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The Prenatal Development effects of Carbon Dioxide (CO2) Exposure in Rats (Rattus norvegicus)

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The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals, " Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996.

The Prenatal Developmental Effects of Carbon Dioxide (CO_2) Exposure in Rats (*Rattus norvegicus*)

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1. Executive Summary

Female Sprague-Dawley rats [Crl: CD(SD)] were exposed to clean air or carbon dioxide (CO_2) gas at exposure levels of 1.5%, 2.0%, 2.5%, and 3.0% during days 6-20 of pregnancy in order to study the effects of inhalation exposure of CO_2 on the adult females and to further determine its influence on embryo-fetal survival rate and prenatal development. Rats were exposed approximately 23 hr/day in whole body inhalation exposure chambers. For the adult female rats, there were no remarkable internal findings due to CO_2 exposure at any of the levels studied, and also no differences in bodyweight changes or food consumption noted for any of the treatment groups. For fetal development, there were no malformations or developmental variations that were attributable to increased levels of CO_2 exposure and intrauterine growth was likewise unaffected.

There were two findings of note with implications for the safe exposure levels of CO_2 during pregnancy. The related findings were a statistically significant mean litter proportion of post-implantation loss (resorptions occurring in the early phase of pregnancy) in the 3.0% CO_2 group and a corresponding statistically significant lower mean litter proportion of viable fetuses for the same group. These results yield a No Observed Adverse Effect Level (NOAEL) of 2.5% for CO_2 and a Lowest Observed Adverse Effect Level (LOAEL) of 3.0% for CO_2.

In order to protect the health of those serving on submarines, the U.S. Navy sets exposure limits (a 90 day continuous exposure limit (CEL) and a 24-hour emergency exposure limit (EEL)) for CO_2 that were developed before the November 2011 addition of female submarine crew. The current study found rat embryo-fetal resorptions occurring in the early phases of pregnancy in rats exposed to an elevated 3% CO_2 atmosphere. Since a 90 day period would potentially cover the early period of a human pregnancy, using the 2.5% NOAEL from this study would be an appropriate point of departure for developing a CEL. Taking into account that the mechanism of action for resorptions due to CO_2 exposure is likely to be similar for both rats and humans, an interspecies uncertainty factor of 3 was applied to yield a recommended CEL of 0.8% for CO_2 based on the current study. Reproductive endpoints that are developmental in nature must be assumed to result from a single day of exposure during gestation, so it is therefore recommended that the 24-hour EEL also be made 0.8% CO_2 based on the results of this study. These recommendations for the CEL and 24-hour EEL should not outweigh any other studies that may derive a lower recommendation based on any other relevant data and endpoints.

2. Introduction

While a submarine is submerged, the atmosphere is recirculated, and the potential exists for gaseous compounds and chemical contaminants to accumulate in the air. The US Navy has conducted many decades of research to identify and characterize the compounds that its personnel may be exposed to aboard submarines, and has also conducted toxicological studies to assess the risks associated with exposure and set safe exposure limits for those compounds. The US Navy currently sets continuous exposure limits (CELs) and emergency exposure limits

(EELs) for over 200 components in submarine atmospheres as part of efforts to protect the health of submariners. However, "*EELs and CELs are developed for a healthy adult male population with little variation in physical qualifications*" (National Research Council, 2009). The advent of females being assigned to submarine crews in November 2011 has made it imperative to re-evaluate exposure limits with an emphasis on potential reproductive and developmental effects. Assessing the health issues for female crew members aboard submarines is a complex and controversial issue (Kane and Horn, 2001). Based on previous efforts (National Research Council 2007, 2008, 2009), the atmospheric component carbon dioxide (CO_2) is of significant concern in submarine atmospheres with a major source of CO_2 on board submarines being from human respiration. The CO_2 levels on board may reach atmospheric levels that are ten times higher (0.3%) than normal ambient air levels (0.03%). Research is needed to better elucidate the impact on reproductive and developmental health as well as to study the potential impact on mission effectiveness.

The derivation of an exposure limit for submarine atmospheres is dependent upon relevant data providing evidence of a level of adverse impact for an atmospheric component of interest. It is useful to have values for a lowest observed adverse effect level (LOAEL) and a no observed adverse effect level (NOAEL) that are not too widely separated from one another. The current study was designed with this in mind in order to potentially yield adverse effect levels that could be used to derive exposure limits.

While it is well known that significantly elevated concentrations of CO_2 can lead to respiratory, cardiovascular and central nervous system complaints in humans, there are limited data available regarding reproductive and developmental effects; therefore, the current study was designed to focus on potential embryo-fetal developmental effects. Some studies have indicated changes in male reproductive function at elevated levels of CO_2 exposure of 2.5-10.0% (Vandemark et al., 1972) or 3.6% (Mukherjee and Singh, 1967). No relevant information concerning human females is available and only sparse data are available in animal models (reviewed in Ema *et al.*, 2010). Available older data indicate that a 24 hr exposure on gestation days (GDs) 5-21 in rats to 6% CO_2 can produce increases in stillbirths/early postnatal death, thymus enlargement and skeletal and cardiac malformations (Haring, 1960), while exposure to 10% CO_2 has been reported to produce increases in external malformations in golden hamsters given a single 8 hr exposure on GD 8 (Storch and Layton, 1971). Resorptions and ectrodactyly in mice were seen following single 8 hr exposures to 20% CO_2 concentrations on GD 10 (Weaver and Scott, 1984), and rabbit vertebral column malformations were seen at 10-13% CO_2 exposure concentrations in GD 7-12 (Grote, 1965). The United States Environmental Protection Agency (US EPA) cited a summary report which stated that concentrations of 0.42-0.48% CO_2 have been associated with miscarriages, microsomic offspring and flaccid hindlimb paralysis of the offspring of guinea pigs repeatedly exposed during pregnancy (US EPA, 1991), but the summary report cannot be located and the original studies have not been identified.

In addition to the effects described above, higher than normal concentrations of CO_2 have been shown to result in increased partial pressure of CO_2 in alveoli and blood (Guillerm and Radziszewski, 1979). Changes in blood pH and activation of respiratory neurons can induce hyperventilation, which can lead to increased inhalation of other toxicants (James, 2008).

The British Royal Navy recently sponsored a study that reports "a slight delay in fetal development" in pregnant female rats exposed to 2.97% CO_2, based on the observation of delayed skeletal ossification. The authors considered the effect to be "potentially reversible" and not to represent an adverse effect (Huntingdon, 2010). The interpretation of skeletal variations as either adverse or not adverse is highly debatable. The described effects were observed only at the highest dose (2.97% CO_2 in air), with a three-fold concentration difference between high and medium doses.

For the current study, timed pregnant female rats [Crl: CD(SD)] were exposed to either clean air or CO_2 gas at exposure levels of 1.5%, 2.0%, 2.5%, and 3.0% during days 6-20 of pregnancy in order to study the effects of inhalation exposure of CO_2 on the adult females and to further determine its effects on embryo-fetal survival and prenatal development. On GD 20, the pregnant female rats underwent a laparohysterectomy with macroscopic examination of the uterus, and the fetuses were examined for visceral and skeletal effects. The study essentially followed the USEPA Health Effects Test Guidelines OPPTS 870.3700, "Prenatal Developmental Toxicity Study", which approximates the approach used by the British Royal Navy CO_2 study referred to previously (Huntingdon, 2010), with the following exception: 90-day inhalation toxicology studies typically utilize an exposure of 6 hr per day 5 to 7 days per week whereas this study used an exposure of 23 hr per day 7 days per week to better simulate the exposure conditions on a submarine.

3. Materials and Methods

3.1. Animals

A total of 120 female Sprague-Dawley (*Rattus norvegicus*) rats [Crl:CD(SD)], aged 10 to 12 weeks, ~225 to 300 g, were purchased from Charles River Laboratories (Wilmington, MA). The rats were received on GD 2, GD 3 or GD 4. Animals were acclimated in quarantine in a separate housing room until the initiation of exposures GD 6. The female rats were divided into four replicate groups of 30 per group. Each group was stagger-started by 1 day to accommodate the production and acquisition of timed pregnant rats and necropsy schedule (Fig. 1).

Rats were provided husbandry conditions consistent with AAALAC practices and in compliance with the NRC's "Guide for the Care and Use of Laboratory Animals. Following the abbreviated acclimation in quarantine, the rats were placed in the cage units and remained in the cage units

for the duration of the inhalation study for approximately 23 hr/day, with ≤ 1 hr allotted for animal husbandry. During the husbandry period, clinical observations of general animal health and well-being were made. Food and water were made available for all animals *ad libitum* during the study, including during inhalation exposures.

3.2. CO_2 Exposure

Rats were exposed to either clean air or test atmospheres of CO_2 by inhalation. All clean air for the control and exposure system was from an air circulating system using a turbine blower (The Spencer Turbine Co., Windsor, CT) with a room air intake through a high-efficiency particulate air filter (HEPA) to replace used air. Seven cylinders (800 cubic feet each) of carbon dioxide (99.995%Weiler Welding, Dayton, OH) were used to generate the four test atmospheres for exposures.

3.3. Inhalation Exposure Chambers

Rats were exposed in a 1 m^3 whole body exposure chamber (1 m^3, H1000, Lab Products, Seaford, DE). One chamber was used for each concentration including a control chamber. Stainless steel caging (R-16, Lab Products, Seaford, DE) was used to contain the animals during inhalation exposures and served as the domiciliary housing during periods of non-exposure. Each R-16 cage unit housed 24 rats; therefore, two R-16 cage units were placed in middle and bottom sections of each 1 m^3 exposure chamber. The dimensions for each rat compartment was 5.7 x 11 x 8 inches (W x L x H) and provided approximately 62.6 square inches of floor space. Stainless steel pans placed under each set of stainless steel cages to collect the urine and feces were changed daily for the duration of the inhalation exposures.

3.4. Inhalation Exposure Chamber Operation

The inhalation exposure chambers were operated as a push-pull system (Fig. 2). Air was pushed into the inlet of the chambers from an air circulating system using a turbine blower (The Spencer Turbine Co., Windsor, CT) with a room air intake through a high-efficiency particulate air filter (HEPA) to replace used air. Air was pulled from the exhaust of the chambers through a manifold using an exhaust fan on the roof on the facility.

The target inlet air flow rate in the mixed atmosphere chambers was set to 200-250 L/min, providing approximately 12-15 air changes per hour. Inlet air flows were a sum of the clean air and CO_2 flow. The inlet air flow for the control chamber was set at the target range of approximately 380 L/min to reduce the amount of CO_2 (approximately 0.22%) produced by the exhaled breath of the animal load. All inlet air flows were controlled by a manually operated gate valve. All inlet air flows were monitored by mass flow monitor (Model HFM-200 LFE, Teledyne-Hastings Instruments, Pittsburgh, PA) connected to a laminar flow element (Model HFM-200 LFE, Teledyne-Hastings Instruments, Pittsburgh, PA). Each of the mass flow monitors were connected to a four-channel power supply (Model THPS-400-115, Teledyne-Hastings Instruments, Pittsburgh, PA).

The chamber exhaust flow for the mixed atmosphere exposure chambers was adjusted with a manually operated gate valve to maintain a slight negative pressure relative to the room during the exposure to prevent the test atmosphere from entering the laboratory area in the event of leaks. The chamber exhaust flow for the control chamber was adjusted with a manually operated gate valve to maintain a slight positive pressure relative to the room to mimic the flow conditions of the experimental CO_2 exposure chambers.

The static pressure for each of the inhalation chambers was determined using two static pressure devices: a magnahelic pressure gauge (Model 2304, Dwyer Instrument Co., Michigan City, IN) provided a large visual display and an electronic pressure sensor (Model ZPS-05-SR09-EZ-ST-D, Building Automation Products, Inc., Gays Mills, WI) provided an electrical signal for data acquisition.

3.5. Temperature and Humidity

Temperature and relative humidity were measured by a temperature/relative humidity probe (Model HF532WB6XD1XX, Model HC2-S, Rotronics Instruments, Inc., Hauppauge, NY) located inside each exposure chamber. The target temperature was between 18 - 26° C (64 and 79° F) and the target relative humidity was between 30 and 70%.

3.6. Test Atmosphere Generation

All test chemical gases for the mixed atmospheres were metered by mass flow meters (Model HFC-202, Teledyne-Hastings Instruments, Pittsburgh, PA) at flow rates appropriate to maintain target concentrations of mixed atmospheres for each of the target doses. Each of the mass flow meters were connected to a four-channel power supply (Model THPS-400-115, Teledyne-Hastings Instruments, Pittsburgh, PA) and manually adjusted using the appropriate channel of the four channel power supply.

3.7. Test Atmosphere Monitoring

The test atmosphere of each of the five inhalation chambers was monitored continuously for CO_2 with a multiple gas analyzer (Model VA-3113, Horiba Instruments, Inc. Moon Township, PA). Each of the five instruments contained a magnetopneumatic (MP) sensor for O_2 measurements and two non-dispersive infrared analyzers (NDIR) for CO and CO_2 measurements. For these exposures only the CO_2 NDIR was calibrated and monitored. Each of the five instruments was calibrated using an N_2 dilution manifold and varying amounts 5% CO_2 in N_2 calibration gas (Airgas, Dayton, OH) for the CO_2 NDIR. Each instrument was zeroed using N_2.

3.8. Automated Alarm System

Certain monitoring devices for the inhalation chambers were electronically connected to an alarm system (Sensaphone, Model FGD-2000, Phonetics, Inc., Aston, PA) to monitor the key parameters of temperature, relative humidity, airflow, CO_2 concentration and O_2 concentration for the duration of the study. Each of the monitoring devices for these key parameters sent an

electronic signal to the Sensaphone. A high and low value was set for each key parameter; if the electronic signal fell outside the set range, the Sensaphone sent a message to the local FAX machine and called the subject matter expert (SME) who was responsible for correcting the incident.

3.9. Data Collection

Data were monitored and recorded by a data acquisition system (LabView Software v.10.0, National Instruments, Austin, TX). Data were collected every 10 seconds during the 24-hour exposure for temperature, humidity, supply air flow, CO_2 concentration and static pressure for all dose groups. In addition, for the experimental CO_2 exposure groups, data were collected every 10 seconds during the 24-hour exposure for CO_2 flow rate. The 24-hour daily data for each dose group was collected from 0900 until 0900 the following day. Periods when the chambers were opened for animal husbandry and animal procedures were included in the daily averages. At the end of each daily collection period, the average, standard deviation, minimum value, maximum value and the total number of data values were calculated for each parameter. For the overall study, each of the daily averages was used to calculate the mean of the daily exposures, standard deviation, minimum daily, maximum daily, and number of daily averages.

In addition, the Sensaphone alarm system logged data every 30 minutes and served as a back-up data collection system in the event the primary data acquisition system failed.

3.10. Study Day

A study day was defined as a 24-hour period generally from approximately 0900 until 0900 the following day. The study days were numbered consecutively from 1 to 17, corresponding to the first day animals were loaded into the five chambers until the last day animals were removed. The exposures were conducted from May 22, 2012 through June 07, 2012.

3.11. Necropsy and Laparohysterectomy

Necropsies were conducted over several days (Fig. 1) by personnel blinded to treatment groups. Dams were euthanized by CO_2 inhalation on GD 20. The thoracic, abdominal and pelvic cavities were opened and the organs examined. The rats then underwent a laparohysterectomy to excise the uterus. Corpora lutea were counted and recorded (a corpus luteum is the remnant of a matured ovum and the counts are indicative of the number of ova released during ovulation). Gravid uterine weights were recorded. The uterus of each dam was opened and the number of viable and nonviable fetuses, early and late resorptions and total number of implantation sites were recorded, and the placentae were also examined. The individual uterine distribution was documented using the following procedure: all implantation sites, including early and late resorptions, were numbered in consecutive fashion beginning with the left distal uterine horn, noting the position of the cervix and continuing from the proximal to the distal right uterine horn. Uteri that appeared nongravid by macroscopic examination were opened and placed in a 10% ammonium sulfide solution as described by Salewski (1964) for detection of early implantation

loss. Maternal tissues were not preserved for this study as it was not deemed to be necessary. The carcasses were disposed of in accordance with established laboratory procedures. Viable fetuses were euthanized using an intraperitoneal (IP) injection of sodium pentobarbital. A fetal examination was conducted to identify any external or internal abnormalities. Carcasses from the fetuses were fixed in ethanol and subsequently stained to examine for the presence of any skeletal malformations. Additional details regarding the procedures for post-mortem maternal and fetal examinations can be found in the "Tables" section. Data were compiled and analyzed to determine dose-response effects related to CO_2 exposure and a NOAEL and LOAEL were determined.

3.12. Post-Mortem Examinations and Data Analysis

The fetal and maternal examinations were conducted jointly by NAMRU-D staff and personnel contracted from the Developmental and Reproductive Toxicology Department at WIL Research Laboratories, LLC (Ashland, OH), who have specialized skills and training in fetal visceral and skeletal examination.

3.12.1. Intrauterine Data

Intrauterine data were summarized using two methods of calculation. An example of each method of calculation follows:

1. Group Mean Litter Basis:

$$Postimplantation\ Loss/Litter = \frac{\#\ Non-Viable\ Fetuses + Resorptions\ (early\ or\ late)}{\#\ Gravid\ Females}$$

2. Proportional Litter Basis:

$$Summation\ Per\ Group\ (\%) = \frac{Sum\ of\ Postimplantation\ \frac{Loss}{Litter}\ (\%)}{\#\ Litters/Group}$$

Where:

$$Postimplantation\ \frac{Loss}{Litter}\ (\%) = \frac{\#Non-Viable\ Fetuses + Resorptions\ (early/\ late)/Litter}{\#\ Gravid\ Females} x100$$

3.12.2. Fetal Morphological Examination

Fetal examinations were conducted by personnel blinded to treatment group. External, internal, and skeletal fetal findings were recorded as developmental variations or malformations. Prenatal data (viable and nonviable fetuses, early and late resorptions, pre- and post-implantation loss, and the fetal sex distribution) are presented on a group mean basis and as proportional data (% per litter).

3.12.3. External Examinations

Each viable fetus was examined in detail, sex determined, weighed and then euthanized by an intraperitoneal injection of sodium pentobarbital and tagged. Non-viable fetuses (the degree of

autolysis is minimal or absent) were examined, crown-rump length was measured, weighed, sex determined and tagged individually. The crown-rump length of late resorptions (advanced degree of autolysis) was measured, the degree of autolysis recorded, a gross external examination was performed (if possible), and the tissue was discarded.

3.12.4. Visceral Examinations

Fetuses were examined for visceral anomalies by dissection in the fresh (non-fixed) state. The thoracic and abdominal cavities were opened and dissected using a technique described by Stuckhardt and Poppe (1984). Fetal kidneys were examined and graded for renal papillae development (Woo and Hoar, 1972), the heart and major vessels were examined, and the sex was confirmed. The heads were removed from approximately one-half of the fetuses in each litter and placed in Bouin's solution for subsequent processing and soft-tissue examination using the Wilson sectioning technique (Wilson, 1965). The heads from the remaining one-half of the fetuses in each litter were examined by a mid-coronal slice. All carcasses, including the carcasses without heads, were eviscerated and fixed in 100% ethyl alcohol for subsequent examination of skeletons.

3.12.5. Skeletal Examinations

Each eviscerated fetus was fixed in 100% ethyl alcohol and shipped to WIL Research Laboratories, LLC for skeletal examinations. Fetuses were stained with Alizarin Red S and Alcian Blue by a method similar to that described by Dawson (1926) and Inouye (1976). The skeletal examination was made following this procedure.

The fetal developmental findings were summarized by: 1) presenting the incidence of a given finding both as the number of fetuses and the number of litters available for examination in the group and 2) considering the litter as the basic unit for comparison and calculating the number of affected fetuses in a litter on a proportional basis as follows:

$$Summation\ Per\ Group\ (\%) = \frac{Sum\ of\ Viable\ Fetuses\ Affected/Litter(\%)}{\#\ Litters/Group}$$

Where:

$$Viable\ Fetuses\ Affected/Litter(\%) = \frac{\#Viable\ Fetuses\ Affected/Litter}{\#\ Viable\ Fetuses/Litter} x100$$

3.13 Derivation of Exposure Limit

The CEL was derived based on the NOAEL and using an uncertainty factor (UF) of 3 for interspecies variation.

NOAEL / UF = CEL

4. Data Analyses

4.1 Statistical Analyses

All analyses were two-tailed for significance levels of 5% and 1%. All statistical tests were performed using the data analysis software SigmaPlot12 (Systat Software, Inc., 2010) or WIL Toxicology Data Management System (WTDMS, WIL Research Laboratories, LLC, PMGSIv4.04, 07/18/2012). Data from nongravid females were excluded from calculation of means and from comparative statistics. The litter, rather than the fetus, is defined as the experimental unit.

4.1.1. Maternal In-Life Data

Continuous data variables [mean body weights (absolute and net), body weight gains (absolute and net) and food consumption of each interval] were analyzed using a parametric one-way analysis of variance (ANOVA) (Snedecor and Cochran, 1980) to determine differences between exposure groups. When the results of the ANOVA were significant ($p<0.05$), Dunnett's Test (Dunnett, 1964) was applied to the data.

4.1.2. Laparohysterectomy Data

The group mean numbers of corpora lutea, implantation sites, viable fetuses, maternal gravid uterine weights, and mean fetal weight (separately by sex, and combined) were analyzed using a parametric one-way ANOVA (Snedecor and Cochran, 1980) and Dunnett's test (1964). The mean litter proportions of prenatal data (% per litter of viable and nonviable fetuses, early and late resorptions, total resorptions, pre- and post-implantation loss and the fetal sex distribution) were analyzed using the Kruskal-Wallis nonparametric one-way ANOVA (Kruskal and Wallis, 1952) to determine differences between groups. If the results of the Kruskal-Wallis test were significant ($p<0.05$), Dunn's Test (Dunn, 1964) was applied to the data.

4.1.3. Fetal Morphology Data

The mean litter proportions (% per litter) of total fetal malformations and developmental variations (external, visceral, skeletal and combined) and of each particular external, visceral and skeletal malformation or variation were tabulated. The mean litter proportions of fetal malformations and developmental variations were analyzed using a Kruskal-Wallis nonparametric ANOVA (Kruskal and Wallis, 1952) followed by the Dunn's Test (1964) (if appropriate) as described above.

5. Results

5.1. Environmental Parameters

The mean temperatures (±standard deviation) for control, low, mid low, mid high and high dose were 23.2 (±0.1), 21.5 (±0.3), 22.2 (±0.5), 22.2 (±0.2) and 22.4 (±0.2) °C, respectively. The mean

humidity levels (±standard deviation) for control, low, mid low, mid high and high dose were 45 (±5), 52 (±5), 54 (±6), 49 (±5) and 51 (±6) %, respectively. The mean supply air flows (±standard deviation) for control, low, mid low, mid high and high dose were 383 (±2), 223 (±1), 221 (±2), 222 (±2) and 223 (±2) L/min, respectively. The mean CO_2 flow rates (±standard deviation) for low, mid Low, mid high and high dose were 2.9 (±0.0), 4.2 (±0.0), 5.8 (±0.1) and 6.8 (±0.1) L/min, respectively. The mean CO_2 concentrations (±standard deviation) for control, low, mid low, mid high and high dose were 0.1 (±0.0), 1.5 (±0.0), 2.0 (±0.0), 2.5 (±0.1) and 3.0 (±0.1) %, respectively. The mean O_2 concentrations (±standard deviation) for control, low, mid low, mid high and high dose were 21.1 (±0.1), 20.3 (±0.1), 20.3 (±0.1), 20.1 (±0.1) and 20.1 (±0.1) ppm, respectively. The mean static pressures (±standard deviation) for control, low, mid low, mid high and high dose were 0.24 (±0.03), -0.01 (±0.06),-0.01 (±0.07),-0.11 (±0.04) and 0.01 (±0.07) in H_2O, respectively. The data for the environmental parameters are shown in Table 1.

5.2. Maternal Bodyweights

Maternal animals were weighed at 3-day intervals during the dosing period and again on the day of necropsy. Results are shown in Table 2 with bodyweight in grams (g). There were no statistically significant differences between the control group and any treatment group for maternal bodyweight changes.

5.3. Maternal Food Consumption

Maternal food consumption was measured at 3-day intervals during the dosing period and again on the day of necropsy. Results are shown in Table 3 with food weight in grams (g). There were no statistically significant differences between the control group and any treatment group for food consumption.

5.4. Maternal Necropsy Data

Maternal necropsy data are shown in Table 4. At the scheduled necropsy on gestation day 20, no remarkable internal findings were observed at exposure levels of 1.5%, 2.0%, 2.5%, and 3.0% CO_2. Macroscopic findings observed in the groups exposed to increased levels of CO_2 occurred infrequently and in a manner that was not exposure-related. One female was determined to be nongravid in each of the 1.5%, 2.0%, and 2.5% CO_2 groups.

5.5. Laparohysterectomy Data

The data for reproductive success are given in Tables 5 and 6.

There were four areas with statistically significant ($p<0.01$) differences between the control and the 3% CO_2 treatment group. The first finding of note is the mean litter proportion of post-implantation loss (entirely early resorptions) in the 3.0% CO_2 group (9.6% per litter), which was slightly higher than the control group (2.7% per litter) as shown in Table 5. A corresponding, significantly lower mean litter proportion of viable fetuses was also noted in this group (90.5%

per litter) when compared to the control group (97.3% per litter) (Table 6). The higher mean litter proportion of post-implantation loss in the 3.0% CO_2 group was due to 21 of 24 litters in this group having at least 1 early resorption compared to only 7 of 24 litters in the control group with at least 1 early resorption. Although post-implantation loss was higher in the 3.0% CO_2 group, no corresponding effect on the mean number of viable fetuses in this group was observed, most likely as a result of a significantly ($p<0.01$) higher mean number of implantation sites in the 3.0% CO_2 group (Table 5). Because implantation occurred prior to the start of elevated CO_2 exposures, the higher mean number of implantation sites in the 3.0% CO_2 group was not attributed to increased CO_2 exposure.

Mean fetal weights, mean male and female placental weights, and fetal sex ratio in the 3.0% CO_2 group were similar to the control group (Table 6). Differences were slight and not statistically significant.

Intrauterine growth and survival in the 1.5%, 2.0%, and 2.5% CO_2 groups were unaffected by increased CO_2 exposure. Mean numbers of corpora lutea and implantation sites and the mean litter proportions of pre-implantation loss were similar across all groups. Differences compared to the control group were slight and not statistically significant with the exception of the previously mentioned higher mean number of implantation sites noted in the 3.0% CO_2 group.

5.6. Fetal Morphological Data

The numbers, types, and percentages of fetal malformations are listed in Tables 7 through 10, respectively. The numbers of fetuses and litters available for morphological evaluation were 282 fetuses from 24 litters for the Control group, 278 fetuses from 23 litters for the 1.5% treatment group, 277 fetuses from 23 litters for the 2.0% treatment group, 284 fetuses from 23 litters for the 2.5% treatment group and 299 fetuses from 24 litters in the 3.0% treatment group. Malformations (external, visceral, or skeletal as described in 5.61-5.63) were observed in 1 fetus from the Control group, 2 fetuses from 2 separate litters for the 1.5% treatment group, 2 fetuses from 2 separate litters for the 2.0% treatment group, 2 fetuses from 2 separate litters for the 2.5% treatment group, and 4 fetuses from 3 separate litters for the 3.0% treatment group. These visceral developmental variations were not considered related to increases in CO_2 exposure as they were noted similarly in the control group, were not statistically significant, and did not occur in an exposure-dependent manner.

Malformations noted for this study were regarded as those structural anomalies that alter general body conformity, disrupt or interfere with normal body function, or may be incompatible with life. Variations were regarded as alterations in anatomic structure that are considered to have no significant biological effect on animal health or body conformity and/or occur at high incidence, representing slight deviations from the norm.

5.6.1. External Malformations and Variations

External malformations were noted in 1 fetus in the Control group, 1 fetus from the 1.5% treatment group, and 3 fetuses from 2 separate litters in the 3.0% CO_2 group (Table 7 or percent per litter in Table 8). In the 3.0% CO_2 group, one fetus had gastroschisis (stomach, several loops of the intestine, and liver protruded through an opening in the ventral midline) and localized fetal edema was noted for 2 fetuses: one for hindlimbs and the other for neck and thorax. In the 1.5% CO_2 group, one fetus was noted with vertebral agenesis (filamentous tail), consisting of all vertebrae posterior to sacral vertebra number 1 absent, and anal atresia (Table 7).

One fetus in the control group had cyclopia (2 eyes situated closely in 1 orbit), proboscis-like nose, mandibular micrognathia, maxillary agnathia, astomia, aglossia, only 2 facial papillae present (malpositioned), and malpositioned pinnae (Table 7). Skeletally, cyclopia consisted of absent zygomatic arches and small, misshapen, and fused squamosal bones; mandibular micrognathia consisted of small, misshapen, and fused mandible bones and malpositioned and fused tympanic rings; maxillary agnathia consisted of absent maxilla and premaxilla bones; and proboscis-like nose consisted of fused and misshapen nasal bones.

These developmental variations were not considered related to increases in CO_2 exposure as they were noted similarly in the control group, were not statistically significant, and did not occur in an exposure-dependent manner.

No gross external developmental variations were observed in fetuses at any exposure level (Table 7).

5.6.2. Visceral Malformations and Variations
No visceral malformations were observed in fetuses at any exposure level (Tables 7, 8).

Visceral developmental variations noted in the increased CO_2 exposure groups consisted of major blood vessel variation (retroesophageal right subclavian artery joining the aortic arch adjacent to the ductus arteriosus [no brachiocephalic trunk] or right carotid and right subclavian arteries arising independently from the aortic arch [no brachiocephalic trunk]), presence of accessory lobules of the liver, and renal papillae not developed and/or distended ureters (Table 9). These visceral developmental variations were not considered related to increases in CO_2 exposure as they were noted similarly in the control group, were not statistically significant, and did not occur in an exposure-dependent manner.

Renal papillae not fully developed (Woo and Hoar Grade 1[Woo and Hoar, 1972]) were noted in 3 fetuses in the Control group, 1 fetus in the 1.5% treatment group, 2 fetuses in the 2.0% group, and 3 fetuses in the 3.0% CO_2 group (Table 9). In addition, areas of dark red discoloration on the adrenal glands were noted for single fetuses in the 2.0% and 3.0% CO_2 groups; these findings were not classified as either a malformation or developmental variation, were not included on the

summary tables, and were not considered to be related to increases in CO_2 exposure because they occurred infrequently, at similar frequencies to the control group, or in a manner that was not exposure-dependent.

5.6.3. Skeletal Malformations and Variations

Skeletal malformations and variations were noted in none of the Control group animals, in 1 fetus from the 1.5% treatment group, 2 fetuses from 2 separate litters for the 2.0% treatment group, 2 fetuses from 2 separate litters in the 2.5% treatment group and 1 fetus in the 3.0% CO_2 groups (Tables 7, 8, 9 and 10). One fetus in the 3.0% CO_2 group was noted with a unilateral bent limb bone (humerus) and bent scapula. Two fetuses in the 2.5% CO_2 group were also noted with a unilateral bent scapula. In the 2.0% CO_2 group, one fetus had a vertebral anomaly without an associated rib anomaly (consisting of an extra site of ossification between cervical arches with a cartilaginous attachment to the cervical arch) and another fetus had sternoschisis (sternal bands no. 6 not joined). In the 1.5% CO_2 group, one fetus was noted with a rib anomaly consisting of malpositioned ribs. These skeletal malformations were not considered related to increases in CO_2 exposure as they were noted in single fetuses, were not statistically significant, or did not occur in an exposure-dependent manner.

These skeletal developmental variations were not considered related to increases in CO_2 exposure as they were noted similarly in the control group, were not statistically significant, and did not occur in an exposure-dependent manner. Skeletal developmental variations in the increased CO_2 exposure groups were not observed in an exposure-dependent manner were not statistically significant or occurred in single fetuses or at similar frequencies to the control group (Tables 9 and 10).

5.6.4. Summary of External, Visceral and Skeletal Examinations

When the total malformations and developmental variations were evaluated on a proportional basis, no statistically significant differences from the control group were noted. Fetal malformations and developmental variations, when observed in the increased CO_2 exposure groups, occurred infrequently or at a frequency similar to that in the control group, and/or did not occur in an exposure-related manner. Based on these data, no fetal malformations or developmental variations were attributed to increased CO_2 exposure.

6. Discussion

CO_2 is a colorless, odorless gas that is a product of animal respiration which makes humans the major source of CO_2 during normal operations on a submarine (Crawl, 2003). Deriving an exposure limit for CO_2 depends on the length of expected exposure, the concentration involved and the population being exposed. The U.S. Navy has previously set the concentration for a CEL

based on a 90-day exposure for a population of healthy males. Since female crewmembers are now serving on submarines, female health factors must be taken into account in deriving a CEL. Because CO_2 has a spectrum of concentration-dependent effects, the relevant effect with the lowest known effective concentration will serve as the starting point for deriving an exposure limit.

Since CO_2 exists in the earth's atmosphere at concentrations of approximately 0.03% by volume (Morey and Shattuck, 1989), any concentrations approaching this would therefore be considered safe, since these levels are inescapable. At the higher end of the spectrum, the most severe toxic effect of CO_2 is asphyxiation, which occurs at concentrations as low as 11% (Hamilton and Hardy. 1974). Between these extremes lie relevant effects that have been used by other agencies to develop exposure limits.

The spacecraft maximum allowable concentration (SMAC) set by NASA for 7-180 day durations was derived from a number of studies that included endpoints such as visual impairment, headache, dyspnea, testicular injury, and hyperventilation tolerability. The final endpoint of hyperventilation tolerability yields a NOAEL of 2%, which was adjusted using an uncertainty factor to yield an acceptable concentration of 0.7% (Wong, 1996). Although the validity of some of these studies has been questioned, the 7-180 day SMAC for CO_2 remains 0.7% (James, 2008).

Based on studies involving visual tracking and depth perception, the National Research Council noted a LOAEL of 2.5%, which was adjusted using an uncertainty factor of 3 due to small number of subjects, resulting in a recommended CEL of 0.8% for submarine atmospheres (NRC, 2007). While this value is directly relevant, none of the studies considered for the final evaluation included any values for female reproductive health.

To this end, Haring (1960) used 6% CO_2 to expose Sprague-Dawley rats during pregnancy from GD 5-21, with increased stillbirths/early postnatal death and cardiac and skeletal malformations of the fetuses noted. The study yielded a LOAEL of 6%. While this is a directly relevant endpoint, the concentration used is so high that without a NOAEL it is difficult to say whether there is a relevant effect at CO_2 concentrations likely to be normally encountered on a submarine. The current study used a highest concentration of 3.0% CO_2 with a LOAEL at 3.0% and a NOAEL at 2.5% CO_2, with the statistically significant adverse endpoints of early fetal resorptions and low mean litter proportion of viable fetuses. It should be noted that a previous study using virtually the same parameters did not report any such adverse findings using a CO_2 concentration of 2.97% even though embryo-fetal resorptions were among the endpoints evaluated (Huntingdon, 2010). A reasonable explanation for the differences noted between the current study and the Huntingdon (2010) study is that biological effects at the highest concentrations used (~3%) are on the borderline of being detectable and might be more evident at higher concentrations. Additional studies would be required for confirmation.

While there are questions that remain unanswered, the support for a NOAEL/LOAEL from the current study is based on a more extensive and relevant set of biological endpoints than some of the studies used to develop exposure limit recommendations such as the SMAC as noted above (James, 2008) and the low number of subjects used (NRC, 2001) to support the CEL recommendation of 0.8%.

One factor to consider when recommending an exposure limit is whether the timeframe covered by the study is relevant to the human exposure scenario for which the exposure limit is being derived. The rat exposures in the current study (17 exposure days) cover nearly the entire range of gestation, with the relevant endpoint of early resorption occurring in the early stages of pregnancy. Since a 90-day CEL represents a period that could cover the early stages of a potential human pregnancy, there is direct relevance of the study's noted adverse endpoint to the establishment of a 90-day CEL.

The endpoint of resorption is thought to be related to placentation, which is a similar process between humans and rats since both undergo hemochorial placentation where maternal blood comes into direct contact with the fetal chorion (reviewed in Soares *et al.*, 2012). Angiogenesis towards oxygen-rich blood is a critical element of placentation, and elevated carbon dioxide levels may disrupt the development of the placenta (Fonseca *et al.*, 2012). Due to these similarities between rats and humans, along with the potential for CO_2 to disrupt placentation, it is reasonable to accept the endpoint of fetal resorption as directly relevant in deriving an exposure limit for CO_2.

The observed adverse effects of the current study are likely due to the experimental variable of CO_2 concentration, leading to the use of the NOAEL of 2.5% as a point of departure for recommending a CEL. The use of an uncertainty factor (UF) was employed to take into account interspecies variation between humans and rats. National Research Council guidance indicates that when the mechanism of action is unlikely to differ between species, in this case between rats and humans, an interspecies UF of 3 is appropriate (NRC, 2001). From these observations we have derived a CEL based on the following formula:

$$\text{NOAEL} / \text{UF} = \text{CEL}$$
$$2.5\% / 3 = 0.83\%$$

Rounding down yields a recommended CEL of 0.8%.

The CEL recommendation based on this study does not imply that any relevant data and endpoints indicating a lower concentration for a CEL should not take priority.

7. Conclusions

No remarkable internal findings were observed in maternal animals at exposure levels of 1.5%, 2.0%, 2.5%, or 3.0% CO_2. Intrauterine growth in the 1.5%, 2.0%, 2.5%, and 3.0% CO_2 groups and survival in the 1.5%, 2.0%, and 2.5% CO_2 groups were unaffected by CO_2 exposure. No fetal malformations or developmental variations were attributed to increased CO_2 exposure.

There were two findings of note with implications for the safe exposure levels of CO_2 during pregnancy. These findings were a statistically significant mean litter proportion of post-implantation loss (resorptions occurring in the early phase of pregnancy) in the 3.0% CO_2 group and a corresponding statistically significant lower mean litter proportion of viable fetuses for the same group. These results yield a No Observed Adverse Effect Level (NOAEL) of 2.5% for CO_2 and a Lowest Observed Adverse Effect Level (LOAEL) of 3.0% for CO_2.

In order to protect the health of those serving on submarines, the U.S. Navy sets a 90-day continuous exposure limit (CEL) for CO_2 that was developed before the November 2011 addition of female submarine crew. The current study addressed these concerns and found rat embryo-fetal resorptions occurring in the early phases of pregnancy when exposed to elevated 3% CO_2 atmospheres. Since a 90-day period would potentially cover the early period of a human pregnancy, the 2.5% NOAEL from this study would be an appropriate point of departure for developing a new CEL. Taking into account the interspecies differences between rats and humans, we recommend an uncertainty factor of 3 be applied to yield a recommended CEL of 0.8% for CO_2. Reproductive endpoints that are developmental in nature must be assumed to result from a single day of exposure during gestation (Willem D. Faber, personal communication 17 September, 2012; Donald G. Stump, personal communication, 21 September, 2012), so it is therefore recommended that the 24-hour EEL also be made 0.8% CO_2 based on the results of this study. These recommendations for the CEL and 24-hour EEL should not outweigh any other studies that may derive a lower recommendation based on any other relevant data and endpoints.

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9. Figures

Figure 1: Exposure Schedule. Exposure replicates were staggered by one day. Necropsies at the end of the study were also staggered by one day for each replicate.

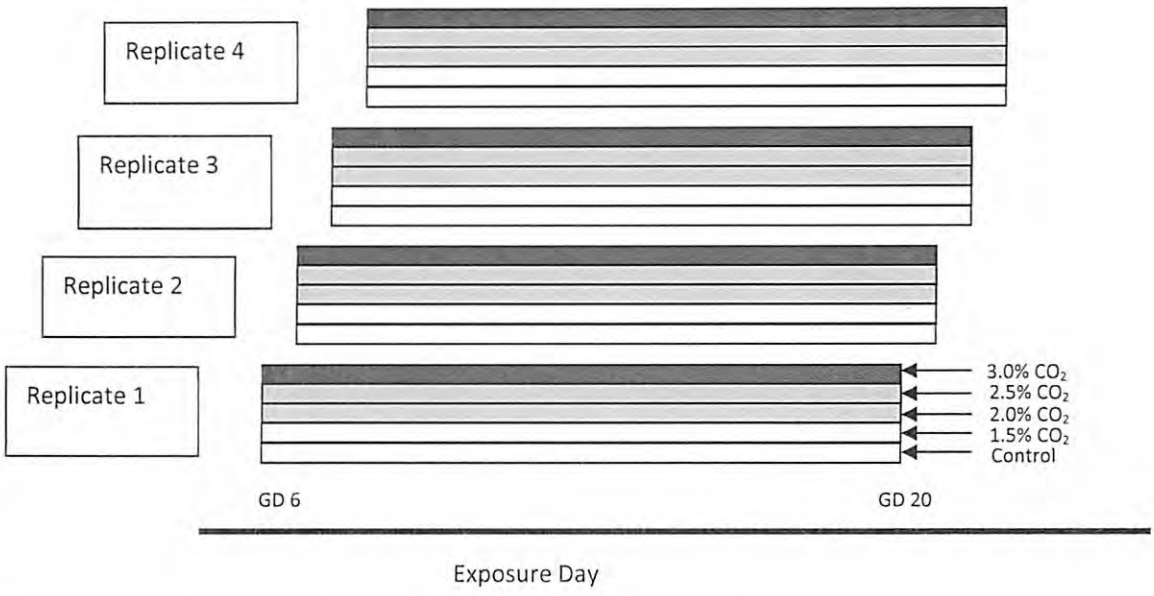
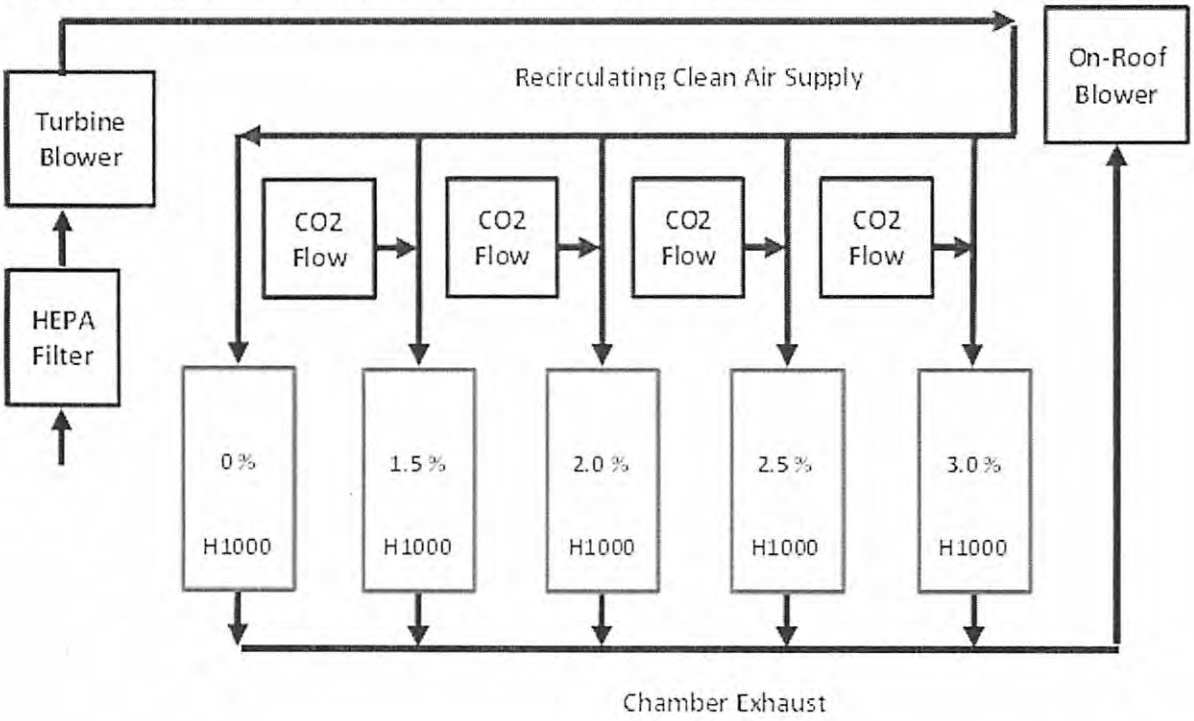


Figure 2: Diagrammatic Representation of the Exposure System



10. Tables

Table 1: Inhalation Exposure Summary Data: Environmental Parameters							
			Group 1	Group 2	Group 3	Group 4	Group 5
			Clean Air	Low	Mid Low	Mid High	High
			Control	Dose	Dose	Dose	Dose
Temp	(Deg C)	**Mean**	**23.2**	**21.5**	**22.2**	**22.2**	**22.4**
		St Dev	0.1	0.3	0.5	0.2	0.2
		Min	22.9	20.9	21.2	21.6	21.8
		Max	23.3	21.8	22.7	22.5	22.7
		Count	17	17	17	17	17
Humidity	(%)	**Mean**	**45**	**52**	**54**	**49**	**51**
		St Dev	5	5	6	5	6
		Min	39	44	43	42	42
		Max	54	61	63	58	60
		Count	17	17	17	17	17
Supply Air Flow Rate	(L/min)	**Mean**	**383**	**223**	**221**	**222**	**223**
		St Dev	2	1	2	2	2
		Min	380	221	219	219	220
		Max	386	225	225	225	227
		Count	17	17	17	17	17
Carbon Dioxide Flow Rates	(L/min)	**Mean**		**2.9**	**4.2**	**5.8**	**6.8**
		St Dev		0.0	0.0	0.1	0.1
		Min	NA	2.9	4.1	5.5	6.6
		Max		3.0	4.3	5.9	6.9
		Count		17	17	17	17
Carbon Dioxide Concentration	(ppm)	**Mean**	**0.1**	**1.5**	**2.0**	**2.5**	**3.0**
		St Dev	0.0	0.0	0.0	0.1	0.1
		Min	0.1	1.4	1.9	2.3	2.8
		Max	0.1	1.5	2.0	2.6	3.1
		Count	17	17	17	17	17
Oxygen Concentration	(%)	**Mean**	**21.1**	**20.3**	**20.3**	**20.1**	**20.1**
		St Dev	0.1	0.1	0.1	0.1	0.1
		Min	20.9	20.1	20.1	20.0	19.9
		Max	21.2	20.5	20.5	20.4	20.3
		Count	17	17	17	17	17
Static Pressure	(" H2O)	**Mean**	**0.24**	**-0.01**	**-0.01**	**-0.11**	**0.01**
		St Dev	0.03	0.06	0.07	0.04	0.07
		Min	0.19	-0.11	-0.11	-0.17	-0.09
		Max	0.29	0.05	0.09	-0.02	0.09
		Count	17	17	17	17	17

Table 2: Maternal Body weights							
Treatment Group	Average Initial Body Weight (g) GD 6 ± SE	Average Body Weight (g) GD 9 ± SE	Average Body Weight (g) GD 12 ± SE	Average Body Weight (g) GD 15 ± SE	Average Body Weight (g) GD 18 ± SE	Average Body Weight GD 20 ± SE	Total Body Weight Gain (g) from GD 6 to GD 20 ± SE
Control	254 ± 4.0	277 ± 4.1	295 ± 4.4	315 ± 4.8	354 ± 5.4	384 ± 6.3	130 ± 3.8
1.5%	253 ± 4.1	276 ± 4.0	293 ± 41	314 ± 4.1	354 ± 4.7	385 ± 5.7	132 ± 2.9
2.0%	253 ± 2.6	276 ± 2.7	294 ± 3.0	311 ± 3.1	348 ± 3.4	378 ± 3.7	125 ±2.7
2.5%	253 ± 3.1	274 ± 2.9	291 ± 3.5	311 ± 4.1	349 ± 4.7	378 ± 4.5	125 ± 2.9
3.0%	255 ± 3.5	275 ± 3.5	294 ± 3.4	314 ± 3.6	354 ± 4.4	384 ± 5.2	128 ± 3.6
SE = Standard Error							

Table 3: Maternal Food Consumption						
Treatment Group	Averaged Initial Food Consumption (g) GD 6 to GD 9 ± SE	Averaged Food Consumption (g) GD 9 to GD 12 ± SE	Averaged Food Consumption (g) GD 12 to GD 15 ± SE	Averaged Food Consumption (g) GD 15 to GD 18 ± SE	Averaged Food Consumption (g) GD 18 to GD 20 ± SE	Average Calculated Daily Food Consumption (g) GD 6 to GD 20 ± SE
Control	21.6 ± 0.45	21.9 ± 0.42	23.5 ± 0.51	25.3 ± 0.62	24.5 ± 0.55	23.4 ± 0.48
1.5%	22.1 ± 0.54	22.4 ± 0.47	23.6 ± 0.51	26.2 ± 0.65	25.2 ± 0.60	23.9 ± 0.46
2.0%	21.9 ± 0.44	21.8 ± 0.50	22.2 ± 0.45	24.2 ± 0.41	23.4 ± 0.88	22.7 ± 0.38
2.5%	21.0 ± 0.31	21.5 ± 0.39	22.6 ± 0.48	25.0 ± 0.68	23.6 ± 0.58	22.9 ± 0.43
3.0%	22.0 ± 0.41	22.7 ± 0.43	23.9 ± 0.47	25.5 ± 0.43	25.2 ± 0.65	23.9 ± 0.38
SE = Standard Error						

Table 4: Maternal Macroscopic Findings											
Treatment Group	Number Examined	Animals with No Significant Changes Observed	Nongravid	Ovaries: Cyst(s)	Placentae: Fused	Kidneys: Dilated Pelvis	Lymph Node, Iliac: Enlarged	Urinary Bladder: Calculi	Urinary Bladder: Thickened	Spleen: Accessory	Placenta(e): Enlarged
Control	24	22	0	0	0	1	1	1	1	0	0
1.5%	24	21	1	1	1	0	0	0	0	1	0
2.0%	24	23	1	0	0	0	0	0	0	0	0
2.5%	24	22	1	0	0	0	0	0	0	0	1
3.0%	24	24	0	0	0	0	0	0	0	0	0

Table 5: Summary of Fetal Data at Necropsy

Treatment Group		Sex		Viable Fetuses	Dead Fetuses	Resorptions		Post Implantation Loss	Implantation Sites	Corpora Lutea	Preimplantation Loss	Fetal Weight Gain (g)	# Gravid Females
		M	F			Early	Late						
Control	Total	131	151	282	0	8	0	8	290	338	48	NA	24
	Mean	5.5	6.3	11.8	0	0.3	0	0.3	12.1	14.1	2	4.1	
	SD	1.53	1.81	1.57	0	0.56	0	0.56	1.59	2.1	2.13	0.23	
	SE	0.31	0.37	0.32	0	0.12	0	0.12	0.32	0.43	0.43	0.05	
1.5%	Total	144	134	278	0	22	3	25	303	311	21	NA	23
	Mean	6.3	5.8	12.1	0	1	0.1	1.1	13.2	14.1	1	4	
	SD	1.94	2.1	1.76	0	1.11	0.34	1.16	1.61	2.27	1.17	0.21	
	SE	0.4	0.44	0.37	0	0.23	0.07	0.24	0.34	0.48	0.25	0.04	
2.0%	Total	134	143	277	0	16	0	16	293	335	42	NA	23
	Mean	5.8	6.2	12	0	0.7	0	0.7	12.7	14.6	1.8	4	
	SD	2.37	2.3	1.92	0	1.06	0	1.06	1.25	1.73	1.7	0.29	
	SE	0.49	0.48	0.4	0	0.22	0	0.22	0.26	0.36	0.35	0.06	
2.5%	Total	154	130	284	0	14	0	14	298	333	35	NA	23
	Mean	6.7	5.7	12.3	0	0.6	0	0.6	13	14.5	1.5	4	
	SD	1.87	1.92	1.67	0	0.72	0	0.72	1.58	2.29	1.65	0.21	
	SE	0.39	0.4	0.35	0	0.15	0	0.15	0.33	0.48	0.34	0.04	
3.0%	Total	139	160	299	0	32	0	32	331	372	41	NA	24
	Mean	5.8	6.7	12.5	0	1.3	0	1.3	13.8*	15.5	1.7	3.9	
	SD	1.84	1.9	1.59	0	0.87	0	0.87	1.61	2.64	1.85	0.26	
	SE	0.38	0.39	0.32	0	0.18	0	0.18	0.33	0.54	0.38	0.05	

* = Significantly different from the control group at 0.01
NA = Not Applicable SD = Standard Deviation SE = Standard Error

Table 6: Summary of Fetal Data at Necropsy (% Per Litter)

Treatment Group		Corpora Lutea	Implantation Sites	Viable Fetuses (%)	Dead Fetuses (%)	Early Resorptions (%)	Late Resorptions (%)	Total Resorptions (%)	Pre-implantation Loss (%)	Post-implantation Loss (%)	Males (%)	Females (%)	Male Fetal Weights (g)	Female Fetal Weights (g)	Combined Fetal Weights (g)	Male Placenta (g)	Female Placenta (g)
Control	Mean	14.1	12.1	97.3	0	2.7	0	2.7	13.1	2.7	46.6	53.4	4.2	4	4.1	0.55	0.53
	% Dif.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	SD	2.1	1.59	4.44	0	4.44	0	4.44	12.75	4.44	12.26	12.26	0.25	0.24	0.23	0.06	0.072
	SE	0.43	0.32	0.91	0	0.91	0	0.91	2.6	0.91	2.5	2.5	0.05	0.05	0.05	0.012	0.015
	N	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
1.5%	Mean	14.1	13.2	91.9	0	7.1	1	8.1	6.1	8.1	52.1	47.9	4.2	3.9	4	0.54	0.53
	% Dif.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	-2.5	-2.4	-1.8	0
	SD	2.27	1.61	8.2	0	7.6	2.73	8.2	6.93	8.2	15.21	15.21	0.23	0.18	0.21	0.06	0.06
	SE	0.48	0.34	1.71	0	1.59	0.57	1.71	1.48	1.71	3.17	3.17	0.05	0.04	0.04	0.012	0.013
	N	22	23	23	23	23	23	23	22	23	23	23	23	23	23	23	23
2.0%	Mean	14.6	12.7	94.1	0	5.9	0	5.9	11.8	5.9	47.8	52.2	4.1	3.9	4	0.55	0.56
	% Dif.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-2.4	-2.5	-2.4	0	5.7
	SD	1.73	1.25	10.37	0	10.37	0	10.37	10.16	10.37	17.95	17.95	0.28	0.26	0.29	0.129	0.273
	SE	0.36	0.26	2.16	0	2.16	0	2.16	2.12	2.16	3.74	3.74	0.06	0.06	0.06	0.027	0.057
	N	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23
2.5%	Mean	14.5	13	95.3	0	4.7	0	4.7	9.5	4.7	54.4	45.6	4.1	3.9	4	0.56	0.54
	% Dif.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-2.4	-2.5	-2.4	1.8	1.9
	SD	2.29	1.58	5.6	0	5.6	0	5.6	9.56	5.6	14.47	14.47	0.24	0.22	0.21	0.052	0.072
	SE	0.48	0.33	1.17	0	1.17	0	1.17	1.99	1.17	3.02	3.02	0.05	0.05	0.04	0.011	0.015
	N	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23
3.0%	Mean	15.5	13.8*	90.5+	0	9.6+	0	9.6+	9.9	9.6+	46.5	53.5	4.1	3.9	3.9	0.52	0.51
	% Dif.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-2.4	-2.5	-4.9	-5.5	-3.8
	SD	2.64	1.61	6.14	0	6.14	0	6.14	9.14	6.14	14.67	14.67	0.26	0.23	0.26	0.043	0.041
	SE	0.54	0.33	1.25	0	1.25	0	1.25	1.87	1.25	2.99	2.99	0.05	0.05	0.05	0.009	0.008
	N	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24

Percentage data compared using Dunn's Test

Fetal weights compared using Dunnett's Test.
* = Significantly different from the control group at 0.01 using parametric analysis
+ = Significantly different from the control group at 0.01 using non-parametric analysis
NA = Not Applicable SD = Standard Deviation SE = Standard Error
% Dif. = Percent Difference N = Number of Animals

Table 7: Summary of Fetuses and Litter with Malformations (Absolute Numbers)

		Fetuses					Litters				
		Control	1.5%	2.0%	2.5%	3.0%	Control	1.5%	2.0%	2.5%	3.0%
External Examination	Number Examined	282	278	277	284	299	24	23	23	23	24
	Cyclopia	1	0	0	0	0	1	0	0	0	0
	Proboscis-Like Nose	1	0	0	0	0	1	0	0	0	0
	Mandibular Micrognathia	1	0	0	0	0	1	0	0	0	0
	Maxillary Agnathia	1	0	0	0	0	1	0	0	0	0
	Astomia	1	0	0	0	0	1	0	0	0	0
	Aglossia	1	0	0	0	0	1	0	0	0	0
	Only 2 Facial Papillae Present	1	0	0	0	0	1	0	0	0	0
	Pinnae Malpositioned	1	0	0	0	0	1	0	0	0	0
	Gastroschisis	0	0	0	0	1	0	0	0	0	1
	Localized Fetal Edema	0	0	0	0	2	0	0	0	0	1
	Vertebral Agenesis	0	1	0	0	0	0	1	0	0	0
	Anal Atresia	0	1	0	0	0	0	1	0	0	0
Visceral Examination	Number Examined	282	278	277	284	299	24	23	23	23	24
	Number With Findings	0	0	0	0	0	0	0	0	0	0
Skeletal Examination	Number Examined	282	278	277	284	299	24	23	23	23	24
	Bent Scapula	0	0	0	2	1	0	0	0	2	1
	Bent Limb Bone(s)	0	0	0	0	1	0	0	0	0	1
	Rib Anomaly	0	1	0	0	0	0	1	0	0	0
	Vertebral Anomaly with or without Rib Anomaly	0	0	1	0	0	0	0	1	0	0
	Sternoschisis	0	0	1	0	0	0	0	1	0	0
Total Numbers with Malformations	External	1	1	0	0	3	1	1	0	0	2
	Soft Tissue	0	0	0	0	0	0	0	0	0	0
	Skeletal	0	1	2	2	1	0	1	2	2	1
	Combined	1	2	2	2	4	1	2	2	2	3

Table 8: Summary of Litter Proportions of Malformations (% Per Litter)

		Control			1.5%			2.0%			2.5%			3.0%		
		24 Examined			23 Examined			23 Examined			23 Examined			24 Examined		
		Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
External Examination	Cyclopia	0.3	1.70	0.35	0	0	0	0	0	0	0	0	0	0	0	0
	Proboscis-Like Nose	0.3	1.70	0.35	0	0	0	0	0	0	0	0	0	0	0	0
	Mandibular Micrognathia	0.3	1.70	0.35	0	0	0	0	0	0	0	0	0	0	0	0
	Maxillary Agnathia	0.3	1.70	0.35	0	0	0	0	0	0	0	0	0	0	0	0
	Astomia	0.3	1.70	0.35	0	0	0	0	0	0	0	0	0	0	0	0
	Aglossia	0.3	1.70	0.35	0	0	0	0	0	0	0	0	0	0	0	0
	Only 2 Facial Papillae Present	0.3	1.70	0.35	0	0	0	0	0	0	0	0	0	0	0	0
	Pinnae Malpositioned	0.3	1.70	0.35	0	0	0	0	0	0	0	0	0	0	0	0
	Gastroschisis	0	0	0	0	0	0	0	0	0	0	0	0	0.3	1.36	0.28
	Localized Fetal Edema	0	0	0	0	0	0	0	0	0	0	0	0	0.6	2.72	0.56
	Vertebral Agenesis	0	0	0	0.4	1.74	0.36	0	0	0	0	0	0	0	0	0
	Anal Atresia	0	0	0	0.4	1.74	0.36	0	0	0	0	0	0	0	0	0
Visceral Examination	Number With Findings	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Skeletal Examination	Bent Scapula	0	0	0	0	0	0	0	0	0	0.8	2.51	0.52	0.3	1.70	0.35
	Bent Limb Bone(s)	0	0	0	0	0	0	0	0	0	0	0	0	0.3	1.70	0.35
	Rib Anomaly	0	0	0	0.3	1.39	0.29	0	0	0	0	0	0	0	0	0
	Vertebral Anomaly with or without Rib Anomaly	0	0	0	0	0	0	0.4	1.74	0.36	0	0	0	0	0	0
	Sternoschisis	0	0	0	0	0	0	0.4	1.74	0.36	0	0	0	0	0	0
Percent Per Litter with Malformations	External	0.3	1.7	0.35	0.4	1.74	0.36	0	0	0	0	0	0	0.8	2.99	0.61
	Soft Tissue	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Skeletal	0	0	0	0.3	1.39	0.29	0.7	2.40	0.50	0.8	2.51	0.52	0.3	1.70	0.35
	Combined	0.3	1.70	0.35	0.7	2.18	0.45	0.7	2.40	0.50	0.8	2.51	0.52	1.2	3.35	0.68

SD = Standard Deviation SE = Standard Error

Table 9: Summary of Fetuses and Litters with Variations (Absolute Numbers)

		Fetuses					Litters				
		Control	1.5%	2.0%	2.5%	3.0%	Control	1.5%	2.0%	2.5%	3.0%
External Examination	Number Examined	282	278	277	284	299	24	23	23	23	24
	Number of Findings	0	0	0	0	0	0	0	0	0	0
Visceral Examination	Number Examined	282	278	277	284	299	24	23	23	23	24
	Major Blood Vessel Variation	0	2	4	0	2	0	1	3	0	2
	Liver – Accessory Lobule(s)	4	0	4	1	1	2	0	3	1	1
	Renal Papilla(e) Not Developed and/or Distended Ureter(s)	1	3	0	3	3	1	2	0	1	2
Skeletal Examination	Number Examined	282	278	277	284	299	24	23	23	23	24
	14th Rudimentary Rib(s)	28	19	26	11	12	11	10	9	7	8
	Cervical Centrum #1 Ossified	93	61	76	53	75	21	16	17	18	17
	Bent Rib(s)	10	6	4	6	5	8	4	4	5	3
	Sternebra(e) Malaligned (slight to moderate)	4	2	3	2	0	4	2	3	2	0
	Reduced Ossification of the 13th Rib	4	4	2	8	3	3	2	1	5	3
	Sternebra(e) # 5 and/or #6 Unossified)	17	23	21	30	24	12	10	12	12	11
	Sternebra(e) #1, #2, #3, and/or #4 Unossified	0	0	0	2	2	0	0	0	2	2
	7th Cervical Rib(s)	3	8	2	0	1	3	3	1	0	1
	27 Presacral Vertebrae	1	0	0	0	1	1	0	0	0	1
	Hyoid Unossified	2	1	1	2	1	1	1	1	2	1
	Reduced Ossification of the Vertebral Arches	0	0	2	1	6	0	0	1	1	3
	25 Presacral Vertebrae	0	4	0	0	0	0	1	0	0	0
	Reduced Ossification of the Skull	1	1	1	2	3	1	1	1	2	3
	14th Full Rib(s)	1	0	0	0	0	1	0	0	0	0
	Unco-Ossified Vertebral Centra	0	0	0	0	1	0	0	0	0	1
	Extra Site of Ossification Ventral to Cervical Centrum #2	0	0	0	0	1	0	0	0	0	1
	Reduced Ossification of the Rib(s)	1	0	1	0	1	1	0	1	0	1

Table 10: Summary of Litter Proportions of Variations (% Per Litter)

		Control			1.5%			2.0%			2.5%			3.0%		
		24 Examined			23 Examined			23 Examined			23 Examined			24 Examined		
External Examination		Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
	Number with findings	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Visceral Examination	Major Blood Vessel Variation	0	0	0	0.7	3.21	0.67	2.5	8.58	1.79	0	0	0	0.7	2.38	0.49
	Liver – Accessory Lobule(s)	1.4	5.31	1.08	0	0	0	1.4	4.04	0.84	0.3	1.49	0.31	0.3	1.70	0.35
	Renal Papilla(e) Not Developed and/or Distended Ureter(s)	0.4	2.04	0.42	1.0	3.47	0.72	0	0	0	0.9	4.47	0.93	0.9	3.31	0.68
Skeletal Examination	14th Rudimentary Rib(s)	9.5	16.41	3.35	6.8	10.72	2.32	9.3	15.94	3.32	3.8	6.81	1.42	3.8	6.25	1.27
	Cervical Centrum #1 Ossified	34.1	25.06	5.12	21.9	21.34	4.45	26.6	27.21	5.67	19.3	16.77	3.50	25.1	28.82	5.88
	Bent Rib(s)	4.0	7.07	1.44	2.2	5.07	1.06	1.4	3.20	0.67	2.1	4.43	0.95	1.6	4.63	0.95
	Sternebra(e) Malaligned (slight to moderate)	1.5	3.55	0.72	0.7	2.18	0.45	1.0	2.67	0.56	0.9	2.88	0.60	0	0	0
	Reduced Ossification of the 13th Rib	1.5	4.28	0.87	1.5	5.47	1.14	0.7	3.21	0.67	2.7	6.19	1.29	1.0	2.61	0.53
	Sternebra(e) # 5 and/or #6 Unossified)	6.6	9.01	1.84	8.3	13.34	2.78	7.8	9.20	1.92	10.0	19.59	4.09	7.4	12.85	2.62
	Sternebra(e) #1, #2, #3, and/or #4 Unossified	0	0	0	0	0	0	0	0	0	0.6	2.14	0.45	0.6	2.03	0.41
	7th Cervical Rib(s)	1.2	3.18	0.65	3.0	10.62	2.22	0.6	2.98	0.62	0	0	0	0.3	1.36	0.28
	27 Presacral Vertebrae	0.3	1.70	0.35	0	0	0	0	0	0	0	0	0	0.3	1.36	0.28
	Hyoid Unossified	1.0	5.10	1.04	0.3	1.49	0.31	0.4	1.90	0.40	0.8	2.75	0.57	0.4	2.04	0.42
	Reduced Ossification of the Vertebral Arches	0	0	0	0	0	0	0.8	3.79	0.79	0.4	1.74	0.36	2.2	6.67	1.36
	25 Presacral Vertebrae	0	0	0	1.4	6.95	1.45	0	0	0	0	0	0	0	0	0
	Reduced Ossification of the Skull	0.3	1.57	0.32	0.4	2.09	0.43	0.4	1.90	0.40	0.6	2.06	0.43	1.1	2.90	0.59
	14th Full Rib(s)	0.3	1.70	0.35	0	0	0	0	0	0	0	0	0	0	0	0
	Unco-Ossified Vertebral Centra	0	0	0	0	0	0	0	0	0	0	0	0	0.3	1.70	0.35
	Extra Site of Ossification Ventral to Cervical Centrum #2	0	0	0	0	0	0	0	0	0	0	0	0	0.3	1.70	0.35
	Reduced Ossification of the Rib(s)	0.5	2.55	0.52	0	0	0	0.4	1.74	0.36	0	0	0	0.3	1.36	0.28
Percent Per Litter with Variations	External	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Soft Tissue	1.8	5.58	1.14	1.6	4.58	0.96	4.0	9.08	1.89	1.2	5.96	1.24	2.0	4.12	0.84
	Skeletal	51.2	23.10	4.71	38.3	23.37	4.87	41.8	25.50	5.32	37.0	21.94	4.57	38.7	25.65	5.24
	Combined	51.5	22.88	4.67	39.0	23.1	4.82	44.3	25.49	5.31	37.3	22.28	4.65	40.0	24.61	5.02

SD = Standard Deviation SE = Standard Error

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Female rats were exposed to clean air or 1.5%, 2.0%, 2.5%, or 3.0% carbon dioxide (CO_2) gas for ~23 hr/day from GD6-20 of pregnancy. There were no remarkable post-exposure findings in adults, including no differences in bodyweight or food consumption. There were no remarkable post-exposure findings in fetal bodyweight, and no malformations or developmental variations following CO_2 exposure. There were two statistically significant findings in the 3.0% CO_2 group: a higher mean post-implantation loss per litter (early resorptions) and a corresponding lower mean proportion of viable fetuses per litter. These results yield a No Observed Adverse Effect Level (NOAEL) of 2.5% and a Lowest Observed Adverse Effect Level (LOAEL) of 3.0% for CO_2. Using the 2.5% NOAEL as a point of departure for a developing a CEL and an interspecies uncertainty factor of 3 yields a recommended CEL of 0.8% for CO_2. Developmental endpoints are assumed to result from a single day of exposure during gestation; therefore, the 24-hour EEL is also recommended to be made 0.8% CO_2. These recommendations should not outweigh any other studies that may derive a lower recommendation based on relevant data and endpoints.

15. SUBJECT TERMS
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