AR226-1104 SRPT 12234

Study No. T-6295.30; ST69
Oxygen Saturation and pO2 Levels in Rat Pups Borne to PFOS Exposed Dams

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Study Objective

This study was the first of a series of exploratory pilot studies designed to investigate the mechanism of perinatal mortality in rats and mice borne to timed-pregnant dams dosed with PFOS during gestation. The hypothesis that PFOS interferes with normal respiration and/or gas exchange in the pups was investigated. In addition, amniotic fluid was analyzed for PFOS levels and specimens were gathered for possible future analysis (i.e., to investigate the ability of PFOS to interfere with the glucose metabolism).

Method Summary

This study was performed in the 3M Strategic Toxicology Laboratory under a defined protocol (1) and classified as a "Class B Study" as explained in TOX SOP 0950, Strategic Toxicology Lab GLP Program Procedure (2). All in-life procedures were approved by the 3M Laboratory Animal Review Committee (3M LARC) and are detailed in animal usage application 2001-0182 (3).

Under the initial study protocol (1), dams were dosed on days 14-17 of gestation to reach of cumulative dose of 100 mg/kg. The intent was to allow these animals to litter, and to then sacrifice both dams and pups within 24 hours of birth. This dosing regime, however, resulted in very high rates of stillborn pups and early deliveries, greatly complicating the study and preventing the endpoints of interest (oxygen saturation and partial oxygen pressure in blood (pO2)) from being examined. In response, the study protocol was amended and a second study as performed. As discussed in the protocol amendment (4), it is thought that an excessive dose of PFOS may have been delivered to the pups and/or that dosing period may have been too late in gestation. Under the amended study protocol, animals were treated via oral gavage with 25 mg PFOS per kg body weight per day for 3 consecutive days (days 12-14 of gestation) to reach a cumulative dose of 75 mg/kg. All animals, including dams, were euthanized via decapitation just prior to delivery. Pups were removed via caesarean-section (c-section), to standardize the time of data collection, and manually stimulated to breath prior to decapitation under a heat lamp set to maintain a temperature of ~ 95 to 98 degrees F. Blood and amniotic fluid (pooled from multiple amniotic sacs using a needle and syringe prior to removing each pup) were collected from each dam for PFOS analysis (see Appendix 1 for details on PFOS analytical method). Liver was also collected from each c-sectioned dam and flash frozen in liquid nitrogen for possible future analysis. Pups were decapitated within 5 minutes being removed from the placental sac. Immediately following decapitation of the pups, blood was drained onto a heparinized weigh boat and pipetted into an I-Stat cartridge for determination of pO2 and other blood gases by the I-Stat method (described in the study protocol, 1). No oxygen saturation measurements using the pulse oximeter were obtained. One additional dam was received from the vendor. It was dosed with PFOS as described above and allowed to litter. This dam and her litter were then monitored to assess viability after birth using this study design.

Results

Initial Study

In-life observations from the initial study can be found in Table 1. Four control animals and six PFOS treated animals were included in this study. One control and one treated animal were not pregnant. Pregnant control animals gained an average of 33.0 ± 7.8 grams from day 14-17 of gestation while treated pregnant animals gained only an average of 8.6 ± 12.2 grams. All control animals littered on day 21 of gestation and all treated animals littered on either day 20 or 21 of gestation. Control litters appeared normal while treated litters were either borne dead or died shortly after birth (within approximately 10 minutes). Liver and sera were gathered from some dams and pups and flash frozen for possible future analysis. An attempt was made to measure oxygen saturation using a pulse oximeter, and pO2 levels with an I-Stat blood gas analyzer on those pups borne alive. No useful data was gathered for either parameter. It was decided that the pulse oximeter method was not suitable for use on such small pups and that future blood gas analysis would be limited to the I-Stat method.

Amended Study

In-life observations from the amended study can be found in Table 2. Three control animals and 4 PFOS treated animals were included in this study. All animals were pregnant. Control animals gained an average of 99.5 ± 0.7 grams from day 12-21 of gestation while treated animals gained only an average of 74.8 ± 11.4 grams. Dams were palpitated on gestation day (GD) 19 by Dr. DeWayne Walker (3M Lab Animal Veterinarian) and pups were determined to be too immature to survive if delivered. One control animal was decapitated and c-sectioned on GD20 and pups (n=12) were unable to breath and survive. C-sectioning of the remaining dams was thus postponed on the recommendation of Dr. Walker until early morning (5:30 – 6:30am) on GD21. The remaining 2 control animals were decapitated and c-sectioned on GD21, and litters of 10 and 11 pups were delivered, all appearing normal. Three of the treated dams were also decapitated and c-sectioned and litters of 10-13 pups were delivered. Blood was evident in the uterine horns of each of the PFOS treated dams. Treated pups had difficulty breathing and some died within minutes after birth. It was determined that these treated pups were not as mature as the control pups. The remaining PFOS dam was allowed to litter. She delivered 13 pups (10 on GD22 am, 3 on GD23 am), all borne dead or dying within minutes of birth.

I Stat data from the pups can be found in Table 3. The only parameter that significantly different between control and PFOS treated groups ($p \leq 0.05$) was pH, with controls averaging 7.14 ± 0.08 (N=2) and PFOS treated averaging 7.07 ± 0.03 (N=8). No significant difference in pO2 levels (mmHg) or O2 saturation (%) was found between treated and control pups. Control pO2 levels averaged 30 ± 4 mmHg while levels in PFOS treated pups averaged 35 ± 6 mmHg. Oxygen saturation averaged 39 ± 1 % in controls and 44 ± 13 % in PFOS treated pups. Systemic pO2 represents the O2 levels at the exact time the blood is collected and is readily altered by a few breaths (5). Literature values for pO2 from control pups delivered from c-sectioned decapitated dams are ~ 60 mmHg when taken within 30 seconds of birth and after at least one breath (5). This is equivalent to vaginally born pup PO2 levels (5). The pO2 levels found in the current study are substantially lower than those cited in the literature. This may be a result of the

increased time the pups were allowed to breath on their own prior to decapitation (5 minutes as opposed to 30 seconds).

Dam sera and amniotic fluid PFOS levels can be found in Table 4. Control sera and amniotic fluid PFOS levels averaged 0.017 ± 0.003 ug/ml and 0.011 ± 0.003 ug/ml respectively. The ratio of sera to amniotic fluid PFOS levels for controls averaged 1.7:1. PFOS treated sera and amniotic fluid PFOS levels averaged 68.3 ± 15.0 ug/ml and 16.4 ± 1.2 ug/ml respectively. The ratio of sera to amniotic fluid PFOS levels for the PFOS treated dams averaged 4.2:1.

Conclusions

The low numbers of animals used in this exploratory pilot study prevents definitive conclusions from being drawn. The results of the I Stat analysis suggest, however, that PFOS does not interfere with normal respiration and/or gas exchange in neonatal rat pups borne to PFOS exposed dams. Results of the dam sera and amniotic fluid PFOS analysis suggest that PFOS distributes more readily into the sera than into the amniotic fluid (approximately 4:1 in PFOS treated animals). PFOS was, however, detectable in the amniotic fluid of all animals tested. This shows that neonatal rat pups borne to PFOS treated dams are exposed to PFOS *in utero* via the amniotic fluid. The dose used in the amended study, 25 mg/kg/day on days 12-14 of gestation (cumulative dose of 75 mg/kg/day) produced death in live borne pups within minutes of birth. A dose producing death within the first 6 - 24 hours of live birth is desired to allow time for data collection and observation. It is also thought that a single dose may produce the same effect as multiple daily doses. Thus, a dose response study will be performed to determine if the dosing regime can be limited to a single administration and to find a dose level that will extend life to 6-24 hours following birth.

List of Tables

Table 1: Initial Study In-Life Data

	Dose (GDs 14-17)		Body Weight (g)							
Animal #	(mg/kg/day)	Cumulative	GD14	GD15	GD16	GD17	GD18	Change GD 14-17	Litter day	Comments
1R05311	0	0	266	272	281	295	NA	29	GD21	(am) no count, all normal
1R05312	0	0	322	331	343	364	NA	42	GD21	(am) no count, all normal
1R05316	0	0	280	287	295	308	324	28	GD21	(am) 13 pups all normal
1R05317	0	0	261	255	258	263	266	2	NA	Not pregnant
1R05313	25	100	296	304	307	306	NA	10	GD20	(pm) no count, pups borne alive but dying within 10 minutes
1R05314	25	100	315	322	330	339	NA	24	GD20	(pm) started to litter, performed C-Sect., all pups dead
1R05315	25	100	318	320	318	310	NA	-8	GD20	9 pups in am, 1 in pm, all borne dead
1R05318	25	100	314	314	325	316	307	2	GD21	(am) 2 pups, borne dead
1R05319	25	100	257	249	235	222	213	-35	NA	Not pregnant
1R05320	25	100	279	288	293	294	278	15	GD21	(am) 1 pup, borne dead

Gestation Day (GD)
Not Applicable or Not Available (NA)

Table 2: Amended Study In-Life Data

Animal #	Dose (GDs 12-14) (mg/kg/day)	Cumulative (mg/kg)	Body Weight (g) GD12	GD13	GD14	GD19	GD20	GD21	Change GD 12-21	Litter day	C-section day	Number pups / litter	comments
1R06028	0	0	241	248	259	320	329	NA	NA	NA	GD20	12	pups not mature enough to breath & survive
1R06029	0	0	280	290	293	350	363	379	99	NA	GD21	10	normal
1R06030	0	0	287	294	296	352	371	387	100	NA	GD21	11	normal
1R06031	25	75	255	269	256	311	330	345	90	NA	GD21	13	not as mature as controls, difficulty breathing, some dying before we could decipatate them (within minutes); blood in uterine horn
1R06032	25	75	255	262	254	302	319	332	77	NA	GD21	11	not as mature as controls, difficulty breathing, some dying before we could decipatate them (within minutes); blood in uterine horn
1R06033	25	75	216	227	219	251	266	281	65	NA	GD21	10	not as mature as controls, difficulty breathing, some dying before we could decipatate them (within minutes); blood in uterine horn
1R06034	25	75	232	232	220	268	291	299	67	GD22 & 23	NA	13	10 on GD22 (am), 3 on GD23 (am), all borne dead or died shortly after birth (within minutes)

Gestation Day (GD)
Not Applicable or Not Available (NA)

Table 3: Amended Study I Stat Data (Pups)

Animal # (dam)	Dose Group	cumulative maternal dose (mg/kg)	pup # *	pH	p CO2 (mmHg)	p O2 (mmHg)	BE (mmol/L)	HCO3(mmol/L)	TCO2 (mmol/L)	O2 sat (%)	Na (mmol/L)	K (mmol/L)	iCa++ (mmol/L)	Hct (%PCV)	Hgb (g/dL)
1R06030	Control	0	1-5	7.08	80.0	32	-8	23	26	38	131	9	0.74	39	13
1R06030	Control	0	6-10	7.20	46.1	27	-9	18	19	39	130	>9	0.33	29	10
Control Average				**7.14**	**63.1**	**30**	**-9**	**21**	**23**	**39**	**131**	**9**	**0.54**	**34**	**12**
Control St Dev				**0.08**	**24.0**	**4**	**1**	**4**	**5**	**1**	**1**	**0**	**0.29**	**7**	**2**
			1-5	7.07	76.5	33	-8	22	25	41	128	>9	0.75	35	12
			6-8	6.99	76.1	24	-13	18	21	20	128	8	0.32	34	12
1R06031	PFOS	75	9-11	7.05	63.7	38	-13	17	19	49	129	>9	0.74	37	13
			1-5	7.10	70.7	34	-7	22	24	44	133	7	0.30	28	10
1R06032	PFOS	75	6-10	7.09	51.3	36	-13	16	17	49	125	8	0.37	29	10
			1-5	7.08	67.7	29	-9	20	22	33	121	9	0.31	20	7
			6-8	7.09	49.3	41	-14	15	17	58	124	>9	0.39	35	12
1R06033	PFOS	75	9-11	7.07	40.5	42	-16	12	13	58	122	8	NA	18	6
PFOS Average				**7.07**	**62.0**	**35**	**-12**	**18**	**20**	**44**	**126**	**8**	**0.45**	**30**	**10**
PFOS St Dev				**0.03**	**13.4**	**6**	**3**	**3**	**4**	**13**	**4**	**1**	**0.20**	**7**	**3**
T-Test control/treated				**0.04**	**0.47**	**0.15**	**0.11**	**0.17**	**0.21**	**0.29**	**0.09**	**0.12**	**0.33**	**0.23**	**0.27**

* Blood samples pooled from multiple pups per litter for each reading.
Not available: NA

Table 4: Amended Study Maternal Sera and Amniotic Fluid PFOS Levels					
Animal #	**Dose Group**	**Cumulative Dose (mg/kg)**	**Serum PFOS (ug/ml)**	**Amniotic Fluid PFOS (ug/ml)**	**sera:amniotic fluid PFOS ratio**
1R06028	control	0	NA	NA	NA
1R06029	control	0	0.019	0.009	2.1:1
1R06030	control	0	0.015	0.013	1.2:1
	Control Average		**0.017**	**0.011**	**1.65:1**
	Control Std Dev		**0.003**	**0.003**	**0.64**
1R06031	PFOS	75	56.0	16.0	3.5:1
1R06032	PFOS	75	64.0	17.7	3.6:1
1R06033	PFOS	75	85.0	15.5	5.5:1
1R06034	PFOS	75	NA	NA	NA
	PFOS Average		**68.3**	**16.4**	**4.2:1**
	PFOS Std Dev		**15.0**	**1.2**	**1.13**
Not Available (NA)					

Signatures:

Prepared By:

Deanna Luebker 12/31/2001

Deanna Luebker, MS
Advanced Research Toxicologist
Study Director

Date

Andrew M. Seacat 1/2/02

Andrew Seacat, Ph.D.
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Date

Reviewed By:

John T. Butenhoff 2/15/2002

John Butenhoff, Ph.D.
Corporate Toxicology Management

Date

Daniel C. Hakes 2/26/02

Dan Hakes
Sponsor Representative

Date

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References

1. 3M Medical Department, Corporate Toxicology Protocol for Study No. T-6295.30 DT-69 Oxygen Saturation and pO2 Levels in Rat Pups Borne to PFOS Exposed Dams, 2001.

2. 3M Medical Department, Corporate Toxicology Strategic Toxicology Laboratory Standard Operating Procedure (TOX SOP) No. 0950 – GLP Program Procedure, 1999.

3. 3M Lab Animal Review Committee Animal Usage Application No. 2001-0182, MECHANISTIC RESEARCH IN TIMED PREGNANT RATS AND MICE – AMENDMENT TO AUA # 2001-0182, 2001.

4. 3M Medical Department, Corporate Toxicology Amendment #1 for Study No. T-6295.30 DT-69 Oxygen Saturation and pO2 Levels in Rat Pups Borne to PFOS Exposed Dams, 2001.

5. Vaillancourt C., Berger N., and Boksa P. (1999) Effects of vaginal birth versus Caesarean section birth with general anesthesia on blood gasses and brain energy metabolism in neonatal rats. Experimental Neurology 160:142-150.

Appendix 1

3M CorporateToxicology

Analytical Laboratory Bldg 236

To: **Deanna Luebker**
Andrew Seacat

From: **David J. Ehresman**

Compound: **Oxygen Saturation and pO2 Levels in Rat Pups Borne to PFOS Exposed Dams**
3M Medical Department Study Number T-6295.30
3M Strategic Toxicology Lab Folder Number DT-69

Date **December 10, 2001**

Pregnant female rats were exposed to PFOS to evaluate the pup fetal lung maturity in an attempt to determine rat pup oxygen levels at birth. Part of this study involved determining the concentration of PFOS in the female rat serum and in the amniotic fluid post exposure to correlate with the Oxygen Saturation levels of the exposed and control pups.

Samples of the rat serum and amniotic fluid from both exposed and control animals were deliver to the Strategic Toxicology Laboratory for analysis by LC-MS. The amniotic fluid samples were collected at the time of Caesarean section delivery for both the control and dosed animals.

Control animals were pregnant females rats with the same approximate gestational status. Control amniotic fluid and serum were collected from them for analysis.

Analytical Method:

PFOS levels were determined from both the rat serum and amniotic fluid using a single extraction in ethyl acetate. The pH of the tested solution (100 uL; serum or amniotic fluid) was adjusted to an approximate pH of 3.0 with the addition of 400 uL of a 0.2 N formic acid buffer solution (pH = 3.0). Internal standard (PFHS) was added prior to the extraction and the mixture was shaken on a mechanical shaker for 1 hour. The tubes were then centrifuged at 3000 rpm's for 5 minutes and the ethyl acetate transferred to a clean polypropylene tube. The ethyl acetate was dried to dryness using a gentle stream of nitrogen gas on the N-Evap drier. The resultant residue was re-dissolved in 250 uL of 25% acetonitrile and 75% 10 mm ammonium acetate buffer. This mixture was then filtered through a 0.2 micron syringe filter and placed in a polypropylene injection vial. 25 uL was injected on to the LC-MS system.

Appendix 1

The liquid chromatograph used was the Finnigan MAT Spectra system P4000. The column used was a Keystone Fluorophase (150 x 2.1 mm) speciality column featuring a selective, fluorinated, branched-chain alkyl packing. The flow rate was maintained at a constant 0.4 ml/min. A gradient flow of acetonitrile was used to
elute the compounds of interest starting with 15% acetonitrile and ending with a concentration of 70 % acetonitrile over a six and one half minute gradient program. There was a constant methanol concentration of 10% methanol with the balance of the mobile phase being 10 mm ammonium acetate buffer. The initial buffer concentrations were returned to starting 6.5 minutes into the run and reached at 8.0 minutes. Column equilibration period was then 2.5 additional minutes before the next injection for a cycle time of 10.5 minutes per injection. All compounds of interest eluted within the first 5 minutes of the gradient run.

The Finnigan TSQ system was operated in the MS/MS mode using the Electro Spray source in the negative ionization mode with a constant source potential of 3.0 kV applied. The reactant gas in the Q2 region of the triple quad system was argon. The method involved the transition of the following mass ions:

Compound	Base Peak (Q1 mass)	MS/MS Ion	Q2 Offset Voltage
Int. Std. (PFHS)	399.0	80	60 volts
PFOS	499.0	80	60 volts

The capillary heater was held at a constant 300 degrees C. All quantitative calculations were based on the MS/MS ion ratios between the compound of interest (PFOS) and the Internal Standard (PFHS).

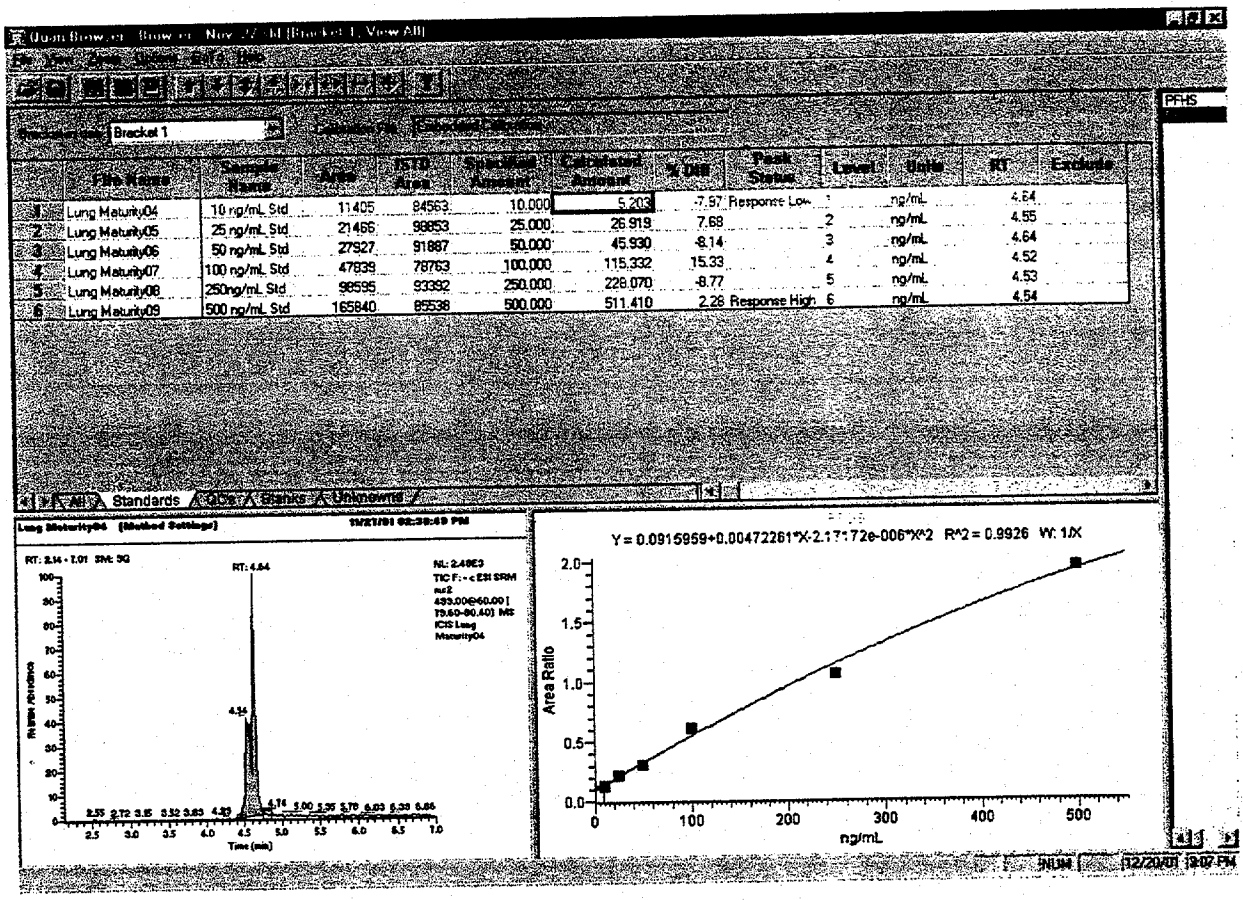
Study Results:

Animal Number	Status	Serum PFOS Level	Amniotic Fluid Level
6029	Control	19 ng/mL	9 ng/mL
6030	Control	15 ng/mL	13 ng/mL
6031	Dosed Animal	56.0 ug/mL	16.0 ug/mL
6032	Dosed Animal	64.0 ug/mL	17.7 ug/mL
6033	Dosed Animal	85.0 ug/mL	15.5 ug/mL

Due to the high concentration of PFOS in the serum a 1:500 dilution was required to bring the concentration down to be within the quantitation limits of the established standard curve for the dosed animals. The dosed animal amniotic fluid was diluted 1:100 before the concentration was within the acceptable curve limitations. The routine standard curves were established using 6 points between 10 ng/mL and 500 ng/mL.

Appendix 1

The R squared on the curve used for the calculated results was 0.9926 for the straight serum and amniotic fluid results run on the control animals and the 1:100 dilution of the dosed animal amniotic fluid extracts. The 1:500 dilution of the dosed rat serum curve had an R squared value of 0.9997.



Straight serum, diluted serum, and control dams amniotic fluid standard curve graph from original Finnigan Xcalibur program.

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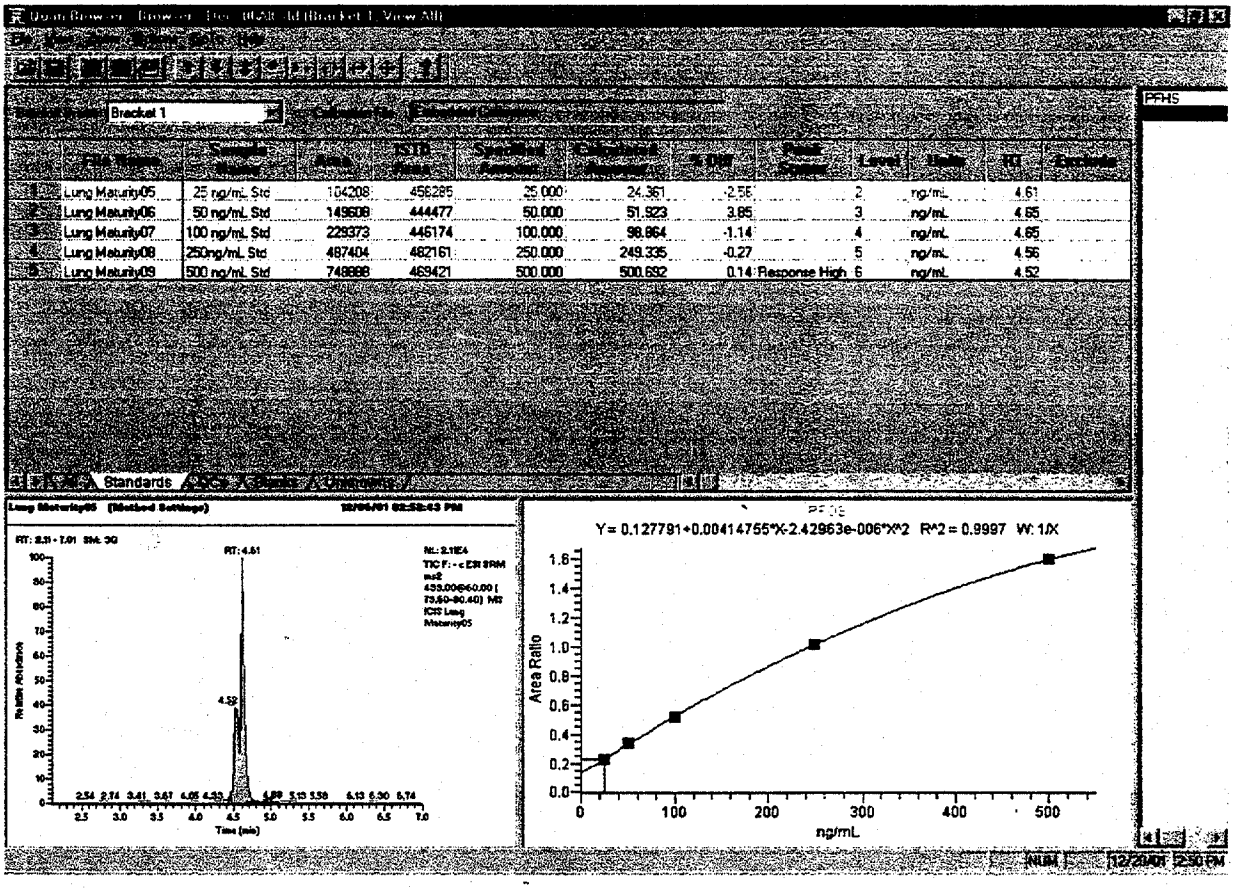
Appendix 1



This graph represents the standard curve used with the 1:500 diluted specimens. Note: This curve was based on 5 points starting at 25 ng/mL through 500 ng/mL. The 10 standard was dropped on this run due to low detector response.

The raw data and printed reports are contained in labeled 3 ring binders in the Bldg. 236 Strategic Toxicology LC-MS Laboratory. All original methods and raw data are stored as files that are backed up to Zip discs from the Finnigan Xcalibur system. These files would need to be re-loaded to a compatible Finnigan Xcalibur system to function properly.

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3M MEDICAL DEPARTMENT, CORPORATE TOXICOLOGY
Protocol for Study No. T-6295.30 DT-69
Oxygen Saturation and pO2 Levels in Rat Pups Borne to PFOS Exposed Dams

***Study Objective*:**
This study is the first of a series designed to investigate the mechanism of perinatal mortality in rats and mice borne to timed-pregnant dams dosed with PFOS during gestation. The hypothesis that will be tested in this study is that PFOS interferes with normal respiration and/or gas exchange in the pups. In addition, specimens will be gathered to investigate the ability of PFOS to interfere with the glucose metabolism.

Research Client: 3M Specialty Chemicals Division
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Saint Paul MN 55133-3220

Sponsor: 3M Specialty Chemicals Division
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Saint Paul MN 55133-3220

Study Location: 3M Strategic Toxicology Laboratory
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Proposed Study Timeline:
In-Life Start Date: September 24, 2001
In-Life End Date: October 2, 2001

Regulatory Compliance:
This study will be performed in the 3M Strategic Toxicology Laboratory in the "spirit of GLP". A defined protocol will be in place and the study will be classified as "Class B" as explained in TOX SOP 0905, Strategic Toxicology Lab GLP Program Procedure. All in-

life procedures have been approved by the 3M Laboratory Animal Review Committee (3M LARC) and are detailed in animal usage application 2001-0182.

Test Material:
Perfluorooctane Sulfonic Acid, Potassium Salt (FC-95) Lot 217
CAS # 2795-39-3
$C_8F_{17}OSO_2^-K^+$

Animals:

Species: Rat
Strain: Sprague Dawley
Source: Charles River Laboratories, Inc.
Age at initiation of treatment: Day 16 of Gestation
Number and sex: . 8, female
Identification: unique tail mark

Husbandry:

Housing:

All rats will be individually housed in standard solid bottom cages and provided nesting material.

Diet/Water:

All rats will be provided tap water and rat chow *ad libitum* throughout the study.

Environment:

Environmental controls for the animal room will be set to maintain a temperature of 72 ± 3°F, humidity of 30-70%, a minimum of 10 exchanges of room air per hour and a 12 hour light/dark cycle.

Dose and Dosing Procedures:

Studies conducted by the EPA NHEERL have demonstrated that the PFOS-induced increases in perinatal mortality observed in previous rat reproduction studies, in which dosing occurred from 42 days prior to mating through end of lactation, can occur in rats and mice by dosing during gestation alone. This effect appears to relate directly to body burden and can be reproduced by dosing during the last several days of gestation. A cumulative dose of roughly 100 mg/kg has been shown to reduce pup viability by approximately 50% within the first 5 days of lactation. These observations provide an effective exposure model for mechanistic research.

Four of the animals will arrive at 3M on gestation day 9, and four animals on gestation day 10. Animals were ordered in this manner so littering would occur one day apart. Dosing will occur following a one-week acclimation period as outlined in Table 1. Four rats will be treated via oral gavage with 25 mg PFOS per kg body weight per day, for four consecutive days (days 16-19 of gestation) to reach a cumulative dose of 100mg/kg. A 5mg/mL stock solution of PFOS in 2% Tween 80 will be prepared immediately prior to

dosing and 5 ml dosing solution / kg body weight will be administered to each rat in the PFOS treatment group. Control rats will receive 5ml/kg 2% Tween 80 on days 16-19 of gestation.

TABLE 1: Dosing Regime

Dose Groups	N	Dosing days	Cumulative Dose (mg/kg)	Sacrifice dates
A. Control (2% Tween 80)	4	A.1: 9/24 - 9/27/01 A.2: 9/25 – 9/28/01	0	A.1: N = 2 on Monday 10/1/01 A.2: N = 2 on Tuesday 10/2/01
B. PFOS treatment (25 mg/kg/day PFOS)	4	B.1: 9/24 - 9/27/01 B.2: 9/25 – 9/28/01	100 mg/kg	B.1: N = 2 on Monday 10/1/01 B.2: N = 2 on Tuesday 10/2/01

Observation of Animals:

Clinical Observations:

Each animal will be observed daily (excluding weekends) for mortality and morbidity and notable findings will be recorded.

Body Weights:

Dams will be weighed immediately prior to each dosing. Terminal body weights will be recorded on dams and litters.

Sample Collection:

Number & Frequency

Animals will be sacrificed within 24 hours of delivery. Dams will be euthanized via CO2 asphyxiation and pups via decapitation. Animals have been ordered such that four will litter Sunday night, September 30th. The remaining four will litter Monday night, October 1st. The animals that litter/are borne on Sunday night will be sacrificed on Monday and the animals that litter/are borne Monday night will be sacrificed on Tuesday, within 24 hour of their birth. It is estimated that 10 to 14 pups will be born per litter. It is presumed that the dams will cannibalize pups that are stillborn or that die during the night. Eight of the remaining pups (if available) per litter will be used in the study. Three of the eight pups from each litter will be identified (marked) as time-course pups. The oxygen saturation in these pups will be monitored at several recorded time points throughout the day using the oximeter method (described below) to determine changes in oxygen saturation over time. The other pups on the study will be monitored for oxygen saturation by the oximeter method, then immediately sacrificed to obtain blood for partial oxygen pressure in blood (pO2) determination by the I-Stat method (described below). Near the end of the study, the time-course pups will be sacrificed to obtain blood for pO2 determination by the I-Stat method. Any remaining pups may be sacrificed to obtain serum and tissues for possible future analysis.

Method of Collection/Measurement

Oxygen saturation will be measured with a pulse oximeter (Ohmeda), and pO2 will be measured with an I-stat blood gas analyzer (Abbot Labs). Oxygen saturation and pulse rate will be measured on each pup using an Ohmeda Biox 3740 Pulse

Oximeter, according to the manufacturers' directions. The pup will then be euthanized by decapitation and blood will be drained onto a slide. The blood will be transferred from the side to an I-Stat cartridge using a heparin-washed syringe. The cartridge will immediately be inserted into the I-Stat analyzer and the pO2 level determined. Systemic blood from each dam (≈ 5 ml) will be collected via the abdominal aorta and transferred to blood collection tubes without anticoagulant. All blood samples will be allowed to clot for a period of 15 to 30 minutes at room temperature and the clot will be spun down in a centrifuge at 1100 x g for 5 minutes. The serum will be transferred to labeled 1.5 ml microfuge tubes and centrifuged again at 2000 x g to remove any remaining red blood cells. The serum will then be transferred to labeled polypropylene microfuge tubes and flash-frozen in liquid nitrogen. Livers from all pups and dams will be removed, weighed, placed individually into labeled tubes and flash frozen in liquid nitrogen.

Sample Handling:
Liver and sera samples will be stored at -70°C in the Strategic Toxicology Lab, building 270.

Analysis:
The oxygen saturation and the pO2 techniques will be compared to determine which method will be used in future studies. The results from each analytical method will be compared between treated and control animals and analyzed to determine statistical differences using the students T-test. These results will be used to determine the validity of the hypothesis that PFOS can interfere with normal respiration and/or gas exchange in the pups. Liver and sera will be retained for analysis of gluconeogenic enzyme activity, glycogen stores, and serum glucose levels. Results will be provided for inclusion in the final report.

Signatures:

Deanna J. Luebker, M.S. — Date
Advanced Research Toxicologist
Study Director

Andrew M. Seacat, Ph.D. — Date
Toxicology Specialist
Study Toxicologist

Dan Hakes — Date
Sponsor Representative

3M MEDICAL DEPARTMENT, CORPORATE TOXICOLOGY
Amendment #1 for Study No. T-6295.30 DT-69
Oxygen Saturation and pO2 Levels in Rat Pups Borne to PFOS Exposed Dams

Sponsor:	3M Specialty Chemicals Division
Study Location:	3M Strategic Alternative Toxicology Laboratory
Study Director:	Deanna J. Luebker, M.S.

Background:
The initial study performed under this protocol resulted in very high rates of stillborn pups and early deliveries. This greatly complicated the study, preventing the endpoints of interest from being examined. It is believe that these complications were a result of a dose being delivered to the pups that was in excess of what was necessary to achieve the desired decrease in pup viability. As was stated in the original study protocol, "studies conducted by the EPA NHEERL have demonstrated that a cumulative dose of roughly 100 mg/kg will reduce pup viability by approximately 50% within the first 5 days of lactation". In the EPA studies, however, Tween 20 was used for a vehicle rather than Tween 80, as has been used in the majority of PFOS oral dosing studies. A greater amount of PFOS is presumed to be absorbed when using Tween 80 as compared to Tween 20. Thus, we believe a cumulative dose of 100 mg/kg with Tween 80 is in excess of what is necessary in these studies. In addition, the EPA has demonstrated that the later in gestation dosing occurs, the greater the rate of pup mortality. The period of dosing used the under the original study protocol, days 14-17 of gestation, may have been too late to administer such a high dose of PFOS.

Objective:
The purpose of this amendment is to adjust the dosing regime used under the original protocol to prevent *in utero* death and early deliveries. In addition, amniotic fluid will be collected and analyzed for PFOS and caesarean-sections (c-sections) will be performed rather than allowing the dams to deliver. The purpose of the c-sections is to standardize the time of data collection. A follow-up study will thus be performed under the original study protocol with the following modifications:

1. **Page 1. Study Objective:**
   In addition to the originally stated objective, amniotic fluid will be collected from c-sectioned rats to measure PFOS levels.

2. **Page 1. Proposed Study Timeline:**
   The in-life start date for the follow-up study will be November 7, 2001 and the in-life end date will be November 14, 2001.

3. **Page 2. Animal Number:**
   Six animals will be used in the follow-up study.

4. **Page 2-3. Dose and Dosing Procedures:**

All six animals will arrive at 3M on day 11 of gestation. Three rats will be treated via oral gavage with 25 mg/kg PFOS per kg body weight per day for 3 consecutive days (days 12-14 of gestation) to reach a cumulative dose of 75 mg/kg. PFOS will be prepared as described in the original protocol. The remaining three rats will serve as controls and receive 5ml/kg body weight per day of 2% Tween 80 on days 12-14 of gestation. Any additional rats received from the vendor will be dosed with PFOS as described above and allowed to litter. Pups and dams will be monitored through day 5 of lactation to assess the dosing regime or sacrificed to collect tissues for additional analyses.

5. **Page 3. Sample Collections, Number and Frequency**
All animals, including dams, will be euthanized via decapitation on gestation day 19. Pups will be removed via c-sectioning.
In addition to that described in the original protocol, amniotic fluid will be collected from each dam following decapitation.
Liver and sera may be collected from some, not all, of the dams and pups.

6. **Page 4. Sample Handling**
Amniotic fluid will temporally be stored at –70°C in the Strategic Toxicology Lab, building 270. For PFOS analysis, the amniotic fluid will be shipped on dry ice to Dave Ehersman, building 236. Liver and sera will be handled as described in the original protocol.

7. **Page 4. Analysis**
In addition to the analysis outlined in the original protocol, PFOS level will be compared between PFOS treated and control dose groups and analyzed for statistical differences.

Signatures:

Deanna J. Luebker, M.S. — Date
Advanced Research Toxicologist
Study Director

Andrew M. Seacat, Ph.D. — Date
Toxicology Specialist
Study Toxicologist

Dan Hakes — Date
Sponsor Representative

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