 30:271–282. [PubMed: 11950171]

- Detmar J, Rennie MY, Whiteley KJ, Qu D, Taniuchi Y, Shang X, Casper RF, Adamson SL, Sled JG, Jurisicova A. Fetal growth restriction triggered by polycyclic aromatic hydrocarbons is associated with altered placental vasculature and AhR-dependent changes in cell death. *American journal of physiology. Endocrinology and metabolism*. 2008; 295:E519–E530. [PubMed: 18559983]
- Hemmingsen JG, Hougaard KS, Talsness C, Wellejus A, Loft S, Wallin H, Moller P. Prenatal exposure to diesel exhaust particles and effect on the male reproductive system in mice. *Toxicology*. 2009; 264:61–68. [PubMed: 19632294]
- Hougaard KS, Jensen KA, Nordly P, Taxvig C, Vogel U, Saber AT, Wallin H. Effects of prenatal exposure to diesel exhaust particles on postnatal development, behavior, genotoxicity and inflammation in mice. *Particle and fibre toxicology*. 2008; 5:3. [PubMed: 18331653]
- Inano H, Onoda M. Radioprotective action of curcumin extracted from *Curcuma longa* LINN: inhibitory effect on formation of urinary 8-hydroxy-2'-deoxyguanosine, tumorigenesis, but not mortality, induced by gamma-ray irradiation. *International journal of radiation oncology, biology, physics*. 2002; 53:735–743.
- Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN, Burton GJ. Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. *The American journal of pathology*. 2000; 157:2111–2122. [PubMed: 11106583]
- Jo E, Seo G, Kwon JT, Lee M, Lee B, Eom I, Kim P, Choi K. Exposure to zinc oxide nanoparticles affects reproductive development and biodistribution in offspring rats. *The Journal of toxicological sciences*. 2013; 38:525–530. [PubMed: 23824008]
- Kasai H, Crain PF, Kuchino Y, Nishimura S, Ootsuyama A, Tanooka H. Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenesis*. 1986; 7:1849–1851. [PubMed: 3769133]
- Kratschmer W, Lamb LD, Fostiropoulos K, Huffman DR. Solid C60: a new form of carbon. *Nature*. 1990; 347:5. [PubMed: 2203967]
- Kraus TA, Engel SM, Sperling RS, Kellerman L, Lo Y, Wallenstein S, Escribese MM, Garrido JL, Singh T, Loubeau M, Moran TM. Characterizing the pregnancy immune phenotype: results of the viral immunity and pregnancy (VIP) study. *Journal of clinical immunology*. 2012; 32:300–311. [PubMed: 22198680]
- Kulvietis V, Zalgeviciene V, Didziapetriene J, Rotomskis R. Transport of nanoparticles through the placental barrier. *The Tohoku journal of experimental medicine*. 2011; 225:225–234. [PubMed: 22052087]
- Langston, C. AAVPT Biennial Symposium/ACVIM Forum. Denver, CO: 2001. Pharmacotherapy in Neonates and Pregnant Animals.
- Li C, Taneda S, Taya K, Watanabe G, Li X, Fujitani Y, Nakajima T, Suzuki AK. Effects of in utero exposure to nanoparticle-rich diesel exhaust on testicular function in immature male rats. *Toxicology letters*. 2009; 185:1–8. [PubMed: 19100318]
- Loccisano AE, Campbell JL Jr, Butenhoff JL, Andersen ME, Clewell HJ 3rd. Evaluation of placental and lactational pharmacokinetics of PFOA and PFOS in the pregnant, lactating, fetal and neonatal rat using a physiologically based pharmacokinetic model. *Reprod Toxicol*. 2012; 33:468–490. [PubMed: 21872655]
- Melnik EA, Buzulukov YP, Demin VF, Demin VA, Gmoshinski IV, Tyshko NV, Tutelyan VA. Transfer of Silver Nanoparticles through the Placenta and Breast Milk during in vivo Experiments on Rats. *Acta naturae*. 2013; 5:107–115. [PubMed: 24307938]
- Mori T, Hori Y, Dizdaroglu M. DNA base damage generated in vivo in hepatic chromatin of mice upon whole body gamma-irradiation. *International journal of radiation biology*. 1993; 64:645–650. [PubMed: 7903331]
- Naismith DJ, Richardson DP, Pritchard AE. The utilization of protein and energy during lactation in the rat, with particular regard to the use of fat accumulated in pregnancy. *Br J Nutr*. 1982; 48:433–441. [PubMed: 7115665]
- Pathmasiri W, Pratt K, Collier D, Lutes L, McRitchie S, Sumner S. Integrating metabolomic signatures and psychosocial parameters in responsivity to an immersion treatment model for adolescent obesity. *Metabolomics Official journal of the Metabolomic Society*. 2012; 8

- Perera FP, Whyatt RM, Jedrychowski W, Rauh V, Manchester D, Santella RM, Ottman R. Recent developments in molecular epidemiology: A study of the effects of environmental polycyclic aromatic hydrocarbons on birth outcomes in Poland. *American journal of epidemiology*. 1998; 147:309–314. [PubMed: 9482506]
- Qi W, Bi J, Zhang X, Wang J, Wang J, Liu P, Li Z, Wu W. Damaging effects of multi-walled carbon nanotubes on pregnant mice with different pregnancy times. *Scientific reports*. 2014; 4:4352. [PubMed: 24619025]
- Ramdhan DH, Ito Y, Yanagiba Y, Yamagishi N, Hayashi Y, Li C, Taneda S, Suzuki AK, Watanabe G, Taya K, Kamijima M, Nakajima T. Nanoparticle-rich diesel exhaust may disrupt testosterone biosynthesis and metabolism via growth hormone. *Toxicology letters*. 2009; 191:103–108. [PubMed: 19699283]
- Rattanapinyopituk K, Shimada A, Morita T, Sakurai M, Asano A, Hasegawa T, Inoue K, Takano H. Demonstration of the Clathrin- and Caveolin-Mediated Endocytosis at the Maternal-Fetal Barrier in Mouse Placenta after Intravenous Administration of Gold Nanoparticles. *Journal of Veterinary Medical Science*. 2014; 76:377–387. [PubMed: 24257253]
- Scrivens WA, Tour JM, Creek KE, Pirisi L. Synthesis of ¹⁴C-Labeled C60, Its Suspension in Water, and Its Uptake by Human Keratinocytes. *Journal of the American Chemical Society*. 1994; 116:2.
- Shimizu M, Tainaka H, Oba T, Mizuo K, Umezawa M, Takeda K. Maternal exposure to nanoparticulate titanium dioxide during the prenatal period alters gene expression related to brain development in the mouse. *Particle and fibre toxicology*. 2009; 6:20. [PubMed: 19640265]
- Sumner S, Snyder R, Burgess J, Myers C, Tyl R, Sloan C, Fennell T. Metabolomics in the assessment of chemical-induced reproductive and developmental outcomes using non-invasive biological fluids: application to the study of butylbenzyl phthalate. *Journal of applied toxicology : JAT*. 2009; 29:703–714. [PubMed: 19731247]
- Sumner SC, Fennell TR, Snyder RW, Taylor GF, Lewin AH. Distribution of carbon-14 labeled C60 ([¹⁴C]C60) in the pregnant and in the lactating dam and the effect of C60 exposure on the biochemical profile of urine. *Journal of applied toxicology : JAT*. 2010a; 30:354–360. [PubMed: 20063269]
- Sumner SCJ, Fennell TR, Snyder RW, Taylor GF, Lewin AH. Distribution of carbon-14 labeled C60 ([¹⁴C]C60) in the pregnant and in the lactating dam and the effect of C60 exposure on the biochemical profile of urine. *Toxicol. J. Appl*. 2010e; 30:354–360.
- Sumner SCJ, Snyder R, Wingard CJ, Mortensen NP, Holland NA, Shannahan JH, Dhungana S, Pathmasiri W, Han L, Lewin AH, Fennell TR. Distribution and biomarkers of Carbon-14 labeled fullerene C60 ([¹⁴C(U)]C60) in female rats and mice for up to 30 days after intravenous exposure. *Journal of Applied Toxicology*. 2015 Epub ahead of print.
- Sumner SJ, Burgess JP, Snyder RW, Popp JA, Fennell TR. Metabolomics of urine for the assessment of microvesicular lipid accumulation in the liver following isoniazid exposure. *Metabolomics : Official journal of the Metabolomic Society*. 2010f; 6:238–249. [PubMed: 21057652]
- Thompson LC, Urankar RN, Holland NA, Vidanapathirana AK, Pitzer JE, Han L, Sumner SJ, Lewin AH, Fennell TR, Lust RM, Brown JM, Wingard CJ. C60 exposure augments cardiac ischemia/reperfusion injury and coronary artery contraction in sprague dawley rats. *Toxicological sciences : an official journal of the Society of Toxicology*. 2014; 138:365–378. [PubMed: 24431213]
- Tsuchiya T, Oguri I, Yamakoshi YN, Miyata N. Novel harmful effects of [60]fullerene on mouse embryos in vitro and in vivo. *FEBS letters*. 1996; 393:139–145. [PubMed: 8804443]
- Vasan RS. Biomarkers of cardiovascular disease: molecular basis and practical considerations. *Circulation*. 2006; 113:2335–2362. [PubMed: 16702488]
- Vidanapathirana AK, Thompson LC, Mann EE, Odom JT, Holland NA, Sumner AJ, Han L, Lewin AH, Fennell TR, Brown JM, Wingard CJ. PVP formulated Fullerene (C60) increases Rho-kinase dependent Vascular Tissue Contractility in Pregnant Sprague Dawley Rats. *Reproductive Toxicology*. 2014a; 49:86–100. [PubMed: 25088243]
- Vidanapathirana AK, Thompson LC, Odom J, Holland NA, Sumner SJ, Fennell TR, Brown JM, Wingard CJ. Vascular Tissue Contractility Changes Following Late Gestational Exposure to Multi-Walled Carbon Nanotubes or their Dispersing Vehicle in Sprague Dawley Rats. *J Nanomed Nanotechnol*. 2014; 5 DOI: <http://dx.doi.org/10.4172/2157-7439.1000201>.

- Wick P, Malek A, Manser P, Meili D, Maeder-Althaus X, Diener L, Diener PA, Zisch A, Krug HF, von Mandach U. Barrier capacity of human placenta for nanosized materials. *Environmental health perspectives*. 2010; 118:432–436. [PubMed: 20064770]
- Yamakoshi YN, Yagami T, Fukuhara K, Sueyoshi S, Miyata N. Solubilization of Fullerenes into Water with Polyvinylpyrrolidone Applicable to Biological Tests. *Journal of the Chemical Society, Chemical Communications*. 1994
- Yamashita K, Yoshioka Y, Higashisaka K, Mimura K, Morishita Y, Nozaki M, Yoshida T, Ogura T, Nabeshi H, Nagano K, Abe Y, Kamada H, Monobe Y, Imazawa T, Aoshima H, Shishido K, Kawai Y, Mayumi T, Tsunoda S, Itoh N, Yoshikawa T, Yanagihara I, Saito S, Tsutsumi Y. Silica and titanium dioxide nanoparticles cause pregnancy complications in mice. *Nature Nanotechnology*. 2011; 6:321–328.
- Yamashita K, Yoshioka Y, Pan H, Taira M, Ogura T, Nagano T, Aoyama M, Nagano K, Abe Y, Kamada H, Tsunoda SI, Aoshima H, Nabeshi H, Yoshikawa T, Tsutsumi Y. Biochemical and hematologic effects of polyvinylpyrrolidone-wrapped fullerene C60 after oral administration. *Die Pharmazie*. 2013; 68:54–57. [PubMed: 23444781]
- Yang H, Sun C, Fan Z, Tian X, Yan L, Du L, Liu Y, Chen C, Liang XJ, Anderson GJ, Keelan JA, Zhao Y, Nie G. Effects of gestational age and surface modification on materno-fetal transfer of nanoparticles in murine pregnancy. *Scientific reports*. 2012; 2:847. [PubMed: 23150793]
- Yoon M, Nong A, Clewell HJ 3rd, Taylor MD, Dorman DC, Andersen ME. Evaluating placental transfer and tissue concentrations of manganese in the pregnant rat and fetuses after inhalation exposures with a PBPK model. *Toxicological sciences : an official journal of the Society of Toxicology*. 2009; 112:44–58. [PubMed: 19726578]

Figure 1. 8-OH-dG (mean $\pm$ standard deviation) in urine of a) pregnant dams and b) lactating dams administered [$^{14}\text{C}(\text{U})$] C_{60} (solid bar) or vehicle (grey bar) via tail vein injection, and in urine of resulting c) male or d) female pups.

GD11-24h

a)

GD11-8d

b)

Figure 2. Metabolites in urine associated with $[^{14}\text{C}(\text{U})]\text{C}_{60}$ (formulated with 5% PVP-saline) exposure in pregnant rats were involved in multiple pathways, showing the ten most significant pathways at a) GD11-24h and b) GD11-8d.

Figure 3.

Metabolites (choline and acetylcholine) were elevated in urine from $[^{14}\text{C}(\text{U})]\text{C}_{60}$ (formulated with 5% PVP-saline) exposed pregnant rats at GD11-24h compared with control. These metabolites map to pathways involved Rho-Rho kinase signaling (RhoA, ROCK, eNOS).

Table 1

Rats received a single tail vein injection of [$^{14}\text{C}(\text{U})$] C_{60} (formulated in a 5% PVP-saline solution) or vehicle, (n = 3).

	Dose Administered	Dose Day – Termination Time
Pregnant dams	Vehicle	GD ^a 11-24h ^b
	Vehicle	GD11-8d
	[$^{14}\text{C}(\text{U})$] C_{60}	GD11-24h
	[$^{14}\text{C}(\text{U})$] C_{60}	GD11-8d
	[$^{14}\text{C}(\text{U})$] C_{60}	GD15-24h
	[$^{14}\text{C}(\text{U})$] C_{60}	GD15-4d
	[$^{14}\text{C}(\text{U})$] C_{60}	GD18-24h
Lactating rats	Vehicle	PND ^c 8-24h
	Vehicle	PND8-11d
	[$^{14}\text{C}(\text{U})$] C_{60}	PND8-24h
	[$^{14}\text{C}(\text{U})$] C_{60}	PND8-3d
	[$^{14}\text{C}(\text{U})$] C_{60}	PND8-11d

^aGD = gestation day

^bHours(h)/days(d) between dose administration and termination

^cPND = postnatal day

Table 2

The percentage of total recovered radioactivity (average $\pm$ standard deviation) in tissue digests and fluids collected from pregnant dams and fetuses following a single tail vein injection of [^{14}C](U)[C₆₀] (formulated with 5% PVP-saline) at different gestation days (GD11, GD15 or GD18) (dams, $n = 3$).

Sample	GD11-24h	GD11-8d	GD15-24h	GD15-4d	GD18-24h
Adipose ^a	0.909 $\pm$ 0.00636	0.800 $\pm$ 0.124	0.885 $\pm$ 0.0844	0.677 $\pm$ 0.130	0.796 $\pm$ 0.0871
Bladder	0.00758 $\pm$ 0.00199	0.00942 $\pm$ 0.00226	0.00995 $\pm$ 0.00493	0.00664 $\pm$ 0.00211	0.00763 $\pm$ 0.00129
Muscle ^b	6.29 $\pm$ 1.77	3.04 $\pm$ 1.37	5.96 $\pm$ 1.05	6.62 $\pm$ 3.19	6.74 $\pm$ 0.901
Blood ^c	5.78 $\pm$ 0.767	0.142 $\pm$ 0.0633	4.61 $\pm$ 0.702	0.619 $\pm$ 0.0551	6.93 $\pm$ 0.993
Spleen	5.67 $\pm$ 0.367	7.50 $\pm$ 2.03	4.87 $\pm$ 0.275	5.02 $\pm$ 1.41	4.18 $\pm$ 0.552
Liver	39.2 $\pm$ 9.97	43.4 $\pm$ 4.02	29.5 $\pm$ 3.38	35.4 $\pm$ 6.08	23.0 $\pm$ 7.35
Kidney	1.49 $\pm$ 0.0644	0.682 $\pm$ 0.173	1.08 $\pm$ 0.112	0.698 $\pm$ 0.129	1.11 $\pm$ 0.0210
Brain	0.0781 $\pm$ 0.0110	0.0138 $\pm$ 0.00866	0.0888 $\pm$ 0.00402	0.0344 $\pm$ 0.0227	0.0920 $\pm$ 0.0139
Heart	0.248 $\pm$ 0.0323	0.150 $\pm$ 0.0874	0.195 $\pm$ 0.0411	0.118 $\pm$ 0.0377	0.254 $\pm$ 0.0450
Lung	23.7 $\pm$ 5.64	30.8 $\pm$ 9.76	41.0 $\pm$ 4.55	39.0 $\pm$ 1.03	42.0 $\pm$ 11.2
Digestive tract ^d	2.63 $\pm$ 0.772	1.79 $\pm$ 1.19	1.34 $\pm$ 0.294	1.09 $\pm$ 0.446	2.00 $\pm$ 0.526
Bone ^e	6.82 $\pm$ 1.96	6.72 $\pm$ 1.78	5.04 $\pm$ 0.413	5.16 $\pm$ 0.625	5.71 $\pm$ 0.916
Marrow	0.0283 $\pm$ 0.0185	0.0339 $\pm$ 0.0193	0.0182 $\pm$ 0.00666	0.0381 $\pm$ 0.0173	0.0317 $\pm$ 0.00960
Ovaries	0.0594 $\pm$ 0.0132	0.0504 $\pm$ 0.0123	0.0630 $\pm$ 0.00910	0.0465 $\pm$ 0.0077	0.0641 $\pm$ 0.00554
Skin ^f	3.87 $\pm$ 0.628	3.15 $\pm$ 0.644	2.59 $\pm$ 0.259	3.08 $\pm$ 0.550	2.42 $\pm$ 0.312
Uterus/Vagina/Cervix	0.313 $\pm$ 0.0872	0.535 $\pm$ 0.139	0.427 $\pm$ 0.0637	0.520 $\pm$ 0.0931	0.340 $\pm$ 0.00729
Thoracic Lymph Nodes	0.0235 $\pm$ 0.0181	0.0185 $\pm$ 0.00608	0.0158 $\pm$ 0.0108	0.0213 $\pm$ 0.0106	0.0155 $\pm$ 0.00662
Mesen Lymph Nodes	0.0501 $\pm$ 0.0147	0.0667 $\pm$ 0.0173	0.0452 $\pm$ 0.00728	0.0474 $\pm$ 0.0128	0.0362 $\pm$ 0.0157
Brown Fat	0.0347 $\pm$ 0.0239	0.0317 $\pm$ 0.0193	0.0168 $\pm$ 0.00704	0.0204 $\pm$ 0.0164	0.00991 $\pm$ 0.00408
Pancreas	0.153 $\pm$ 0.0145	0.280 $\pm$ 0.0735	0.207 $\pm$ 0.0134	0.187 $\pm$ 0.0732	0.152 $\pm$ 0.0483
Placenta	0.0493 $\pm$ 0.0252	0.498 $\pm$ 0.181	0.693 $\pm$ 0.00915	0.992 $\pm$ 0.122	2.30 $\pm$ 0.725
Pooled Fetuses ^g	0.204 $\pm$ 0.0552	0.0242 $\pm$ 0.00790 ⁱ	0.0248 $\pm$ 0.0183	0.0463 $\pm$ 0.00943	0.0372 $\pm$ 0.0136
Fetal Blood	N.C. ^h	0.00124 $\pm$ 0.000217 ⁱ	N.C.	0.00141 $\pm$ 0.000670	0.00157 $\pm$ 0.000394
Fetal Liver	N.C.	0.00201 $\pm$ 0.000417 ⁱ	0.00160 $\pm$ 0.000081	0.00261 $\pm$ 0.00052	0.00435 $\pm$ 0.00132

^a Adipose tissue were estimated as; GD12=8.5271%, GD16=9.0362%, and GD19=9.4179%.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

b_j Muscles were estimated as a total of 40.43% of body weight.

c_j The total estimated blood radioactivity assuming 7.4% body weight as blood for rats.

d_j Digestive Tract comprised of stomach, cecum, small and large intestines and digestive content.

e_j Bones were assumed to be 7.3% of body weight.

f_j Skin was estimated to be 19.03% of total body weight.

g_j The average total radioactivity recovered in all fetuses from each litter.

h_j N.C. = not collected

i_j Average and standard deviation of two litters.

Table 3

Concentration (ng/g tissue) of [$^{14}\text{C}(\text{U})$] C_{60} (average $\pm$ standard deviation) in tissue digests and fluids collected from pregnant dams and fetuses following a single tail vein injection of [$^{14}\text{C}(\text{U})$] C_{60} (formulated with 5% PVP-saline) at different gestation days (GD11, GD15 or GD18) (dams, $n = 3$).

Sample	GD11-24h	GD11-8d	GD15-24h	GD15-4d	GD18-24h
Adipose	15.1 $\pm$ 2.61	14.5 $\pm$ 1.94	19.6 $\pm$ 3.04	13.8 $\pm$ 1.85	14.2 $\pm$ 1.40
Bladder	32.7 $\pm$ 7.37	31.3 $\pm$ 2.03	49.8 $\pm$ 5.25	29.3 $\pm$ 1.84	36.2 $\pm$ 5.46
Muscle	21.2 $\pm$ 0.614	12.6 $\pm$ 4.61	29.3 $\pm$ 3.51	31.0 $\pm$ 12.5	28.1 $\pm$ 5.54
Blood	110 $\pm$ 16.1	3.20 $\pm$ 1.22	124 $\pm$ 13.5	16.1 $\pm$ 0.670	156 $\pm$ 18.3
Spleen	3,194 $\pm$ 1,199	5,328 $\pm$ 649	4,568 $\pm$ 1,727	4,303 $\pm$ 891	3,610 $\pm$ 363
Liver	1,534 $\pm$ 752	1,319 $\pm$ 77.5	1,602 $\pm$ 402	1,414 $\pm$ 341	831 $\pm$ 182
Kidney	254 $\pm$ 59.3	150 $\pm$ 30.2	317 $\pm$ 76.2	186 $\pm$ 32.3	298 $\pm$ 34.0
Brain	16.7 $\pm$ 5.06	3.23 $\pm$ 1.89	26.7 $\pm$ 1.93	9.83 $\pm$ 5.59	27.1 $\pm$ 3.15
Heart	108 $\pm$ 16.2	65.8 $\pm$ 32.0	110 $\pm$ 11.0	60.4 $\pm$ 17.8	132 $\pm$ 21.7
Lung	7,748 $\pm$ 2,387	12,645 $\pm$ 6,264	19,192 $\pm$ 2,291	18,144 $\pm$ 2,700	21,033 $\pm$ 10,208
Digestive tract ^a	313 $\pm$ 42.1	321 $\pm$ 109	253 $\pm$ 30.6	175 $\pm$ 14.1	359 $\pm$ 41.8
Bone	128 $\pm$ 14.6	155 $\pm$ 26.7	139 $\pm$ 24.4	136 $\pm$ 11.1	130 $\pm$ 6.73
Ovaries	172 $\pm$ 83.0	107 $\pm$ 10.2	189 $\pm$ 31.5	110 $\pm$ 11.2	158 $\pm$ 11.2
Marrow	600 $\pm$ 169	679 $\pm$ 168	530 $\pm$ 162	642 $\pm$ 54.4	635 $\pm$ 56.4
Skin	28.3 $\pm$ 2.47	28.3 $\pm$ 5.43	27.2 $\pm$ 2.92	31.1 $\pm$ 3.55	21.3 $\pm$ 2.32
Uterus/Vagina/Cervix	71.2 $\pm$ 20.3	56.0 $\pm$ 5.89	82.8 $\pm$ 16.2	70.4 $\pm$ 2.79	39.5 $\pm$ 8.76
Thoracic Lymph Nodes	255 $\pm$ 70.0	199 $\pm$ 35.2	241 $\pm$ 13.4	206 $\pm$ 33.7	191 $\pm$ 9.63
Mesen Lymph Nodes	369 $\pm$ 249	315 $\pm$ 21.2	296 $\pm$ 35.4	300 $\pm$ 70.6	218 $\pm$ 78.9
Brown Fat	61.8 $\pm$ 42.0	57.5 $\pm$ 27.2	58.0 $\pm$ 15.2	58.9 $\pm$ 31.5	42.7 $\pm$ 17.0
Pancreas	43.7 $\pm$ 16.4	76.9 $\pm$ 16.6	80.1 $\pm$ 10.7	58.1 $\pm$ 7.34	56.7 $\pm$ 14.6
Placenta	94.3 $\pm$ 18.9	29.8 $\pm$ 2.90	122 $\pm$ 6.85	94.9 $\pm$ 8.24	174 $\pm$ 1.05
Pooled Fetuses	83.1 $\pm$ 4.58	0.789 $\pm$ 0.00424 ^c	7.84 $\pm$ 5.18	3.21 $\pm$ 0.717	2.13 $\pm$ 0.292
Fetal Blood	N.C. ^b	1.86 $\pm$ 0.281 ^c	N.C.	3.46 $\pm$ 1.21	9.69 $\pm$ 4.873
Fetal Liver	N.C.	1.282 $\pm$ 0.0120 ^c	7.19 $\pm$ 1.54	5.83 $\pm$ 0.557	5.07 $\pm$ 1.41

^a Digestive Tract comprised of stomach, cecum, small and large intestines and digestive content.

^b N.C. = not collected

^c Average and standard deviation of two liters.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 4

The percentage of total recovered radioactivity (average $\pm$ standard deviation) in tissue digests and fluids collected from the lactating rats and their pups following a single tail vein injection of [$^{14}\text{C}(\text{U})$] C_{60} (formulated with 5% PVP-saline) to dams at postnatal day 8 (adult rats, $n = 3$ and pups, $n = 30$).

Sample	Rats		
	PND8-24h	PND8-3d	PND8-11d
Adipose ^a	1.13 $\pm$ 1.51	0.986 $\pm$ 0.526	1.82 $\pm$ 2.12
Bladder	0.00317 $\pm$ 0.000475	0.00383 $\pm$ 0.000789	0.00789 $\pm$ 0.00502
Muscle ^b	4.09 $\pm$ 1.13	4.13 $\pm$ 0.613	16.2 $\pm$ 19.2
Blood ^c	2.17 $\pm$ 0.311	0.839 $\pm$ 0.0827	1.04 $\pm$ 0.819
Spleen	5.42 $\pm$ 1.27	5.72 $\pm$ 0.726	4.75 $\pm$ 0.651
Liver	69.7 $\pm$ 3.15	71.0 $\pm$ 0.598	68.7 $\pm$ 20.0
Kidney	0.785 $\pm$ 0.0725	0.855 $\pm$ 0.0270	0.762 $\pm$ 0.320
Brain	0.0401 $\pm$ 0.0227	0.0221 $\pm$ 0.00358	0.0875 $\pm$ 0.0934
Heart	0.135 $\pm$ 0.0476	0.0913 $\pm$ 0.0143	0.0743 $\pm$ 0.0588
Lung	11.3 $\pm$ 1.72	8.98 $\pm$ 0.841	7.91 $\pm$ 2.65
Digestive tract ^d	0.665 $\pm$ 0.116	1.10 $\pm$ 0.396	0.855 $\pm$ 0.200
Bone ^e	2.75 $\pm$ 0.894	2.92 $\pm$ 1.07	1.95 $\pm$ 0.712
Marrow	0.00610 $\pm$ 0.000452	0.00833 $\pm$ 0.00309	0.0111 $\pm$ 0.00505
Ovaries	0.0122 $\pm$ 0.00560	0.0169 $\pm$ 0.000904	0.0156 $\pm$ 0.00378
Skin ^f	1.20 $\pm$ 0.265	2.12 $\pm$ 0.362	1.73 $\pm$ 0.548
Uterus/Vagina/Cervix	0.0187 $\pm$ 0.00482	0.0236 $\pm$ 0.00200	0.0792 $\pm$ 0.0626
Thoracic Lymph Nodes	0.00597 $\pm$ 0.00392	0.00823 $\pm$ 0.00770	0.0182 $\pm$ 0.0124
Mesen Lymph Nodes	0.0291 $\pm$ 0.00999	0.0292 $\pm$ 0.00991	0.0286 $\pm$ 0.00633
Brown Fat	0.00915 $\pm$ 0.00740	0.0159 $\pm$ 0.0155	0.00571 $\pm$ 0.000889
Mammary Tissue	0.0207 $\pm$ 0.0131	0.0150 $\pm$ 0.00811	0.0103 $\pm$ 0.00558
Milk	0.000640 $\pm$ 0.000135	0.000386 $\pm$ 0.000139	<LOQ ^g
Pup Liver	0.00338 $\pm$ 0.00115	0.0181 $\pm$ 0.0199	0.0232 $\pm$ 0.0174 ⁱ
Pup Spleen	0.000680 $\pm$ 0.000306	0.00161 $\pm$ 0.000600	0.00358 $\pm$ 0.00315 ⁱ
Pup Lung	0.00150 $\pm$ 0.000938	0.00365 $\pm$ 0.00124	0.0207 $\pm$ 0.0234 ⁱ
Pup Digestive Tract	0.0408 $\pm$ 0.0480	0.0772 $\pm$ 0.0434	0.0186 $\pm$ 0.00900 ⁱ
Pup M Blood	0.000578 ^h	0.00433 $\pm$ 0.000562	0.00309 $\pm$ 0.00125
Pup F Blood	0.000763 ^h	0.00426 $\pm$ 0.000690	0.00761 $\pm$ 0.00889

^a Adipose tissue were estimated as; PND9=9.97%, PND11=8.80%, and PND19=7.21%

^b Muscles were estimated as a total of 40.43% of body weight.

^c The total estimated blood radioactivity assuming 7.4% body weight as blood for rats.

^d Digestive Tract comprised of stomach, cecum, small and large intestines and digestive content.

^e Bones were assumed to be 7.3% of body weight.

^f Skin was estimated to be 19.03% of total body weight.

^g Radioactivity below LOQ

^h One litter.

ⁱ Average and standard deviation of two litters

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 5

Concentration (ng/g tissue) of [$^{14}\text{C}(\text{U})$] C_{60} (average $\pm$ standard deviation) in tissue digests and fluids collected from lactating rats and pups following a single tail vein injection of [$^{14}\text{C}(\text{U})$] C_{60} (formulated with 5% PVP-saline) at PND8 and tissue collection at different time points (PND9, PND11 and PND19) (adult rats, n = 3, pups = 30).

Sample	Dams		
	PND8-24h	PND8-3d	PND8-11d
Adipose	20.2 $\pm$ 27.2	11.4 $\pm$ 3.98	15.5 $\pm$ 20.9
Bladder	14.3 $\pm$ 2.08	9.24 $\pm$ 2.10	28.3 $\pm$ 24.2
Muscle	17.9 $\pm$ 5.27	11.2 $\pm$ 3.24	24.5 $\pm$ 33.6
Blood	47.7 $\pm$ 4.53	12.4 $\pm$ 3.06	9.66 $\pm$ 9.35
Spleen	5,288 $\pm$ 1,577	3,367 $\pm$ 902	2,548 $\pm$ 1,113
Liver	2,832 $\pm$ 382	1,480 $\pm$ 324	1,374 $\pm$ 829
Kidney	203 $\pm$ 18.4	133 $\pm$ 31.4	99.9 $\pm$ 26.6
Brain	11.5 $\pm$ 6.03	4.10 $\pm$ 1.10	9.61 $\pm$ 11.1
Heart	63.9 $\pm$ 11.7	26.9 $\pm$ 2.17	18.9 $\pm$ 11.8
Lung	5,172 $\pm$ 892	2,475 $\pm$ 662	2,524 $\pm$ 2,000
Digestive tract ^a	74.6 $\pm$ 19.8	94.3 $\pm$ 60.7	39.9 $\pm$ 41.3
Bone	66.3 $\pm$ 21.9	42.2 $\pm$ 13.4	24.7 $\pm$ 2.69
Ovaries	56.7 $\pm$ 4.91	42.7 $\pm$ 3.14	38.1 $\pm$ 18.4
Marrow	257 $\pm$ 21.9	221 $\pm$ 90.2	161 $\pm$ 61.7
Skin	11.2 $\pm$ 2.63	11.8 $\pm$ 0.795	7.06 $\pm$ 6.21
Uterus/Vagina/Cervix	19.0 $\pm$ 1.80	15.0 $\pm$ 0.720	28.7 $\pm$ 27.1
Thoracic Lymph Nodes	159 $\pm$ 38.2	138 $\pm$ 42.3	194 $\pm$ 121
Mesen Lymph Nodes	226 $\pm$ 57.3	197 $\pm$ 121	170 $\pm$ 126
Brown Fat	26.2 $\pm$ 10.1	21.0 $\pm$ 4.87	10.7 $\pm$ 11.3
Mammary Tissue	30.4 $\pm$ 4.99	18.3 $\pm$ 3.64	14.9 $\pm$ 11.3
Milk	2.08 $\pm$ 0.23	0.631 $\pm$ 0.058	<LOQ ^b
Pup Liver	2.86 $\pm$ 0.00291	6.15 $\pm$ 0.0139	4.12 $\pm$ 1.93 ^d
Pup Spleen	2.79 $\pm$ 0.00131	0.00166 $\pm$ 0.00116	5.35 $\pm$ 2.97 ^d
Pup Lung	0.0220 $\pm$ 0.0401	2.74 $\pm$ 0.884	12.4 $\pm$ 15.9 ^d
Pup Digestive Tract	4.20 $\pm$ 3.77	15.9 $\pm$ 6.30	1.26 $\pm$ 0.448 ^d
Pup M Blood	0.827 ^c	4.91 $\pm$ 0.758	3.48 $\pm$ 0.408
Pup F Blood	1.154 ^c	4.86 $\pm$ 1.24	7.26 $\pm$ 7.06

^a Digestive Tract comprised of stomach, cecum, small and large intestines and digestive content.

^b Radioactivity below LOQ

^c Average and standard deviation of two litters.

^d Average and standard deviation of two litters.

Table 6

Cytokine/Chemokine expression in pregnant dams. All data are reported as mean $\pm$ standard deviation pg/mL of plasma duplicates (n = 3-4 dams).

Treatment	Eotaxin	IL-1 β	IL-4	IL-5	IL-6	MCP-1	TNF- α	VEGF
Vehicle GD11-24h	583 $\pm$ 968	n.d. ^a	164 $\pm$ 209	65.4 $\pm$ 113	1,438 $\pm$ 2,390	237 $\pm$ 172	n.d.	n.d.
Vehicle GD11-8d	82.8 $\pm$ 144	n.d.	n.d.	n.d.	271 $\pm$ 322	130 $\pm$ 22.3	n.d.	n.d.
[¹⁴ C(U)]C ₆₀ GD11-24h	275 $\pm$ 329	n.d.	19.3 $\pm$ 38.6	n.d.	574 $\pm$ 689	110 $\pm$ 79.2	n.d.	n.d.
[¹⁴ C(U)]C ₆₀ GD11-8d	41.2 $\pm$ 82.5	n.d.	n.d.	n.d.	114 $\pm$ 159	82.8 $\pm$ 92.9	n.d.	n.d.
[¹⁴ C(U)]C ₆₀ GD15-24h	12.5 $\pm$ 24.9	n.d.	n.d. ^b	n.d.	n.d.	37.4 $\pm$ 28.7	n.d.	n.d.
[¹⁴ C(U)]C ₆₀ GD15-4d	50.6 $\pm$ 49.6	n.d.	n.d.	n.d.	120 $\pm$ 141	49.0 $\pm$ 58.2	n.d.	n.d.
[¹⁴ C(U)]C ₆₀ GD18-24h	14.6 $\pm$ 17.3	n.d.	0.90 $\pm$ 1.81	0.84 $\pm$ 1.67	72.0 $\pm$ 87.7	11.9 $\pm$ 8.26 ^b	n.d.	6.73 $\pm$ 3.86 ^c

^a n.d. = not detected, '0' was used in the statistical analysis.

^b Analysis GD11-24h vehicle vs treatment, p < 0.05

^c Analysis GD11-8d vehicle vs treatment, p < 0.05

Cytokine/Chemokine expression in lactating rats and pups. All data are reported as mean $\pm$ standard deviation pg/mL of plasma duplicates (n = 3-4 adult rats).

Table 7

Treatment	Plasma	Eotaxin	IL-1 β	IL-4	IL-5	IL-6	MCP-1	TNF- α	VEGF
Vehicle PND8-24h	Dam	1,367 $\pm$ 2,261	n.d. ^a	1,183 $\pm$ 2,049	874 $\pm$ 1,514	1,288 $\pm$ 2,090	1,176 $\pm$ 1,634	1,642 $\pm$ 2,844	n.d.
	Pup, Male	n.d.	n.d.	n.d.	13.7 $\pm$ 23.7	12.1 $\pm$ 21.0	495 $\pm$ 143	n.d.	n.d.
	Pup, Female	n.d.	n.d.	3.82 $\pm$ 6.62	n.d.	n.d.	672 $\pm$ 197	n.d.	n.d.
Vehicle PND8-11d	Dam	132 $\pm$ 140	n.d.	143 $\pm$ 115	n.d.	752 $\pm$ 232	210 $\pm$ 155	n.d.	n.d.
	Pup, Male	13.7 $\pm$ 23.7	n.d.	42.7 $\pm$ 64.3	n.d.	300 $\pm$ 330	208 $\pm$ 57.1	n.d.	n.d.
	Pup, Female	n.d.	n.d.	3.82 $\pm$ 6.62	n.d.	81.2 $\pm$ 38.3	217 $\pm$ 27.9	n.d.	n.d.
[¹⁴ C(U)]C ₆₀ PND8-24h	Dam	182 $\pm$ 329	n.d.	82.6 $\pm$ 141	25.4 $\pm$ 50.8	315 $\pm$ 585	596 $\pm$ 441	n.d.	n.d.
	Pup, Male	n.d.	n.d.	n.d.	n.d.	178 $\pm$ 254	364 $\pm$ 45	n.d.	n.d.
	Pup, Female	n.d.	n.d.	n.d.	n.d.	n.d.	488 $\pm$ 235	n.d.	n.d.
[¹⁴ C(U)]C ₆₀ PND8-3d	Dam	198 $\pm$ 273	n.d.	9.40 $\pm$ 18.8	n.d.	n.d.	315 $\pm$ 94.5	n.d.	n.d.
	Pup, Male	n.d.	n.d.	121 $\pm$ 205	5.38 $\pm$ 10.8	1,038 $\pm$ 1,346	142 $\pm$ 186 ^b	n.d.	n.d.
	Pup, Female	21.9 $\pm$ 43.7	n.d.	14.0 $\pm$ 28.0	n.d.	29.3 $\pm$ 36.4	505 $\pm$ 143	n.d.	n.d.
[¹⁴ C(U)]C ₆₀ PND8-11d	Dam	205 $\pm$ 238	n.d.	28.0 $\pm$ 55.9	n.d.	560 $\pm$ 647	143 $\pm$ 77.0	n.d.	n.d.
	Pup, Male	n.d.	n.d.	n.d.	n.d.	n.d.	239 $\pm$ 84.9	n.d.	n.d.
	Pup, Female	n.d.	n.d.	n.d.	n.d.	33.9 $\pm$ 67.7	163 $\pm$ 46.1	n.d.	n.d.

^a n.d. = not detected, '0' was used in the statistical analysis.

^b Analysis PND8-24h vehicle vs treatment, p < 0.05

Table 8

Markers of cardiovascular injury in pregnant dams. All data are reported as mean $\pm$ standard deviation. PAI-1 in pg/ ml and vWF in ng/ml plasma duplicates (n = 3-4 dams).

Treatment	PAI-1	vWF
Vehicle GD11-24h	217 $\pm$ 264	204 $\pm$ 57.1
Vehicle GD11-8d	879 $\pm$ 335	317 $\pm$ 238
[¹⁴ C(U)]C ₆₀ GD11-24h	103 $\pm$ 142	175 $\pm$ 90.7
[¹⁴ C(U)]C ₆₀ GD11-8d	869 $\pm$ 179	180 $\pm$ 104
[¹⁴ C(U)]C ₆₀ GD15-24h	733 $\pm$ 201 ^a	211 $\pm$ 35.7
[¹⁴ C(U)]C ₆₀ GD15-4d	1,317 $\pm$ 249	223 $\pm$ 81.4
[¹⁴ C(U)]C ₆₀ GD18-24h	1,165 $\pm$ 274	215 $\pm$ 78.0

^a Analysis GD11-24h vehicle vs treatment, p <0.05

Table 9

Markers of cardiovascular injury in lactating dams and pups. All data are reported as mean $\pm$ standard deviation. PAI-1 in pg/ml and vWF in ng/ml plasma duplicates from (n = 3-4 adults rats).

Treatment	Plasma	PAI-1	vWF
Vehicle PND8-24h	Dam	n.d. ^a	112 $\pm$ 44.9
	Pup, Male	n.d.	650 $\pm$ 430
	Pup, Female	n.d.	248 $\pm$ 58.8
Vehicle PND8-11d	Dam	200 $\pm$ 108	90.5 $\pm$ 56.8
	Pup, Male	n.d.	538 $\pm$ 218
	Pup, Female	55.4 $\pm$ 95.9	683 $\pm$ 339
[¹⁴ C(U)]C ₆₀ PND8-24h	Dam	164 $\pm$ 328	135 $\pm$ 87.5
	Pup, Male	n.d.	751 $\pm$ 545
	Pup, Female	17.0 $\pm$ 34.1	332 $\pm$ 235
[¹⁴ C(U)]C ₆₀ PND8-3d	Dam	130 $\pm$ 157	298 $\pm$ 239
	Pup, Male	25.3 $\pm$ 43.8	750 $\pm$ 659
	Pup, Female	n.d.	963 $\pm$ 531
[¹⁴ C(U)]C ₆₀ PND8-11d	Dam	295 $\pm$ 25	153 $\pm$ 42.1
	Pup, Male	n.d.	360 $\pm$ 209
	Pup, Female	n.d.	206 $\pm$ 95.9 ^b

^a n.d. = not detected, '0' was used in the statistical analysis.

^b Analysis PND8-11d vehicle vs treatment, p <0.05

Table 10

Metabolites that are deemed to be important for separating pregnant dams administered a single tail vein injection of [$^{14}\text{C}(\text{U})$] C_{60} from vehicle (GD11-24h and GD11-8d).

Metabolite	Direction	
	GD11-24h	GD11-8d
2-Hydroxyglutarate	- ^a	↓ ^b
2-Oxocaproate	-	↓
4-pyridoxate	-	↑ ^c
Butyrate	-	↓
Choline	↑	-
Creatine	-	↑
Creatinine	↑	-
Fructose	↑	-
Gluconate	↑	-
Glycerol	-	↑
Glycylproline	↑	-
Guanidoacetate	-	↑
Hippurate	↓	↓
Isoleucine	↑	-
Isopropanol	-	↓
Lactate	↑	↑
Leucine	↑	↑
Methionine	-	↓
Methylamine	-	↓
Na-Acetyllysine	↑	-
O-Acetylcholine	↑	-
Pantothenate	↑	-
Pyridoxine	-	↑
Sarcosine	-	↑
Succinylacetone	-	↑
Sucrose	↑	↑
Taurine	-	↑
trans-aconitate	↓	↑
Trigonelline	↑	-
Trimethylamine	-	↑

^a - = not important for separation

^b ↓ = decreased in [$^{14}\text{C}(\text{U})$] C_{60} administered group compared to those administered vehicle

^c ↑ = increased in [$^{14}\text{C}(\text{U})$] C_{60} administered group compared to those administered vehicle